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Differential Abundance Analysis of Gut Microbiome between Healthy Controls and Colorectal Cancer Patients

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A B S T R A C T

Colorectal cancer (CRC) is a significant public health burden, accounting for approximately 10% of all new cancer cases worldwide, making it the world's third most deadly cancer. The causes of CRC are complex and environmental factors play a stronger role than genetic factors. The gut microbiome has been linked to several bowel cancers, such as CRC. The present study aimed to estimate differential abundance analysis of CRC versus HC groups of the colorectal microbiome. Biopsy samples were taken from the normal mucosa of 13 healthy controls (HC) and the tumor of 17 patients with CRC during colonoscopy. The microbiome of tumor tissue and normal mucosa was evaluated by 16S rRNA gene amplicon sequencing. Differential abundance analysis of CRC versus HC groups showed that *Enterobacteriaceae*, *Bacteroides fragilis*, *Prevotella*, *Fusobacterium*, *Leptotrichia*, *Akkermansia muciniphila*, *Streptococcus*, and *Parabacteroides* have drastic fold changes ($P \leq 0.05$). A heat map and dendrogram of the 20 ascending operational taxonomic units (OTUs) based on the FDR (False Discovery Rate) p-value were constructed to visualize the similarity between CRC and HC samples. The significant difference in the differential abundance of bacteria taxa in CRC versus HC groups indicates that these bacteria can be important pathogens in the development and progression of CRC.

Introduction

We know that the decoding of the human genome by the Human Genome Project is not enough to understand human biology because there are tremendous microorganisms living in and on the human body with a great diversity of genes that live in and on the human body during the life cycle, which can greatly affect health or diseases. Therefore, the decoding of the Human Microbiome Project, which is considered the "second human genome project", can effectively contribute to the scientific research community ("The integrative

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human microbiome project," 2019). Inside and on our bodies, there are trillions of microorganisms, including bacteria, viruses, fungi, and other life forms, collectively known as the microbiome. A microbiome is the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space. Different organs have distinct microbial inhabitants, but the group that has attracted the most attention in biomedical research is that in the gut (Brody, 2020).

Colorectal cancer (CRC) is a heterogeneous disease of the intestinal epithelium and is the third most common cause of cancer-related death worldwide (Chhikara et al,.2023). The causes of CRC are complex and diverse. Genetic factors have been shown to account for only 10-30% of CRC risk, and environmental factors play an important role in the development of CRC (Gagnière et al., 2016; Zhou et al., 2016). Gut microbiota plays an important role in the absorption of nutrients and minerals, synthesis of vitamins, amino acids, and enzymes, and production of short-chain fatty acids (SCFAs). Fermentation byproducts of SCFAs such as acetate, propionate, and butyrate are important for gut health and provide energy to epithelial cells, enhance epithelial barrier integrity, and provide immunity and protection against pathogens (Deleu et al., 2021; Siddiqui & Cresci, 2021). The gut microbiome is associated with several intestinal and extraintestinal disorders. Many studies have been conducted on the investigation of the gut microbiome and its relationship with some diseases, the gastrointestinal (GI) tract, such as celiac disease (Leonard et al., 2021), inflammatory bowel diseases (IBDs) (Tamboli, Neut, Desreumaux, & Colombel, 2004), irritable bowel syndrome (IBS)(Mars et al., 2020), colorectal cancer (CRC)(Abdi et al., 2022b; An et al., 2022; Coker, Wu, Wong, Sung, & Yu, 2020; Zhou et al., 2016), chronic diseases of the liver (Tilg, Cani, & Mayer, 2016)and chronic diseases of the pancreas (Adolph, Mayr, Grabherr, Schwärzler, & Tilg, 2019). Changes in the gut microbiome known as dysbiosis through inflammatory diseases, microbial metabolites, or virulence factors cause the development and progression of CRC (Sobhani et al., 2011). Pathogenic bacteria in the gut can cause host DNA damage by genotoxic substances. The activity of these bacteria can also promote CRC by affecting host signaling pathways, such as E-cadherin/β-catenin, TLR4/MYD88/NF-kB, and SMO/RAS/p38 MAPK. Some of these bacteria help tumor cells escape from the immune system and contribute to the progression of CRC by suppressing the immune system and creating a pro-inflammatory environment (Li et al,.2022).

