Clearance of Circulating Bacteria and Recovery of Microbiota Diversity Following Adoptive Cellular Therapy in Advanced Colorectal Cancer Patients

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Abstract

Objective: There is increasing evidence of a human blood-microbiome that may have an immune modulatory effect. Anticancer therapies including adoptive cellular therapy (ACT) may influence this blood microbiome. The purpose of this study was to investigate impacts of biological ACT infusions on the blood microbiota.

Materials and Methods: Plasma was collected from colorectal cancer patients (CRC) treated with either chemotherapy alone (oxaliplatin and capecitabine) (XELOX alone group, CT, n=19), or a mixed dendritic cell/cytokine induced killer cell product (DC-CIK) + XELOX (group, ICT, n=20). Circulating microbiota analysis was performed by amplifying and sequencing variable regions V3~V4 of bacterial 16S rRNA genes. The association of microbiota with clinical immunotherapeutic response was evaluated.

Results: The plasma bacterial DNA copy number decreased and diversity of plasma microbiota significantly increased after administration of the DC-CIK product. The plasma bacterial DNA copy number correlated with the CD3⁺/CD16⁺/CD56⁺ NK cells in circulation. The diversity index was associated with CD3⁺/CD16⁺/CD56⁺ and CD8⁺/CD28⁺ cells infused. A more diverse blood microbiome including *bifidobacterium*, *lactobacillus* and *enterococcus* was identified among responders to immunotherapy compared with non-responders.

Conclusions: Cellular immunotherapy can affect the plasma microbiota's diversity in a manner favorable to clinical responses. Sustainable bacteria diversity was associated with benefit from immune therapy in CRC patients.

Background

Immunotherapy alone or in combination with other standard anti-cancer therapies has gained widespread use across many malignancies(1). For malignancies with a limited endogenous cellular immune response, delivery of cellular products such as autologous T cells collected by apheresis may restore impaired anti-tumor immunity(2). One of such adoptive cellular immunotherapy (ACT) termed DC-CIK, a combination of ex vivo generated, autologous dendritic cells (DC) and cytokine-induced killer cells (CIK) has become widely used in China for cellular immunotherapy due to rapid and simple processes for production and the non-MHC-restricted function of the product (3–5). Previous studies have demonstrated the effectiveness of DC-CIK infusions in patients with solid tumors including lung, breast, gastric, pancreatic, hepatocellular and renal cell carcinomas, and glioblastomas (5).

The role of the microbiome of the gastrointestinal tract, mouth, skin, and vagina in health and disease has been well established, but more recently, it has been observed that the gut microbiome also affects the anti-tumor efficacy and toxicity of immune checkpoint inhibitors (ICIs) (6–9). Specifically, the composition and diversity of gut microbiota varies between clinical responders and no-responders (10). Pre-clinical mouse models suggest that gut microbiota can modulate tumor responses to adoptive T cell therapy (11, 12) as well; however, there is little data regarding the interaction of adoptive cell therapy and

the microbiome of cancer patients. We therefore wished to study the interaction of the microbiome and immune response of CRC patients undergoing ACT with DC-CIK.

Although the gut microbiome would be attractive to study, we reasoned that because the adoptively transferred cells traffic intravenously to sites of malignancy, they would interact with the microbiome more directly in the bloodstream. Although the bloodstream is often thought to be “sterile”, recent data suggests the presence of a bloodstream microbiome as well (13). The blood microbiome may also modulate clinical activity of immunotherapies (14). Therefore, we investigated whether there was an association between the bloodstream microbial composition and the efficacy of DC-CIK therapy in colorectal cancer patients.

Materials And Methods

Setting and participants

This was a retrospective cohort study based on Clinical Trial NCT01906632. The study was approved by the Ethics Committee of Beijing Shijitan Hospital (2019KYLS(51)). All patients gave written informed consent for participation. From January 1, 2014 to January 31, 2019, 39 colorectal cancer patients hospitalized in Beijing Shijitan Hospital, Capital Medical University Cancer Center, who met the following criteria were enrolled in this study: treatment with 2 cycles of standard capecitabine/oxaliplatin chemotherapy (CT group, n=19) or 2 cycles of capecitabine/oxaliplatin chemotherapy combined with 2 cycles of DC-CIK therapy (Immunochemotherapy (ICT) group, n=20). (as previously described (14)). Patients who had received prior immunotherapy or chemotherapy within 8 weeks before the first cycle of XELOX were not included.

