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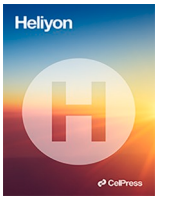

Research article

Endoscopic brush sampling identifies mucosa associated microbiota in colorectal adenomas

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ABSTRACT

The intestinal microbiome plays a crucial role in colorectal adenomas and the mucosa associated microbiota are thought to play a more critical role in interactions with the host immune system. Current omics approaches, offer a holistic assessment of the gut microbiome and the human host interaction. To enhance the value of data from these sequencing methods, appropriate sample collection is vital. We evaluated the potential use of endoscopic brush samples for mucosal microbiota analysis in colorectal adenomas and compared it with direct adenoma tissue sequencing in terms of microbial gene sequencing. The results showed a significant increase in microbial diversity in samples collected by the endoscopic brush, which did not interfere with pathological biopsy. This study found that utilizing endoscopic brush sampling for the microbiome analysis of colorectal adenomas offers several advantages over the direct examination of microbiomes within tumor tissues, including the capacity to accurately collect gut microbiome from different locations in the intestine, circumventing interference from tissue genes, providing more abundant microbial data and enabling inclusion of small adenomas without disrupting pathological biopsies.

1. Introduction

The human gut microbiota comprises a diverse community of microorganisms residing in the gastrointestinal tract, including bacteria, fungi, archaea, and certain viruses. This microbial community plays a crucial role in gut physiology, particularly in energy metabolism and immune response. Disruptions in their relative abundance can perturb the gut's homeostatic balance and are associated with a spectrum of inflammatory, metabolic, and neoplastic diseases [1,2]. Colorectal cancer (CRC), a multifactorial disease, is influenced more by environmental factors than genetic predispositions. The gut microbiota has emerged as a significant factor in CRC development, with dysbiosis potentially increasing the risk of CRC [3–5]. CRC patients exhibit altered gut microbiota compositions, suggesting its potential as a therapeutic target [6,7]. The tumor microenvironment's microbial community has garnered increasing research interest as an environmental factor in disease pathogenesis. High-throughput genomics and 16S rRNA gene sequencing have facilitated a more profound comprehension of the human gut microbiome's role in disease. Representative sample collection is crucial for omics techniques, necessitating a method that is straightforward, efficient, cost-effective, and yields high-quality data [8]. While fecal samples dominate microbiota research, they may not accurately reflect intra-individual variation in microbial composition. The mucus layer of the gut is the primary interface between the host and its microbiota, underscoring the importance of studying the mucus of adenoma tissue to understand its relationship with the gut microbiota [9,10].

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The gut's microbial community composition varies by anatomical site, emphasizing the importance of considering sample collection methods in microbiota studies [11,12]. A recent paper discussed the pros and cons of stool sampling, tissue biopsy, and endoscopic brushing, highlighting endoscopic brushing as superior to mucosal biopsy [8]. However, stool sampling, the standard method, does not accurately reflect the microbes in contact with tumors due to the presence of luminal bacteria, mucus layer secretions, and host genetic material that can confound subsequent genomic and transcriptomic analyses [13–15]. Mucosal tissue biopsy is constrained by cost, invasiveness, and patient discomfort. To address these issues, we advocate for endoscopic cell brush sampling as an innovative approach to studying the interactions between colorectal tumor tissue and its surface microbiota. This non-invasive method allows for the precise collection of intestinal microbes, enabling a direct investigation of microorganisms interacting with intestinal epithelial tissue without the interference of host DNA. Furthermore, by comparing mucosal samples from adenomatous tissue surfaces to those from normal colon tissue surfaces in adenoma patients, we can discern the microbial community disparities between the two tissue types.

2. Methods

2.1. Sample collection

Mucus of adenoma: Individuals who presented for colonoscopy with indications of screening for, or a prior history of, colorectal polyps were asked to participate in the study. Exclusion criteria included IBD and IBS and the use of antibiotics 1 month prior to surgery. Written and informed consent was obtained from each subject and was required for participation. In total, with reference to similar studies [8,16], 7 participants with no history of colon adenomas were recruited for this study (Clinical information can be found in Supplementary Table 1, STROBE flow diagram can be found in Supplementary Fig. 1). Before a colonoscopy, subjects were asked to adhere to a clear liquid diet for 24 h. Bowel cleansing was done using polyethylene glycol with electrolytes administered as a split dose, 12 and 5 h before the procedure. Patients prepared for colonoscopy by standard protocol the night prior. Following conscious sedation per routine care, one cytology brush (Micro-tech (nanjing), MTN-XB-40) reaches the colon through colonoscopy, pressed against the adenoma tissu, and rotated two full turns. The brush was then placed into 0.5 mL of RNAlater (Thermo Fisher) in a 1.5 mL Eppendorf tube and immediately were stored at −80 °C immediately. The study was approved by the Cancer Hospital Affiliated to Chongqing University Ethics Committee under the study number CZLS2023010-A.

