The effect of treatment on the microbiota of patients diagnosed with colonic lesions

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Abstract

Background. Colorectal cancer (CRC) is a worldwide health problem. Despite growing evidence that members of the gut microbiota can drive tumorigenesis, little is known about what happens to the microbiota after treatment for an adenoma or carcinoma. This study tested the hypothesis that treatment for adenoma or carcinoma alters the abundance of bacterial populations associated with disease to those associated with a normal colon. We tested this hypothesis by sequencing the 16S rRNA genes in the feces of 67 individuals before and after treatment for adenoma (N=22), advanced adenoma (N=19), and carcinoma (N=26).

Results. There were large changes to the bacterial communities associated with treatment across the three groups. The communities from patients with carcinomas changed significantly more than those with adenoma following treatment (P-value=5.4e-05); however, there was not a significant difference between those with advanced adenoma and those with adenoma or carcinoma (P-value > 0.05). Although treatment brought about large intrapersonal changes, the change in the abundance of individual OTUs to treatment was not consistent within diagnosis groups (P-value > 0.05). Because the distribution of OTUs across patients and diagnosis groups was patchy, we used the Random Forest machine learning algorithm to identify groups of OTUs that allowed us to successfully distinguish between pre- and post-treatment samples for each of the diagnosis groups. However, across the three models, there was little overlap between the OTUs that were indicative of treatment. Next, we used a larger cohort that contained individuals with normal colons and those with adenomas, advanced adenomas, and carcinomas to determine whether individuals who underwent a treatment were more likely to have OTUs associated with normal colons. We again built Random Forest models and measured the change in the positive probability of having one of the three diagnoses. Although we could clearly differentiate pre- and post-treatment communities from the three diagnosis groups,

only those patients that initially had carcinomas experienced a significant decrease in positive probability of having a carcinoma (P-value < 0.05). Finally, we tested whether the type of treatment impacted the microbiota of those diagnosed with carcinomas and were unable to detect any significant differences in characteristics of these communities between individuals treated with surgery alone and those treated with chemotherapy or chemotherapy and radiation (P-value > 0.05).

Conclusions. Further exploration of the relationship between diagnosis, treatment, and the impact on the microbiota will yield improvements in disease management.

Keywords

microbiota; colorectal cancer; polyps; treatment; risk factor.

Background

Colorectal cancer (CRC) is the third most common cause of cancer deaths in the United States [1,2]. Disease mortality has significantly decreased, thanks to improvements in screening [1]. Despite this improvement, there are still approximately 50,000 deaths from the disease per year [2]. Current estimates indicate that 20-30% of those who undergo treatment will experience recurrence and 30-50% of those patients will die [3,4]. Identification of methods to assess patients' risk of recurrence is of great importance to reduce mortality and healthcare costs.

There is growing evidence that the gut microbiota is involved in the progression of CRC. Mouse-based studies have identified populations of *Bacteroides fragilis*, *Escherichia coli*, and *Fusobacterium nucleatum* that alter disease progression [5–9]. Furthermore, studies that alter the structure of the microbiota through the use of antibiotics or inoculation of germ free mice with human feces has shown that varying community compositions can result in varied tumor burden [10–12]. Collectively, these studies hypothesize that the microbiota is altering the amount of inflammation in the colon and with it the rate of tumorigenesis [13].

Building on this evidence, several human-based studies have identified unique signatures of colonic lesions [14–19]. One line of research has identified community-level differences between those bacteria that are found on and adjacent to colonic lesions and have supported a role for *Bacteroides fragilis*, *Escherichia coli*, and *Fusobacterium nucleatum* in tumorigenesis [20–22]. Others have proposed feces-based biomarkers that could be used to diagnose the presence of colonic adenomas and carcinomas [23–25]. These studies have associated *Fusobacterium nucleatum* and other oral pathogens with lesions (adenoma, advanced adenoma, and carcinoma). They have also noted that the loss of bacteria generally thought to produce short chain fatty acids, which can suppress inflammation, is associated with colonic lesions. Gut bacteria have a role in tumorigenesis

and there is promise that these populations may be useful biomarkers for aiding in the early detection of disease.

Despite advances in understanding the role between the gut microbiota and colonic tumorigenesis, we still do not understand how treatments including resection, chemotherapy, and/or radiation affect the composition of the gut microbiota. If the community drives tumorigenesis then one would hypothesize that treatment to remove a lesion would affect the microbiota and risk of recurrence. To test this hypothesis, we addressed two related questions: Does treatment affect the colonic microbiota in a predictable manner? If so, does the treatment alter the community to more closely resemble that of individuals with normal colons?

