ADDITIONAL MATERIALS

Tumour-associated and non-tumour-associated microbiota: Addendum

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ABSTRACT

In a recent study we reported that tissue-associated microbial Co-abundance Groups (CAGs) were differentially associated with colorectal cancer (CRC). Two of the CAGs, which we named Pathogen CAG and Prevotella CAG, were correlated with a gene expression signature indicative of a TH17 response. A TH17 response has been associated with decreased survival in patients with CRC, and members of the Pathogen CAG such as Fusobacterium nucleatum, Escherichia coli, and Bacteroides fragilis have been repeatedly reported to be associated with CRC development. Thus we hypothesized that the abundance of these CAGs may be associated with poor survival. In this Addendum we extend our analysis of the at-surgery microbiota to microbiota profiles obtained after surgery for CRC which we analyzed in the context of survival data for patients with CRC. Surprisingly we found that high tissue-associated abundance of the previously defined Prevotella- and Pathogen-CAGs at surgery was associated with longer survival. Furthermore, we detected an association of the Bacteroidetes CAG in pre-surgery faecal microbiota with stability of the microbiota after surgery.

KEYWORDS

colorectal cancer; gut microbiota; survival

Results

Survival after surgery may be associated with the microbiota profile at surgery

In the current analysis we tested whether tissue-associated microbiota profiles at surgery were predictive of survival. An overview of the studied individuals follows. The median follow-up time was 1371 days post diagnosis, the minimum follow-up time was 630 days. During the follow-up period 16 individuals died. We obtained microbiota data from at-surgery tumour tissue for 47 individuals. For more patient-related details please see Supplementary Table 1.

We then stratified individuals by their American Joint Committee on Cancer (AJCC) tumour stage and we repeated CAG-clustering of the microbiota for this cohort. As expected, using a Cox proportional hazards model we found that AJCC stage had a strong effect on survival (P = 0.015, Likelihood ratio test of the overall model), with stage IV being particularly discriminative (P = 0.026, HR = 12.44, CI[1.36, 113.57]). Similarly, Kaplan-Meier curves and a log-rank test demonstrated a strong association between AJCC stage and survival (Fig. 1). We then extended our model by including the relative abundance of five tissue-associated bacterial CAGs (see Supplementary Fig. 1 for CAG profiles of the cohort) while adjusting for tumour stage, age, gender, treatment with chemo- and/or radiotherapy and site of the cancer. Surprisingly, Pathogen- and Prevotella-CAG-type microorganisms, which were again detected in this cohort, were associated with longer survival (P = 0.12, HR = 0.8, CI[0.6, 1.06] and P = 0.075, HR = 0.36, CI [0.12, 1.1], respectively). Fig. 2, panel A shows the Kaplan-Meier estimator for individuals stratified by below-median and above-median abundance of the Prevotella-CAG while keeping all other variables constant. The Bacteroidetes CAG was also associated with longer survival (P = 0.078, HR = 0.75, CI[0.58, 1.03]). The only bacterial CAG associated with shorter survival was the Firmicutes CAG 2 (P = 0.17, HR = 1.52, CI[0.84, 2.75]; Fig. 2, panel B),
which we also found to be more abundant in individuals with CRC in our previous study.¹

**Overall microbiota profiles are similar before and after surgery**

If the pre-surgery microbiota is a risk factor or biomarker for CRC, then the post-surgery microbiota might be relevant for relapse or survival. We performed principal coordinate analysis on the faecal microbiota profiles from 28 individuals, measured before and after surgery. The overall microbiota composition did not change to a statistically significant degree after surgery (Fig. 3). Similarly, abundance of only one OTU was statistically significantly different between before and after surgery samples (ANCOM, FDR < .1). Despite these global similarities we observed that the faecal microbiota of several individuals differed strongly after surgery, which was correlated with the overall microbiota profile before surgery (Fig. 4). We then grouped faecal OTUs into CAGs as previously and

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**Figure 1.** AJCC staging is a strong prognostic marker for survival. The graph shows the Kaplan-Meier curves of 67 individuals, stratified below by tumour stage at surgery. Time is in days. Significance values were calculated using a Log-rank test.

