Mucosa-associated Cultivable Aerobic Gut Bacterial microbiota among Colorectal Cancer Patients Attending at the Referral Hospitals of Amhara Regional State, Ethiopia

Authors:

Yesuf Adem Siraj[1,4,#], Email: yesufadems@yahoo.com

Melese Gebeyehu Byadgelegn[2], Email: gbmelese@gmail.com

Mensur Osman Yassin[3], Email: omensur@yahoo.com

Yohannes Zenebe Chekol[1], Email: yohabt22@gmail.com

[1] Department of Medical Laboratory Sciences, School of Health Sciences, College of Medicine and Health Sciences, Bahir Dar University, P.O.Box 79, Bahir Dar, Ethiopia

[2] Department of General Surgery, School of Medicine, College of Medicine and Health Sciences, Bahir Dar University, P.O.Box 79, Bahir Dar, Ethiopia

[3] Department of Surgery, School of Medicine, College of Medicine and Health Sciences, University of Gondar, P.O.Box 196, Gondar, Ethiopia

[4] CDT – Africa, College of Health Sciences, Addis Ababa University, P.O.Box 9086, Addis Ababa, Ethiopia

 # Corresponding author:

Yesuf Adem Siraj

Email: yesufadems@yahoo.com

College of Medicine and Health Sciences, Bahir Dar University, P.O.Box79, Bahir Dar, Ethiopia

Abstract

Background: Colorectal cancer is one of the top ten cancer death in the world. Despite an increased prevalence of colorectal cancer has been documented from developing countries, reports on gut microbiota among colorectal cancer patients are none especially in Ethiopia. Therefore, the current study evaluated cultivable aerobic bacterial distributions among malignant tissue of colorectal cancer and its adjacent normal biopsies.

Methods: Fifteen CRC patients who were undergoing colorectal cancer resection surgery during April 2017 to February 2018 at Felege Hiwot Referral and University of Gondar Teaching Hospitals were included. Biopsy specimens were taken from malignant and its adjacent normal tissues. Bacterial cultivation, quantification and characterization of saline washed biopsies were performed under aerobic and candle jar conditions. Differences in bacterial microbiota compositions between malignant and normal tissue biopsies were evaluated and analyzed using Microsoft excel 2010 and GraphPad Prism5 statistical software.

Results: Fifteen CRC patients were participated with a mean age of 53.8 $\pm$ 10.8 years old and majorities (73.3%) of patients were in between the age groups of 40 and 60 years old. The mean $\pm$ SD bacterial microbiota of malignant biopsies ($3.2x10^5 \pm 1.6x10^5$ CFU/ml) was significantly fewer than that of adjacent normal tissue biopsies ($4.0x10^5 \pm 2.2x10^5$ CFU/ml). This dysbacteriosis is positively correlated with the occurrence of CRC (p=0.019). Proteobacteria (55.6%), Firmicutes (33.3%) and Fusobacteria (11.1%) were the most frequently isolated phyla from non-malignant biopsies while only Proteobacteria (58.8%) and Firmicutes (41.2%) were from malignant ones. Family level differences were observed among phyla (Firmicutes and Proteobacteria) isolated from the study participants. For instance, the relative abundance of family Bacillaceae from malignant

(26%) was lower than the normal biopsies (39%). On other hand, family Enterobacteriaceae was twice more abundant in malignant tissues (45%) than in its matched normal tissues (23%). Furthermore, the family Enterococcaceae (14%) of family Firmicutes was solely isolated from malignant tissue biopsies.

Conclusion: The overall microbial composition of normal and malignant tissues was considerably different among the study participants. Further culture independent analysis of mucosal microbiota will provide detail pictures of microbial composition differences and pathogenesis of CRC in Ethiopian settings.

Key words: Gut microbiota, culture-based, mucosal biopsies, colon cancer, Ethiopia

1. Background:

Colorectal cancer (CRC) is the fourth most common causes of cancer deaths in the world with almost 900, 000 deaths annually (1) next to lung cancer (2). It accounts for approximately 10% of cancer-related mortality in western countries (3). Although a population based data is unavailable from Ethiopia, colorectal cancer is a problem of significant magnitude with unresectable tumor (4). Based on a single cancer registry data of Addis Ababa City, the Global cancer statistics center reported 4,716 (7%) new CRC cases in 2018 (2) which makes CRC ranked at the third of cancer cases in Ethiopia.

