CODEN [USA]: IAJPBB ISSN : 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES
SJIF Impact Factor: 7.187

Avalable online at: http://www.iajps.com *Research Article*

GUT MICROBIOTA ALTERATION IN COLORECTAL CANCER AND ITS CLINICAL IMPLICATIONS

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Article Received: November 2020 **Accepted:** December 2020 **Published:** January 2021

Abstract:

Introduction: *Colorectal cancer is the second commonest cancer arising in the world. Colorectal cancer can present with an array of symptoms and approximately 35–48% of patients diagnosed with colorectal cancer have experienced rectal bleeding.*

Objectives: *The main objective of the study is to analyse the role of gut microbiota alteration in colorectal cancer and its clinical implications.*

Material and methods: *This is basically a descriptive study which was conducted in Allied Hospital Faisalabad during January 2019 to November 2019. Several studies have shown that numerous bacterial species appear to be associated with the pathogenesis of CRC and recent studies have provided a mechanism for the participation of gut microbiota in the progress of CRC.*

Results: *The median age of the patients was 56 years (range: 20–86), and 73% were male. Most patients were married [85.6%], and more than half of the participants were high school educated or higher [77.8%] and unemployed 52.8%. The differences in microbial community abundance between the two groups were examined by statistical methods, and the significance of the differences was evaluated by FDR (false discovery rate). We screened out the species that caused the difference in the composition of the two groups of samples.*

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Please cite this article in press Muhammad Rasim et al, ***Gut Microbiota Alteration In Colorectal Cancer And Its Clinical Implications.,*** *Indo Am. J. P. Sci, 2021; 08(1).*

INTRODUCTION:

Colorectal cancer is the second commonest cancer arising in the world. Colorectal cancer can present with an array of symptoms and approximately 35–48% of patients diagnosed with colorectal cancer have experienced rectal bleeding. Even though the positive predictive value of rectal bleeding for colorectal cancer is low (<3%), it is regarded as an alarm symptom in persons over the age of 40 years [1]. Meanwhile, the majority of individuals who experience rectal bleeding do not report it to their general practitioner (GP). More surprisingly, studies have shown that colorectal cancer patients, who had experienced rectal bleeding, delayed help-seeking more often than patients who had not experienced rectal bleeding [2].

The possible association between rectal bleeding and patient delay differentiates colorectal cancer from most other cancers where bleeding appears to be associated with a short patient interval. Therefore, it is imperative that the factors contributing to this are examined and understood [3]. It has been assumed that the revealed association between rectal bleeding and long patient intervals is a consequence of patients attributing the rectal bleeding to benign causes such as hemorrhoids. Meanwhile, the results of one study of 93 patients who presented with rectal bleeding to their GP suggested that the relationship between rectal bleeding and the patient interval appeared to be modified by personal experiences [4]. Thus, it was found that those patients who had experienced rectal bleeding before and may had suffered from known benign rectal disorders were *less* likely to delay help-seeking than those who had never experienced rectal bleeding before. The proportion of patients who considers cancer when experiencing rectal bleeding is not known [5].

The gut contains a complicated environment that is settled by bacteria, fungi, and viruses. The total number may reach 100 trillion, and the number of microbe cells is estimated to be 10-fold more than the human cells. This densely resident microbial community consistently communicates with the host and also enhances the epithelial defense against pathogens, accelerates the maturity of the immune system, and absorbs the nutrition from ingested foods [6]. Despite the mucus layer, which consists of various macromolecules and secreted antimicrobial molecular and intercellular tight connection proteins, the gut microbiota also possess the capacity to defend pathogens by inducing IgG antibodies through recognition of their conserved antigen part of gram-negative bacteria [7]. The gut microbiota not only protect the local homeostasis, but also mediate the related organ. For example, an in-vivo experiment proved that the gut microbiota was manipulated by intestinal lectins to decrease alcohol-associated steatohepatitis [8]. Along with the evolution of gut microbiota, body cells also demonstrate effective pathways for avoiding the pathogen infection.

An updated estimation reveals that more than 376, 000 new cases of CRC and 191,000 deaths occur every year in China. CRC has long been investigated and it is classified into two typical types: colitis-associated colorectal cancer (CAC) and sporadic colorectal cancer (SCC), according to genomic mutation diversity. Hereditary syndrome has been identified with a total of fourteen mutations. The inner involved signal pathways are totally different between these two relatively independent phenotypes, but they also share a few sequential genetic mutations [9].