Clinical and laboratory data were extracted from the hospital electronic medical record and included: age; sex; colorectal cancer stage; diabetes and hyperlipidemia (which was defined as cholesterol and/or triglycerides higher than normal level before the first cycle of XELOX); results of CEA, CA19-9, CA-153, and CA-125 before therapy; phenotypes of lymphocytes before therapy and after therapy; results of clinical assessment after 2 cycles of XELOX or XELOX combined with 2 cycles of DC-CIK. Responses were assessed by RECIST1.1. Patients were deemed to have clinical benefit if they had a CR (complete response), PR (partial response), or SD (stable disease) and these patients were considered responders (R). They were considered non-responders (NR) if they had progressive disease (PD). Characteristics of all patients before therapy are detailed in Table 1. Study flow is presented in Figure S1).

Sample collecting and preservation

Time points of sample collection are shown in Figure S1. Fasting venous blood was collected in K2 EDTA tubes (BD Vacutainer) and centrifuged at 1500rpm for 20 minutes to isolate the plasma fraction, which was placed in DNA-free, sterile collecting tubes and stored frozen at -80°C until further analysis.

DNA extraction and microbiota analysis

DNA extraction

All microbial DNA isolation procedures were performed in a biological flow cabinet and all consumables used were sterile. QIAamp UCP Pathogen Mini Kit (Cat No. 50214, Qiagen Inc, Venlo, Netherlands) was used to isolate microbial DNA from plasma following the manufacturer's instructions. DNA was quantified with a Qubit Fluorometer by using the Qubit® dsDNA BR Assay kit (Invitrogen, USA) and the quality was checked by running aliquots on 1% agarose gels.

Quantification of total bacterial 16S DNA gene copy number

Total bacterial 16S DNA gene copy number in plasma was quantified using TaqMan qPCR as described previously (15). Extraction blanks (DNA-free water) served as environmental DNA contamination controls.

Bacterial 16S rRNA sequencing

The plasma microbial composition and diversity were assessed by real-time full-length 16S rRNA gene sequencing (BGI Genomics, Shenzhen, China). All samples were coded before they were sent to BGI so the technicians performing the assays would be blinded to the study group with which a particular sample was associated.

(1) Library construction

Variable regions V3~V4 of bacterial 16S rRNA gene were amplified with degenerate PCR primers, 341F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Forward and reverse primers were both tagged with Illumina pad, adapter and linker sequences. PCR enrichment was performed in 50 µL reaction that contained fusion PCR primer, PCR master mix and 30ng template. PCR cycling conditions were: 94 °C for 3 minutes, 30 cycles of 94 °C for 30 seconds, 56 °C for 45 seconds, 72 °C for 45 seconds and final extension for 10 minutes at 72 °C for 10 minutes. AmpureXP beads were used for PCR product purification and purified product was eluted in Elution buffer. Libraries were qualified with an Agilent 2100 bioanalyzer (Agilent, USA). The validated libraries were sequenced on an Illumina MiSeq platform (BGI, Shenzhen, China) following standard pipelines of Illumina, and generating 2 × 300 bp paired-end reads.

(2) Sequencing and bioinformatics analysis

Raw reads were filtered to remove adaptors, low-quality and ambiguous bases. Then paired-end reads were added to tags using Fast Length Adjustment of Short reads program (FLASH, v1.2.11) (16) to get the tags. With a cutoff value of 97%, the tags were clustered into OTUs using UPARSE software (v7.0.1090) (17). The chimera sequences were detected by comparing with the Gold database using UCHIME (v4.2.40) (18). Using Ribosomal Database Project (RDP) Classifier v.2.2 with a minimum confidence threshold of 0.6, the OTU representative sequences were taxonomically classified. Then they were trained on the Greengenes database v201305 by QIIME v1.8.0 (19). All the Tags were compared back to OTU by

USEARCH_global (20) to get the OTU abundance statistics table of each sample. Alpha and beta diversity were estimated by QIIME (v1.8.0) (19) and MOTHUR (v1.31.2) (21) at the OTU level, respectively. GraPhlan map of species composition was created using GraPhlAn. Linear discriminant analysis effect size (LEfSe) cluster analysis was conducted by LEfSe. Barplot and heatmap and different classification levels was plotted with R package v3.4.1 and Morpheus (<https://software.broadinstitute.org/morpheus/>), respectively.

