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Composition and Diversity Differences between Colon Microbiome of Colorectal Cancer Patients and Healthy Individuals by Age

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ABSTRACT

Colorectal cancer (CRC) is the third cause of cancer death globally. New evidence suggests that colorectal microbiome dysbiosis may be involved in the cause and development of CRC. This study aimed to investigate the differences in bacterial composition and diversity between CRC samples and healthy individuals (HC) based on age through high-throughput 16S rRNA sequences. Biopsy samples were obtained from 17 CRC patients and 13 healthy controls (HC). We analyzed the colon microbiome composition and diversity by alpha and beta diversity. The results showed that colon microbial diversity was significantly higher in the CRC-32-50 and CRC-50-75 groups than in the healthy controls. Still, on the other hand, the diversity of group HC-32-50 was lower than all other groups. *Prevotella*, *Faecalibacterium*, *Fusobacterium*, and *Akkermansia* were overrepresented in the CRC-32-50, while *Bacteroides* were in the HC-32-50 group. Our results showed that the diversity and composition of the two groups, HC-32-50 and CRC-32-50, were significantly different. These findings suggest that dysbiosis is more common in CRC patients under the age of 50 than in those over 50. Further studies on the colon microbiome are needed to determine the diversity and composition of the colon microbiome in age-related colorectal cancer to complete our understanding of the impact of the microbiome on the progression of colon cancer.

Introduction

Colorectal cancer (CRC) has been common cancer worldwide; CRC is the second leading cause of cancer death and the third most common cancer with 1.8 million new cases in 2018 (Bray et al. 2018, DeSantis et al. 2017). As a well-

known multifactorial disease, CRC may be due to individual genetic background, environmental factors (such as diet and medication), lifestyle, and a dynamic imbalance between intestinal microbiota. The gut microbiome has been considered an “invisible organ” of the human body, supporting the host to promote health or



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initiate disease. Host microbiome has been shown to play a key role in the onset and progression of cancer (Hakansson and Molin 2011, Gagnière et al. 2016, Zhou et al. 2016). Changes in the composition of the gut microbiome, called dysbiosis, can increase the risk of chronic inflammation and the production of carcinogenic metabolites, leading to intestinal neoplasia. Microbial composition and intestinal diversity are formed in the first years of life and are caused by contact with environmental factors such as age, sex, race, immune system, diet, use of antibiotics, and so on (Flint et al. 2012, Martin et al. 2014).

Studying the colon microbiome composition of CRC patients can open up new inspection methods for tumor screening. The advent of NGS methods in the last decade has led to metagenomic studies using methods such as 16S rRNA sequencing to identify the diversity and composition of microbes in different parts of the body without the need for microbial culture (Weinstock 2012, Goodrich et al. 2014).

Research on the intestinal microbiome in patients with CRC has been performed in the past; however, CRC-related bacteria have not yet been accurately identified, so further studies are needed to understand these bacteria better. Previous studies have evaluated differences in microbial composition and diversity between CRC and healthy controls. Still, rarely the microbial composition and diversity of patient CRC and HC groups based on age have been investigated.

Materials and Methods

Sample collection and DNA extraction

Biopsy samples were obtained from 17 CRC patients and 13 healthy controls (HC) in Imam Ali Research Hospital between June 2019 and January 2020. Participants in the study had not taken antibiotics or probiotics in the past two months and had no family history of colorectal cancer. Biopsy samples were then transferred into a refrigerator at -20°C for further analysis. According to the manufacturer's instructions, DNA from biopsy samples was extracted using NucleoSpin Microbial DNA Mini kit (MN, Germany). DNA stored at -20°C for subsequent analysis.

Amplification of 16S rRNA Gene

The V4 region of 16S rRNA genes in Microbial DNA was amplified by PCR using 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) primers (metabion, Germany). Amplification was performed in triplicate, meaning that each sample was amplified in three separate 25 μl reactions, and then the amplicons were mixed for each sample. PCR with 25 μl reactions was performed with the following conditions: initial denaturation of 94°C for 3 min; followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were first observed on 2% agarose gel electrophoresis and sent to Macrogen Company (South Korea) for 16S rRNA Illumina platform paired-end sequencing.

Sequencing data and diversity analysis

This study used the QIAGEN CLC Genomics Workbench (v.21.0.4) software to analyze 16S rRNA sequencing data. The reads were clustered into Operational Taxonomic Units (OTUs) with 97% sequence similarity (Edgar 2010). Classification of representative sequences for each OTU was carried out, and taxonomic data were then assigned to each representative sequence and showed the community composition of each sample at various classification levels of phylum, class, order, family, genus, and species. To estimate alpha and beta diversity, the phylogenetic tree was reconstructed using a Maximum Likelihood approach based on a Multiple Sequence Alignment (MSA) of the OTU sequences, including the 100 most abundant OTUs, generated by MUSCLE.