CAC is always associated with inflammatory bowel disease, an inflamed disorder phenotype in the young population. SCC is usually used to refer to the common colorectal cancer that considered without family heredity. CRC is a malignant disease which involves multiple factors during its multi-stage development. The initiating events of CRC have been proved to be APC mutation in SCC and TP53 mutation in CAC. The etiology of CRC has been investigated using large cohorts and confirmed by animal models, and the consensus conclusion contains genetic background and environmental risk factors such as diabetes, cholecystectomy, obesity, high fat diet, and processed and red meat [10]. However, a large number of studies have recently reported that the gut microbiota may also participate as an essential contributor factor in the initiation and development of CRC.

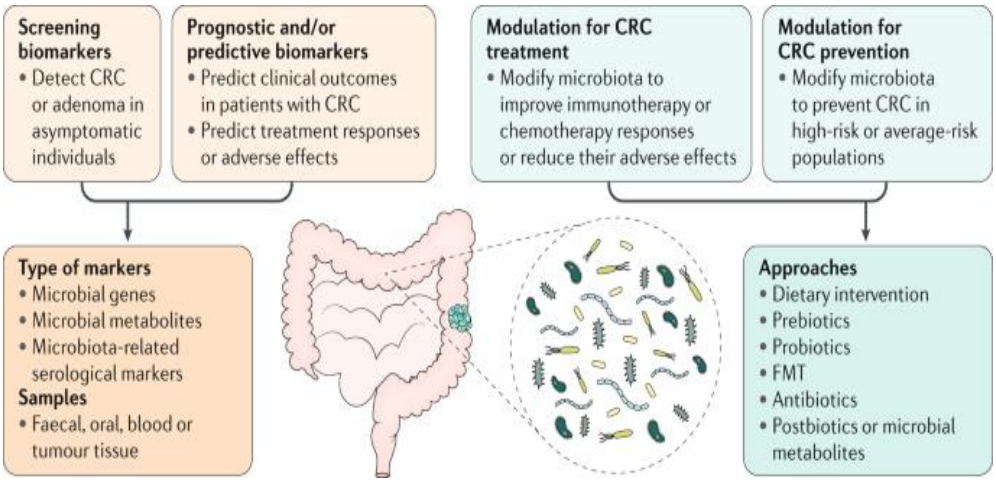


Figure 1: Gut microbiota and colorectal cancer

Objectives:
The main objective of the study is to analyse the role of gut microbiota alteration in colorectal cancer and its clinical implications.

MATERIAL AND METHODS:

This is basically a descriptive study which was conducted in Allied Hospital Faisalabad during January 2019 to November 2019. Several studies have shown that numerous bacterial species appear to be associated with the pathogenesis of CRC and recent studies have provided a mechanism for the participation of gut microbiota in the progress of CRC.

Inclusion criteria:

- All those patients who were ready to participate in this study and having confirmed CRC.

Exclusion criteria:

- Those who had used antibiotics or microecological agents within 2 months before enrolment.
- Those who suffer from chronic diseases such as hypertension, heart disease, and diabetes.

Data collection:
All patients were interviewed and examined by a gastroenterologist. Accordingly, patients' informed through written consent was obtained from each patient before placing interview according to the strategies of the local institutes. After clinical assessment, all patients suffered anal examination and digital rectal review. The subjects were divided into two groups, one was CRC patients and second was control group. Data were collected in the clean environment of fresh feces (not less than 6 g), put in an aseptic sampling tube and sent to the laboratory, and kept at −80°C for inspection.

Methodology of sample collection:
Stool samples were collected before surgery and electronic colonoscopy in patients with rectal cancer and during the collection process, the samples should not be contaminated by urine or sewage. After two weeks of colonoscopy, participants were informed to collect fecal samples with sterile containers and the samples were stored at −80 °C as soon as processed. Fecal samples from 10 patients with CRC (CRC) and 10 healthy controls (Control) were mixed at equal weight, respectively. One gram of the mixed feces was diluted in 5 mL sterile PBS solution, then initial filtered, concentrated, homogenized, step by step filtered, and centrifuged. The supernatant was collected, isolated, purified and equally repackaged before kept in −80 °C refrigerator.

Identification of microbiome:
For pragmatic reasons, fecal specimens are frequently used as proxies for gut microbiota. Fecal specimens are naturally collected, non-invasive and can be sampled repeatedly, so they are the source of samples for most intestinal microbiota studies. For using preservatives to store fecal specimens stably, 95% ethanol and RNAlater are worthy of recommendation. We estimated the relative abundance of gut microbes using the GRAMMy algorithm. We then identified differentially abundant microbes by the RAIDA algorithm which uses a modified ZIL model to account for ratios with zeros.