Statistical analysis

Statistical analysis of patient characters

The non-normal continuous variables were expressed as median (range) and compared using Kruskal-Wallis tests. Categorical variables were compared using chi-square tests.

Statistical analysis for bacteria-related results

Bacterial 16S DNA copy number was compared between different groups using Kruskal-Wallis tests. Alpha diversity (including Chao, Shannon and Simpson) was compared between different groups using the Mann-Whitney test. Pairwise comparisons of taxonomic abundances based on the response to therapy were also conducted using the Mann-Whitney test. The spearman's correlation analysis was used for correlation analysis. The ability of significant bacterial variables in predicting response to ICT was assessed by computing receiver operating characteristic (ROC) curves. All statistical evaluations were carried out using SPSS software (Statistical Package for the Social Science, version 17.0, SPSS Inc.) and GraphPad Prism 5 (Version 5.01, GraphPad Software, Inc.). A value of $p < 0.05$ was considered to be statistically significant.

Results

Bacterial DNA quantity was reduced following immunochemotherapy and negatively correlated with the CD3⁺/CD16⁺/CD56⁺ NK cell proportion in peripheral blood.

All the pre- and post-therapy samples were tested for bacterial 16S gene copy number. Before therapy, the bacterial DNA copies in the plasma of the CT and ICT groups were not significantly different (Fig. 1A).

After one and two cycles of standard chemotherapy, compared with pre-CT, bacterial DNA copies in the samples of post-CT-1 (after 1 cycle of CT) and post-CT-2 (after two cycles of CT) were not significantly different, respectively (Fig. 1B). There were only 18 samples in the post-CT-2 group that were positive for 16S DNA). For the patients who were treated with two cycles of standard chemotherapy and 2 cycles of DC-CIK therapy, significantly lower bacterial DNA copies were found in the peripheral blood samples of the group post-ICT-2 (after two cycles of CT + DC-CIK; $n = 16$) compared with pre-ICT ($p = 0.002$) and post-ICT-1 (after one cycle of CT + DC-CIK; $p = 0.020$. Figure 1C).

Lower amounts of plasma bacterial DNA were associated with higher proportions of CD3⁻/CD16⁺/CD56⁺ cells in peripheral blood as shown by a negative Spearman's correlation both in the CT group ($r=-0.544$, $p = 0.0005$) and the ICT group ($r=-0.783$, $p < 0.0001$). The CD3⁻/CD16⁺/CD56⁺ T cell subset of peripheral blood significantly decreased in post-CT-2 compared with pre-CT ($p = 0.045$) but it did not decrease statistically after therapy in the ICT group (Fig. 1D-G). These data suggest that peripheral blood CD3⁻/CD16⁺/CD56⁺ cells, which are maintained by DC-CIK therapy, may reduce bacteria in the bloodstream.

Enhanced bacterial diversity associated with the number of CIK cells infused.

Due to DNA quantity and quality tested before and after construction of full-length 16S rRNA gene libraries, only pre-CT ($n = 19$), post-CT-1 ($n = 18$), pre-ICT ($n = 20$), post-ICT-1 ($n = 20$) were subjected to next generation sequencing and microbiota statistical analysis (Table S2).

After 1 cycle of therapy, pre-ICT and post-ICT-1 showed significant differences for Shannon ($p = 0.025$) and Simpson ($p = 0.014$) at OTU level (Fig. 2E-F). The Shannon and Simpson index of post-ICT-1 increased compared with that of pre-ICT. In contrast, there was no statistical difference for bacterial diversity between pre-CT and post-CT-1 (Fig. 2A-C).

At the operational taxonomic unit (OTU) level, the Chao, Shannon and Simpson diversity were not significantly different between the CT group and ICT before therapy, respectively, but the Chao ($p = 0.027$), Shannon ($p = 0.001$) and the Simpson ($p = 0.003$) indices were statistically different respectively between the CT group and ICT after therapy (Figure S4).

We also observed that the elevated Shannon index in post-ICT was related to the number of CIK cells infused as shown by a positive Spearman's correlation ($r = 0.577$, $p = 0.008$) and was closely related to the amount of CD3⁻/CD16⁺/CD56⁺ ($r = 0.542$, $p = 0.013$) and CD8⁺/CD28⁺ ($r = 0.473$, $p = 0.035$) cells infused, respectively (Fig. 2G-I).