We evaluated alpha diversity based on the total number, Chao 1 bias-corrected, Chao 1, Shannon, and Simpson's indexes to examine differences in bacterial diversity between different groups. The Kruskal-Wallis H test was used to compare microbial abundance and diversity between different groups. Beta diversity analysis was evaluated by principal coordinate analysis (PCoA) on the UniFrac distances (Nardelli et al. 2020) to determine differences in species diversity between samples.

Statistical Analysis

We used the Kruskal-Wallis H test to compare microbial abundance and alpha diversity between different groups. PERMANOVA was used to measure the effect size and importance of beta variability. P values < 0.05 were considered statistically significant.

Results

Diversity in CRC and HC based on Age

We estimated alpha diversity based on phylogenetic diversity, total number, Chao 1, Shannon entropy, and Simpson's index between the age groups of 32 to 50 years and the group of 50 to 75 years, in both CRC patients and HC groups. According to our alpha diversity analysis, a significant difference was observed between most groups. The results indicated that colon microbial phylogenetic diversity was significantly higher in the CRC-32-50 and CRC-50-75 groups than in the healthy controls. Still, on the other hand, the diversity of group HC-32-50 was lower than all other groups (Fig. 1a). The results of the total number index in alpha diversity showed that CRC-32-50 group was significantly higher than HC-32-50 group ($p=0.002$) (Fig.1b), at the species ($p=0.0008$), genus ($p=0.0008$), family ($p=0.001$), order ($p=0.009$), and class ($p=0.005$) levels (Fig. 1c-g). The results indicated that the richness and diversity of group HC-32-50 were less than the other three groups. Beta diversity was calculated using the unweighted UniFrac method and the PCoA was carried out to display the microbiome space between the samples of different groups. The results showed a gently separated distribution of the colon microbial communities among HC-32-50 and HC-50-70 groups; it was also observed between HC-50-75 and CRC-50-75 groups. The PERMANOVA analysis supported that the colon microbiome significantly different between HC-32-50 and CRC-32-50 ($P=0.0001$), HC-50-75, and CRC-50-75 ($P=0.001$), HC-50-75 and CRC-32-50 ($P=0.001$), HC-32-50 and CRC-50-75 ($P=0.00004$) groups.

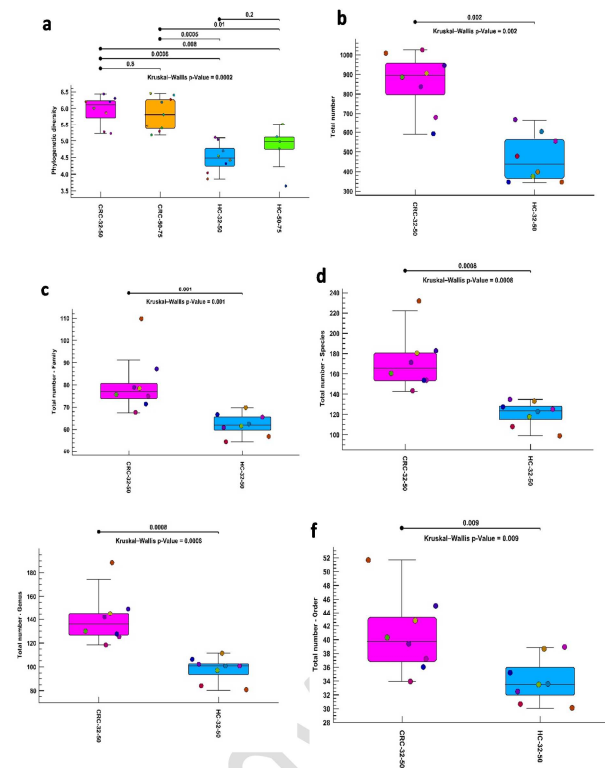


Fig. 1) Alpha diversity between CRC-32-50, CRC-50-75, HC-32-50, and HC-50-75 groups. a) phylogenetic diversity b) Total number index in alpha diversity showed that CRC-32-50 group was significantly higher than HC-32-50 group. c) Total number index in alpha diversity at the family ($p=0.001$), and d) genus ($p=0.0008$).

Microbiome composition in CRC and HC based on Age

In CRC patients and HC, the number of OUT was 2563 and 1839 OTUs, respectively. Proteobacteria and Actinobacteria in the phylum level showed a higher amount in HC-32-50 group (38% and 17%) than in CRC-32-50 group (2% and 1%) and other groups. Furthermore, *Bacteroidetes* and *Fusobacteria* in CRC-50-75 group (45% and 12%) were higher abundances than in HC-50-75 group (37% and 0.002%) and the other three groups at the phylum level (Fig. 2a). Comparing the abundance of bacteria between HC-32-50 and CRC-50-75 groups at the family level, we found that *Bacteroidaceae* (27%), *Enterobacteriaceae* (26%), and *Lachnospiraceae* (10%) in HC-32-50 are more than CRC-50-75 group (Fig. 2b). The abundance assessment of the CRC-32-50 versus HC-32-50

was the relative abundance of different genera as follows: *Bacteroides* (20% vers. 27%), *Prevotella* (14% vers. 0.005%), *Faecalibacterium* (4% vers. 3%), *Fusobacterium* (5% vers. 0.001%), and *Akkermansia* (2% vers. 0.000003%) (Fig. 2c). Figure 2d shows the relative abundance of different bacteria for 13 healthy samples and 17 patient samples based on age at the class level.

