Differences in the fecal Microbiome Before and After Colorectal Cancer Treatment

Running Title: Human Microbiome and Colorectal Cancer

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Abstract

Background: Colorectal cancer (CRC) continues to be a worldwide health problem with previous research suggesting that a link may exist between the fecal bacterial microbiome and CRC. The overall objective of our study was to test the hypothesis that changes in the bacterial microbiome occur after surgery in patients with lesions (i.e. adenoma or carcinoma). Specifically, we wanted to identify what within the community was different before and after surgical removal of said lesion.

Results: The bacterial microbiome in the pre and post surgery samples of the individuals with adenoma were similar while those with carcinoma were not (P-value = 0.000472). There was no difference in the relative abundance of any OTU between the pre and post surgery samples (P-value > 0.151). A model with a total of 53 variables was able to classify lesion (AUC = 0.811 - 0.866) while a model built to classify samples as before surgery had 70 variables (AUC = 0.641 - 0.805). The post surgery sample for both models had a decrease in the positive probability for either lesion or before surgery sample (P-value = 3.28e-04 and 6.71e-12). In total there were 23 OTUs that were common to both models and the majority of these classified to commensal bacteria (Bacteroides, Clostridiales, Blautia, and Ruminococcaceae).

Conclusions: Our data supports the hypothesis that there are differences in the bacterial microbiome between pre and post surgical samples. With individuals with carcinoma having more drastic changes to the overall community then those with adenoma. However, changes to commensal bacteria were common to individuals with either adenoma or carcinoma suggesting that these bacteria may be central to initial polyp formation and transition to carcinoma.

24 **Keywords**

25 bacterial microbiome; colorectal cancer; polyps; FIT; post surgery; risk factors

Background

Colorectal cancer (CRC) continues to be a leading cause of cancer related deaths and is the second most common cancer death among men aged 40-79 years of age [1,2]. Over the last few years death due to the disease has seen a significant decrease, thanks mainly to improvements in screening [1]. However, despite this improvement there are still approximately 50,000 deaths from the disease a year [2].

Recently, there has been promising work on the bacterial microbiome and it's ability to be able to complement existing screening methods such as Fecal Immunoglobulin Test (FIT) or act alone as a screening tool [3,4]. There has also been research into how this microbiome could be altered directly on tumor tissue itself [5]. A few studies have also shown how this microbiome [6] or specific members within it [7] could be directly involved with the pathogenesis of CRC. These studies have helped to provide a tantalizing link between the bacterial microbiome and CRC. Yet there remains limited information on the bacterial microbiome before and after surgery for removal of lesion (adenoma or carcinoma) and whether the community changes at all.

In this study we tested the hypothesis of whether that the bacterial microbiome changes after surgery for individuals with a lesion. Our analysis included both alpha and beta diversity analysis along with investigation of individual operational taxonomic units (OTUs). We also utilized Random Forest models and observed how these models as well as specific OTUs within them performed pre (initial) and post (follow up) surgery. We also used these models to look for similar important OTUs to identify the crucial OTUs for not only classifying initial and follow up samples but also lesion or normal.

Results

Bacterial Community and FIT We first wanted to test whether there were any broad differences between initial and follow up samples based on lesion being either adenoma or carcinoma. What we found was that the bacterial community in those with carcinoma were more dissimilar (as measured by thetayc) to their initial sample then those with adenoma (P-value = 0.000472) [Figure 1a]. We also found that there were larger changes in fecal blood (measured by FIT) for those with carcinoma versus adenoma (P-value = 2.15e-05) [Figure 1b]. The broad shift in bacterial community structure before and after surgery was visualized using NMDS for both adenoma [Figure1c] (PERMANOVA = 0.999) and carcinoma [Figure 1d] (PERMANOVA = 0.005). Interestingly, when initial and follow up samples were compared to each other, regardless of whether they were adenoma or carcinoma (lesion), there was no significant overall difference between them (PERMANOVA = 0.09). When investigating more broad alpha diversity metrics there was no difference found between initial and follow up samples for lesion, adenoma, or carcinoma for any metric tested [Table S1]. We also observed that there was no difference in OTU relative abundance between initial and follow up samples for lesion, adenoma, or carcinoma [Figure S1].