We answered these questions by sequencing the V4 region of 16S rRNA genes amplified from fecal samples of individuals with adenoma, advanced adenoma, and carcinomas pre- and post-treatment. We used both classical community analysis to compare the alpha and beta-diversity of communities pre- and post treatment. Next, we generated Random Forest models to identify bacterial populations that were indicative of treatment for each diagnosis group. Finally, we measured the predictive probability of diagnosis models to determine whether treatment would alter the probability of being diagnosed as having a normal colon or the colon of the original diagnosis group. We found that treatment alters the composition of the gut microbiota; however, we only observed a shift from diseased to normal colon for those initially diagnosed with carcinomas. Understanding how the community responds to these treatments could be a valuable tool for identifying biomarkers to quantify the risk of recurrence.

Results

Treatment alters the bacterial community structure of patients diagnosed with colonic lesions. Within our 67-person cohort we tested whether the microbiota of patients with adenoma (N=22), advanced adenoma (N=19), or carcinoma (N=26) had any broad differences between pre- and post-treatment samples [Table 1]. The structure of the microbial communities of the pre- and post-treatment samples differed [Figure 1A]. We found that the communities obtained pre- and post-treament among the patients with carcinomas changed significantly more than those patients with adenoma (P-value = 5.4e-05). There were no significant differences in the amount of change observed between the patients with adenoma and advanced adenoma or between the patients with advanced adenoma and carcinoma (P-value > 0.05). Next, we tested whether there was a consistent direction in the change in the community structure between the pre and post-treatment samples for each of the diagnosis groups [Figure 1B-D]. We only observed a consistent shift in community structure for the patients with carcinoma when using a PERMANOVA test (adenoma P-value0.999, advanced adenoma P-value0.945, and carcinoma P-value0.005). Finally, we measured the number of observed OTUs, Shannon evenness, and Shannon diversity in the pre and post-treatment samples and did not observe a significant change for any of the diagnosis groups (P-value > 0.05) [Table S1].

The effects of treatment are not consistent across treatment groups. We used two approaches to identify those bacterial populations that change between the two samples for each diagnosis group. First, we sought to identify individual OTUs that could account for the change in overall community structure. However, using a paired Wilcoxon test we were unable to identify any OTUs that were significantly different in the pre and post-treatment groups [Figure S1]. It is likely that high inter-individual variation and the patchy distribution of OTUs across individuals limited the statistical power of the test. To overcome these problems we developed Random Forest models to identify collections of OTUs that would

allow us to differentiate between pre and post-treatment samples from each of the diagnosis groups. Because of the relatively small number of subjects in each group, we restricted our models to only incorporate 10 OTUs to limit the likelihood that that models would overfit the data. Despite this restriction, the models performed well (adenoma AUC=0.69 - 0.92, advanced adenoma AUC=0.8 - 1, carcinoma AUC=0.82 - 0.98). Interestingly, the 10 OTUs that were used for each model had little overlap with each other [Figure 2]. These results support the earlier community-wide analysis where we observed that the treatment had an impact on the overall community structure; however, the effect of treatment was not consistent across patients and diagnosis groups.

Post-treatment samples from patients with carcinoma change towards a microbiota associated with normal Next, we determined whether treatment changed the microbiota in a way that the post-treatment communities resembled that of patients with normal colons. To test this, we used an expanded cohort of 423 individuals that were diagnosed under the same protocol as having normal colons or colons with adenoma, advanced adenoma, or carcinoma [Table 2]. We then constructed Random Forest models to classify the pre and post-treatment samples as having their original diagnosis or having a normal colon. The models performed well (adenoma AUC=0.62 - 0.72, advanced adenoma AUC=0.68 - 0.77, carcinoma AUC=0.84 - 0.9; Figure S2). The OTUs that were incorporated into the adenoma and advanced adenoma models largely overlapped and those OTUs that were used to classify the carcinoma samples were largely distinct from those of the other two models [Figure 3A]. Among the OTUs that were shared across the three models were those populations commonly considered as commensals (e.g. *Faecalibacterium*, *Lachnospiraceae*, *Bacteroides*, *Dorea*, *Anaerostipes*, and *Roseburia*) [Figures 3B]. Although many of these OTUs were also included in the model differentiating between patients with normal colons and those with carcinoma, this model also included OTUs affiliated with populations that have previously been associated with carcinoma (*Fusobacterium*, *Porphyromonas*, *Parvimonas*) [Figure S3]. Finally, we applied these three

