Revisiting Short-Chain Fatty Acids and the Microbiota in Colorectal Cancer

Running title: SCFAs and Colorectal Cancer

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Abstract

Introduction

Results

Discussion

Conclusions

Materials and Methods

Study design and sampling. The overall protocol has been described in detail previously (1, 2). In brief, this study used fecal samples obtained at either a single cross-sectional time point (n=490) or from before and after treatment for their tumor (n=67). For patients undergoing treatment for their tumor the length of time between their initial and follow up sample ranged from 188 - 546 days. Our use of treatment has been previously defined as encompassing removal of a tumor with or without chemotherapy and radiation (1). Diagnosis of tumor was made by colonoscopic examination and histopathological review of biopsies obtained (1, 2). The University of Michigan Institutional Review Board approved the study and informed consent was obtained from all participants in accordance to the guidelines set out by the Helsinki Decleration.

Measuring specific SCFAs. Our protocol for the measurement of acetate, butyrate, and propionate followed a previously published protocol (3). The following changes to this protocol included the use of frozen fecal samples suspended in 1ml of PBS instead of fecal suspensions in DNA Genotek OmniGut tubes, and the use of the acutal weight of fecal samples instead of the average weight for SCFA concentration normalizations. These changes did not affect the overall median concentrations of these SCFAs between the two studies (see Table 1 (3) and Figure 1 in this report).

16s rRNA gene sequencing. The workflow and processing have been described previously (1, 4, 5). The major differences from these previous reports include: the use of version 1.39.5 of the mothur software package and clustering Operational Taxonomic Units (OTUs) at 97% similarity used the OptClust algorithm (6).

Generating imputed metagenomes. The use of PICRUSt version 1.1.2 with the recommended standard operating protocol (7) was used. Briefly, the mothur shared file and metadata was converted into a biom formated table using the biom convert function, the subsequent biom file was processed with the normalize_by_copy_number.py function, and subsequent imputed metagenomes created using the predict_metagenomes.py function.

Obtaining OPFs from metagenomes. A subset of the cross-sectional group (n=490) containing a total of 85 individuals (normal n=29 normal, adenoma n=28, and carcinoma n=28) was shotgun

sequenced on an Illumina HiSeq with 125 bp paired end reads using a previously described method (8). Briefly, the sequences were quality filtered and sequences aligning to the human genome were removed prior to contig assembly with MEGAHIT (9). Open Reading Frames (ORFs) were identified using Prodigal (10), counts generated using Diamond (11), subsequent clustering into Operational Protein Families (OPFs) used mmseq2 (12), and OPF gene alignment used the KEGG database (13).

Pulling genes involved with SCFA synthesis. Specific genes located near the end of the pathways involved in the synthesis of acetate, butyrate, and propionate were analyzed for any differences between individuals with normal colons and those with tumors. These genes were based on pathways from KEGG as well as previous research (13, 14) and a list can be found in the supplemental material [Table S1].

Statistical analysis workflow. All analysis was performed using the statistical language R (15). Generally, differences between the different disease groups used a Kruskal-Walis rank sum test with a Dunn's post-hoc test. We next assessed whether SCFAs added to Random Forest models built with OTU data significantly improved classification. Next, models to classify high or low SCFA concentration based on 16S rRNA gene sequencing data were created using Random Forest (16). Regression models to classify the exact SCFA concentration based on 16S rRNA gene sequencing data also were built using the Random Forest algorithm. The measured SCFA concentrations were first tested for differences between groups. The ability of 16S rRNA gene sequencing to classify these concentrations were then assessed. Next, the imputed gene counts of important mediators of SCFA creation were tested. Finally, the counts generated for OPFs that matched important genes involved with SCFA creation were analyzed.

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Figure 1. Using HPLC no change in SCFA measurements was observed between normal, adenoma, and carcinoma individuals. Acetate concentrations in fecal samples of individuals with normal colons, adenomas, and carcinomas (A). Butyrate concentrations in fecal samples of individuals with normal colons, adenomas, and carcinomas (B). Propionate concentrations in fecal samples of individuals with normal colons, adenomas, and carcinomas (C). The black links indicate the median SCFA concentration. Acetate concentrations in fecal samples before and after treatment for adenoma (yellow) and carcinoma (red) (D). Butyrate concentrations in fecal samples before and after treatment for adenoma (yellow) and carcinoma (red) (E). Propionate concentrations in fecal samples before and after treatment for adenoma (yellow) and carcinoma (red) (F). The black dots and lines represent the median change in SCFA concentration.

Figure 2. SCFAs do not improve OTU-based Random Forest models. Difference between the area under the curve of 100 different 80/20 split OTU-based normal versus adenoma 10-fold CV models with and without SCFAs (A). Difference between the area under the curve of 100 different 80/20 OTU-based normal versus carcinoma 10-fold CV models with and without SCFAs (B). The black linke represents the median AUC. The dotted line highlights an AUC of 0.5.

Figure 3. No change in butyrate producing genes identified between normal, adenoma, and carcinoma individuals. Imputed gene relative abundance of important butyrate pathway genes using PICRUSt (A). Counts per million (corrected for size and number of contigs in an OPF) for the Butyrate Kinase gene (B). The other genes from the PICRUSt analysis did not align to any of the OPFs in the metagenome analysis.

Figure S1. OTU-based Random Forest models of SCFA concentrations. Classification Random Forest train and tests of 100 different 80/20 OTU-based models with 10-fold CV based on higher or lower than the medain SCFA concentration (A). The top 10 OTUs based on mean decrease in accuracy (MDA) for each model, colored by their lowest taxonomic identification (B). Regression Random Forest train and tests of 100 different 80/20 OTU-based models with 10-fold CV based on correlation to actual SCFA concentration (C). The top 10 OTUs based on mean decrease in accuracy (MDA) for each model, colored by their lowest taxonomic identification (D).