Various environmental factors are commonly associated with the occurrence of colorectal cancer. These include; dietary change, smoking habit, heavy alcohol consumption, and history of inflammatory bowel diseases [Crohn's diseases and ulcerative colitis] (5). Colitis-associated cancer (CAC) is a chronic intestinal inflammation that possibly as a result of defective intestinal barrier function and host-microbiota interaction (6).

In spite of microbial composition of the human intestine is obviously correlated to the health conditions, human gut microbiota have emerged as a major environmental factor that modulate the risk of colorectal cancer. Dysbiosis of gut microbiota (7)(8) is now assumed to be an underlying factor in the development of colorectal cancer. Currently several researches are trying to associate the change in the composition of human intestinal microbiota with colorectal cancer occurrence. However, most studies might not show strong association due to different constraints including use of non-intestinal biopsy investigations and convenience of specimen (9). Mucosa-associated microbiota potentially affects CRC risk primarily through direct interaction with the host (10) and its significantly differed organization in CRC patients and healthy individuals (11).

Granting an increase in prevalence of colorectal cancer has been documented from developing countries (2)(12), reports on gut microbiota in relation to colorectal cancer are not yet issued particularly in Ethiopia. Phenotypic, genotypic and toxin gene analyses of gut microbiota composition have not yet been done among colorectal cancer patients in the study area and in Ethiopia at large. Therefore, this study is aimed at determining the microbial distribution and characterizing cultivable aerobic gut mucosal associated bacteria among cancerous and adjacent apparently normal tissues of colorectal cancer patients.

2. Method and materials

2.1. Patient recruitment and mucosal biopsy

At Gasteroenterology and Digestive Clinics of Felege Hiwot and University of Gondar Teaching Hospitals, 15 confirmed CRC patients who underwent surgical resections of cancerous tissues were enrolled in the study during April 2017 to February 2018. Ethical clearance was obtained from the ethical review committee of College of Medicine and Health Sciences, Bahir Dar University and Amhara National Regional Health Bureau. Informed consent from each study participant was obtained and information was kept confidential. Two biopsies (with 5-7 x 5-7mm dimensions) were obtained from malignant and adjacent normal areas of the colorectal lumen of CRC patients during colorectal cancer open resection surgery. Each biopsy specimen was aseptically collected and immediately transported to the bacteriology laboratory. Saline washed biopsy suspensions were used for aerobic cultivation. Biopsy specimens were preserved at 4^{o}C where delayed analysis was unavoidable. Findings were analyzed and interpret accordingly using statistical software.

2.2. Bacterial count and identification

All collected biopsies were intensively washed with 5 ml of normal saline. Twenty µl suspension of each saline-washed specimen was suspended on to each three plates of meat peptone agar. MacConkey agar, a selective media, was also employed to isolate common pathogenic bacteria like *Salmonella* and *Shigella* species. Colony forming unit (CFU) count, morphological characteristics of bacterial isolates at average logarithmic growth phase and identification of bacterial species using a series of biochemical tests were aseptically performed. Sterility and performance of the prepared media were checked by parallel inoculation of localy available control strains of American Type Culture Collection: *S. aureus* (ATCC®-25923), *P. aeruginosa* (ATCC®-27853) and *E. coli* (ATCC®-25922).

2.3. Statistical Analysis

Statistical data analysis and plotting were performed by means of Microsoft excel 2010 and GraphPad Prism5 software. Statistically significant level was considered at $p \leq 0.05$.

3. Result

Fifteen colorectal cancer patients were recruited from two referral hospitals: Felege Hiwot Referral Hospital (n=8) and University of Gondar Teaching Hospital (n=7). Nine (60%) were males with a male to female ratio of 1.5:1. The cumulative mean age $\pm$ SD of the study participants were 53.8 $\pm$ 10.8 years with a range of 38 and 79 years old. Eleven (73.3%) of the study participants were between the age groups of 40 and 60 years while elders with $\geq$60 years old were only 4 (26.7%) (Table 1).