models to the pre and post-treatment samples for each diagnosis group and quantified the change in the positive probability of the model. A decrease in the positive probability would indicate that the colon more closely resembled that of a patient with a normal colon. There was no significant change in the positive probability for the adenoma or advanced adenoma groups [Figure 4]. The positive probability for the pre and post-treatment samples from patients diagnosed with carcinoma significantly decreased. In fact, only 6 of the 26 patients (23.08%) that were initially diagnosed with a carcinoma had a higher positive probability after treatment. One of those was re-diagnosed with carcinoma on the follow up visit. These results indicate that although there are changes in the microbiota associated with treatment, those experienced by the patients in the carcinoma group were the only ones where the change was directed towards what would be found in a normal colon

Difficult to identify effects of specific treatments on the change in the microbiota. The type of treatment that the patients received varied across diagnosis groups. Those with adenomas and advanced adenomas received surgical resection (adenoma, N=4; advanced adenoma, N=4) or polyp removal during colonoscopy (adenoma, N=18; advanced adenoma, N=15) and those with carcinomas received surgical resection (N=12), surgical resection with chemotherapy (N=9), and surgical resection with chemotherapy and radiation (N=5). We focused on the patients with carcinoma and pooled those patients that received chemotherapy with those that received chemotherapy and radiation to improve our statistical power. We did not observe a significant difference in the effect of these treatments on the number of observed OTUs, Shannon diversity, or Shannon evenness (P-value > 0.05). Furthermore, there was not a significant difference in the effect of the treatments on the amount of change in the community structure (P= 1). Finally, the change in the positive probability was not significantly different between the two treatment groups (P=1). Due to the relatively small number of samples in each treatment group, it is difficult to make a definitive statement regarding the specific type of treatment on the amount of change in the structure of the microbiota

Discussion

This study builds upon previous work that demonstrated a role for the microbiota in tumorigenesis and that the microbiota contains biomarkers to diagnosis colonic lesions [5–9,23–27]. Our study focused on comparing the microbiota of patients diagnosed with adenoma, advanced adenoma, and carcinoma before and after treatment. For all three groups of patients we observed changes in their microbiota. The microbiota of patients diagnosed with carcinoma changed significantly more than the other groups. This change resulted in communities that more closely resembled those of patients with a normal colon. For the patients diagnosed with carcinomas there was a larger change between their initial and follow up samples than was observed for patients diagnosed with either adenoma or advanced adenoma. The changes in the community structure of those diagnosed with carcinoma were similar and resulted in communities that more closely resembled those found in patients with normal colons.

Understanding the effect of treatment on the microbiota of those initially diagnosed with carcinomas may have important implications into understanding disease recurrence. The microbiota changes associated with treatment among patients initially diagnosed with carcinomas resulted in community structures that resembled patients with normal colons. This would suggest that treatment for carcinoma is not only successful for removing the carcinoma but also at removing bacteria associated with them. Interestingly, 6 of the 26 patients diagnosed with carcinomas had an elevated probability of having carcinomas after treatment. This leads us to hypothesize that these individuals may be at a high risk of recurrence. Interestingly, the 23.08% prevalence of increased carcinoma probability from our study is within the expected rate of recurrence (20-30% [3,4]), which would support our hypothesis . Unfortunately, because of the design of our study, it was not possible to determine whether individuals who had an increased positive probability for carcinoma experienced recurrence. Regardless, it is intriguing to think of using biomarkers from

the microbiota to not only predict the presence of lesions, but to also assess the risk of developing them.

It is interesting that those patients diagnosed with adenoma and advanced adenoma did not experience a shift towards a community structure that resembled those with normal colons. This may be due to the fundamental differences between the features of adenomas and advanced adenomas and carcinoma. Specifically, carcinomas may create an inflammatory milieu that would impact the structure of the community and removal of that stimulus would alter said structure. In addition, it is possible that the difference between the microbiota of patients with adenoma and advanced adenoma and those with normal colons is subtle. This is supported by the reduced ability of our models to correctly classify patients with adenomas and advanced adenomas relative to those diagnosed with carcinomas [Figure S2]. Given the patchy distribution of microbiota across patients in the different diagnosis groups, it is possible that we lacked the statistical power to adequately characterize the change in the communities following treatment. A final hypothesis is that the specific type of treatment altered the structure of the microbiome. The treatment to remove adenomas and advanced adenomas was either polyp removal or surgical resection whereas it was surgical resection alone or in combination with chemotherapy or with chemotherapy and radiation for individuals with carcinoma. Because chemotherapy and radiation target rapidly growing cells, these treatments would be more likely to cause a turn over of the colonic epithelium driving a more significant change in the structure of the microbiota. Although, we were able to test for an effect across these specific types of treatment, the number of patients in each treatment group was relatively small.