Next-generation sequencing (NGS) technologies such as 16S rRNA sequencing provide useful data for characterizing the microbial composition of an ecosystem. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution), and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes. Determining the abundant microbes between two or more environments, known as "differential abundance analysis"(Lin & Peddada, 2020), is an important parameter to consider in microbiome analysis studies. Differential abundance analysis aims to find the differences in the abundance of individual taxonomic groups between two classes of subjects or samples (for example patient vs control groups), assigning a significance value to each comparison.

In the previous research (Abdi, Kordi-Tamandani, Lagzian, & Bakhshipour, 2021, 2022a; Abdi et al., 2022b), we reported the composition and diversity of the gut microbiome based on gender and age, showing that pathogenic bacteria were increased in patients with CRC. In this article, we estimate the differential abundance analysis of the colon microbiome in two groups of healthy controls and patients with CRC to show the significant difference in some microbial taxa in these two groups.

Materials and methods

Sampling and 16S rRNA amplification

Biopsy samples were collected from healthy controls (HC) and CRC patients referred to the colonoscopy department of Imam Ali Zahedan Research Hospital according to the protocol described in the previous study (Abdi et al., 2022b). DNA extraction from biopsy samples was

performed using the NucleoSpin Mini Microbial DNA Kit (MN, Germany), then the DNA was stored at -20°C.

Amplification of 16S rRNA gene was done using primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2012), which target the V4 region. The details of the PCR steps were explained in the previous study (Abdi et al., 2021). The PCR products were determined by the run on 2% agarose gel electrophoresis, then were purified (Expin PCR SV-mini, Gene All), and final products were sent to Macrogen Company (South Korea) for high-throughput sequencing on the 16S rRNA Illumina platform paired-end sequencing (Illumina sequencer, Macrogen).

Bioinformatics analysis and differential abundance analysis

Analysis of 16S rRNA sequence data was performed using QIAGEN CLC Genomics Workbench (v.21.0.4) software. Reads were filtered based on sequence size and then grouped into operational taxonomic units (OTUs) with 97% sequence similarity (Edgar, 2010). The resulting output contains an abundance table for sequences clustered with OTUs from the annotated reference database. To enhance the visualization of the OTU abundance table, it was decorated with metadata. This allowed multiple samples to be pooled based on specific characteristics.

To further assess the similarity between samples, we performed differential abundance analysis to find the OTUs with the highest differential abundance across all samples. For the estimate of differential abundance analysis, we chose the obtained OTU table and Across groups (ANOVA-like) as Comparisons. We then sorted the OTU table (which is a differential abundance analysis) in ascending order for the FDR (False Discovery Rate) P-value column. Next, we highlighted the number of 20 most different OTUs across all samples. Constructing a heatmap and dendrogram of these 20 OTUs, helped to evaluate the similarity between samples.

Results

Differential abundance analysis

Differential abundance analysis in our study revealed microbiological markers for CRC versus HC. Among the different universal taxa with distinguishable differential abundance between CRC and HC groups, eight taxa (The first row of Table 1) detected by 16S rRNA gene sequencing data were identified as important microbiological markers for CRC versus HC. *Enterobacteriaceae* family with 3 times lower differential abundance was observed in the CRC group than in the healthy group. *Bacteroides fragilis, Prevotella, Fusobacterium, Leptotrichia, Akkermansia muciniphila, Streptococcus*, and *Parabacteroides* were observed with a differential frequency several times higher in the CRC group than in the healthy group. These taxa of bacteria showed drastic fold changes in differential abundance analysis (Table 1).