Table 1: Socio-demographic characteristics of colorectal cancer cases

		1st Quartile	2nd Quartile	3rd Quartile	Total
		N(%)	N(%)	N(%)	N(%)
		4(26.7)	7(46.6)	4(26.7)	15(100)
Age [in years]	Mean ± SD	40.8 ± 3.6	54.6 ± 4.7	65.5 ± 9.1	53.8 ± 10.8
	Median	39.5	56	61.5	56
	Minimum	38	48	60	38
	Maximum	46	59	79	79
Sex	Male N (%)	3(75)	4(57)	2(50)	9(60)
	Female N (%)	1(25)	3(43)	2(50)	6(40)
	Male : Female ratio		1.5 : 1		

The overall abundance of cultivable aerobic bacteria was recovered from triplicate culture plates and compared with types of biopsies. The mean ± SD population of aerobic bacteria cultivated from normal-featuring biopsies was approximately $4.0x10^5 \pm 2.2x10^5$ CFU/ml while it was $3.2x10^5 \pm 1.6x10^5$ CFU/ml from malignant tissues. According to the Pearson r test, significant correlation was observed between a reduced bacterial microbiota (dysbacteriosis) of washed malignant tissue suspensions and the occurrence of colorectal cancer ($p=0.019$, Pearson $r=0.596$, 95% CI=0.120–0.849) (Figure 1).

As the Box-Whiskers appearance indicates, the mean bacterial population of malignant was significantly different from the adjacent normal tissues biopsies at $p<0.05$ (Figure 1). The relative abundance of bacteria at family or genus level in each cancerous specimen was much smaller compared to the other equivalent normal tissue biopsies. The upper range value of bacterial abundance of malignant tissues [$6.8x10^5$ CFU/ml] was reduced at a minimum by $2.0x10^5$ CFU/ml

of washed biopsy suspension from its matched normal tissue biopsies count [$8.6x10^5$ CFU/ml]. Similarly, the lower range value of washed malignant tissue biopsies [$1.7x10^5$ CFU/ml] was also $2.0x10^4$ CFU/ml fewer than its equivalent counts of adjacent normal tissues [$1.9x10^5$ CFU/ml] (Figure 1).

Figure 1: Box – Whisker plot of bacterial microbiota abundance in normal and malignant tissue biopsies of CRC patients.

The plot shows median values, means (+ sign in boxes), interquartile ranges (IQR) (boxes) and 1.5 x IQR (whiskers). Bacterial population isolated from paired biopsies of CRC patients was significantly associated with the occurrence of tumor at (*$p \leq 0.05$) or being normal tissue at (**$p \leq 0.01$).

Comparing the mucosal microbiota of malignant niche to its matched adjacent normal tissues indicated varied bacterial compositions over those two groups of samples of CRC patients. Three bacterial phyla; Proteobacteria (55.6%), Firmicutes (33.3%) and Fusobacteria (11.1%) were over represented in non-malignant tissues of CRC patients (Figure 2) while only two phyla; Firmicutes (41.2%) and Proteobacteria (58.8%) were recovered from malignant biopsies of CRC patients (Figure 3). In addition, more bacterial diversity has been observed from apparently healthy tissue specimens group than its equivalent particularly among the age groups of 55 to 65 years (Figure 2 and 3).

Figure 2: Age - bacterial phylum distribution isolated from normal tissue biopsies of CRC patients

Figure 3: Age - bacterial phylum distribution isolated from malignant tissue of biopsies of CRC patients

Though most members of phylum Fusobacteria are obligate anaerobic bacteria, a genus *Streptobacillus* (Figure 4) under a family Leptotrichiaceae (20%) with a microaerophilic nature was recovered only from the normal tissue biopsies using CO_2 enriched cultivation. Phyla Firmicutes and Proteobacteria recovered from both groups of tissues showed no difference while bacterial family level differences between groups were observed. The relative abundance of family Bacillaceae isolated from non-malignant tissue biopsies was at (39%) of the total isolated bacterial families while it was much lower proportion (26%) from malignant tissue biopsies. On the other hand, the relative abundance of family Enterobacteriaceae (45%) isolated from malignant tissue was twice higher than from the matched control biopsies (23%). Furthermore, the family Enterococcaceae (14%) was isolated only from malignant biopsies (Figure 4).