It was not clear why 6 of the 26 patients with carcinoma had a higher probability of having carcinomas after treatment. We hypothesized that these individuals may have had more severe tumors; however, the tumor severity of these 6 individuals (3 with Stage II and 3 with Stage III) was similar to the distribution observed among the other 20 patients. We

also hypothesized that we may have sampled these patients later than the rest and their communities may have reverted to a carcinoma-associated state; however, there was not a statistically significant difference in the length of time between sample collection among those whose probabilities increased or decreased (Wilcoxon Test; P-value=0.56). Finally, it is possible that these patients may not have responded to treatment as well as the other 20 patients diagnosed with carcinoma and so the microbiota may not have been impacted the same way. Again, further studies looking at the role of the microbiota in recurrence are needed to understand the dynamics following treatment.

This study builds upon existing work that has established a role for the microbiota in tumorigenesis and that demonstrated the utility of microbiome-based biomarkers to predict the presence of colonic lesions. The most exciting future direction from the current study is the possibility that markers within the microbiota could be used to evaluate the effect of treatment and predict recurrence for those initially diagnosed with carcinoma. If such an approach is effective, it might be possible to even target the microbiota as part of combined therapies including currently standard approaches. Our data provides additional evidence on the importance of the microbiota in tumorigenesis by addressing the recovery of the microbiota after treatment and opens interesting avenues of research into how these changes may affect recurrence.

Methods

Study Design and Patient Sampling. Sampling and design have been previously reported in Baxter, et al [23]. Briefly, 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 one and two weeks after initial colonoscopy. The bacterial community has been shown to normalize back to a pre-colonoscopy community within this time period [28]. Our study cohort consisted of 67 individuals with an initial sample as described and a follow up sample obtained between 188 - 546 days after treatment of lesion [Table 1]. Patients were diagnosed by colonoscopic examination and histopathological review of biopsies. Patients were classified as having advanced adenoma if they had an adenoma greater than 1 cm, more than three adenomas of any size, or an adenoma with villous histology. 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.

16S rRNA Gene Sequencing. Sequencing was completed as described by Kozich, et al. [29]. 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. The pre- and post-treatment samples were sequenced on the same run.

Sequence Processing. The mothur software package (v1.37.5) was used to process

the 16S rRNA gene sequences and has been previously described [29]. The general workflow using mothur included merging paired-end reads into contigs, filtering for low quality contigs, aligning to the SILVA database [reference], screening for chimeras using UCHIME [reference], classifying with a naive Bayesian classifier using the Ribosomal Database Project (RDP)[reference-Wang], and clustered into Operational Taxonomic Units (OTUs) using a 97% similarity cutoff with an average neighbor clustering algorithm [reference-Westcott&SchlossAEM]. The number of sequences for each sample was rarefied to 10523 to minimize the impacts of uneven sampling.

Model Building. The Random Forest [30] algorithm was used to create the model used to create the three models used. The adenoma model classified normal versus adenoma, advanced adenoma was normal versus advanced adenoma, and carcinoma was normal versus carcinoma. The toal number of individuals in this data set was 423 individuals [Table 1]. There were a total of 239 individuals in the adenoma model, 262 individuals in the advanced adenoma model, and 266 indivdiuals in the carcinoma model [Table 1]. Each model was then applied to our 67-person cohort testing prediction of adenoma intial (adenoma n = 22) versus adenoma follow up (adenoma n = 0), advanced adenoma initial (advanced adenoma n = 19) versus advanced adenoma follow up (advanced adenoma n = 0), carcinoma initial (carinoma n = 26) versus carcinoma follow up (carcinoma n = 1).

