Making Sense of the Noise: Leveraging Existing 16S rRNA Gene Surveys to Identify Key Community Members in Colorectal Tumors

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Abstract

Background. An increasing body of literature suggests that both individual and collections of bacteria are associated with the progression of colorectal cancer. As the number of studies investigating these associations increases and the number of subjects in each study increases, a meta-analysis to identify the associations that are the most predictive of disease progression is warranted. For our meta-analysis, we analyzed previously published 16S rRNA gene sequencing data collected from feces (1737 individuals from 8 studies) and colon tissue (492 total samples from 350 individuals from 7 studies).

Results. We quantified the odds ratios for individual bacterial genera that were associated with an individual having tumors relative to a normal colon. Among the stool samples, there were no genera that had a significant odds ratio associated with adenoma and there were 8 genera with significant odds ratios (ORs) associated with carcinoma. Similarly, among the tissue samples, there were no genera that had a significant odds ratio associated with adenoma and there were 3 genera with significant odds ratios associated with carcinoma. Among the significant odds ratios, the association between individual taxa and tumor diagnosis was equal or below 7.11. Because individual taxa had limited association with tumor diagnosis, we trained Random Forest classification models using only the taxa that had significant ORs, using the entire collection of taxa found in each study, and using operational taxonomic units defined based on a 97% similarity threshold. All training approaches yielded similar classification success as measured using the Area Under the Curve. The ability to correctly classify individuals with adenomas was poor and the ability to classify individuals with carcinomas was considerably better using sequences from stool or tissue.

2

Importance. This meta-analysis confirms previous results indicating that individuals with adenomas cannot be readily classified based on their bacterial community, but that those with carcinomas can. Regardless of the dataset, we found a subset of the fecal community that was associated with carcinomas was as predictive as the full community.

Keywords

3

Background

Colorectal cancer (CRC) is a growing world-wide health problem in which the microbiota has been hypothesized to have a role in disease progression (1, 2). Numerous studies using murine models of CRC have shown the importance of both individual microbes (3–7) and the overall community (8–10) in tumorigenesis. Numerous case-control studies have characterized the microbiota of individuals with colonic adenomas and carcinomas in an attempt to identify biomarkers of disease progression (6, 11–17). Because current CRC screening recommendations are poorly adhered to due to socioeconomic status, test invasiveness, and frequency of tests, development and validation of microbiome-associated biomarkers for CRC progression could further attempts to develop non-invasive diagnostics (18).

Recently, there has been an intense focus on identifying microbiota-based biomarker yielding a seemingly endless number of candidate taxa. Some studies point towards mouth-associated genera such as Fusobacterium, Peptostreptococcus, Parvimonas, and Porphyromonas that are enriched in people with carcinomas (6, 11–17). Other studies have identified members of *Akkermansia*, *Bacteroides*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Mogibacterium*, *Streptococcus*, and *Providencia* are also associated with carciomas (13–15). Additionally, *Roseburia* has been found in some studies to be more abundant in people with tumors but in other studies it has been found to be either less abundant or no different than what is found in subjects with normal colons (14, 17, 19, 20). There are strong results from tissue culture and murine models that Fusobacterium nucleatum, pks-positive strains of Escherichia coli, Streptococcus gallolyticus, and an entertoxin-producing strain of Bacteroides fragilis are important in the pathogenesis of CRC (5, 14, 21–24). These results point to a causative role for the microbiota in CRC pathogenesis as well as their potential as diagnostic biomarkers.

4

Most studies have focused on identifying biomarkers in patients with carcinomas but there is a greater clinical need to identify biomarkers associated with adenomas. Studies focusing on broad scale community metrics have found that measures such as the total number of Operational Taxonomic Units (OTUs) are decreased in those with adenomas versus controls (25). Other studies have identified *Acidovorax*, *Bilophila*, *Cloacibacterium*, *Desulfovibrio*, *Helicobacter*, *Lactobacillus*, *Lactococcus*, *Mogibacterium*, and *Pseudomonas* to be enriched in those with adenomas (25–27). There are few genera that are enriched in patients with adenoma or carcinoma tumors.