LEfSe cluster analysis identified that the relative abundance of bacteroidetes at phylum level was different between pre- and post ICT. The relative abundance of bacteroides at the genus level was increased after ICT compared with pre-ICT (Fig. 2G-H), but it was decreased after CT compared with pre-CT (Figure S5).

Baseline highly rich and diverse plasma microbiome identified in responders and higher abundance of bifidobacterium, lactobacillus and enterococcus in pre-ICT-R plasma than in pre-ICT-NR.

To better understand the impact of the plasma microbiota on efficacy of immune therapies, the microbial composition of sub-group pre-R and pre-NR was analyzed.

At the OTU level, the Chao and Shannon result of pre-ICT-R and pre-ICT-NR were statistically different (Chao: $p = 0.011$; Shannon: $p = 0.039$), respectively (Fig. 3A-C) while the bacterial richness and diversity between pre-CT-R and pre-CT-NR was not different significantly (Figure S7A-C).

At the genus level, relative abundance showed distinctive variation in microbial composition based on clinical assessment in the ICT group (Fig. 3D, S6), while in the CT group, there was no clear distinctive profile based on clinical assessment (Figure S7D). We found that the relative abundance of *bifidobacterium*, *lactobacillus* and *enterococcus* at the genus level was significantly higher in pre-ICT-R than in pre-ICT-NR ($p = 0.016$, 0.016 and 0.029 , respectively) and relative abundance of *pseudomonas* ($p = 0.036$) was statistically lower in pre-ICT-R than in pre-ICT-NR (Fig. 3E). There was no significant difference in the abundant of these 4 bacterial subgroups between pre-CT-R and pre-CT-NR (Figure S7D-F). These data suggest that the relative abundance of the different bacteria affects clinical response when ACT is used but this relative abundance does not seem to have an impact on clinical response when only chemotherapy is used.

Discussion

Immunotherapy is now the fourth pillar of cancer therapy and has been increasingly combined with other standard anti-cancer treatments (22, 23). Although single agent immune checkpoint blockade may affect a single aspect of the immune response to malignancy, adoptive cellular immunotherapy has the capability for direct cytotoxicity against cancer cells, for modulation of immunosuppressive T cell populations, and for enhancing recovery of exhausted T cells *in vivo* (24–26). We have previously reported the significant anti-tumor activity of CIK therapy (3, 5) alone and in conjunction with chemotherapy. A number of host and tumor factors govern the efficacy of immunotherapy and in many cases limit efficacy. The gut microbiome, for example, affects responsiveness to immune checkpoint blockade (10). Little is known about the interactions of peripheral blood bacteria with ACT that may affect ACT efficacy. In the present study, the bacterial richness, diversity and composition (at OTU level) in the peripheral blood of colorectal cancer patients was analyzed before and after initiating immunochemotherapy (ICT) to explore the relationship between the blood microbiota and the clinical efficacy of ICT. Bacteria 16S DNA copy number in plasma was also measured pre-and post-therapy in order to understand the effect of ICT on permeability of the gut barrier and bacteremia.

We found that the diversity of plasma bacteria in CRC patients was affected by ICT such that greater diversity of plasma microbiota was found after ICT therapy compared with that before therapy. Greater diversity of microbiota is associated with improved immune responses (27). Less diversity in microbiota can lead to impaired local and systemic immune responses with breakdown of mucosal barriers and incitement of a profound inflammatory state both locally and systemically (28). Further, studies indicate that the anti-tumor immune response may be impaired by disrupting the gut microbiota (29). Thus, the diversity of the microbiome may be positively affected by ICT therapy and may in turn, enhance the efficacy of local and systemic anti-tumor immunity.

The DC-CIK product contains multiple cell types. We observed that the Shannon index in post-ICT was directly related to the number of CIK cells infused and the $CD3^{-}/CD16^{+}/CD56^{+}$ and $CD8^{+}/CD28^{+}$ proportion within the infused CIK. $CD3^{-}/CD16^{+}/CD56^{+}$ cells play a critical role in combating transformed and malignant cells (30, 31). Increased $CD8^{+}/CD28^{+}$ T cells predict better early response to tumor therapy

(32) and are associated with progression-free survival (PFS) and overall survival (OS) time (33). Our results suggest that modifications to increase the number of CIK cells infused and the proportion of these two T cell subtype within the infused cellular product might have a positive effect on the diversity of microbiota.