Table 1: Differential Abundance Analysis of CRC versus HC group. CRC: Colorectal cancer, HC: Healthy control.

Differential Abundance Analysis		
CRC versus HC		
Taxa name	**Fold change**	**P-value**
Enterobacteriaceae	**-3**	**0.07**
Bacteroides fragilis	**10**	**0.001**
Prevotella	**65**	**7E-07**
Fusobacterium	**4015**	**8.36E-21**
Leptotrichia	**15772**	**1.50E-22**
Akkermansia muciniphila	**545**	**1.85E-14**
Streptococcus	**103**	**1.84E-09**
Parabacteroides	**3083**	**2.26E-17**

E means "times ten raised to the power of" (which would be written as "× 10n")

$P \leq 0.05$ is statistically significant difference.

Heat map for abundance OUT table

We constructed a heatmap and dendrogram of the 20 most abundant OTUs to assess similarities and dissimilarities between samples (Figure 1). Above the heat map, 30 samples of CRC and HC groups (with different numbers) are designed, the CRC group is distinguished by red color and the HC group by blue color. The CRC group on the left side and the HC group on the right side of the heat map are almost distinguished from each other.

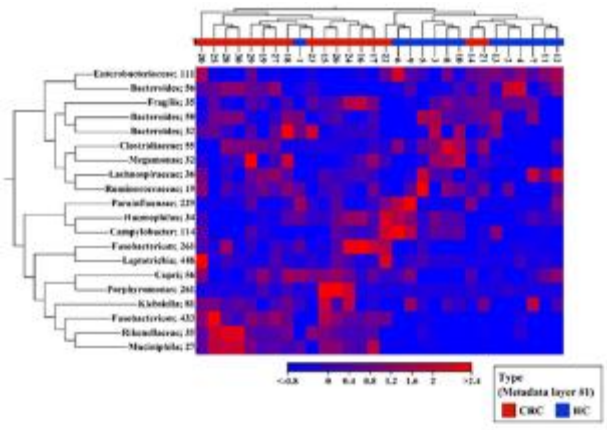

Figure 1: Heat map and dendrogram of the 20 most abundant OTUs. Blue indicates low abundance and red indicates high abundance. CRC: Colorectal cancer, HC: Healthy control.

The differential abundance of different bacteria is indicated by blue color (low abundance) to red (high abundance) in the heat map. Some bacteria are more abundant in the samples belonging to the CRC group and some in the HC group (red color). *Prevotella copri*, *Fusobacterium*, *Leptotrichia*, *Akkermansia muciniphila*, and *Kelebsiella* were more abundant in the CRC group than in the HC group.

Discussion

The composition of intestinal microbiome is influenced by environmental and genetic factors. Studies have shown that approximately 80-90% of CRC occurs sporadically (Hope, Hold, Kain, & El-Omar, 2005; Yamagishi, Kuroda, Imai, & Hiraishi, 2016). The main determinants of microbiota composition are lifestyle, geography, race, diet, drug use, age, gender, and host genotype, and dysbiosis in the gut bacterial composition is associated with microbiota-induced cancer risk (Goodrich et al., 2014; Nakatsu et al., 2015). We aimed to study the differential abundance analysis of the colon microbiome in two groups of HC and patients with CRC to show the significant difference in some microbial taxa in these two groups.

