LETTER

Characterization of intratumor microbiome in cancer immunotherapy

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Received: March 23, 2023; Accepted: July 10, 2023; Published Online: July 12, 2023; https://doi.org/10.1016/j.xinn.2023.100482

Citation: Zhang Z., Gao Q., Ren X., et al., (2023). Characterization of intratumor microbiome in cancer immunotherapy. The Innovation **4(5)**, 100482.

Dear Editor,

As an illuminating cancer hallmark, polymorphic microbiomes profoundly impact cancer phenotypes by promoting or repressing cancer initiation and progression.[1] Diversity and composition in the gut microbiome are significantly associated with the response rate of anti-PD1 immunotherapy in melanoma.[2] In addition to the gut microbiome, a large number of microbiomes colonizing in human tumors have been shown to play significant roles in cancer development.[3] However, a comprehensive understanding of intratumor microbiomes in cancer immunotherapy is lacking, largely due to the challenge of investigating intratumor microbiomes in anti-cancer immunotherapy.

We obtained 1,296 intratumor microbiome genera across samples from The Cancer Genome Atlas (TCGA), which were classified into 303 families and 151 orders in a previous study.[4] We capitalized on this comprehensively filtered data by Poore et al., obtaining normalized intratumor microbial abundance data that have been filtered for contaminant species (for 32 tumor types from the online data repository: ftp://ftp.microbio.me/pub/cancer_microbiome_analysis). We investigated associations (Spearman correlation with false discover rate [FDR] to adjust p value) between microbiome abundance and the expression of 40 immune checkpoints[5] across cancer types and observed 14,542, 3,440, and 1,660 associations at the genus level, family level, and order level, respectively ($|Rs| > 0.2$ and $FDR < 0.05$). We also used four methods, TIMER, ImmuCellAI, CIBERSORT, and GSVA, to estimate the abundance of tumor-infiltrating immune cells in the tumor microenvironment and investigated associations between microbiome abundance and immune cells (Spearman correlation with FDR). In total, we observed 34,674 significant genus-level associations between the microbiome and immune cells (5,167 for TIMER, 7,749 for ImmuCellAI, 1,861 for CIBERSORT, and 19,897 for GSVA), 3,561 significant family-level associations (689 for TIMER, 861 for ImmuCellAI, 320 for CIBERSORT, and 1,691 for GSVA), and 1,758 significant order-level associations (345 for TIMER, 430 for ImmuCellAI, 170 for CIBERSORT, and 813 for GSVA) ($|Rs| > 0.2$ and $FDR < 0.05$). These analyses provide a comprehensive landscape of associations between the intratumor microbiome and immune features.

To further investigate potential roles of intratumor microbiomes in cancer immunotherapy, we collected objective response rate (ORR) data from real-world datasets across 16 cancer types.[6] We then divided these cancer types into ORR-high (CESC, LUAD, BLCA, KIRC, SKCM, LUSC, LIHC, and CRC) and ORR-low (HNSC, UVM, SARC, BRCA, MESO, GBM, STAD, and OV) groups and detected the abundance alteration of each microbiome (with the most stringent filtering data) between the two groups (Wilcoxon method, consider $FDR < 0.1$ as significance). We observed 50 genera, 17 families, and 9 orders that were significantly associated with ORR. Furthermore, we developed a user-friendly data portal, the Intratumor Microbiome for ImmunoTherapy (IMIT, https://hanlab.tamhsc.edu/IMiT or https://hanlaboratory.com/IMiT). IMIT has four well-organized modules, intratumor microbiome, immune checkpoints, infiltrating immune cells, and patient ORR. In each module, users can enter "microbiome," "immune checkpoints," or "immune cell type" to query microbiome-associated immune features. For example, when the user selects BRCA and enters *Desulfonatronospira* and then clicks to search in the microbiome module, IMIT will list six tables regarding immune checkpoints associated with *Desulfonatronospira*, associated immune cells across four methods, and different levels of abundance of *Desulfonatronospira* between ORR-high and ORR-low groups.