Western blotting assay:
Western blot determined the proteins quantities in cell cycle and apoptosis-related biomarkers. The tissue were exposed to angiotensin II of for varying periods

of time and then the proteins were extracted and rinsed two times with cold PBS and lysed in test load up buffer. The expression of the load up buffer was 1.5 percent SDS, 10 percent glycerol, 5 mM β-mercaptoethanol, bromophenol blue and 75 mM Tris (pH 7.0). Whole cell lysates were separated by SDS-PAGE of 12% gel and moved proteins onto a polyvinylidene fluoride membrane. The immunoblots were established and seen by ECL Western blot medium (Thermo Fisher Scientific). U6 and GAPDH were used as internal control. The analysis of each group was repeated in three fold. The image J detection system was employed to determine the concentration of the bands.

Statistical analysis:

Each experiment was repeated three times and all data were displayed in mean±SD and analyzed through SPSS 19.0 (IBM, USA). T-test and one-way ANOVA were applied for measuring comparison among groups. $P<0.05$ was considered to have statistical meaning.

RESULTS:

The median age of the patients was 56 years (range: 20–86), and 73% were male. Most patients were married [85.6%], and more than half of the participants were high school educated or higher [77.8%] and unemployed 52.8%.

Table 01: Baseline characteristics

	***N* = 50**	**%**
Age		
Median	56	
Range	20–86	
Smoking		
Smoker	16	20.1
Non-Smoker	34	79.9
Marital status		
Married	19	85.6
Single	15	7.4
Widowed	12	5.2
Divorced	4	1.7
Educational level		
Elementary school	14	10.5
Middle school	7	11.8
High school	16	37.6
Undergraduate	7	32.3
Graduate school	6	7.9
Employment status		
Full-time job	22	35.8
Part-time job	6	11.4
Unemployed	22	35.8
Histology		
Tubular adenocarcinoma	16	70.3
Signet ring cell carcinoma	8	25.3
Mucinous carcinoma	3	2.2
Others	25	22
Adjuvant chemotherapy		
Platinum-based doublet (SP or FP)	36	67.5
TS-1 monotherapy	14	26.5

None of the patients were asymptomatic when they sought medical help, and a total of 81 patients (60%) had experienced rectal bleeding during the patient interval. Additional to rectal bleeding, the most commonly reported symptoms, i.e. symptoms reported by 20% or more of the sample, were changes in bowel habits (65%), fatigue (47%), pain (35%), weight loss (21%), and general indisposition (20%). Among the rarely reported symptoms were dizziness (13.2%), lack of appetite/nausea (11.8%) and fever (5.2%). A total of 14 (10%) patients had experienced rectal bleeding without co-occurrence of any of the other five

commonly reported symptoms. The median patient intervals in days are reported for patients, who reported changes in bowel habits, fatigue, pain, weight loss, and general indisposition either in combination with rectal bleeding or not in combination with this symptom.

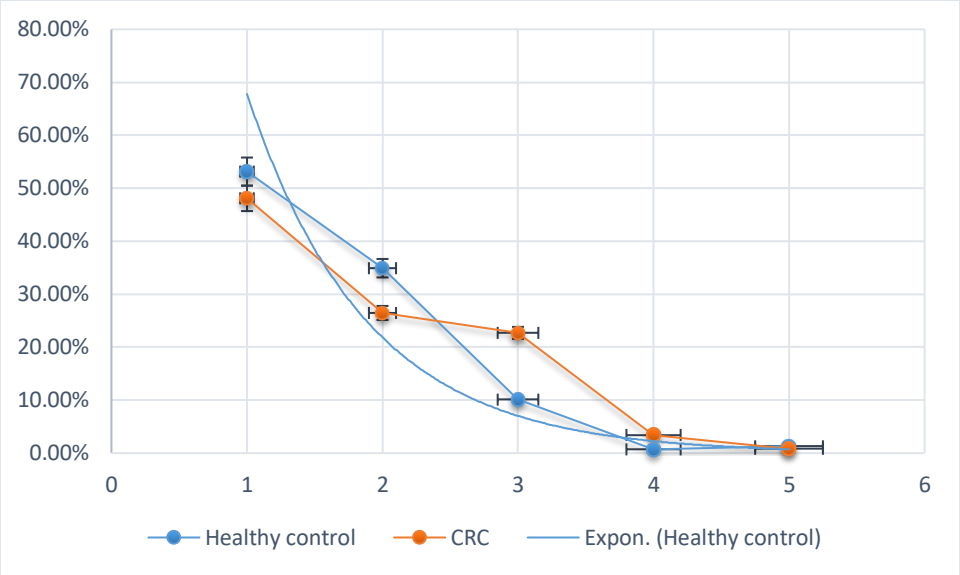
Table 02: Median patient interval (in days) for the five symptoms occurring in ≥20% of the sample