Previously Associated Cancer Bacteria Previous literature has suggested that a number of oral microbes may be important in CRC pathogenesis [8]. So we next examined whether there were differences in previously well described carcinoma associated OTUs. These included the OTUs that aligned with *Porphyromonas asaccharolytica* (Otu000202), *Fusobacterium nucleatum* (Otu000442), *Parvimonas micra* (Otu001273), and *Peptostreptococcus stomatis* (Otu001682). There was a difference in relative abundance in initial and follow up samples for lesion and carcinoma for *Parvimonas micra* (P-value = 0.0116), and *Porphyromonas asaccharolytica* (P-value = 0.00842). In contrast, there was no difference in relative abundance in any of these OTUs for those

with adenoma [Figure 2]. We also observed that only a small percentage of those with adenoma or carcinoma were positive or had an appreciable relative abundance of these respective OTUs [Figure 2].

Full and Reduced Model We next wanted to identify if there were any common bacterial microbiome changes in individuals with adenoma or carcinoma. In order to investigate this we created two different models: one to classify lesion versus normal and one to classify pre versus post samples based on the bacterial community and FIT. The lesion model had an AUC range of 0.73 to 0.797 while the initial sample model had an AUC range of 0.485 to 0.686 after 100 iterations of 20 repeated 10-fold cross validations. By identifying the most important variables for each respective model and then reducing them to only these factors we were able to increase the AUC in the lesion model (0.811 - 0.866) and initial sample model (0.641 - 0.805).

The test set AUC range for the full and reduced lesion model were similar to that reported for the training set AUC ranges and the ROC curve ranges overlap with each other [Figure 3a]. The ROC curve for the final lesion model used was within the range of both the full and reduced lesion model [Figure 3a]. Interestingly, the test set AUC range for the initial sample model performed much better then the training set AUCs. Both the full and reduced initial sample models overlapped with each other [Figure 3b] but there was a marked decrease in the ROC curve for the final before sample model used.

Common OTUs to both Models The reduced models were built based on the most important variables to either classification model. For the lesion model there were a total of 53 variables [Figure S2] whereas for the initial sample model there were a total of 70 variables [Figure S3]. For both models FIT resulted in the largest decrease in MDA [Figure S2a & S3a]. There were a total of Bacteroides, Clostridiales, Blautia, and Ruminococcaceae. The majority of these OTUs had classifications to bacteria typically thought of as commensal [Table S2].

Positive Probability after Lesion Removal If there were common OTUs for individuals with adenoma and carcinoma that were different versus normal controls we would expect to find a decrease in the positive probability of the follow up sample to be either lesion or a before sample. This is what we observed regardless of model used (lesion or before sample) or whether it was built on the full or reduced variable data set [Figure 4 & S4] (full lesion P-value = 1.62e-04, reduced lesion P-value = 3.28e-04, initial and follow up P-value = 6.71e-12, and reduced initial and follow up P-value = 6.71e-12).

Although there was a decrease in positive probability for all models there were differences on whether the models classified the samples as still having lesion. This was most evident for the full and reduced lesion model where there was a large difference between predicted and actual (P-value = 4.88e-01 and 4.7e-01, respectively). However, the before sample model did not have this disparity between predicted and actual (P-value = 1.00 and 1.00). Even though the lesion model varied greatly between predicted and actual it was able to correctly keep the one individual who still had a carcinoma on follow up above the cut off threshold [Figure 4a & S4a] for a positive call while the before sample models did not [Figure 4b & S4b].