Our study showed that members of the *Enterobacteriaceae* family with a differential frequency of 3 times less in the CRC group compared to the healthy group. The majority of *Enterobacteriaceae* in the gut is considered commensals, as they perform beneficial for the host, although some genera of this family are also pathogenic (Kang et al., 2018). The results of this study showed that *Bacteroides fragilis*, *Prevotella*, *Fusobacterium*, *Leptotrichia*, *Akkermansia muciniphila*, *Streptococcus*, and *Parabacteroides* were observed with a differential frequency several times higher in the CRC group. Previous studies showed a significant correlation between the presence of enterotoxigenic *Bacteroides fragilis* (ETBF) in the stool or colonic biopsy specimens and CRC (Ulger Toprak et al., 2006; Wu, Morin, Maouyo, & Sears, 2003; Wu et al., 2006). *B. fragilis* toxin (BFT) which is a zinc-dependent metalloprotease toxin, cleaves the extracellular domain of cell surface protein E-cadherin and results in the complete degradation of the E-cadherin. The cytoplasmic domain of E-cadherin is associated with the nuclear signaling protein β-catenin that induces c-myc expression and IL-8 secretion (Wu et al., 2003). Long-term colonization of colonic epithelial cells with *B. fragilis* can increase the risk of CRC due to oxidative DNA damage, epithelial barrier damage and activation of STAT3/Th17 immune responses(Purcell et al., 2017; Yu, Wei, & Ni, 2018).

A marked higher abundance of *Prevotella* has been similarly reported in Iranian individuals in a previous study (Sarhadi et al., 2020). Increased abundance of *Prevotella* bacteria in the colon is associated with IL-17 and IL-9 producing cells in the mucosa of CRC patients (Niccolai et al., 2020; Sobhani et al., 2011). Previous research has shown that *Prevotella* was associated with high expression levels of CXCL1 (growth-related oncogene). On the other hand, it has been shown that the expression of CXCL1 increases the survival of cancer cells and promotes angiogenesis in CRC (D. Wang et al., 2006). Much evidence has shown that *Fusobacterium nucleatum* affects different stages of CRC progression. *F. nucleatum* promotes the initiation and development of CRC through adherence to epithelial cells by the unique protein FadA (Xu et al., 2007). The FadA factor binds to vascular endothelial cadherin and alters endothelial integrity and causes CRC development (Fardini et al., 2011). The second virulence factor of *F. nucleatum* is an autotransporter protein called Fap2, which can promote CRC progression by

inhibiting the activity of immune cells (Gur et al., 2015). This bacterium increases the expression of several inflammatory genes such as NF-κB and cytokines, including IL-6, IL-8, and IL-18. F. nucleatum also increases the release of inflammatory cytokines, especially IL-8, IL-10, and TNF-α in the pro-inflammatory environment that accelerates colorectal tumor progression (Quah, Bergenholtz, & Tan, 2014).

Some bacteria are more compatible with the intestinal tumor environment and may increase in abundance to inhibit CRC (Tjalsma, Boleij, Marchesi, & Dutilh, 2012), which indicates that these bacteria are a suitable option for therapeutic purposes against CRC (Chia et al., 2018). *Akkermansia muciniphila* can support the microbial flora in the mucosal environment by increasing the availability of mucin sugars. Although mucin degradation is a pathogenic behavior, *A. muciniphila* does not show any pathogenicity, it only resides in the outer mucous layer and does not reach the inner mucous layer. By producing SCFA, this bacterium provides the necessary energy for colon epithelial cells to produce more mucin, which causes the mucin to become thicker. Increasing the thickness of mucin provides more protection against the entry of external factors from the intestinal environment into the internal environment of the body, in other words, it increases the permeability of the intestinal barrier (Gu et al., 2021). Interestingly, in 2022, the opposite role of *A. muciniphila* in the development and spread of cancer was reported in a mouse model, which, contrary to previous studies, indicates that this bacterium is pathogenic (F. Wang et al., 2022). It has been reported that mice receiving *A. muciniphila* had more severe weight loss, shorter colon length, higher inflammatory cytokine gene expression and more intestinal tumors than control mice. The increase in the differential frequency of this bacterium in CRC compared to HC probably indicates the effectiveness of this bacterium on CRC. More studies are needed to resolve this uncertainty.

Conclusion

Differential abundance analysis showed that some taxa of bacteria have a drastic fold change in patients with CRC compared to HC. The differential abundance of bacteria in CRC versus HC groups was significant, indicating that these bacteria can be important pathogens in the development and progression of CRC.

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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