Figure 4: Bacterial family distribution in Normal and Malignant biopsies of CRC patients. Numbers are in percentage of the toatal family coverage.

Discussion

The variability of microbial population of gastrointestinal tract is currently correlated to the occurrence of different disorders including colorectal cancer. Though several recent advanced researches use metagenomic approaches to measure microbial cells in feacal or mucosal specimens, there is no any published data related to the overall microbiota profile of mucosal or feacal specimens of CRC patients in Ethiopia. Therefore, the current study was aimed at determining the distribution of at least cultivable aerobic bacterial microbiota of cancerous and normal-featuring tissues of CRC patients.

The dysbiosis of bacterial microbiota abundance and distribution in malignant tissues from adjacent normal biopsies is currently become an indicative in the diagnosis and prognosis of

CRC patients. These alterations are also demonstrated in our study by the presence of abundant bacterial microbiota in normal biopsies [$\bar{x}$=4.0x10^5 CFU/ml] while much smaller bacterial population [approximately by 2.0x10^5 CFU/ml less] from malignant tissue biopsies of CRC patients (Figure 1).

In this study, we found higher abundance of bacterial composition of phyla; Proteobacteria (55.6%), Firmicutes (33.3%) and Fusobacteria (11.1%) in normal biopsies of CRC patients (Figure 2). However, it is much different from a study reported by Eckburg *et al.* (13), in which 90% of bacterial composition of normal luminal microbiota belongs to the phyla; Firmicutes and Bacteriodes, the remaining minor constituents were Proteobacteria and Actinobacteria.

Among members of phylum Fusobacteria (14), only a genus *Streptobacillus* in the family Leptotrichiaceae was isolated from normal tissues of CRC patients (Figure 2). It could be due to the alternative method we employed, candle jar for fastidious bacterial cultivation, probably supported the growth of microaerophilic bacteria. Other most fusobacterial members strictly require anaerobic environment to grow (15) and are associated greatly with cancer tissues than in normal tissues (16). Despite the genera Bacteriodes (17), Leptotrichia species (18)(19) and Fusobacteria (17)(20) were the most frequently identified and reported bacteria from malignant tissues of colorectal cancer, our study didn't showed any above mentioned species while we employed candle jar cultivation.

According to the author Lau *et al.* (21), *Streptobacillus hongkongensis* is a novel bacterial species that permanently found in human oropharynx and there might be more other *Streptobacillus* species probably also residing in human oropharynx. This genus might get easy access to the lumen of the colorectal regions (22).

The microbial abundance of family Bacillaceae in malignant biopsies (26%) was lower than the abundance in non-malignant tissues (39%) while the family Enterobacteriaceae, a member of phylum Proteobacteria (23) was over-represented (45%) from malignant group of tissue (Figure 4). This observation could be supported by the fact that family Enterobacteriaceae is considerably a member of the carcinogenic bacteria that constitute Lipopolysaccharides (LPS), D-Lactate and other bacterial components which positively correlated with the incidence and progression of inflammatory bowel diseases (IBD) as well as colorectal cancer (24)(25)(26).

Our study also revealed that significant abundance of family Enterococcaceae was identified only from malignant biopsies (Figure 4). This finding supports previously reported evidences that patients with ulcerative colitis and Crohn's disease have larger members of family Enterococcaceae than healthy controls (27)(28)(29).

The imbalance of these bacteria and their gene products (30)(31) that underlies mucosal surface of intestinal microvilli would facilitate the replication of opportunistic pathogens which might have direct contribution in the onset and progression of severe gastrointestinal inflammation leading to colorectal cancer. Hence, these findings could be a focus of future investigations on potential pro-oncogenic pathogens of gasterointestinal cancers in the study area.