Confirming some of these previous findings, a recent meta-analysis found that 16S rRNA gene sequences from members of the *Akkermansia*, *Fusobacterium*, and *Parvimonas* were fecal biomarkers for the presence of carcinomas (28). Contrary to previous studies they found sequences similar to members of *Lactobacillus* and *Ruminococcus* to be enriched in patients with adenoma or carcinoma relative to those with normal colons (12, 15, 16). In addition, they found 16S rRNA gene sequences from members of *Haemophilus*, *Methanosphaera*, *Prevotella*, *Succinovibrio* were enriched in patients with adenoma and *Pantoea* were enriched in patients with carcinomas. Although this meta-analysis was helpful for distilling a large number of possible biomarkers, the aggregate number of samples included in the analysis (n = 509) was smaller than several larger case-control studies that have since been published (12, 27)

Here we provide an updated meta-analysis using 16S rRNA gene sequence data from both feces (n = 1737) and colon tissue (492 samples from 350 individuals) from 14 studies (11–17, 19, 20, 23, 25–27, 29) [Table 1 & 2]. We expand both the breadth and scope of the previous meta-analysis to investigate whether biomarkers describing the bacterial community or specific members of the community can more accurately classify patients as having adenoma or carcinoma. Our results suggest that the bacterial community changes as disease severity worsens and that that a subset of the microbial community can be

5

81 used to diagnose the presence of carcinoma.

Results

Lower bacterial diversity is associated with increased odds ratio (OR) of tumors:
We first assessed whether variation in broad community metrics like total number of
operational taxonomic units (OTUs) (i.e. richness), the evenness of their abundance, and
the overall diversity was associated with disease stage after controlling for study and
variable region differences. In stool, there was a significant decrease in both evenness and
diversity as disease severity progressed from normal to adenoma to carcinoma (P-value
= 0.025 and 0.043, respectively) [Figure 1]; there was not a significant difference for
richness (P-value = 0.21). We next tested whether the decrease in these community
metrics translated into significant ORs for having an adenoma or carcinoma. For fecal
samples, the ORs for richness were not significantly greater than 1.0 for adenoma or
carcinoma (P-value = 0.40) [Figure 2A]. The ORs for evenness were significantly higher
than 1.0 for adenoma (OR = 1.3 (1.02 - 1.65), P-value = 0.035) and carcinoma (OR = 1.66
(1.2 - 2.3), P-value = 0.0021) [Figure 2B]. The ORs for diversity were only significantly
greater than 1.0 for carcinoma (OR = 1.61 (1.14 - 2.28), P-value = 0.0069), but not for
adenoma (P-value = 0.11) [Figure 2C]. Although these OR are significantly greater than
1.0, it is doubtful that these are clinically meaningful values.

Similar to our analysis of sequences obtained from stool samples, we repeated the
analysis using sequences obtained from colon tissue. There were no significant changes in
richness, evenness, or diversity as disease severity progressed from control to adenoma to
carcinoma (P-value > 0.05). We next analyzed the OR, for matched (i.e. where unaffected
tissue and tumors were obtained from the same individual) and unmatched (i.e. where
unaffected tissue and tumor tissue were not obtained from the same individual) tissue
samples. The ORs for adenoma and carcinoma by any measure were not significantly
different from 1.0 (P-value > 0.05) [Figure S1 & Table S1]. This is likely due to the
combination of a small effect size, as suggested from the results using stool, and the

7

relatively small number of studies and size of studies used in the analysis.

Disease progression is associated with community-wide changes in composition

and abundance: Based on the differences in evenness and diversity, we next asked

whether there were community-wide differences in the structure of the communities

associated with different disease stages. We identified significant bacterial community

differences in the stool of patients with adenomas relative to those with normal colons

in 1 of 4 studies and in patients with carcinomas relative to those with normal colons in

6 of 7 studies (PERMANOVA; P-value < 0.05) [Table S2]. Similar to the analyses using

stool samples, there were significant differences in bacterial community structure between

subjects with normal colons and those with adenoma (1 of 2 studies) and carcinoma (1 of

3 studies) [Table S2]. For studies that used matched samples no differences in bacterial

community structures were observed [Table S2]. Combined, these results indicate that

there consistent and significant community-wide changes in the fecal community structure

of subjects with carcinomas. However, the signal observed in subjects with adenomas or

when using tissue samples was not as consistent. This is likely due to a smaller effect

size or the relatively small sample sizes among the studies that characterized the tissue

microbiota.