Treatment and Time Differences After observing these changes in positive probability we wanted to assess whether additional treatments, such as chemotherapy and radiation, could have an impact on the results that we observed. There was no difference in the amount of change in positive probability for either the full or reduced lesion model for either chemotherapy (P-value = 0.184 and 0.184) or radiation therapy (P-value = 0.942 and 0.839). Although the before sample model was similar there was a significant difference in decreased positive probability for those treated with chemotherapy (P-value = 1.27e-03 and 6.18e-04). Finally, we wanted to know if the length of time between the initial and follow up sample could be a possible con-founder. Within our study there was a significant difference in the time elapsed in the collection of the follow up sample between adenoma

and carcinoma samples (uncorrected P-value = 7.59e-05) with time passed being lower for adenoma (253 +/- 41.3 days) then carcinoma (351 +/- 102 days).

Discussion

From our results there were some large observed differences in the bacterial microbiome between pre and post surgery samples based on whether the individual had an adenoma or carcinoma. There was much larger difference between initial and follow up samples based on the thetayc distance metric and in fecal blood as measured by FIT for individuals with carcinoma versus adenoma [Figure 1]. However, there were no differences between initial and follow up samples for Shannon Diversity, observed OTUs, or evenness regardless of whether the individual had an adenoma or carcinoma [Table S1]. There was also no differences in relative abundance of any specific OTU for lesion, adenoma, or carcinoma [Figure s1].

Although there were no differences when investigating all OTUs, when looking specifically at four OTUs that taxonomically classified to previously suggested cancer causing microbes we found that only 3/4 had a decrease in relative abundance between initial and follow up for those with carcinoma and 0/4 had differences for those with adenoma. This data would suggest that these specific OTUs may be important in the transition of an adenoma to a carcinoma but less so in the initiation of an adenoma from benign tissue.

We next created a model that incorporated FIT and the bacterial microbiome to either be able to classify lesions (adenoma or carcinoma) or initial samples in order to find common OTUs in the community that change for both adenoma and carcinoma. What we found was that the commonly associated CRC bacteria were not higly represented within our models but rather that OTUs that made up the most important variables overwhelmingly belonged to commensal bacteria. With only the lesion model having a single OTU from a previously associated cancer bacterium (*Porphyromonas asaccharolytica*). Using only these important OTUs and FIT both models (lesion and before sample) significantly decreased positive probability of either lesion or being an initial sample on follow up [Figure

4 & S4]. Further confirmation of the importance of the changes of commensal bacteria to these classifications was that a total of 23 OTUs were common to both models and the vast majority belonged to regular residents of our gut community.

Problematic paragraph need to amend

There was no difference for the majority of models tested for differences in positive probability based on whether chemotherapy or radiation was received. There was a difference in the length of time between initial and follow up sample between adenoma and carcinoma. These results would indicate that the findings described were specific to the surgical intervention and that differences observed between carcinoma and adenoma samples can not be simply attributed to collection time between samples.

This study builds upon previous work from numerous labs that have looked into the bacterial microbiome as a potential screening tool [3,4] by exploring what happens to the bacterial community after surgical removal of a lesion. Based on previous work by Arthur, et al. [9] it may not be surprising to have E.coli as one of the most important OTUs and one that was common to both models. Interestingly, many of the most important OTUs had taxonomic identification for resident gut microbes. This could suggest that the bacterial community is one of the first components that could change during the pathogenesis of disease. These bacterial microbiome changes could be the first step in allowing more inflammatory bacterium to gain a foothold within the colon [8].

Curiously, we observed that the typical CRC associated bacteria were not predictive within our models. There are a number of reasons why this may have occurred. First, one potential explanation is that even with surgery and a shift of the bacterial community these specific bacteria still persisted within the colon. Second, the bacteria even if they were reduced in relative abundance they were still present within the gut. Third, is that they were not present in enough individuals to be able to classify those with and without disease with

a high degree of accuracy. Fourth, is that it is possible that our Random Forest models were able to get the same information from measures such as FIT or other OTUs. Finally, it is also possible that all of these potential explanations could have played a role. Regardless, our observations would suggest that an individual's resident bacteria have a large role to play in disease initiation and could change in a way that allows predictive models to lower the positive probability of a lesion after surgery [Figure 4]. It should be noted that our study does not argue against the importance of these CRC associated bacteria in the pathogenesis of disease but rather that the models do not utilize these specific bacteria for classification purposes (lesion or before sample). In fact, it is possible that these CRC associated bacteria are important in the transition from adenoma to carcinoma and would be one explanation as to why in our data we not only see high initial relative abundances, in certain individuals, but also large decreases in relative abundance in those with carcinoma but not in those with adenoma after surgery [Figure 2].