Using this data resource, we were able to identify intratumor microbiomes that may contribute to cancer immunotherapy. We first calculated the diversity of intratumor microbiomes and observed significantly higher diversity in the ORR-high group compared with the ORR-low group (Wilcoxon test, $p = 0.028$), which aligns well with a previous study that reported that a higher diversity of gut microbiomes may lead to better treatment response rates,[2] with the caveat that this is the first such observation regarding the intratumor microbiome. More importantly, there are 11 significant genera that show significantly altered abundance between the ORR-high group vs. the ORR-low group (Figure 1A). Among them, four genera, including *Eudoraea* (Wilcoxon test, FDR = 0.066) and *Desulfonatronospira* (Wilcoxon test, FDR = 0.092), have significantly higher abundance in the ORR-high group. Alterations of abundance at the family level and the order level of these genera demonstrated similar patterns. We collected two independent datasets with immune checkpoint blockade (ICB)-treated melanoma patient samples, from GSE91061 (n = 109) and PRJEB23709 (n = 91), to further investigate alterations in the microbiome in cancer immunotherapy and observed that 18 and 3 genera showed significant increases in patients who benefited from the treatment compared to patients who did not benefit from the treatment, respectively (Figure 1B). Among these, *Eudoraea* (Wilcoxon test, $p = 0.044$, $p = 0.028$) and *Desulfonatronospira* (Wilcoxon test, $p = 0.042$, $p = 0.028$) showed significant increase in patients who benefited from the treatment, suggesting that increasing abundance of these two genera in tumors may improve the outcome of ICB treatment.

To characterize the effects of intratumor microbiomes in cancer immunotherapy, we further applied *Eudoraea* (bought from Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ, product number 19308], and the genomic scaffold was available in GenBank [https://www.ncbi.nlm.nih.gov/nuccore/KB907546.1]) in combination with anti-PD-1 antibody in a mouse model of B16F10 melanoma. Lab mice were injected subcutaneously with B16F10 tumor cells (5×10^5). After 1 week, the mice were randomized into six groups with different treatments: control (IgG + PBS), anti-PD-1 (anti-PD-1 + PBS), *Eudoraea* (IgG + *Eudoraea*), combination of anti-PD-1 and *Eudoraea* (anti-PD-1 + *Eudoraea*), *E. coli* (IgG + *E. coli*), and combination of anti-PD-1 and *E. coli* (anti-PD-1 + *E. coli*) (Figure 1C). *E. coli* was a well-studied nonpathogenic bacterium that would not result in severe side effects in mice[7] and was not significantly associated with ORR at all levels (FDR = 0.44 in the genus *Escherichia*, FDR = 0.57 in family *Enterobacteriaceae*, and FDR = 0.57 in the order *Enterobacteriales*), so we used *E. coli* as a control to show that *Eudoraea* is one specific genus in improving ICB treatment. Different strategies were used to treat the mice on days 5, 9, 12, and 15, and tumors were captured on day 17. We observed a significant decrease in tumor size from the combined treatment of anti-PD-1 and *Eudoraea* compared with other treatments, i.e., compared to the anti-PD-1 group ($p = 0.028$ in size and $p = 0.046$ in weight, Figures 1C and 1D), suggesting that *Eudoraea* could enhance anti-PD-1 immunotherapy in melanoma. Treatment with