Individual taxa are associated with significant ORs for carcinomas: Next we

identified those taxa were associated with ORs that were significantly associated with

having a normal colon or the presence of adenomas or carcinomas. No taxa had a

significant OR for the presence of adenomas when we used data collected from stool or

tissue samples (Table S3 & S4). In contrast, 8 taxa had significant ORs for the presence of

carcinomas using data from stool samples. Of these, 4 are commonly associated with

the oral cavity: *Fusobacterium* (OR = 2.74 (1.95 - 3.85)), *Parvimonas* (OR = 3.07 (2.11

- 4.46)), *Porphyromonas* (OR = 3.2 (2.26 - 4.54)), and *Peptostreptococcus* (OR = 7.11

(3.84 - 13.17)) [Table S3]. The other 4 were *Clostridium XI* (OR = 0.65 (0.49 - 0.86)),

134 *Enterobacteriaceae* (OR = 1.79 (1.33 - 2.41)), *Escherichia* (OR = 2.15 (1.57 - 2.95)), and

135 *Ruminococcus* (OR = 0.63 (0.48 - 0.83)). Among the data collected from tissue samples,

136 only unmatched carcinoma samples had taxa with a significant OR. Those included *Dorea*

137 (OR = 0.35 (0.22 - 0.55)), *Blautia* (OR = 0.47 (0.3 - 0.73)), and *Weissella* (OR = 5.15 (2.02

138 - 13.14)). Mouth-associated genera were not significantly associated with an increased

139 OR for carcinoma in tissue samples [Table S4]. For example, *Fusobacterium* had an

140 OR of 3.98 (1.19 - 13.24; however, due to the small number of studies and considerable

141 variation in the data, the Benjimani-Hochberg-corrected P-value was 0.93 [Table S4]. It is

142 interesting to note that *Ruminococcus* and members of *Clostridium group XI* in stool and

143 *Dorea* and *Blautia* in tissue had ORs that were significantly less than 1.0, which suggests

144 that these populations are protective against the development of carcinomas. Overall,

145 there was no overlap in the taxa with significant OR between stool and tissue samples.

146 **Individual taxa with a significant OR do a poor job of differentiating subjects with**

147 **normal colons and those with carcinoma:** We next asked whether those taxa that had

148 a significant OR associated with having a normal colon or carcinomas could be used

149 individually, to classify subjects as having a normal colon or carcinomas. OR values were

150 caluclated based on whether the relative abundance for a taxon in a subject was above

151 or below the median relative abundance for that taxon across all subjects in a study. To

152 measure the ability of these taxa to classify individuals we instead generated receiver

153 operator characteristic (ROC) curves for each taxon in each study and calculated the area

154 under the curve (AUC). This allowed us to use a more fluid relative abundance threshold

155 for defining disease status. Using data from stool samples, the 8 taxa did no better at

156 classifying the subjects than one would expect by chance (i.e. AUC=0.50) [Figure 3A]. The

157 taxa that performed the best included *Clostridium XI*, *Ruminococcus*, and *Escherichia*,

158 however, these had median AUC values less than 0.588. Likewise, in unmatched tissue

159 samples the 8 taxa with significant ORs taxa were marginally better than one would

160 expect by chance [Figure 3B]. The relative abundance of Dorea was the best predictor of

9

carcinomas and its median AUC was only 0.62. These results suggest that although these taxa are associated with a decreased or increased OR for the presences of carcinomas, individually, they do a poor job of classifying a subject's disease status.

Combined taxa model classifies subjects better than using individual taxa: Instead of attempting to classify subjects based on individual taxa, next we combined information from the individual taxa and evaluated the ability to classify a subject's disease status using Random Forest models. For data from stool samples, the combined model had an AUC of 0.75, which was significantly higher than any of the AUC values for the individual taxa (P-value < 0.033). For the full taxa models using stool, *Bacteroides* and *Lachnospiraceae* were the most common taxa in the top 10% mean decrease in accuracy (MDA) across studies [Figure S2]. Of the 3 taxa with significant ORs, all 3 were among the top 10% most important taxa as measured by mean decrease in accuracy, in at least one study. The most important taxa across study within the significant OR taxa only models for stool were *Ruminococcus* and *Clostridium XI* [Figure 5A]. Similarly, using data from the unmatched tissue samples, the combined model had an AUC of 0.77, which was significantly higher than the AUC values for Blautia and Weissella (P-value < 0.037). For the full taxa models using unmatched tissue, *Lachnospiraceae*, *Bacteroidaceae*, and *Ruminococcaceae* were the most common taxa in the top 10% mean decrease in accuracy across studies [Figure S3]. For the singificant OR taxa unmatched tissue models both *Dorea* and *Blautia* were the important based on mean decrease in accuracy [Figure 5B]. Pooling the information from the taxa with significant ORs results in a model that outperforms classifications made using individual taxa.