Limitations:

Since our study employed a culture-based aerobic cultivation, huge segment of mucosal-associated microbiota; obligate anaerobes, fungal agents and uncultivable microbes were not addressed. Microbial distributions in relation to anatomic positions of colorectal biopsies, cancer stage, anticancer or antibiotic use, comorbid diseases and long term dietary habit were not well considered. However, with these limitations, the study will provide base line information for

future development of culture independent studies of gut microbiota in the study area.

Conclusion

The findings presented in the current study suggested a relative abundance and distributions of cultivable aerobic bacterial microbiota of malignant tissues were significantly different from its adjacent normal tissue biopsies. Our study also showed that families of Enterobacteriaceae and Enterococcaceae were the most frequently recovered bacterial family from malignant tissues while detail considerations of these bacteria in the initiation and progression of colorectal cancer remains unclear. Therefore, large scale and deep metagenomic analysis of gut microbiota differences in Ethiopian population play key roles in the future development of advanced diagnostic, prognostic and therapeutic strategies of colorectal cancer patients.

List of Abbreviations:

CAC – Colitis-associated cancer

CFU – Colony forming unit

CRC – Colorectal cancer

IBD – Inflammatory bowel disease

IQR – Interquartile range

IRB – Institutional Review Board

Declarations:

Ethics approval and consent to participate

Ethical clearance was obtained from Institutional Review Board (IRB) of Amhara Regional

Health Bureau, Bahir Dar. Then permission letter was obtained from Felege Hiwot Referral Hospital and University of Gondar Teaching Hospital. Informed consent was obtained from study participants after explaining the objective of the study. Any study participant who was not willing to participate in the study was not forced to participate. Data obtained from the study participants were kept confidential using only codes.

Data Availability Statement

The datasets collected and analyzed in the present study are available with the first author up on request.

Competing interests

We authors declare that we have no competing interest.

Funding

This research was funded by Bahir Dar University, College of Medicine and Health Sciences – Staff Research Grant / 2016-17.

Authors' contributions

YA contributed to study conception and design, laboratory investigations, acquisition of data, data analysis and interpretation and wrote the first draft and final version of manuscript; MG and MO contributed to study participant enrolment, demonstrated the quality of biopsies, final manuscript preparation. YZ contributed to study conception and design, laboratory investigations, acquisition of data, final manuscript preparation. All authors read and approved the final manuscript.

Acknowledgments

We would like to express our deepest gratitude to Felege Hiwot Referral Hospital Laboratory staffs for their generous support during laboratory investigations. Our appreciation also goes to

Felege Hiwot Referral Hospital and Amahara Public Health Institute for their kind provision of available control ATCC strains.

References:

1. Dekker E, Tanis PJ, Vleugels JLA, Kasi PM, Wallace MB. Colorectal cancer. Vol. 394, The Lancet. 2019.
2. 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).
3. Kuipers EJ, Grady WM, Lieberman D, Seufferlein T, Sung JJ, Boelens PG, et al. Colorectal cancer. Nat Rev Dis Prim. 2015;
4. Zemenfes D, Kotisso B. A Two-year review of Colorectal Cancer at Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia. East Cent African J Surg. 2015;20(2):10–6.
5. Haggar FA, Boushey RP. Colorectal cancer epidemiology: Incidence, mortality, survival, and risk factors. Clin Colon Rectal Surg. 2009;22(4):191–7.
6. Waldner MJ, Neurath MF. Mechanisms of Immune Signaling in Colitis-Associated Cancer. CMGH. 2015.
7. Thursby E, Juge N. Introduction to the human gut microbiota. Biochemical Journal. 2017.
8. Ni J, Wu GD, Albenberg L, Tomov VT. Gut microbiota and IBD: Causation or correlation? Nature Reviews Gastroenterology and Hepatology. 2017.
9. Pagnini C, Delle Fave G. Letter: investigating the intestinal mucosa-associated microbiota – relevance and potential pitfalls. Aliment Pharmacol Ther. 2016;44(6):647–8.
10. Chen W, Liu F, Ling Z, Tong X, Xiang C. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. PLoS One. 2012;

