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Tongue images and tongue coating microbiome in patients with colorectal cancer

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ABSTRACT

Background: Tongue diagnosis, as a unique method of traditional Chinese medicine (TCM), discriminates physiological functions and pathological conditions by observing the changes of the tongue coating. **Aims**: To evaluate the differences of tongue images and tongue coating microbiome between patients with colorectal cancer and healthy people. **Methods**: The tongue diagnostic information acquisition system was used to photograph the tongue images and analyze the thickness of the tongue coatings in patients with colorectal cancer and healthy people. The next-generation sequencing technology was used to determine the V2–V4 hypervariable region of 16S rDNA to investigate the microbial community structure and diversity on the tongue coating. **Results**: The tongue coatings in patients with colorectal cancer were obvious thickening compared with tongue images in healthy people. The microbial community structure on the tongue coating was different between patients with colorectal cancer and healthy people. **Conclusion**: Tongue diagnosis may provide important leads towards novel microbiome-related diagnostic tools and tongue coating microbiome may be a novel biomarker for characterizing patient with colorectal cancer.

1. Introduction

The traditional Chinese medicine (TCM) is a complete set of the theoretical system is completely different from modern medicine. The TCM based on the theory of Yin and Yang (the two opposing principles in nature) and the five elements (wood, fire, soil, gold, water). The TCM explore the cause, nature, position and pathogenesis of the disease by the four methods of diagnosis (Observation diagnosis, auscultation, interrogation and feeling the pulse). The approach of curing the disease is that making a balance of Yin and Yang in the patients body through a variety of treatments such as drugs, acupuncture, moxibustion, massage, Cupping therapy, manipulation, breathing technique therapy, dietetic therapy and so on. Tongue diagnosis, a unique method to assess the health by investigation of tongue images and tongue coating, is one of the important element in the observation diagnosis [1].

Tongue diagnosis was used to reflect physiological and clinicopathological changes of inner parts of the body by clinical practitioners of TCM for at least 3000 years [2]. However, Tongue diagnosis was judged mainly through experienced doctor of traditional Chinese medicine using their naked eye in the past. Due to the influence of the factors such as doctor's condition, patients' position, the light and so on, its objectivity and reproducibility had been questioned [3]. The tongue diagnostic information acquisition system is a computer aided tongue diagnosis system. The system is constituted by two main components: Tongue Image Acquisition Module is used to capture qualified tongue images; Image Processing Module is utilized to analyze the thickness, color, fissures, spots and other characteristics of tongue coating. In order to overcome this defect, the tongue images were analyzed by the tongue diagnostic information acquisition system in the present research, this method had been proved to be effective and steady in experiments on tongue image [2].

Colorectal cancer is one of the commonest causes of death among all types of cancers [4]. Both environmental and genetic factors contribute to colorectal cancer. The mechanisms associated with colorectal cancer causation and prevention are largely unknown. Many of the proposed mechanisms implicate the metabolic activities of the bacterial biota normally resident in the intestinal tract [5]. Numerous evidences supports that *Streptococcus bovis* [6–8], *Helicobacter pylori* [9], *Escherichia coli* [10] and *Clostridium septicum* [11] are closely related to the colorectal cancer. Also, certain mucosa-associated bacterial species play an important role

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in the pathogenesis of colorectal cancer [5]. However, Most of the researches were aimed at enterobacteria, Research on the relationship between tongue coating microbiome and colorectal cancer are still poorly understood and lack systematic investigation.

The next-generation sequencing (NGS) technology was emerging as the optimal method of choice for detecting and identifying microbiome [12,13]. The NGS had greatly increased sequencing throughput via the use of massively parallel sequencing [14]. It can analyze a large amount of sequences, enabling sequencing of 16S rRNA to identify complex bacteria species of pathogens and probiotic bacteria [15]. It had recently emerged as a powerful tool to examine bacterial communities in a variety of matrices, including small intestine [16], saliva [17], cervix [18], oral cavity [19], feces [20], waters [21] and soils [22]. Also, this technology was used to detect the tongue coating bacteria species [1].

In the present study, we try to explore the difference of the thickness of the coating tongue between the colorectal cancer patients and the healthy people. Furthermore, we characterized the tongue coating microbiome to evaluate the differences of tongue coating microbiome in patients with colorectal cancer and that in healthy people using the NGS technology.

2. Materials and methods

2.1. Patients and healthy controls

From September 2013 to April 2014, 47 patients with Colorectal Cancer confirmed by pathology (22 with rectal cancer and 25 with colon cancer) were recruited from the Cancer Center of First Affiliated Hospital of Wannan Medical College, and 45 healthy volunteers were recruited as the normal control group. The healthy volunteers have no gastrointestinal diseases, no oral disease, no malignant tumor and cancer related symptoms in the last two years. The characteristics of study participants were investigated. All patients and healthy volunteers signed informed consent under the guideline approved by the Medical Ethical Committee of Wannan Medical College.