	Changes in bowel habits	Pain	Weight loss	Fatigue	General indisposition
Median (IQI) patient interval when presented without rectal bleeding	16 (5–31)	14 (3–28)	18 (4–29)	17 (4–29)	10 (0–29)
	N=30 (22.1%)	N=25 (18.4%)	N=17 (12.5%)	N=26 (19.1%)	N=11 (8.1%)
Median (IQI) patient interval when presented together with rectal bleeding	61 (12–112)	31 (13–119)	38 (22–74)	34 (5–96)	31 (0–57)
	N=58 (42.6%)	N=22 (16.2%)	N=12 (8.8%)	N=38 (27.9%)	N=16 (11.8%)

The differences in microbial community abundance between the two groups were examined by statistical methods, and the significance of the differences was evaluated by FDR (false discovery rate). We screened out the species that caused the difference in the composition of the two groups of samples. According to our existing research findings and combined with accumulated research, there is unequivocal evidence linking gut dysbiosis to CRC development. We identified that the microbial structures of the CRC patients and healthy individuals differed significantly. However, gut dysbiosis and the occurrence of CRC, which occur first, is not yet very clear.

Table 03: Relationship between Gut Microbiome and Colorectal Cancer Susceptibility

Phylum	Mean		value	FDR
	CC	HC		
Firmicutes	25.36	35.87	0.00035	0.00231
Fusobacteria	3.45	0.59	0.00000	0.00001
Proteobacteria	22.36	9.32	0.00079	0.00344
Spirochaetes	0.01	0	0.01342	0.03489
Synergistetes	0.19	0.004	0.00564	0.01833



	HC	CC
Bacteroidetes	53.14%	48.10%
Firmicutes	34.91%	26.46%
Proteobacteria	10.10%	22.70%
Fusobacteria	0.71%	3.44%
Actinobacteria	1.29%	0.81%

Figure 2: The trend chart based on the main microbial composition for each group at the phylum level. On the phylum level, the abundance of Bacteroidetes and Firmicutes is decreasing with the development of health-polyp-adenomas-CRC, and the abundance of Proteobacteria is increasing.

Western Blot Analysis:

Western blot examined relative expression. Overexpression of CTSK was observed in eight out of 12 CRC tissues compared with matched non-tumor tissues ($P = 0.0016$). IHC results further demonstrated that CTSK was significantly upregulated in 79% (31/39) of CRC tissues examined compared to that in 15% (6/39) of the adjacent normal tissues. Moreover, western blot analysis demonstrated that CTSK protein is overexpressed in mCRC tissues compared with that of nmCRC tissues. To evaluate the clinical significances of CTSK, IHC assay was further performed in 42 nmCRC tissues and 27 mCRC tissues. CTSK was overexpressed in 38% (16/42) of nmCRC tissues and a much higher rate was observed in 81% (22/27) of mCRC samples.

Kaplan–Meier survival curve:

Kaplan–Meier survival analysis of a previously published CRC dataset (NCBI/GEO/GSE 39582, $n = 534$) revealed that CTSK expression is closely correlated with patients' survival rate and life expectancy. Patients with low CTSK expression have a significantly better prognosis.