Adenoma group: Biopsy samples from 7 individuals with adenoma were obtained in enteroscopy process. Mostly, adenoma tissue was collected from individuals with adenoma larger than 0.5 cm because the small adenoma sample was reserved for examination by a pathologist. Mucus group: Before adenoma patients undergo adenoma resection surgery, mucosal samples from the surface of the adenomas were collected using a endoscopic brush. Normal group: the Normal group refers to intestinal mucus from normal intestinal tissue of the patient.

2.2. 16S rRNA gene sequencing

Purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina NovaSeq PE250 platform (Illumina, San Diego,USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). After the sequencing, all reads were sorted, screened, and filtered to ensure quality and length. After demultiplexing, the resulting sequences were quality filtered with fastp (0.19.6) and merged with FLASH (v1.2.11) [17]. Then the high-quality sequences were de-noised using DADA2 [4,5] plugin in the Qiime2 [6] (version 2020.2) pipeline with recommended parameters, which obtains single nucleotide resolution based on error profiles within samples. Then the high-quality sequences were de-noised using DADA2 plugin in the Qiime2 (version 2020.2) pipeline with recommended parameters, which obtains single nucleotide resolution based on error profiles within samples. Based on default parameters, use the DADA2 plugin in the Qiime2 workflow to denoise the optimized sequences after quality control and joining. The sequences processed through denoising by DADA2 are typically referred to as ASVs (Amplified Sequence Variants). Exclude all sequences annotated as chloroplast and mitochondrial sequences in all samples. To minimize the impact of sequencing depth on subsequent Alpha diversity and Beta diversity data analysis, rarefy all sample sequences to 20,000. Even after rarefaction, the average sequence coverage (Good's coverage) of each sample remains at 99.09 %. Utilize the Naive bayes classifier in Qiime2 based on the SILVA 16S rRNA gene database (v138) for taxonomic classification analysis of the ASVs.

2.3. Quantification of human and microbial DNA proportion

DNA was extracted from microbial samples using the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's protocol. The concentration and quality of extracted DNA were assessed using a Qubit 4 Fluorometer (Thermo Fisher Scientific) and Nanodrop Spectrophotometer (Thermo Fisher Scientific). Quantification of specific microbial targets was performed using quantitative real-time PCR (qPCR) on a Bio-Rad CFX Connect Real-Time PCR Detection System. Design primers based on quantitative research on microorganisms [18]. Each qPCR reaction was carried out in triplicate using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The bacterial load was determined by qPCR targeting the 16S rRNA gene. Comparing cycle threshold values of each sample to a standard quantification curve resulted in the total number of 16S rRNA gene copies.

2.4. Data analysis

In this study, analysis of all omics data was performed using a free online platform of Majorbio Cloud Platform, https://cloud.majorbio.com (Shanghai Majorbio Bio-pharm Technology Co., Ltd). The alpha diversity was determined by the Wilcoxon rank-sum test between the groups. And the beta diversity (PCoA graphs) was based on the ASV table and bray-curtis distance algorithm to analyze the structural changes of the microbial community at the genus level. Statistical comparisons of taxa abundances at the genus levels for each group were performed and displayed as Veen and Bar plots. To discriminate significant differences relative to the normal group, one-way analysis of variance (ANOVA) was then performed followed by the Duncan test using SPSS 22.0 software. $p < 0.05$ was considered to be a significant difference between treatments and the normal group.

3. Results

Endoscopic brush sampling provides improved bacterial DNA recovery and microbial diversity.

Consistent with prior investigations, our data indicate a decrease in the abundance of intestinal microbiome in adenoma patients. Endoscopic brush sampling demonstrates superior alpha diversity, suggesting that this methodology may more effectively preserve microbial data and facilitate a deeper understanding of the interactions between microbial communities and their host (Fig. 1A–D). Our results support the assertion that endoscopic brush sampling yields a more comprehensive collection of bacterial DNA compared to biopsy sampling, a distinction that may be crucial for studies aimed at bacterial taxa identification.