Performance of models based on taxa relative abundance in full community are better than those based on taxa with significant ORs: Next, we asked whether a Random Forest classification model built using all of the taxa found in the communities would outperform the models generated using those taxa with a significant OR. Similar to

10

our inability to identify taxa associated with a significant OR for the presence of adenomas, the median AUCs to classify subjects as having normal colons or having adenomas using data from stool or tissue samples were only marginally better than 0.5 for any study (median AUC = 0.5486298 [0.3671667 - 0.971]) [Figure 4A & S4A]. In contrast, the models for classifying subjects as having normal colons or having carcinomas using data from stool or tissue samples yielded AUC values meaningfully higher than 0.5 [Figure 4B & S4B-C]. When we compared the models based on all of the taxa in a community to models based on the taxa with significant ORs, the results were mixed. Using the data from stool samples we found that the AUC for 6 of 7 studies increased by an average of -14.8%) and AUC for the Flemer study decreased by 0.54%). The overall improvement in performance was statistically significant (mean = 12.61%, one-tailed paired T-test; P-value = 0.005). Similarly, using the data from unmatched tissue samples we found that the AUC of studies increased by an average of 19.11% when we used all of the taxa rather than the 3 taxa with significant ORs (one-tailed paired T-test; P-value = 0.03). Although the significant OR taxa models can classify indidivuals with and without carcinoma tumors, they are still missing taxa from the full community models that can increase the model accuracy.

Performance of models based on OTU relative abundance in full community are not significantly better than those based on taxa with significant ORs: The previous models were based on relative abundance data where sequences were classified to coarse taxonomic assignments (i.e. typically genus or family level). To determine whether model performance improved with finer scale classification, we assigned sequences to operational taxonomic units (OTUs) where the similarity among sequences within an OTU was more than 97%. We again found that classification models built using all of the sequence data for a community did a poor job of differentiating between subjects with normal colons and those with adenomas (median AUC: 0.53 [0.37- 0.56]), but did a good job of differentiating between subjects with normal colons and those with carcinomas (median AUC: 0.71 [0.5- 0.9]). The OTU-based models performed similarly to those constructed using the taxa

11

with significant ORs (one-tailed paired T-test; P-value = 0.966) and those using all taxa (one-tailed paired T-test; P-value = 0.146) [Figure 4]. Among the OTUs that had the highest mean decrease in accuracy for the OTU-based models, we found that OTUs that affiliated with all of the 8 taxa that had a significant OR were within the top 10% for at least one study. This result was surprising as it indicated that a finer scale classification of the sequences and thus a larger number of features to select from, did not yield improved classification of the subjects.

Generalizability of taxon-based models trained on one dataset to the other datasets: Considering the good performance of the Random Forest models using taxa with a significant OR and using all of the taxa, we next asked how well the models would perform when given data from a different subject cohort. For instance, if a model was trained using data from the Ahn study, we wanted to know how well it would perform using the data from the Baxter study. We found the models trained using the taxa with a significant OR all had a higher median AUC than the models trained using all of the taxa when tested on the other datasets [Figure 6 & S5]. As might be expected, the difference between the performance of the modelling approaches appeared to vary with the size of the training cohort (R^2 = 0.66) [Figure 6]. These data suggest that given a sufficient number of subjects with normal colons and carcinomas, Random Forest models trained using a small number of taxa can accurately classify individuals from a different cohort.