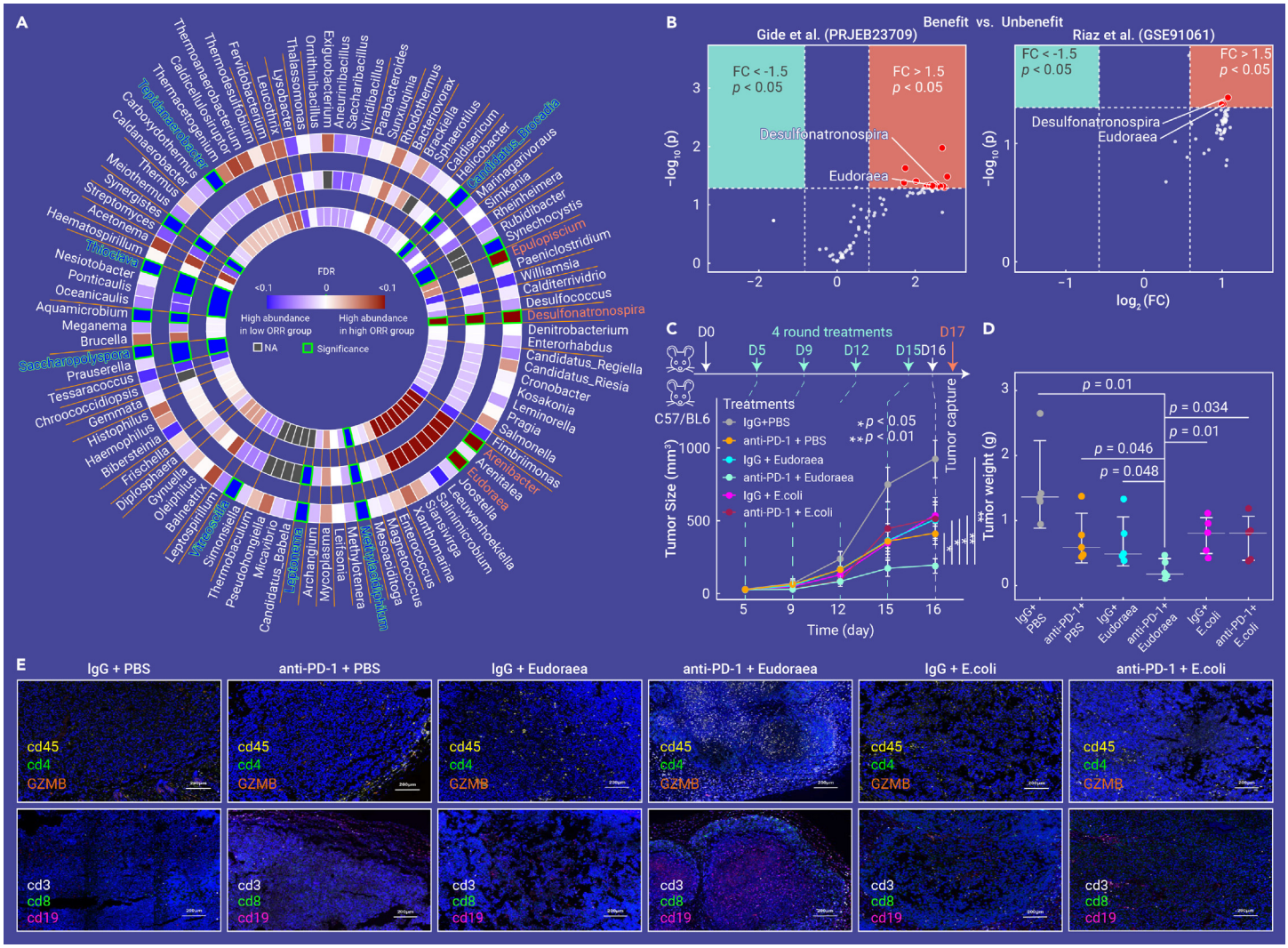

Figure 1. Comprehensive characterization of associations between intratumor microbiomes and cancer immune features and functional role of intratumor microbiome in immunotherapy (A) Associations between intratumor microbiome abundance and ORR at genus level (outer circle), family level (middle circle), and order level (inner circle). (B) Significantly altered intratumor microbiome in patient samples between patients who benefit compared to patients who do not benefit from the immunotherapy treatment, from two independent datasets. (C) Workflow of the combination treatment of *Eudoraea* and anti-PD1 in a melanoma mouse model (upper) and tumor size at different time points across treatment groups (bottom). B16F10 cells (5×10^5) were injected subcutaneously in a volume of 100 μL medium into 6-week-old female C57BL/6 mice. 1 week later, mice were randomly divided into different groups (n = 5 in each group). Tumor volume was measured using digital calipers, and tumor growth was monitored every other day till the tumor size reached the endpoint. When we observed the efficacy of microbiota treatment, *Eudoraea* treatment group (1.5×10^7 colony forming unit [CFU]) and *E. coli* treatment group (1.5×10^7 CFU), microbiota solution was injected intratumorally in a volume of 50 μL. Meanwhile, mouse- PD-1 mAb or IgG isotype control was utilized in mice to test whether *Eudoraea* enhanced the effect of PD-1 mAb therapy. Error bars denote standard error. (D) Tumor weight on day 17 across treatment groups. Error bars denote standard error. (E) Immunofluorescence staining of immune cell (CD45), T cell (CD3), CD4 T cell (CD4), CD8 T cell (CD8), cytotoxic T lymphocyte cell (GZMB) and B cell (CD19) across treatment groups. The antibody panel was stained in the following order, with antibody stripping between positions. Each primary antibody was incubated for 60 min, followed by 10 min incubation with a secondary antibody, followed by the application of the tertiary TSA-amplification reagent for 10 min. Subsequently, slides were stained with Spectral DAPI for 5 min, rinsed, and mounted with coverslip with Prolong Gold Antifade reagent. After curing for 24 h at room temperature in the dark, images were acquired on a Vectra Polaris automated quantitative pathology imaging system.

Eudoraea alone or in combination with anti-PD-1 did not result in significant changes in spleen size and body weight, suggesting limited toxicity of treatment with *Eudoraea*. Furthermore, treatment with *E. coli* did not significantly enhance anti-PD-1 immunotherapy, suggesting that the enhancement of immunotherapy resulted from a specific genus, e.g., *Eudoraea* in our study.