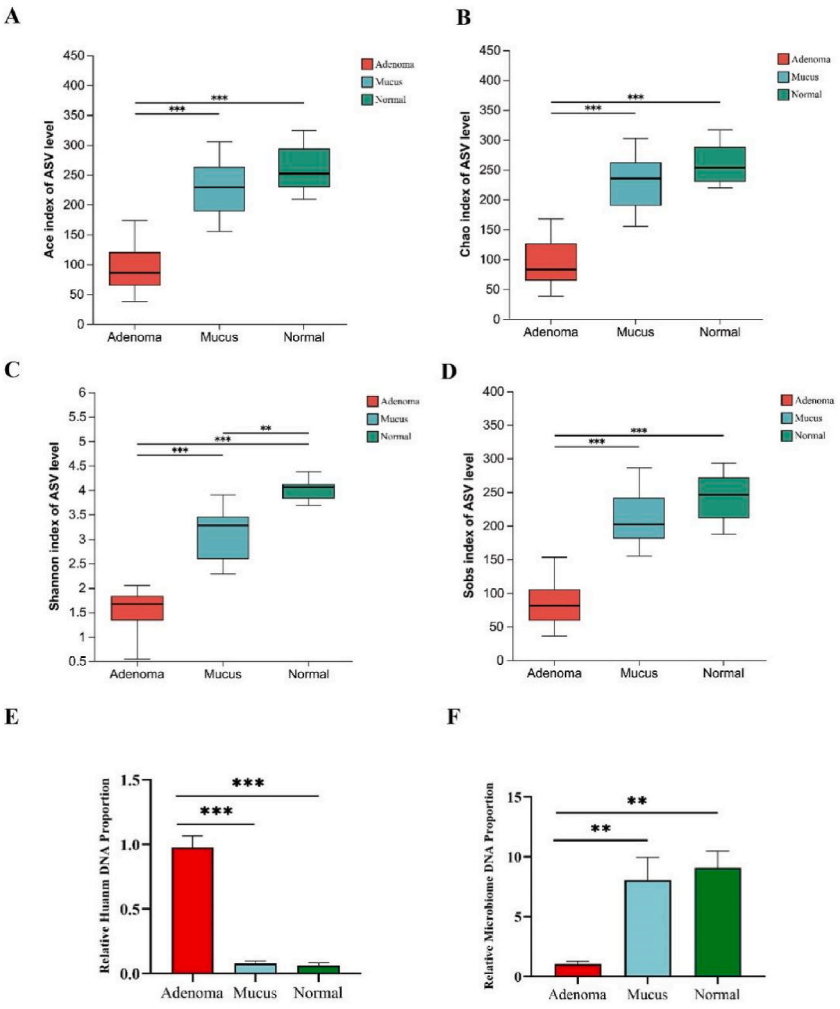

Fig. 1. Microbial differences between mucus, adenoma samples and normal intestinal mucus. (A–D) Bacterial communities alpha diversity between endoscopic brush sampling and adenoma samples. (E, F) The proportion of microorganisms and human genes between different groups.

3.1. Endoscopic brush sampling provides more microbial information than adenoma samples

Endoscopic brush sampling provides more sequencing results of microbial date than adenoma tissue (Fig. 2A). Principal-coordinate analysis (PCoA) revealed pronounced separation between the three sampling methods with good intragroup correlation and reveals that the gut microbiota of distinct adenoma patients exhibit greater dispersion along the coordinate axis in comparison to the control cohort (Fig. 2B). The bar map visualizes the differences in microorganisms between the three sampling methods, with direct collection of adenoma tissue for sequencing significantly affecting the detection of less abundant microorganisms (Fig. 2C).

We performed a statistical analysis of the top 10 microorganisms in terms of abundance. Our results clearly demonstrate that the percentage of microorganisms with lower abundance in cell brush sampling is significantly higher than in direct sequencing of adenoma tissue. This indicates that the low-abundance microbes may be overlooked if adenoma tissue is sequenced directly. Meanwhile, our study found that patients with adenomas had significantly increased levels of *Escherichia Shigella* and *Acinetobacter* compared to the normal group (Fig. 2D). Subsequently, we conducted a comparative analysis of the intestinal mucus and fecal microbiota, revealing significant differences in alpha diversity and PCoA analysis (Supplementary Fig. 2). These findings underscore the research significance of investigating the gut microbiota that directly interfaces with tissues.

