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Original Article

Fecal Microbiota Characteristics of Patients with Colorectal Adenoma Detected by Screening: A Population-based Study

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ABSTRACT

Background: Screening for colorectal cancer (CRC) and precancerous colorectal adenoma (CRA) can detect curable disease. However, participation in colonoscopy and sensitivity of fecal heme for CRA are low.
Methods: Microbiota metrics were determined by Illumina sequencing of 16S rRNA genes amplified from DNA extracted from feces self-collected in RNAlater. Among fecal immunochemical test-positive (FIT+) participants, colonoscopically-defined normal versus CRA patients were compared by regression, permutation, and random forest plus leave-one-out methods.
Findings: Of 95 FIT+ participants, 61 had successful fecal microbiota profiling and colonoscopy, identifying 24 completely normal patients, 20 CRA patients, 2 CRC patients, and 15 with other conditions. Phylum-level fecal community composition differed significantly between CRA and normal patients (permutation $P = 0.02$). Rank phylum-level abundance distinguished CRA from normal patients (area under the curve = 0.767, permutation $P = 0.006$). CRA prevalence was 59% in phylum-level cluster B versus 20% in cluster A (exact $P = 0.01$). Most of the difference reflected 3-fold higher median relative abundance of Proteobacteria taxa (Wilcoxon signed-rank $P = 0.03$, positive predictive value = 67%). Antibiotic exposure and other potential confounders did not affect the associations.
Interpretation: If confirmed in larger, more diverse populations, fecal microbiota analysis might be employed to improve screening for CRA and ultimately to reduce mortality from CRC.

1. Introduction

Screening the general adult population for colorectal cancer (CRC) and precancerous colorectal adenoma (CRA) can detect curable disease and reduce mortality. However, all the screening methods in current use have substantial limitations (Kuipers et al., 2013; Lieberman, 2014). Screening by colonoscopy or detection of fecal occult blood greatly reduces long-term CRC mortality (Zauber et al., 2012), but at least half of CRC mortality in the U.S.A. can be attributed to avoidance of screening (Meester et al., 2015). Colonoscopy, the primary modality used in the U.S.A., is costly and invasive, and its efficacy depends on the endoscopist's skill and the patient's bowel preparation. Computed tomographic colonography has different challenges, and the requirement to pursue detected lesions with colonoscopy limits its use for primary screening (Kuipers et al., 2013). Detecting occult blood in feces, particularly with the fecal immunochemical test (FIT) for human hemoglobin, has reasonable acceptability, cost, and accuracy for detecting CRC (single-test sensitivity, 60%–85%; specificity, >90%) (Lieberman, 2014). Improving the predictive value of a positive FIT (FIT+) with molecular analyses of feces or serum is a high priority (Ahlquist et al., 2012; Carmona et al., 2013; Goedert et al., 2014b; Imperiale et al., 2014; Kuipers et al., 2013; Lieberman, 2014), especially because the sensitivity of FIT for CRA is less than 50% and because many FIT+ patients decline follow-up colonoscopy (Lieberman, 2014).

Research on differences or alterations in the distal gut microbiota has focused on the pathogenesis of CRC (Collins et al., 2011; Schwabe and Jobin, 2013; Sears and Pardoll, 2011; Tjalsma et al., 2012), and such differences may ultimately prove to be helpful for screening. Comprehensive comparisons of the fecal microbiota have been reported by four studies, totaling 176 CRC cases and 241 controls (Ahn et al., 2013; Wang et al., 2012; Weir et al., 2013; Wu et al., 2013; Zeller et al., 2014). Four of these studies reported that butyrate-producing bacteria were significantly less abundant in feces from CRC cases compared to

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controls, although the particular taxa varied. In addition, the three larger studies reported that CRC cases had significantly higher carriage or abundance of potentially pathogenic Fusobacteria and Proteobacteria (Ahn et al., 2013; Wang et al., 2012; Zeller et al., 2014). Similar or different bacteria may contribute to the CRA stage of neoplasia (Tjalsma et al., 2012), and data to address this hypothesis are starting to emerge. Abundance of several bacterial taxa was reported to differ in rectal mucosa of CRA cases compared to healthy controls (McCoy et al., 2013; Mira-Pascual et al., 2014; Sanapareddy et al., 2012; Shen et al., 2010), including two studies in which well characterized cases' unaffected mucosa had higher levels of Proteobacteria taxa than did the controls' mucosa (Mira-Pascual et al., 2014; Shen et al., 2010).

With fecal DNA, Chen and colleagues reported that the overall composition of the microbiota differed between 47 CRA cases and 47 colonoscopically normal, age- and sex-matched controls (Chen et al., 2013). Nominally significant differences in relative abundance, not adjusted for multiple comparisons, were reported for six bacterial genera — two increased (*Enterococcus* and *Streptococcus*) and four decreased (*Bacteroides*, *Clostridium*, *Roseburia*, and *Eubacterium*) in CRA cases (Chen et al., 2013). Likewise, Zackular et al. identified a dozen of differentially abundant fecal taxa in CRA cases, although they did not consider multiple comparisons, overall diversity or composition (Zackular et al., 2014). In two very small studies, no large or statistically significant differences in the fecal microbiota of CRA cases were found (Brim et al., 2013; Mira-Pascual et al., 2014). The fecal microbiota of patients with small, benign adenoma (polyp) resembles that of normal controls (Zeller et al., 2014).