11. Mandal RS, Saha S, Das S. Metagenomic Surveys of Gut Microbiota. Vol. 13, Genomics, Proteomics and Bioinformatics. Beijing Genomics Institute; 2015. p. 148–58.
12. Feuchtner J, Mathewos A, Solomon A, Timotewos G, Aynalem A, Wondemagegnehu T, et al. Addis Ababa population-based pattern of cancer therapy, Ethiopia. PLoS One. 2019;
13. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Microbiology: Diversity of the human intestinal microbial flora. Science (80-). 2005;
14. Bergey DH. Bergey's Manual of Systematic Bacteriology - Vol 4: Bacteroidetes. Springer-Verlag New York Inc. 2010.
15. Kasper SH, Morell-Perez C, Wyche TP, Sana TR, Lieberman LA, Hett EC. Colorectal cancer-associated anaerobic bacteria proliferate in tumor spheroids and alter the microenvironment. Sci Rep. 2020;
16. Jahani-Sherafat S, Alebouyeh M, Moghim S, Amoli HA, Ghasemian-Safaei H. Role of gut microbiota in the pathogenesis of colorectal cancer; A review article. Gastroenterology and Hepatology from Bed to Bench. 2018.
17. Edmiston CE, Avant GR, Wilson FA. Anaerobic bacterial populations on normal and diseased human biopsy tissue obtained at colonoscopy. Appl Environ Microbiol. 1982;
18. Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, et al. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. Genome Res. 2012;
19. Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, et al. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. Genome Res. 2012;
20. Warren RL, Freeman DJ, Pleasance S, Watson P, Moore RA, Cochrane K, et al. Co-

occurrence of anaerobic bacteria in colorectal carcinomas. Microbiome. 2013;

21. Lau SKP, Chan JFW, Tsang CC, Chan SM, Ho ML, Que TL, et al. Human oropharynx as natural reservoir of Streptobacillus hongkongensis. Sci Rep. 2016;

22. Zhao L, Zhang X, Zuo T, Yu J. The Composition of Colonic Commensal Bacteria According to Anatomical Localization in Colorectal Cancer. Engineering. 2017.

23. D H Bergey; William B Whitman; M Goodfellow; Peter Kämpfer; Hans-Jürgen. Bergey's Manual® of Systematic Bacteriology: Volume Two: The Proteobacteria. New York Springer, ©2005. 2005;

24. Liu X, Cheng Y, Shao L, Ling Z, Huang Y. Alterations of the Predominant Fecal Microbiota and Disruption of the Gut Mucosal Barrier in Patients with Early-Stage Colorectal Cancer. Biomed Res Int. 2020;

25. Altomare A, Putignani L, Del Chierico F, Cocca S, Angeletti S, Ciccozzi M, et al. Gut mucosal-associated microbiota better discloses inflammatory bowel disease differential patterns than faecal microbiota. Dig Liver Dis. 2019;

26. Tlaskalová-Hogenová H, Štěpánková R, Hudcovic T, Tučková L, Cukrowska B, Lodinová-Žádníková R, et al. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. Immunology Letters. 2004.

27. Růžičková M, Vítězová M, Kushkevych I. The characterization of Enterococcus genus: resistance mechanisms and inflammatory bowel disease. Open Med. 2020;

28. Zhou Y, Chen H, He H, Du Y, Hu J, Li Y, et al. Increased Enterococcus faecalis infection is associated with clinically active Crohn disease. Med (United States). 2016;

29. Van Der Wiel Korstanje JAA, Winkler KC. The faecal flora in ulcerative colitis. J Med

Microbiol. 1975;

30. Graessler J, Qin Y, Zhong H, Zhang J, Licinio J, Wong ML, et al. Metagenomic sequencing of the human gut microbiome before and after bariatric surgery in obese patients with type 2 diabetes: Correlation with inflammatory and metabolic parameters. Pharmacogenomics J. 2013;

31. Dhakan DB, Maji A, Sharma AK, Saxena R, Pulikkan J, Grace T, et al. The unique composition of Indian gut microbiome, gene catalogue, and associated fecal metabolome deciphered using multi-omics approaches. Gigascience. 2019;