3.2. Endoscopic brush sampling does not interfere with patient rights

The collection of mucus from adenoma tissue using an endoscopic brush is a non-invasive procedure for the patient and does not interfere with the colonoscopy. Furthermore, the endoscopic brush does not cause any damage to the intestinal lumen after the collection is completed (Fig. 3A). We counted 100 cases in which adenoma tissue could be sequenced. However, some smaller adenoma tissues could not be directly sequenced to ensure that pathology biopsies were not compromised. Endoscopic brush sampling does not necessitate the use of adenoma tissue for study, does not interfere with pathology, and can be performed on all adenoma patients. In

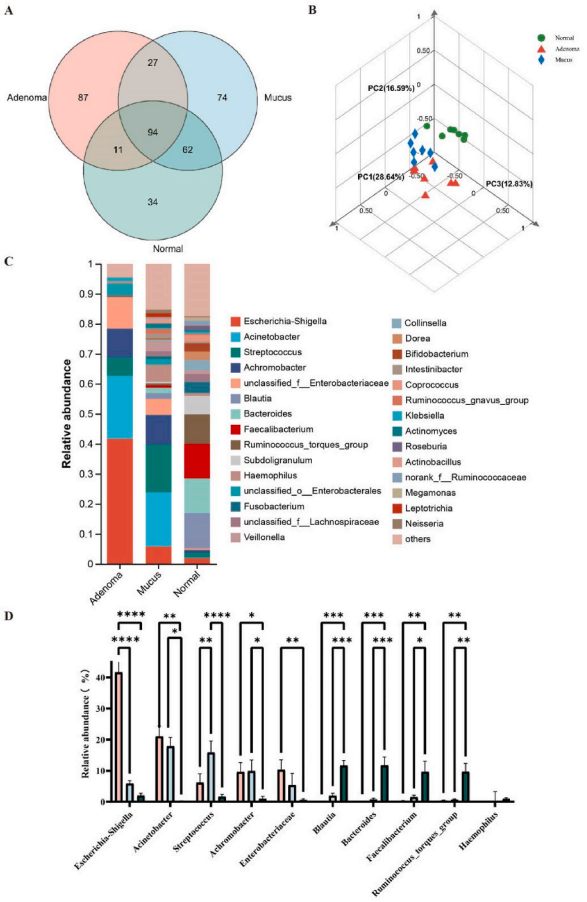

Fig. 2. Community composition analysis between mucus samples, adenoma samples and normal intestinal mucus samples. (A) Venn map analysis of mucus sampling, adenoma samples and normal intestinal mucus in genus level. (B) PCOA analysis of mucus, adenoma samples and normal intestinal mucus. (C) The top 20 identified bacteria based on *t*-test demonstrated as a bar map in genus level. (D)Comparison of mucus sampling and adenoma with normal intestinal mucus microbiota according to relative abundance.

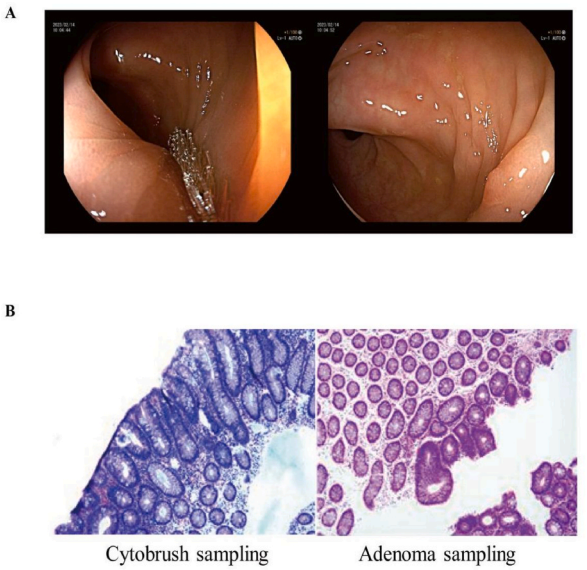

Fig. 3. The impact of endoscopic brush on patient rights. (A)Photos of mucus sampling at adenoma and after sampling. (B) Adenoma tissue sampling affects the picture of pathology detection.

contrast, adenoma tissue sequencing cannot be studied for small size adenomas in order to ensure that it does not interfere with pathological examination. Due to the partial removal of adenoma tissue for intestinal flora sequencing, the integrity of the remaining tissue was compromised, precluding comprehensive pathological examination of the entire adenoma. (Fig. 3B).