We also found that the difference of bacterial composition before and after ICT therapy was mainly manifest within the *Bacteroides* subgroup. The relative abundance of bacteroides in plasma of CRC patients increased after ICT compared with pre-ICT while it decreased after CT. Studies have provided evidence that bacteroides are beneficial to human health and play key roles in interacting with host immunity (34). The relative abundance of bacteroides may be increased by ICT and this might contribute to a greater anti-tumor response.

Studies have indicated that gut barrier would be broken by chemotherapy (35–37). In this study, we found plasma bacteria copy numbers increased slightly after 1 cycle of CT or ICT, but after 2 cycles of therapy, plasma bacterial copy numbers were statistically decreased in the ICT group while they were not decreased significantly in the CT group.

Peripheral blood nature killer (NK) cells can localize to the gut and are essential for gut mucosal epithelial cell survival and barrier remodeling (38). We analyzed immune cell subsets in the peripheral blood before and after ICT and found that, after 2 cycles of therapy, the proportion of $CD3^{-}/CD16^{+}/CD56^{+}$ NK cells significantly decreased in the CT group while it did not decrease statistically in the ICT group. Correlation analysis indicated that after therapy, the $CD3^{-}/CD16^{+}/CD56^{+}$ cell subset in peripheral blood was negatively correlated with bacteria copy number, both in the CT and the ICT groups. These data suggest that the $CD3^{-}/CD16^{+}/CD56^{+}$ cells might be increase by ICT and they may have a role in repairing the gut barrier.

It has been confirmed that gut microbiota are associated with outcome following ICI (7, 10, 39, 40). Greater richness and diversity of gut microbiota are found in ICI responders. Pre-clinical mouse models suggest that gut microbiota may modulate tumor response to adoptive T cell therapy (11, 12). In this study, we found plasma microbiota before therapy in ICT responders was significantly different from that in ICT non-responders. A highly rich and diverse plasma microbiota was found in responders to ICT before therapy. In the CT group, the richness and diversity of plasma microbiota was not significantly different between responders and non-responders before therapy. We infer from these results that richness and diversity of microbiota is related to outcome of DC-CIK therapy in CRC patients.

When we analyzed the composition of plasma microbiota according to response to ICT, we found that before therapy, the relative abundance of *bifidobacterium*, *lactobacillus* and *enterococcus* in responders was significantly higher than that in non-responders, respectively, but the relative abundance of these specific bacteria was not statistically different between responders and non-responders in the CT group. It has been demonstrated that specific probiotic strains, including *lactobacillus rhamnosus*, *bifidobacterium longum* and *enterococcus faecium* may influence the patient's response to ICI (7, 10, 39, 40). Indeed the

current study demonstrated a relationship between probiotics and outcome of DC-CIK therapy in CRC patients.

One limitation of this study is that there is no group of DC-CIK therapy alone. After retrospectively analyzing CRC patients hospitalized during the study period, we found only 4 patients treated with DC-CIK alone who were without any anti-tumor therapy within 8 weeks before DC-CIK therapy. Of these, only two had pre-therapy samples satisfactory for microbiota analysis. Therefore, the group of DC-CIK alone was abandoned in subsequent analyses because of the limited number of samples. Nevertheless, we believe that a comparison of patients treated with chemotherapy alone and immunochemotherapy would still provide insight on the impact of the immunotherapy alone.

Mapping and modulating the microbiota to predict and improve therapeutic outcomes are areas of intense study in cancer immunotherapy (43, 44). In this study, we estimated receiver operating characteristic curves (ROC) for *Bifidobacterium* ($p = 0.016$), *Lactobacillus* ($p = 0.013$) and *Enterococcus* ($p = 0.013$) in predicting response to ICT. Follow-up clinical trials are needed to confirm the predictive effect of probiotics on ICT outcomes. Further, the influence on outcomes of DC-CIK by probiotics need to be verified by animal models and large clinical trials.

Conclusion

Recent clinical studies revealed the presence of a bloodstream microbiome and this may modulate clinical activity of immunotherapies (14, 45). This study suggested that cellular immunotherapy might affect the plasma microbiota's diversity in a manner favorable to clinical responses and sustainable plasma bacteria diversity was associated with benefit from immune therapy in CRC patients. We expect this result to facilitate adoptive cellular immunotherapy for cancer.