**Figure 2.** Tumour-associated microbiota composition may be associated with disease prognosis in CRC. Shown are the Kaplan-Meier curves for individuals stratified by cancer-tissue-associated relative abundance of the *Prevotella*-CAG (A) and Firmicutes CAG 2 (B). All other variables (tumour stage, age, treatment, site) were kept constant. Strata: 1 = below or equal median abundance, n = 24; 2 = above median abundance, n = 23.
again found grouping of bacteria into Firmicutes, Bacteroidetes and Prevotella CAGs (Supplementary Fig. 2), thus resembling enterotypes. Strikingly, the abundance of the luminal Bacteroidetes CAG was significantly negatively correlated with microbiota profile alterations after surgery (Fig. 5, middle panel). Conversely, the collective abundance of bacteria colonizing less than 37% of individuals (which we named Sporadic Colonizers), was positively correlated with change (Fig. 5, left panel). On the level of the bacterial genus, Lachnospira abundance before surgery was associated with microbiota stability (Fig. 5, right panel).

**Conclusions**

It is striking that the mucosal Bacteroidetes CAG was associated with improved survival and the luminal Bacteroidetes CAG was associated with greater stability of the microbiota. These two CAGs both comprised major microbiota components viz. the genera Alistipes and Bacteroides (Supplementary Fig. 1 and Supplementary Fig. 2). Thus it is tempting to speculate that the stability of the microbiota after surgery is also associated with improved survival. Unfortunately it was not possible for us to directly test this hypothesis because re-sampling of the microbiota after surgery obviously depends on patient survival. Future strategic sampling of faecal samples at pre-defined time-points after surgery will enable testing of this hypothesis.

Our current finding that microbiotas of the Prevotella- and Pathogen-CAGs were associated with improved survival was not expected. We previously found correlations of these microbiotas with the expression of host genes associated with an inflammatory response indicative of a TH17 response. Tosolini et al. reported reduced survival for individuals with CRC that had an increased

**Figure 3.** A CRC patient’s faecal microbiota after surgery is similar to their microbiota before surgery. Shown is the PCoA based on the unweighted (top left panel) or weighted (top right panel) UniFrac distance and the Bray-Curtis distance (bottom left panel). P-values were determined using PERMANOVA. after: faecal microbiota of samples collected after surgical resection; before: faecal microbiota of samples collected before surgical resection.
Figure 4. The faecal microbiota profile before surgery correlates with stability of the microbiota after surgery. The plot shows the PCoA based on the Bray-Curtis distance. Circles: faecal microbiota before surgery. Arrow heads: faecal microbiota after surgery. The Bray-Curtis distance of the samples after surgery from their pre-surgery counterpart is smaller for samples in the left-bottom corner of the PCoA. Pearson’s correlation coefficient $r$ and $P$-values are given for the correlation between distance of samples after surgery from their pre-surgery counterpart and location of the pre-surgery sample on the PCoA. The colour of the arrows and the circles is associated with the Bray-Curtis distance of the sample after surgery from the sample before surgery (log-scale), as per the colour scale to the right.

Figure 5. The stability of the faecal microbiota after surgery is positively associated with the pre-surgery abundance of sporadic colonizers and negatively associated with the Bacteroidetes CAG and genus *Lachnospira* abundance. Graphs are the scatter-plots of the Bray-Curtis distance of samples after surgery compared to their pre-surgery sample (x-axis) and the combined centered log-ratio, pre-surgery abundance (y-axis) of bacteria found in less than 37% of individuals (i.e. bacteria which only sporadically colonize the human gut; panel to the left), of the Bacteroidetes CAG as defined in Supplementary Figure 2 (middle panel) and the genus *Lachnospira* (panel to the right). OTUs of this genus were mainly found in the Bacteroidetes CAG and had the highest negative correlation of all single genera with microbiota change after surgery. Linear regression trendlines are displayed.
TH17 response. Furthermore, *F. nucleatum*, one of the members of the Pathogen CAG, has been linked with shorter survival and recurrence after chemotherapy. More comprehensive testing of the inflammatory response in our sample set may yet reveal a rather general activation of the immune system associated with elevated levels of the Pathogen- and *Prevotella*-CAGs which in turn may positively affect cancer-related survival.

We concede that our current findings are preliminary because we could not adjust for factors such as specific tumour genotypes (e.g. microsatellite instability which has been linked with improved disease outcome), and the fact that many of the studied individuals have been followed up for less than two years. Larger and more long-term studies are currently under way at the APC Microbiome Institute.

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**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Author contributions**

BF: Study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis; study supervision. MH: Acquisition of data; analysis and interpretation of data. MOR: Study concept and design and acquisition of data. FS: Study concept and design; drafting of the manuscript; critical revision of the manuscript for important intellectual content; obtained funding; study supervision. POT: Study concept and design; drafting of the manuscript; critical revision of the manuscript for important intellectual content; obtained funding; study supervision.

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