4. Discussion

Fecal samples have been the chosen source for most bacterial flora studies due to their convenient and non-invasive nature. However, even the content of microbiota in the lower digestive tract, which is closest to fecal samples, differs significantly from those found in feces. Recent studies have suggested a role for gut microbiota in the pathogenesis of colorectal tumors, with decreased abundance of probiotic bacteria and increased abundance of Bacteroides observed in stool samples and adenoma tissue from patients with colorectal tumors [15–17]. The role of mucosa-associated microbiota in colorectal tumorigenesis remains unclear, as current studies may not accurately reflect the microbiota that directly interact with the tissue. Furthermore, defining intestinal microbial changes in early-stage, small-volume adenomas is challenging due to the prioritization of pathological examinations.

In this study, we used an endoscopic brush to collect intestinal mucus, thereby avoiding subject injury associated with intestinal mucosal biopsy. Previous studies relied on intestinal mucosal biopsy to collect samples. However, this technique is invasive and increases the risk of bleeding. There is a potential for an increase in postoperative patient discomfort [19,20]. Moreover, both intestinal mucosal biopsies and tumor tissue biopsies, which are composed of human tissue cells, contain only a limited number of microorganisms, which can easily interfere with histological testing. Endoscopic brush sampling of intestinal mucus is virtually non-invasive and independent of adenoma tissue size and avoids interference from human cells. This sampling method facilitates genomic studies, and, as it does not require tumor tissue, allows for the entirety of the tumor tissue to be used for pathological biopsy, which is beneficial for determining the pathological nature of the tumor and whether it is adequately excised or not. Moreover, comparative analyses of mucosal samples from adenomatous tissue surfaces and normal colon tissue in adenoma patients have revealed significant discrepancies between the microbiota of adenomatous and corresponding normal intestinal tissues. The meticulous collection of gut microbiome from various anatomical sites using endoscopic brush sampling presents a novel approach to exploring the interactions between gut microbiome and their host. This method offers the advantage of comparing microorganisms at the tumor site with those in the patient's normal intestinal mucus, providing a opportunity to investigate the role of gut microbiome in colorectal tumorigenesis and potentially benefiting patients.

In aggregate, our findings indicate that endoscopic brush sampling is an appropriate method for assessing mucosal microbiota. The alpha diversity was significantly higher in the endoscopic brush group relative to the biopsy group. Additionally, sequencing of the brush samples disclosed a richer bacterial nucleic acid content and a more extensive diversity within the microbiome compared to the biopsy samples. These observations are likely attributable to a higher bacterial concentration at the time of sequencing, as well as a reduced presence of human genomic contaminants due to the exclusion of interfering human tissue genes. Consequently, endoscopic brush sampling emerges as a more informative and less invasive technique for microbiome analysis, with the added advantage of enhanced patient comfort. However, it is imperative to acknowledge a limitation inherent in our study. Although we took into account sample sizes from similar studies [8], the number of participants in this study is relatively modest, which may not fully account for the influence of confounding variables.

5. Conclusion

Our study aimed to identify alternative sampling methods for intestinal biopsy in tumor microecology studies. We found that endoscopic brush sampling can be a viable alternative to tumor tissue sampling and offers improved clinical benefits to patients. Our findings underscore the importance of sampling methods in tumor microecology studies. In conclusion, we demonstrated the advantages of endoscopic brush sampling in tumor microecology studies, facilitating research on the interaction between gut microbes and intestinal tissues. Additionally, endoscopic brush sampling provides a more cost-effective and convenient method for future studies of gut microbes and tumor pathogenesis.

Ethics approval and consent to participate

Patient sample and data were collected under approval by the Cancer Hospital Affiliated to Chongqing University Ethics Committee under the study number CZLS2023010-A. Patients provided written informed consent upon enrollment.

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Data availability statement

The raw sequencing reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP482816).

CRediT authorship contribution statement

Juncheng Liu: Writing – original draft, Visualization, Methodology, Formal analysis, Conceptualization. **Kexu Xiang:** Writing – review & editing, Investigation. **Huan He:** Data curation. **Weiqing Chen:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38901.

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