The model included only OTU data obtained from 16S rRNA sequencing. 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 making 100 different 80/20 (train/test) splits of the data where normal and adenoma, normal and advanced adenoma, or normal and carcinoma were represented in the same proportion within both the whole data set and the 80/20 split. For each of the different splits, 20 repeated 10-fold cross validation was performed on the 80% component to optimize the mtry hyper-parameter by maximizing the AUC (Area

Under the Curve of the Receiver Operator Characteristic). The resulting model was then tested on the hold out data obtained from the 20% component. All three models had an optimized mtry of 2.

Assessment of the most important OTUs to the model involved counting the number of times an OTU was present in the top 10% of mean decrease in accuracy (MDA) for each of the 100 different splits run. This was then followed with filtering of this list to variables that were only present in more than 50% of these 100 runs. The final collated list of variables was then run through the mtry optimization again. Once the ideal mtry was found the entire sample set specific to normal versus adenoma, normal versus advanced adenoma, or normal versus carcinoma was used to create the final Random Forest model on which classifications on the 67-person cohort was completed. For all three models the final optimized mtry was 2. The hyper-parameter, mtry, defines the number of variables to investigate at each split before a new division of the data was created with the Random Forest model.

Statistical Analysis. The R software package (v3.3.2) was used for all statistical analysis. Comparisons between bacterial community structure utilized PERMANOVA [31] in the vegan package (v2.4.1). Comparisons between probabilities as well as overall OTU differences between pre- and post-treatment samples utilized a paired Wilcoxon ranked sum test. Where multiple comparison testing was appropriate, a Benjamini-Hochberg (BH) correction was applied [32] 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. Model rank importance was determined by obtaining the median MDA from the 100, 20 repeated 10-fold cross validation and then ranking from largest to smallest MDA.

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 Adenoma, Advanced Adenoma, and Carcinoma Groups After Treatment.*. A) A significant difference was found between the adenoma and carcinoma group for thetayc (P-value = NULL). Solid black points represent the median value for each diagnosis group. B) NMDS of the pre- and post-treatment samples for the adenoma group. C) NMDS of the pre- and post-treatment samples for the advanced adenoma group. D) NMDS of the pre- and post-treatment samples for the carcinoma group.

Figure 2: The 10 OTUs used to classify treatment for Adenoma, Advanced Adenoma, and Carcinoma. A) Adenoma OTUs. B) Advanced Adenoma OTUs. C) Carcinoma OTUs.

Figure 3: Treatment Response Based on Models Built for Adenoma, Advanced Adenoma, or Carcinoma. A) Positive probability change from initial to follow up sample in those with adenoma. B) Positive probability change from initial to follow up sample in those with advanced adenoma. C) Positive probability change from initial to follow up sample in those with carcinoma..

Figure 4: Common OTUs to All Models. A) Venn diagram showing the OTU overlap between each model. B) For each common OTU the lowest taxonomic identification and importance rank for each model run is shown.

Table 1: Demographic Data of Pre and Post Treatment Cohort

Table 2: Demographic Data of Training Cohort

Figure S1: Distribution of P-values from Paired Wilcoxson Analysis of All OTUs Before and After Treatment

Figure S2: ROC Curves of the Adenoma, Advanced Adenoma, and Carcinoma Models. A) Adenoma ROC curve: The light green shaded areas represent the range of values of a 100 different 80/20 splits of the test set data and the dark green line represents the model using 100% of the data set and what was used for subsequent classification. B) Advanced Adenoma ROC curve: The light yellow shaded areas represent the range of values of a 100 different 80/20 splits of the test set data and the dark yellow line represents the model using 100% of the data set and what was used for subsequent classification. C) Carcinoma ROC curve: The light red shaded areas represent the range of values of a 100 different 80/20 splits of the test set data and the dark red line represents the model using 100% of the data set and what was used for subsequent classification.

Figure S3: Summary of Important OTUs for the Adenoma, Advanced Adenoma, and Carcinoma Models. A) MDA of the most important variables in the adenoma model. The dark green point represents the mean and the lighter green points are the value of each of the 100 different runs. B) Summary of Important Variables in the advanced adenoma model. MDA of the most important variables in the SRN model. The dark yellow point represents the mean and the lighter yellow points are the value of each of the 100 different runs. C) MDA of the most important variables in the carcinoma model. The dark red point represents the mean and the lighter red points are the value of each of the 100 different runs.

Declarations

Ethics approval and consent to participate

The University of Michigan Institutional Review Board approved this study, and all subjects provided informed consent. This study conformed to the guidelines of the Helsinki Declaration.

Consent for publication

Not applicable.

Availability of data and material

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.

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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