Discussion

Although we expected that the full OTU models would perform the best at classifying individuals with and without carcinomas, our observations suggest that both the full and significant OR taxa models performed equally well. These results suggest that lower level classification to species and strain may not add extra useful information with respect to prediction models. This has been suggested in previous literature where metagenomics did not perform better than 16S rRNA gene sequencing data at classifying individuals with normal colons and those with carcinomas (30). One possible reason as to why lower level classification may not result in better models is that the communities are patchy and higher level taxonomic information pools some of this patchiness, allowing for better prediction models. There may also be a fair bit of data redundancy within models that utilize more of the community. An example of this redundancy would be when we trained models on one study and tested it on the other studies and the AUCs of the models created with the select OR taxa performed as well as full taxa models [Figure 6B].

Our observations also suggest that a small collection of taxa can classify disease as well as full OTU-based models but that these taxa individually perform quite poorly [Figure 3]. This result supports the contention that there might be redundancy of function amongst the taxa included in the significant OR models. As an example, multiple different microbes could be similarily stimulating the activation of inflammatory pathways and by doing so exacerbate disease progression. Multiple reports within the literature have found that different bacteria, such as *Escherichia coli* and *Fusobacterium nucleatum*, can similarly worsen inflammation in mouse models of tumorigenesis (5, 6, 21). Although the inflammatory taxa were patchy in their importance and presence across studies those that were not typically associated with inflammation were consistently important for every study [Figure 6]. The loss of these taxa (*Ruminococcus* and *Clostridium XI* in stool and *Dorea* and *Blautia* in unmatched tissue) is particularly interesting because many are commonly thought to be beneficial due

13

to their involvement in production of short chain fatty acids (31–33).

The adenoma models as a whole performed poorly in classifying individuals with and without adenomas. This outcome is not inconsistent with what has been published previously (27, 34). However, the modeling results are at odds with results obtained in Baxter, *et al.* (12). There are some major differences between the models generated in this meta-analysis and what was used in Baxter, *et al*. First, the prevoius report's models investigated the classification of lesions (individuals with adenoma or carcinoma) and not adenoma alone. The Baxter, *et al.* models also contained Fecal Immunoglobulin Test data while our meta-analysis models only contained 16S rRNA gene sequencing data. Although being able to classify individuals with adenomas is important, the classification of advanced adenomas is a more clinically meaningful diagnostic tool (i.e. those that are at highest risk of progressing to a carcinoma). It is possible that we might have been able to detect differences in the bacterial community if advanced adenomas were separated from adenomas but that data was not available for the majority of studies analyzed. Additionally, the initial changes to the bacterial community could be focal to where the initial adenoma develops and would not be easily assessed with a fecal sample.

Although stool represents an easy and less invasive way to assess risk, it is not clear how well this sample reflects adenoma- and carcinoma- tissue associated microbial communities. The colon tissue-based studies did not provide a clearer understanding of how the microbiota may be associated with tumors. Generally, the full OTU-based models of unmatched and matched colon tissue samples were concordant with stool samples showing that GI resident microbes were the most prevalent in the top 10 most important variables across study [Figure S3]. *Fusobacterium* was not consistently identified across studies and this could be due to both a small number of studies and a small sample size within these studies. The majority of the colon tissue-based results were also study specific with many of the top 10 taxa being present only in a single study. Additionally, the presence

14

of genera associated with contamination, within the top 10 most important variables for the genera and OTU models is worrying (e.g. *Novosphingobium*, *Acidobacteria Gp2*, *Sphingomonas*, etc. (35)). The low bacterial biomass of tissue samples coupled with potential contamination and small sample sizes could explain why these results seem to be more sporadic than the stool results.

One important caveat to this study is that even though genera associated with certain species such as *Bacteroides fragilis* and *Streptococcus gallolyticus* subsp. *gallolyticus* were not identified, it does not necessarily mean that these specific species are not important in human CRC (22, 24). There are reports that *Bacteroides fragilis*, positive for the enterotoxigenic gene, are found at specific locations along the colon but the samples we were able to use in this meta-analysis could not identify these types of differences (36). Additionally, since we are limited in our aggregation of the data to the genus level, it is not possible to clearly delineate which species are contributing to overall disease progression. Our observations are not inconsistent with the previous literature on either *Bacteroides fragilis* or *Streptococcus gallolyticus* subsp. *gallolyticus*. As an example, the stool-based full community models consistently identified the *Bacteroides* taxa to be important model components across studies. This suggests that even though *Bacteroides* may not increase the OR of individuals having an adenoma or carcinoma and may not vary in relative abundance, like *Fusobacterium*, it is still important in CRC.