2.2. Tongue images analysis

All tongue coatings of patients and healthy volunteers were photographed in the morning prior to breakfast. All participants were required to rinse their mouth before photographing. The tongue coatings of all participants were photographed and the thickness of the tongue coatings was analyzed by the DS01-B tongue diagnostic information acquisition system (DAOSH Co., Shanghai, China). The tongue diagnostic information acquisition system was proved to be effective in the past [23].

2.3. Tongue coating samples collection

All tongue coatings of patients and healthy volunteers were sampled in the morning prior to breakfast. All participants were required to rinse their mouth before sampling. Thirty one of the forty five patients with Colorectal Cancer and forty of the forty seven healthy volunteers were withdrawn from the study due to non-standard operations. Resulting in a final study group of 7 controls and 14 study patients.

2.4. MiSeq sequencing methods

2.4.1. RNA extraction and PCR

Total RNA from each the tongue coating samples were extracted using a TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's recommended protocol. The quality and quantity of the purified RNA were determined by measuring the absorbances at 260 nm and 280 nm (A260/A280) using a Nanodrop ND-1000 spectrophotometer (LabTech, Washington, DC,USA). RNA integrity was further verified by electrophoresis through a 1.0% (w/v) agarose gel. Isolated DNA was used as a template for the amplification of the V2–V4 region of 16S rRNA. DNA samples were amplified in 20 µl PrimerStar HS Premix (AP221-02; TransGen, Beijing, China) that contained 4 µl FastPfu Buffer, 2 µl dNTPs, 0.4 µl Fastfu Polymerase, 10 ng template DNA and a final primer concentration of 5 µM. PCR amplification for extracting V2–V4 regions, the following parameters were used: 95 °C for 2 min; then 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and, finally, 72 °C for 10 min. The sequences of the primers used were as follows: V2–V4 (338F–806R): 338F: ACTCCTACGGGAGGCAGCA; 806R: GGACTACHVGGGTWTCTAAT. All quantitative PCRs for each gene used three biological replicates, with three technical replicates per experiment.

2.4.2. cDNA library construction and sequencing

The tongue coating microbiome in patients with colorectal cancer and that in healthy people was detected by the next-generation sequencing methods. PCR products are ligated with Y adapter. Magnetic nanoparticles are applied to eliminate self-ligated Y adapters. PCR amplification is carried out for library enrichment, and single-stranded DNA sequences are generated after being treated by NaOH solution. After purification and quantification, a mixture of amplicons was used for sequencing on the MiSeq platform at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China) according to standard protocols [24].

2.4.3. Sequencing data analysis

The software Trimmomatic was used to acquire high-quality, non-chimeric V2–V4 tags from raw paired-end reads. The reads with more than 10% of bases with a quality score of $Q < 20$, non-coding RNA (such as rRNA, tRNA and miRNA), ambiguous sequences represented as “N” and adapter contamination were removed. UCHIME [25] (version 4.2.40 http://drive5.com/usearch/manual/uchime_algo.html) was used to detect chimeras from high-quality V2–V4 tags. Classification of operational taxonomic units (OTUs) at 97% identity using furthest neighbor clustering was done using the Uparse [26]. (version 7.1 http://drive5.com/uparse/) Calculations for Shannon, nonparametric Shannon, and Simpson diversity indices were performed using the software Usearch (version 7.1 http://qiime.org/).

Table 1
Characteristics of study participants.

	Controls ($n = 47$)	Patients ($n = 45$)	*P* value
Males, *n*	25	28	NA
Age, years	51.57 ± 8.01	53.24 ± 9.70	0.369
BMI, kg/m^2	25.11 ± 2.80	26.00 ± 3.42	0.178
Current smoker, *n*	18	22	0.306
Known diabetes, *n*	4	9	0.114
Known hypertension, *n*	1	6	0.102
Surgical treatment, *n*	0	44	NA
Chemotherapy, *n*	0	38	NA
Radiotherapy, *n*	0	0	NA
Thickness of the tongue coating	130.60 ± 96.53	394.7 ± 243.35	<0.01

NA: not applicable due to selection criteria.
The characteristic of tongue images of healthy people and patients with colorectal cancer was described. The tongue diagnostic information acquisition system was used to describe thickness of the tongue coating objectively. There was a significant statistical difference between healthy people and patients with colorectal cancer on the thickness of tongue coating ($P < 0.01$).