To investigate the effect of different treatments on the tumor microenvironment, we performed an immunofluorescent staining assay and observed significantly increased immune cell abundance for the combined treatment of anti-PD-1 and *Eudoraea* compared to other groups (Figure 1E). In particular, that combination treatment strikingly increased the percentage of infiltrating $CD8^+$ T cells (ANOVA test, $p = 2.6 \times 10^{-5}$) and cytolytic T cells (ANOVA test, $p = 4.7 \times 10^{-8}$). We also used flow cytometry assay to investigate alterations in tumor-infiltrating immune cells and observed significant increases in the percentage of immune cells in the tumor (CD45+/all, ANOVA test, p = 0.0011), percentage of $CD8^+$ T cells in the T cells ($CD8^+/CD3^+$, ANOVA test, $p = 4.0 \times 10^{-4}$), and percentage of cytolytic T cells in the $CD8^+$ T cells ($GZMB^+/CD8^+$, ANOVA test, p = 0.048) for the combination of anti-PD-1 and *Eudoraea*. These results suggest that *Eudoraea* may improve immunotherapy by increasing the active immune cells in the tumor microenvironment. We performed RNA-seq (raw sequencing data deposited in NCBI's Gene Expression Omnibus with accession number GSE205896) to further understand the regulation of *Eudoraea* in cancer immunotherapy. Using ImmuCellAI to impute infiltrating immune cells based on gene expression, we observed results consistent with those achieved by immunofluorescent staining and flow cytometry. We detected 458 upregulated and 10 downregulated genes in the combination therapy of anti-PD-1 and *Eudoraea* vs. anti-PD-1. Gene set enrichment analysis showed significant enrichment of gene alterations in immune-related pathways, including interferon gamma response and Il2 STAT5 signaling, which suggests that *Eudoraea* may increase active immune cells by enhancing immune pathways.

Overall, we comprehensively investigated associations between intratumor microbiomes and immune features, including ORR, expression of immune checkpoints, and abundance of infiltrated immune cells, for each cancer type. We also developed a user-friendly data portal, IMIT, which will be a useful resource for understanding the impact of intratumor microbiomes in cancer

immunotherapy. The microbiome in TCGA cohort was calculated by a robust statistical framework to measure and mitigate the potential effects of contamination and used a series of strategies to eliminate potential confounder factors,[4] and the related data have been used in a significant number of following studies. Utilizing this valuable resource, we identified multiple genera that may enhance cancer immunotherapy. We also observed qualitatively similar results by analyzing several other independent datasets (GEO: GSE91061 and PRJEB23709, Figure 1B), which further reinforcing our findings. It is very unlikely that we would observe a similar pattern if it were driven by confounding factors such as low read counts and/or contaminants. Release of large ICB cohorts across different cancer types would provide more opportunity to identify more intratumor microbes and cancer-type-specific microbes in improving immunotherapy in the future. We further demonstrated that the combination treatment of anti-PD-1 with *Eudoraea* could significantly enhance the outcome of immunotherapy in the *in vivo* mouse model, potentially through the activation of $CD8^+$ T cells and cytolytic T cells. We used specific pathogen-free mice for experimental characterizations, as described in a previous study.[7] Furthermore, accumulated evidences revealed that microbiome would impact response to cancer immunotherapy,[8] while the gnotobiotic models, e.g., animals that either were born and raised in germ-free condition or treated by antibiotic, have a significant decrease of gut microbiomes,[9] which may cause other confounding factors in investigating functional roles of microbiome, e.g., *Eudoraea*, in cancer immunotherapy. There are significant challenges in the characterization of microbiome, especially low-biomass microbiomes (e.g., intratumor microbiomes). Ideally, confirmation of the taxa in the tumor is necessary, but it is extremely challenging.[10] However, our work focuses on identifying microbiomes to promote immunotherapy, which will be insightful and clinically meaningful even if the identified microbiomes are not endogenous. From a clinical perspective, factors, even exogenous ones, that could enhance the efficacy and/or reduce the toxicity should be considered to maximize the benefits for patients. Furthermore, identifying intratumor microbiomes that may serve as biomarkers in predicting the outcomes of immunotherapy would be a great advance, but this will require significant effort, e.g., a large number of patient samples with immunotherapy. Recent studies also quantified fungi and viruses across human cancers. It would be interesting to further investigate their functional roles in immunotherapy. Taken together, these results demonstrated the power of rigorous analysis of large-scale data, including both omics data and real-world data, to accelerate progress in anti-cancer therapy, in particular regarding the functional significance of intratumor microbiomes in cancer immunotherapy.

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ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program of China (2019YFE0120800, 2019YFA0111600), the Natural Science Foundation of China for outstanding Young Scholars (82022060), the National Natural Science Foundation of China (81902149, 81874242, 31800979, 82073145), the Natural Science Foundation of Hunan Province for outstanding Young Scholars (2019JJ30040), Talent Young Scholars of Hunan Province (2019RS2009), Natural Science Foundation of Hunan Province (2020JJ5892), and the Shanghai Pujiang Program (21PJ1401700), the Natural Science Foundation of Hunan Province for outstanding Young Scholars (2019JJ30040), and Talent Young Scholars of Hunan Province (2019RS2009). We regret that page limitations have prevented us from including all the relevant studies in this letter.

DECLARATION OF INTERESTS

The authors declare no competing interests.