Despite these limitations to the findings, meta-analyses are a useful tool in microbiota research because they can both validate existing research and make new discoveries by pooling many independent investigations together. Yet, it is still difficult to perform these studies because of inaccessible 16S sequencing data, missing or vague metadata (e.g. which samples are carcioma and which are not), varying sequence quality, and multiple small data sets. Better attention to these specific problems could help to increase the reproducibility and replicability of microbiota studies and make it easier to perform

15

these crucial meta-analyses. Moving forward, meta-analyses will be important tools to help aggregate and find commonalities across studies when investigating the microbiota in the context of a specific disease and more are needed (28, 37–39).

By aggregating together a large collection of studies analyzing both fecal and colon tissue samples, we are able to provide evidence supporting the importance of the bacterial community in carcinoma tumors. Although further validation of the biomarkers presented here need to be undertaken, the replicability of the AUC of a specific collection of taxa across multiple studies suggests a strong potential for the use of the microbiota as a risk stratification tool for individuals with carcinomas.

Methods

Datasets: The studies used for this meta-analysis were identified through the review articles written by Keku, et al. (40) and Vogtmann, et al. (41). Additional studies, not mentioned in those reviews were obtained based on the authors' knowledge of the literature. Studies were included that used tissue or feces as their sample source for 454 or Illumina 16S rRNA gene sequencing. Some studies (N = 12) were excluded because they did not have publicly available sequences or did not have metadata in which the authors were able to share. We were able to obtain sequence data and metadata from the following studies: Ahn, *et al.* (11), Baxter, *et al.* (12), Brim, *et al.* (29), Burns, *et al.* (15), Chen, *et al.* (13), Dejea, *et al.* (20), Flemer, *et al.* (17), Geng, *et al.* (19), Hale, *et al.* (27), Kostic, *et al.* (42), Lu, *et al.* (26), Sanapareddy, *et al.* (25), Wang, *et al.* (14), Weir, *et al.* (23), and Zeller, *et al.* (16). The Zackular (43) study was excluded because their 90 individuals were contained within the larger Baxter study (12). The Kostic study was excluded because after we processed the sequences, all of the case samples had 100 or fewer sequences. The final analysis included 14 studies (Tables 1 and 2). There were seven studies with only fecal samples (Ahn, Baxter, Brim, Hale, Wang, Weir, and Zeller), five studies with only tissue samples (Burns, Dejea, Geng, Lu, Sanapareddy), and two studies with both fecal and tissue samples (Chen and Flemer). After curating the sequences, 1737 stool samples and 492 tissue samples remained in the analysis [Tables 1 and 2].

Sequence Processing: Raw sequence data and metadata were primarily obtained from the Sequence Read Archive (SRA) and dbGaP. Other sequence and metadata were obtained directly from the authors (n = 4, (17, 23, 25, 27)). Each dataset was processed separately using mothur (v1.39.3) (44) using the default quality filtering methods for both 454 and Illumina sequence data. If it was not possible to use the defaults because the sequences were trimmed too much, then the stated quality cut-offs from the original study were used. Chimeric sequences were identified and removed using VSEARCH (45). The

17

curated sequences were assigned to OTUs at 97% similarity using the OptiClust algorithm

(46) and classified to the deepest taxonomic level that had 80% support using the naïve

Bayesian classifier trained on the RDP taxonomy outline (version 14, (47)).

Community analysis: We calculated alpha diversity metrics (i.e. OTU richness, evenness,

and Shannon diversity) for each sample. Within each dataset, we ensured that the data

followed a normal distribution using power transformations. Using the transformed data,

we tested the hypothesis that individuals with normal colons, adenomas, and carcinomas

had significantly different alpha diversity metrics using linear mixed-effect models. We

also calculated the OR for each study and metric by considering any value above the

median alpha diversity value to be positive. We measured the dissimilarity between

individuals by calculating the pairwise Bray-Curtis index and used PERMANOVA (48) to

test whether individuals with normal colons were significantly different from those with

adenomas or carcinomas. Finally, after binning sequences into the deepest taxa that

the naïve Bayesian classifier could calssify the sequences, we quantified the ORs for

individuals having an adenoma or carcinoma and corrected for multiple comparisons using

the Benjamini-Hochberg method (49). Again, for each taxon, if the relative abundance was

greater than the median relative abundance for that taxon in the study, the individual was

considered to be positive.