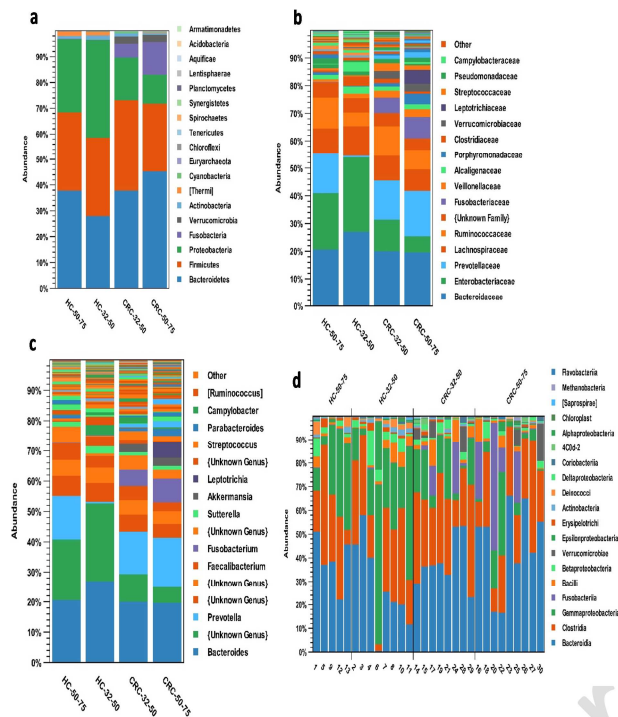


Fig. 2) Relative abundance of microbial composition in CRC patients and HC group based on age at three levels: a) Phylum b) Family c) Genus. d) Relative abundance of different bacteria for 13 healthy samples and 17 patient samples based on age at the class level.

Discussion

Studies are essential to understand the impact of geographical and cultural differences on the potential role of the colon microbiome on CRC (Allali et al. 2018). So far, no study has been performed on the population of Iran to determine the colon microbiome composition. The recent advent of high-throughput sequencing methods enabled assessing the characteristics of bacterial communities and their relationship with health and disease (Gioula et al. 2018).

In this study, we observed that the microbial composition and diversity in CRC and HC groups

varied according to age. Alpha diversity results indicated that the richness and diversity of the HC-32-50 group were less than the other three groups; in contrast, the CRC-32-50 were higher than the others. In other words, the diversity and composition of the two groups, HC-32-50 and CRC-32-50, were significantly different. Alpha diversity analysis indicated that the richness and diversity of group HC-32-50 were less than the other three groups. These results suggest that dysbiosis in CRC patients is more likely to occur in the age group of 32 to 50 years. According to the results, *Bacteroides*, *Prevotella*, *Fusobacterium*, and *Akkermansia* were overrepresented in the CRC-32-50 than HC-32-50 group at the genus level.

In contrast to a previous study (Wu et al. 2019), our results showed that the abundance of Fusobacteria phylum in the young group is lower than in the adult group, while Firmicutes in the young group was higher than in the elder group. According to our finding, the abundance of *Fusobacterium* was significantly higher in the CRC-32-50 than HC-32-50 group. The association between *Fusobacterium* and CRC progression has been well studied (Feng et al. 2015, Park et al. 2016, Tahara et al. 2014). *Fusobacterium nucleatum* can bind to E-cadherin on the surface of colon cells through the virulence factor FadA. This bacterium can produce an inflammatory and carcinogenic response by activating Wnt / B-catenin signaling (Gallimidi et al. 2015, Rubinstein et al. 2013). Overpresent of *Prevotella* in the colon is linked with elevated IL-17 (Sobhani et al. 2011) and IL-9 (Niccolai et al. 2020) producing cells in the mucosa of CRC patients. Studies have shown that *Akkermansia muciniphila* can enhance immunotherapeutic therapy based on programmed death 1 (PD-1) against CRC (Routy et al. 2018). However, this bacterium has a probiotic effect. Due to its enrichment in the colon of CRC patients, it is suggested that it be further studied as an anti-cancer probiotic.

This is the first study on CRC-associated colon microbiome in the Iranian population. One of the limitations of this study was the small number of samples; however, the advantage of this study was the 16S deep sequencing, which led to unique results on microbiome composition and diversity.

Conclusion

In conclusion, this study indicated that the diversity and composition of the two groups, HC-32-50 and CRC-32-50, were significantly different. These findings suggest that dysbiosis is more common in CRC patients under the age of 50 than in those over 50. Further studies are needed to confirm the findings of this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethical statement

Sample collections were conducted under approvals from the research ethics committee at the Zahedan University of Medical Sciences (IR.ZAUMS.REC.1398.051), Approval Date: 2019-04-29.

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