One limitation of our study is that we do not know whether individuals who were still classified as positive by the lesion model eventually had a subsequent CRC diagnosis. This information would help to strengthen the case for our Random Forest based model to have kept a number of individuals above the cutoff threshold even though at follow up they were diagnosed as no longer having a lesion. Another limitation is that we do not know if adding modern tests such as the stool DNA test [10] could help improve our overall AUC. Another limitation is that this study drew heavily from those with Caucasian ancestry. The results may not be immediately representative of those with either Asian or African ancestry. Finally, although our training and test set are relatively large we still run the risk of over-fitting or having a model that may not be immediately extrapolate-able to other populations. We've done our best to safeguard against this by not only running 10-fold cross validation but also having over 100 different 80/20 splits to try and mimic the type of variation that might be expected to occur. The time difference in collection of sample between adenoma and carcinoma that was observed could have affected our observed

results. However, our random forest based model, specifically the lesion trained model, were still able to adjust positive probabilities for both adenoma and carcinoma to similar amounts despite this.

Another interesting outcome was that within figure 3 the before sample model showed better test AUC results then the training set AUC. This may have occurred because the training AUC that was determined from 20 repeated 10 fold cross validation removed samples at random and did not take into account that they were matched samples. Another potential reason is that the model itself may be over-fit since the total number of samples was not that large. However, the lesion model did not suffer from these discrepancies and similar conclusions can be drawn solely from this model. Regardless, further independent studies will need to be carried out to verify our findings since not only are we dealing with feces, which could be very different than the communities present on the actual tissue, but also are dealing with correlations that may not be representative of the true pathogenesis of disease.

Despite these limitations we think that these findings significantly add to the existing scientific knowledge on CRC and the bacterial microbiome. The ability for machine learning algorithms to take bacterial microbiome data and successfully lower positive probability after either adenoma or carcinoma removal provides evidence that there are specific signatures, mostly attributable to commensal organisms, associated with these lesions. It also shows that these algorithms can not only successfully react to successful treatment regimens but also may be able to one day stratify CRC disease risk with a high level of accuracy.

Methods

Study Design and Patient Sampling The sampling and design of the study was similar to that reported in Baxter, et al [3]. In brief, study exclusion involved those who had already undergone surgery, radiation, or chemotherapy, had colorectal cancer before a baseline fecal sample could be obtained, had IBD, a known hereditary non-polyposis colorectal cancer, or Familial adenomatous polyposis. Samples used to build the models for prediction were collected either prior to a colonoscopy or between 1 - 2 weeks after. The bacterial microbiome has been shown to normalize within this time period [11]. Our follow up data set had a total of 67 individuals that not only had a sample as described but also a follow up sample between 188 - 546 days after surgery and treatment had been completed. This study was approved by the University of Michigan Institutional Review Board. All study participants provided informed consent and the study itself conformed to the guidelines set out by the Helsinki Declaration.

FIT and 16S rRNA Gene Sequencing FIT was analyzed as previously published using both OC FIT-CHEK and OC-Auto Micro 80 automated system (Polymedco Inc.) [12]. 16S rRNA gene sequencing was completed as previously described by Kozich, et al. [13]. In brief, DNA extraction used the 96 well Soil DNA isolation kit (MO BIO Laboratories) and an epMotion 5075 automated pipetting system (Eppendorf). The V4 variable region was amplified and the resulting product was split between three sequencing runs with normal, adenoma, and carcinoma evenly represented on each run. Each group was randomly assigned to avoid biases based on sample collection location.