Abbreviations

ACT: adoptive cellular therapy; CRC: colorectal cancer patients; DC-CIK: dendritic cell/cytokine induced killer cell; ICIs: immune checkpoint inhibitors; CT: chemotherapy; ICT: Immunochemotherapy; NR: non-responders; R: responders; RECIST: Response Evaluation Criteria in Solid Tumors; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; OTU: operational taxonomic unit; PFS: Progression-free survival; OS: overall survival; IQR: Interquartile range; LDA: Linear discriminant analysis; LEFSe: LDA Effect Size; ROC: receiver operating characteristic curves.

Declarations

Ethics approval and consent to participate

This was a retrospective cohort study based on Clinical Trial NCT01906632. The study was approved by the Ethics Committee of Beijing Shijitan Hospital (2019KYLS(51)). All patients gave written informed consent for participation.

Consent for publication

Not applicable.

Availability of data and material

Please contact author for data requests.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DY, XW, XZ, SW, JZ, HY, MAM, AH, JR, and HKL conceived and designed the project and contributed to the interpretation of data. DY, JW, YY, SL and JR contributed to the acquisition and analysis of data. DY, MAM, JR and HKL drafted the manuscript. JR and HKL jointly supervised this work.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

The bacterial DNA quantity of plasma was reduced by therapy in the ICT group. Bacterial 16S DNA baseline in pre-CT (n=19) and pre-ICT (n=20) samples (A). Comparison of plasma quantities of bacterial 16S DNA in paired pre-therapy and post-therapy CT and ICT patients (B, C) (post-CT-2: n=18; post-ICT-2: n=16). Median for 16S DNA copies was indicated per each group. Comparison of CD3⁺/CD16⁺/CD56⁺ T-cell proportion in peripheral blood before and after two cycle of therapy (D, E). Median for proportion of CD3⁺/CD16⁺/CD56⁺ cells was indicated per each group. Spearman's correlation analysis on proportion of CD3⁺/CD16⁺/CD56⁺ cells and bacterial 16S DNA copy numbers (F, G), displaying linear fit curve (solid

line) and 95% CI (dashed line). Kruskal-Wallis test with Dunn's multiple comparison test was used for statistical analysis. (* $p < 0.05$, ** $p < 0.01$). CT, chemotherapy; ICT, Immunotherapy combined with chemotherapy; pre-, before therapy; post-, after therapy; post-therapy-1, after 1 cycle of treatment; post-therapy-2, after 2 cycle of treatment.

Figure 2

Bacterial diversity is increased by ICT and is associated with the number of CIK cells infused. Pairwise comparison of Chao (A, D), Shannon (B, E) and Simpson (C, F) at operational taxonomic unit (OTU) level between pre- and post- therapy in the CT and ICT groups. Shannon ($p=0.025$) and Simpson ($p=0.014$) at OTU level were statistically different respectively between pre- and post- ICT however those were not significantly different in group CT. Graphlan representing linear discriminant analysis effect size results which identified main features that are differentially abundant between pre- and post-therapy in group ICT (G). Median for relative abundance of OTUs at genus level was shown in different pre- and post-ICT groups by bar plots (H). Spearman's correlation analysis on Shannon (post-ICT-1) and the number of CIK cell infused, CD3-/CD16+/CD56+ and CD8+/CD28+ T-cell proportion in CIK infused (I-K), displaying linear fit curve (solid line) and 95% CI (dashed line). Statistical comparisons between groups were made using Mann-Whitney test with $*p<0.05$, $**p<0.01$ and $***p<0.001$. CT, chemotherapy; ICT, Immunotherapy combined with chemotherapy. pre-, before therapy; post-, after therapy; post-therapy-1, after 1 cycle of treatment.

Figure 3

Microbial analysis of pre-ICT samples according to clinical assessment. Chao (A), Shannon (B) and Simpson (C) at operational taxonomic unit (OTU) level according to clinical assessment of group pre-ICT were displayed. Median for relative abundance of OTUs at genus level (D) is shown in R- and NR sub-

groups by bar plots. Median for relative abundance of Bifidobacterium, Lactobacillus, enterococcus, pseudomonas at genus level was displayed by R and NR sub-groups (E). Statistical comparisons between sub-groups were made using Kruskal-Wallis test or Mann-Whitney test with * $p < 0.05$ and ** $p < 0.01$. ICT, Immunotherapy combined with chemotherapy; R, response; NR, no-response; pre-, before therapy.

Supplementary Files

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