Random Forest classification analysis: To classify individuals as having normal colons

or tumors, we built Random Forest classification models for each dataset and comparison

using taxa with significant ORs (after multiple comparison correction), all taxa, or OTUs.

Because no taxa were identified as having a significant OR associated with adenomas

using stool samples or tissue samples, classification models based on OR data were

not constructed to classify individuals as having normal colons or adenomas. Within the

training dataset, 10-fold cross validation (5-fold cross validation for small datasets) was

used to build a model using the default mtry setting that was then evaluated on the testing

18

set. For the models constructed using the taxa with significant ORs, the default mtry setting was used to train the model and this model was tested on the other datasets in the meta-analysis. The reported AUC values are the average AUCs for the application of the model on the test sets. For the OTU-based models, hyperparameters were fit splitting the dataset into training (80% of samples) and testing (20%) sets. Within the training dataset, 10-fold cross validation was used to identify the best hyperparameters, which were then used to build a model that was then evaluated on the testing set. The original 80/20 split and fitting was repeated 100 times. The Mean Decrease in Accuracy (MDA), a measure of the importance of each taxon to the overall model was used to rank the taxa used in each model.

Statistical Analysis: All statistical analysis after sequence processing utilized the R (v3.4.3) software package (50). For OTU richness, evenness, and Shannon diversity analysis, values were power transformed using the rcompanion (v1.11.1) package (51) and then Z-score normalized using the car (v2.1.6) package (52). Testing for OTU richness, evenness, and Shannon diversity differences utilized linear mixed-effect models created using the lme4 (v1.1.15) package (53) to correct for study, repeat sampling of individuals (tissue only), and 16S hyper-variable region used. Odds ratios (OR) were analyzed using both the epiR (v0.9.93) and metafor (v2.0.0) packages (54, 55) by assessing how many individuals with and without disease were above and below the overall median value within each specific study. OR significance testing utilized the chi-squared test. Diversity differences measured by the Bray-Curtis index utilized the creation of distance matrix and testing with PERMANOVA executed with the vegan (v2.4.5) package (56). Random Forest models were built using both the caret (v6.0.78) and randomForest (v4.6.12) packages (57, 58). All figures were created using both ggplot2 (v2.2.1) and gridExtra (v2.3) packages (59, 60).

Reproducible Methods: The code and analysis can be found at https://github.com/

398 SchlossLab/Sze_CRCMetaAnalysis_Microbiome_2017. Unless otherwise mentioned, the

399 accession number of raw sequences from the studies used in this analysis can be found

400 directly in the respective batch file in the GitHub repository or in the original manuscript.

Declarations

Ethics approval and consent to participate

Ethics approval and informed consent for each of the studies used is mentioned in the respective manuscripts used in this meta-analysis.

Consent for publication

Not applicable.

Availability of data and material

A detailed and reproducible description of how the data were processed and analyzed for each study can be found at https://github.com/SchlossLab/Sze_CRCMetaAnalysis_ Microbiome_2017. Raw sequences can be downloaded from the SRA in most cases and can be found in the respective study batch file in the GitHub repository or within the original publication. For instances when sequences are not publicly available, they may be accessed by contacting the corresponding authors from whence the data came.

Competing Interests

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

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Authors' contributions

All authors helped to design and conceptualize the study. MAS identified and analyzed the data. MAS and PDS interpreted the data. MAS wrote the first draft of the manuscript and both he and PDS reviewed and revised updated versions. All authors approved the final manuscript.