Sequence Processing The mothur software package (v1.37.5) was used to process the 16S rRNA gene sequences. This process has been previously described [13]. The general processing workflow using mothur was as follows: Paired-end reads were first merged into contigs, quality filtered, aligned to the SILVA database, screened for chimeras,

classified with a naive Bayesian classifier using the Ribosomal Database Project (RDP), and clustered into Operational Taxonomic Units (OTUs) using a 97% similarity cutoff with an average neighbor clustering algorithm. The number of sequences for each sample was rarefied to 10523 in an attempt to minimize uneven sampling.

Lesion Model Creation The Random Forest [14] algorithm was used to create the model used for prediction of lesion (adenoma or carcinoma) with the main testing and training of the model completed on a data set of 490 individuals. This model was then applied to our follow up data set of 67 individuals. The model included data on FIT and the bacterial microbiome. Non-binary data was checked for near zero variance and OTUs that had near zero variance were removed. This pre-processing was performed with the R package caret (v6.0.73). Optimization of the mtry hyper-parameter involved taking the samples and making 100 different 80/20 (train/test) splits of the data where normal and lesion were represented in the same proportion within both the whole data set and the 80/20 split. Each of these splits were then run through 20 repeated 10-fold cross validations to optimize the mtry hyper-parameter by maximizing the AUC (Area Under the Curve of the Receiver Operator Characteristic). This resulting model was then tested on the 20% of the data that was originally held out from this overall process. Once the ideal mtry was found the entire 490 sample set was used to create the final Random Forest model on which classifications on the 67-person cohort was completed. The default cutoff of 0.5 was used as the threshold to classify individuals as positive or negative for lesion. The hyper-parameter, mtry, defines the number of variables to investigate at each split before a new division of the data is created with the Random Forest model.

Before Sample Model Creation We also investigated whether a model could be created that could identify before and after surgery samples. The main difference was that only the 67-person cohort was used at all stages of model building and classification. Other than this difference the creation of this model and optimization of the mtry hyper-parameter was

completed using the same procedure that was used to create the lesion model. Instead of classifying samples as positive or negative of lesion this model classified samples as positive or negative for being a before surgery sample.

Selection of Important OTUs In order to assess which variables were most important to all the models we counted the number of times a variable was present in the top 10% of mean decrease in accuracy (MDA) for each of the 100 different 80/20 split models and then filtered this list to variables that were only present more than 50% of the time. This final collated list of variables was what was considered the most important for the lesion or before sample models.

Statistical Analysis The R software package (v3.3.2) was used for all statistical analysis. Comparisons between bacterial community structure utilized PERMANOVA [15] in the vegan package (v2.4.1). Comparisons between probabilities as well as overall OTU differences between initial and follow up samples utilized a paired Wilcoxson ranked sum test. Where multiple comparison testing was needed a Benjamini-Hochberg (BH) correction was applied [16] and a corrected P-value of less than 0.05 was considered significant. Unless otherwise stated the P-values reported are those that were BH corrected.

Analysis Overview Initial and follow up samples were analyzed for differences in alpha and beta diversity. Next, differences in FIT between initial and follow ups for either adenoma or carcinoma were investigated. From here, all OTUs that were used in either model were then analyzed using a paired Wilcoxson test. We then investigated the relative abundance of specific previously associated CRC bacteria, specifically, OTUs that taxonomically classified to *Fusobacterium nucleatum*, *Parvimonas micra*, *Peptostreptococcus assacharolytica*, and *Porphyromonas stomatis*. We wanted to test if there were any differences based on whether the individual had an adenoma or carcinoma. From here the lesion model was then tested for accuracy in prediction and whether it reduced the positive probability of lesion after surgery. The most important OTUs for this

were used to build a reduced model and it was assessed for similarity to the original model. We then used the before sample model to assess whether it could classify samples better then the lesion model. The most important OTUs were then identified from this model and used to create a reduced feature before sample model. This reduced feature model, as was done with the lesion model, was compared to the full model for loss of accuracy. Finally, a list of common OTUs were found for the two different models used.