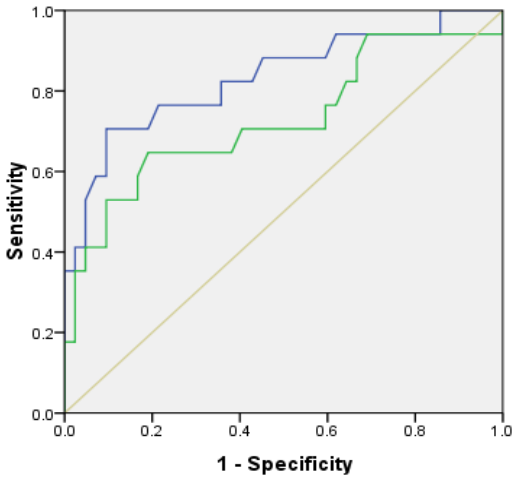


Figure 1 ROC curve of group A

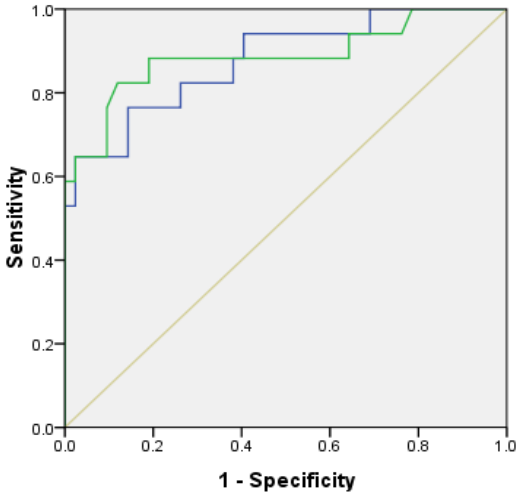


Figure 2 ROC curve of group B.

DISCUSSION:

Several studies have shown that numerous bacterial species appear to be associated with the pathogenesis of CRC and recent studies have provided a mechanism for the participation of gut microbiota in the progress of CRC [9]. Some bacterial species like *Clostridium septicum*, *Enterococcus faecalis*, *Streptococcus bovis*, *Bacteroides fragilis*, *Helicobacter pylori*, *Escherichia coli* and *Fusobacterium* spp. have been detected and supposed to play a role in colorectal pathogenesis [10].

For example, *Streptococcus gallolyticus* (In the past *Streptococcus bovis*) is reported in nearly 20–50% and 5% of colon tumors and normal colon respectively. In CRC patients *Ruminococcus bromii*, *Clostridium clostridioforme* and *Bifidobacterium longum* have low prevalence compared to normal population [11]. Furthermore, in different studies a notably increase number of the *Bacteroides/Prevotella* and *Fusobacterium nucleatum* population is described in CRC population [12].

In a study by Gao *et al*. no significant difference was observed between proximal and distal colon microbiota in 30 healthy compare to 31 cancer patients; nevertheless, in colorectal cancer patients, *Firmicutes* and *Fusobacteria*, *Lactococcus* a nd *Fusobacterium* were more prevalent and *Proteobacteria*, *Pseudomonas* and *Escherichia–Shigella* were less frequent in tissues samples compared to control group [13].

Several studies showed higher prevalence of *F. nucleatum* in CRC tissue compare to a matched normal tissue. *F. nucleatum* is showed as a probable candidate for CRC predisposition. *F. nucleatum* adheres to colonic epithelial cells through its FadA adhesion. FadA binds to E-cadherin, activates β-catenin signaling, and differentially regulates the inflammatory and oncogenic responses [14]. Fap2 protein of *F. nucleatum* can stimulates CRC expansion by inhibition of the antitumor immune cell activity via TIGIT.

Enterococcus faecalis (E. faecalis), a commensal microorganism in the intestinal tract, has been repeatedly found in colorectal cancer patients [15]. *E.faecalis* has recently been considered as a human pathogen. Balamurugan et al. had reported statistically significant higher levels of *E. faecalis* from the feces of patients with CRC compared to healthy volunteers [16]. These bacteria can produce reactive oxygen and nitrogen species (RONS) that directly lead to DNA break, point mutation and chromosomal instability. These functions demonstrated this common colonic commensal has rendered an organism with the potential to contribute to oncogenic transformation in the colon [17].

Controversial result have been reported regarding the role of *H. pylori* in CRC. Zumkeller et al., in their meta-analysis study, reported a 1.4 time increased risk of CRC in patients with a *H. pylori* infection around the world [18]. Guo *et al*, in a meta-analysis study of 7679 Asian patients, recommended a carcinogenic role of *H. pylori* at a primary phase of carcinogenesis [19]. Bacterial cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) are encoded in some *H. pylori* strains and induce the activation of inflammation pathways. There is also another hypothesis that direct and indirect production of

RONS by some strains could participate in tumorigenesis in the colon [20].

CONCLUSION:

It is concluded that diet is associated with increased incidence of CRC. Diet shapes the microflora and affects its metabolites and functions. Excessive intake of animal protein and fat (especially red meat and processed meat) will produce excessive secondary bile acid and hydrogen sulfide, leading to barrier dysfunction, inflammation, DNA damage, genotoxicity, and so on, which may increase the risk of CRC.

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