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Table 1: Total Individuals in each Study Included in the Stool Analysis

Study	Data Stored	Region	Control (n)	Adenoma (n)	Carcinoma (n)
Ahn	DBGap	V3-4	148	0	62
Baxter	SRA	V4	172	198	120
Brim	SRA	V1-3	6	6	0
Flemer	Author	V3-4	37	0	43
Hale	Author	V3-5	473	214	17
Wang	SRA	V3	56	0	46
Weir	Author	V4	4	0	7
Zeller	SRA	V4	50	37	41

Table 2: Studies with Tissue Samples Included in the Analysis

Study	Data Stored	Region	Control (n)	Adenoma (n)	Carcinoma (n)
Burns	SRA	V5-6	18	0	16
Chen	SRA	V1-3	9	0	9
Dejea	SRA	V3-5	31	0	32
Flemer	Author	V3-4	103	37	94
Geng	SRA	V1-2	16	0	16
Lu	SRA	V3-4	20	20	0
Sanapareddy	Author	V1-2	38	0	33

Figure 1: Significant Bacterial Community Metrics for Adenoma or Carcinoma in Stool. A) Adenoma evenness. B) Carcinoma evenness. C) Carcinoma Shannon diversity. Blue represents controls and red represents either adenoma (panel A) or carcinoma (panel B and C). The black lines represent the median value for each repsective group.

Figure 2: Odds Ratio for Adenoma or Carcinoma based on Bacterial Community Metrics in Stool. A) Community-based odds ratio for adenoma. B) Community-based odds ratio for carcinoma. Colors represent the different variable regions used within the respective study.

Figure 3: The AUC of Indivdiual Significant OR Taxa to classify Carcinoma. A) Stool samples. B) Unmatched tissue samples. The larger circle represents the median AUC of all studies and the smaller circles represent the individual AUC for a particular study. The dotted line denotes an AUC of 0.5.

Figure 4: Stool Random Forest Model Train AUCs. A) Adenoma random forest model AUCs between all genera, all OTU, and select model based on significant OR taxa. B) Carcinoma random forest model AUCs between all genera, all OTU, and select model based on significant OR taxa. The black line represents the median AUC for the respective group. If no values are present in the singificant OR taxa group then there were no significant taxa identified and no model was tested.

Figure 5: Most Important Members in Significant OR Taxa Carcinoma Models. A) Common taxa in the top 10 percent for carcinoma Random Forest stool-based models. B) Common taxa in the top 10 percent for carcinoma Random Forest unmatched tissue-based models. Blue represents less important and red represents more important to the Random Forest Model. White means that particular taxa was not in the top 10%.

Figure 6: Stool Random Forest Genus-Based Model Test AUCs. A) Test AUCs of adenoma models using all genera across study. B) Test AUCs of carcinoma models using

34

652 all genera or significant OR taxa only. The black line represents the AUC at 0.5. The red

653 lines represent the median AUC of all test AUCs for a specific study.

Figure S1: Odds Ratio for Adenoma or Carcinoma based on Bacterial Community Metrics in Tissue. A) Community-based odds ratio for adenoma. B) Community-based odds ratio for carcinoma. Colors represent the different variable regions used within the respective study.

Figure S2: Most Common Taxa Across Carcinoma Full Community Stool Study Models. A) Common taxa in the top 10 percent for carcinoma Random Forest all taxa-based models. B) Common taxa in the top 10 percent for carcinoma Random Forest all OTU-based models. Blue represents less important and red represents more important to the Random Forest Model. White means that particular taxa was not in the top 10%.

Figure S3: Most Common Genera Across Full Community Tissue Study Models. A) Common genera in the top 10 percent for matched carcinoma Random Forest all genera-based models. B) Common genera in the top 10 percent for unmatched carcinoma Random Forest all genera-based models. C) Common genera in the top 10 percent for matched carcinoma Random Forest all OTU-based models. D) Common genera in the top 10 percent for unmatched carcinoma Random Forest all OTU-based models. Blue represents less important and red represents more important to the Random Forest Model. White means that particular taxa was not in the top 10%.

Figure S4: Tissue Random Forest Model Train AUCs. A) Adenoma random forest model AUCs between all genera, all OTU, and select model based on significant OR taxa in unmatched and matched tissue. B) Carcinoma random forest model AUCs between all genera, all OTU, and select model based on significant OR taxa in unmatched and matched tissue. The black line represents the median AUC for the respective group. If no values are present in the singificant OR taxa group then there were no significant taxa identified and no model was tested.

Figure S5: Tissue Random Forest Genus-Based Model Test AUCs. A) Test AUCs of adenoma models using all genera across study. B) Test AUCs of carcinoma models using all genera for matched tissue studies. C) Test AUCs of carcinoma models using all genera or significant OR taxa only for unmatched tissue studies The black line represents the AUC at 0.5. The red lines represent the median AUC of all test AUCs for a specific study.