The current project had two objectives. We evaluated the feasibility of adding fecal microbiota analysis to the ongoing, population-based CRC screening program in Shanghai, and we sought to identify fecal microbial and other differences between well characterized CRA cases and colonoscopically normal controls from the same population.

2. Methods

2.1. Overview

The research protocol and procedures were reviewed and approved by institutional review boards at the U.S. National Cancer Institute and at the Shanghai Municipal Center for Disease Control and Prevention (SCDC). It is registered at ClinicalTrials.gov (identifier: NCT01778595) and conforms with the STROBE guidelines for reporting standards in observational cohort and case–control studies (http://www.strobe-statement.org/).

Staff at the Minhang and Xuhui district community health centers were trained and provided supplies for 100 participants. Consecutive patients aged 50–74 years who presented for CRC screening, in whom heme was detected by the fecal immunochemical test [FIT+, Baso Diagnostics Inc. (www.baso.com.cn/en/), prevalence = 14%], were scheduled for colonoscopy at the local hospital and also were invited to participate in the research study. The objectives and required procedures were presented and discussed individually, signed informed consent was obtained, and a CRC risk factor questionnaire was completed. The participant was instructed to collect the fecal specimens within the next few days, and well before bowel cleansing for colonoscopy which was scheduled about 2 weeks later. Colonoscopy and histopathology results, based on review by a single pathologist at the Fudan University Cancer Center, were electronically transmitted by the local hospital to the community health center and the SCDC.

2.2. Fecal Specimen Collection

The participant was provided with written and illustrated instructions, a cup for catching stool, and bar-coded 20 mL fecal collection vials (SARSTEDT, Nümbrecht, Germany). The vials for the current project had been preloaded with 5 mL of RNAlater (QIAGEN, Hilden, Germany) supplemented with kanamycin 300 μg/mL. In this media, fecal microbiota diversity and composition are unaltered and stable for up to seven days at room temperature (submitted for publication). The participant recorded the time of collection and promptly brought the specimens to the community health center where they were immediately frozen at −20 °C. The specimens were transferred weekly on dry ice to the SCDC repository where they were inventoried and stored at −80 °C. One vial from each participant was shipped overnight on dry ice to BGI for testing.

2.3. DNA Extraction and 16S rRNA Amplification, Sequencing, and Analysis

DNA isolation and purification were performed as described previously (Qin et al., 2012). One sample was found to be completely degraded. For unbiased representation of the fecal microbiota as described previously in detail (Fadrosh et al., 2014), approximately 469 bp of the 16S rRNA gene V3–V4 hypervariable region of the fecal DNA was amplified with primers that included a linker sequence (suitable for the Illumina MiSeq 250PE instrument), a 12 bp index sequence, a heterogeneity spacer (to minimize bias with low-diversity amplicons), and 16S rRNA universal primers 319F/806R. DNA products were quantified by a Qubit fluorometer (Life Technologies, Grand Island New York, U.S.A.). The amplicons were sequenced in a single pool in one run with the MiSeq 250PE, generating approximately 2.22 Gb of data. The raw sequences were processed to concatenate forward and reverse reads and to sort and match paired end sequences and barcodes. Using the pipeline of the Institute of Genome Sciences, University of Maryland Medical School, the processed reads were clustered, and the operational taxonomic units (OTUs) were assigned to taxa by matching to the Ribosomal Data Project naïve Bayesian classifier (Wang et al., 2007). Richness (number of observed species) and alpha diversity metrics (Chao1, Shannon index, and Phylogenetic Diversity whole-tree) were calculated using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010).

2.4. Statistical Analysis

Primary analyses compared microbiota diversities, both alpha diversity and beta diversity, with CRA (any combination of tubular or villous histology or ≥1 cm diameter) versus normal colonoscopy. Secondary analyses compared CRA patients to all participants and also excluded participants who had received an antibiotic within 24 weeks of enrollment. Linear regression was used to assess the association of alpha diversity metrics. Quantile–quantile (QQ) plots of P-values were used to evaluate the global association patterns for taxa. Wilcoxon rank-sum tests were used to assess associations of individual taxon relative abundances, without adjustment for multiple comparisons. Differences of all taxon relative abundances in CRA cases, normals and other participants are provided but, these were not formally analyzed because of lack of statistical power considering the small sample size and heavy multiple testing burden.

We performed receiver operating characteristic (ROC) analysis to assess the potential of differentiating CRA/normal status based on microbiota composition. We used random forest (RF), a powerful supervised learning algorithm, to build classifiers based on taxon relative abundances with or without sex. Because of the small sample size, we could not partition the data into discovery and validation. For unbiased area-under-the-curve (AUC) assessment, we performed leave-one-out (LOO) analysis. Briefly, for each given participant, we built a classifier with all other participants and used the classifier to calculate the posterior probability for the given participant to be a CRA case. The AUC was then calculated based on the posterior probabilities. We also performed permutation analysis to investigate whether the method for assessing AUC was biased upward, i.e., higher than 0.5, for non-discriminative features. In each permutation, we randomly permuted the CRA/normal