Reproducible Methods. A detailed and reproducible description of how the data were processed and analyzed can be found at https://github.com/SchlossLab/Sze_followUps_2017. Raw sequences have been deposited into the NCBI Sequence Read Archive (SRP062005 and SRP096978) and the necessary metadata can be found at https://www.ncbi.nlm.nih.gov/Traces/study/ and searching the respective SRA study accession.

Figure 1: General Differences between the Adenoma or Carcinoma Group. A) A significant difference was found between the adenoma and carcinoma group for thetayc (P-value = 0.000472). B) A significant difference was found between the adenoma and carcinoma group for change in FIT (P-value = 2.15e-05). C) NMDS of the initial and follow up samples for the Adenoma group. D) NMDS of the initial and follow up samples for the Carcinoma group. For C) and D) the teal represents initial samples and the pink represents follow up samples.

Figure 2: Previously Associated CRC Bacteria in Initial and Follow up Samples. A) Carcinoma initial and follow up samples. There was a significant difference in initial and follow up sample for the OTUs classified as *Peptostreptococcus stomatis* (P-value = 0.0496) and *Porphyromonas asaccharolytica* (P-value = 0.00842). B) Adenoma initial and follow up samples. There were no significant differences between initial and follow up.

Figure 3: Graph of the Receiver Operating Characteristic Curve for lesion and Before Sample Models. The shaded areas represents the range of values of a 100 different 80/20 splits of the test set data using either all variables (grey) or reduced variable (red) models. The blue line represents the reduced variable model using 100% of the data set. A) Lesion model. B) Before sample model

Figure 4: Breakdown by Carcinoma and Adenoma of Prediction Results for Lesion and Before Sample Reduced Variable Models A) Lesion positive probability adjustment of those with carcinoma from initial to follow up sample B) Initial follow up positive probability adjustment of those with carcinoma from initial to follow up sample C) Lesion positive probability adjustment of those with adenoma as well as those with SRN and the probability adjustment from initial to follow up sample. D) Initial follow up positive probability adjustment of those with adenoma as well as those with SRN and the probability adjustment from initial to follow up sample. The dotted line represents the threshold used to make the decision of whether a sample was positive or not.

Figure S1: Distribution of P-values from Paired Wilcoxson Analysis of OTUs in Initial versus Follow Up

Figure S2: Summary of Important Variables in the Lesion Model A) MDA of the most important variables in the lesion model. The black point represents the median and the different colors are the different runs up to 100. B) The total number of appearances of each variable in the 100 different lesion models. The cutoff of 50% was used to assess importance.

Figure S3: Summary of Important Variables in Before Sample Model A) MDA of the most important variables in the lesion model. The black point represents the median and the different colors are the different runs up to 100. B) The total number of appearances of each variable in the 100 different lesion models. The cutoff of 50% was used to assess importance.

Figure S4: Breakdown by Carcinoma and Adenoma of Prediction Results for Lesion and Before Sample Full Variable Models A) Lesion positive probability adjustment of those with carcinoma from initial to follow up sample B) Initial follow up positive probability adjustment of those with carcinoma from initial to follow up sample C) Lesion positive probability adjustment of those with adenoma as well as those with SRN and the probability adjustment from initial to follow up sample. D) Initial follow up positive probability adjustment of those with adenoma as well as those with SRN and the probability adjustment from initial to follow up sample. The dotted line represents the threshold used to make the decision of whether a sample was positive or not.

Figure S5: Thetayc Graphed Against Time of Follow up Sample from Initial

Declarations

Ethics approval and consent to participate

Consent for publication

Availability of data and material

Competing Interests

All authors declare that they do not have any relevant competing interests to report.

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Authors' contributions

All authors were involved in the conception and design of the study. MAS analyzed the data. NTB processed samples and analyzed the data. All authors interpreted the data. MAS and PDS wrote the manuscript. All authors reviewed and revised the manuscript. All authors read and approved the final manuscript.

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