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Tumor Microbiome Diversity Influence Papillary Thyroid Cancer invasion

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Abstract

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer with higher incidence, the difficulty considered in PTC therapeutic process could be the property. Therefore, it is so important to know the factors that may indicate the progression course of cancer. In this study, we dissect the PTC intra-tumor microbiota and its role in influencing the tumor progression by 16S RNA sequences. The role of the PTC intra-tumor microbiome composition in 80 PTC patients was explored in this study. Overall substantial abundance of microbiome in PTC tumors from all patients were detected. We found that PTC patients with advanced lesion (T3 or T4) had significantly higher tumor bacteria diversity than the patients with relative mild lesion (T1/T2). Importantly, we identified a signature of three tumor bacterial taxa (Pseudomonas, Rhodococcus, Sphingomonas) highly predictive of PTC tumor invasion status. These data suggest that microbial host factors, independent of the genomic composition of the tumor, may determine tumor behaviors and patients' outcomes. Furthermore, the correlation between specific bacterial genus and thyroid hormones or autoimmune thyroid disease-related antibodies may also indicate the potential contribution of microbiome in the relation between autoimmune thyroid disease or the irregular thyroid function and PTC progression.

Introduction

Thyroid cancer (TC) is the most common malignancy of theendocrine organs[1]; its incidence is increasing faster than any other soli tumor in recent years. Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer, most patient with PTC are curable with 5-year survival of over 95%[2], but more than 3-5% of them still experience recurrence or metastatic disease[3], showing poor prognosis.Therefore, the difficulty considered in PTC therapeutic process could be the propertywhich can result in the destruction of the neoplastic foci and inhibition of the natural course of the disease[4]. Evidences have supported that activating somatic alterations of genes may contributed to the thyroid tumor development and progression[5]. And the molecular subtypes of the diseases may improve the pathological classification and better inform the management of PTC.

Recent studies reported that the commensal microorganisms alterations are strongly associated with the oncogenesis and tumor progression[6].Substantial evidences suggested that the gut microbes may confer susceptibility to certain cancers and may also influence response to therapeutics[7]. Moreover, the altered composition of intra-tumor microbiota have been observed in cancers, such as head and neck squamous cell carcinoma (HNSCC)[8], pancreatic cancer[9], lung cancer[10],urothelial cancer[11], cholangiocarcinoma[12], cervical cancer[13] and breast cancer[14]*et al*,which alsopresenttheireffect in determining tumor behaviors and patients' outcomes independent of the genomic composition of the tumor.

Yet, the link between thyroid cancer and microorganisms has not been well delineated.The germ-free rats, which are raised in sterile conditions and lack gut bacteria altogether, have smaller thyroid glands than conventionally raised rats, suggesting a crucial role for these microbes in thyroid health[15]. It has also

been reported that intestinal imbalances attribute to low thyroid function and thyroid autoimmunity[16]. The gut bacteria support conversion of T4 into T3 thyroid hormonesin the intestine and modulation of both Th1 and Th2 immune responses[17],however characters of gut microorganisms in PTC remained explored. Furthermore,the composition of the human PTC intra-tumor microbiome that may contribute to the natural history of PTC deservestudied.In this study, the bacteria of thyroid cancer tissue were analyzed by 16S RNA sequences, and a deeper understanding of host-microbiome interactions was explored, which might be critical to realization of the full potential of such approaches.

Material And Methods

Study cohort

The study was approved by Institutional Review Board and Human Ethics Committee of Air Force Military medical University (Xi`an, China). Tumor samples from 80 papillary thyroid carcinoma (PTC) patients were obtained from Tangdu Hospital.All PTC patients were newly diagnosed and histologically confirmed to PTC. They were offered participation, and were required to provide informed consent. Written permission was obtained from all the participants.Demographics and clinical variables were collected during the clinic visits.

Sample collection and DNA extraction

The tissue samples were cut into 0.3 cm, and quick-frozen in liquid nitrogen, and then kept in -80℃. DNA was extracted using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's instructions. The concentration of bacterial DNA was measured using Nanodrop 2000 (Thermo Scientific, USA).**16S rRNA gene sequencing**

The hypervariable region V3-V4 of the bacterial 16S rRNA gene were amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R(5'-GGACTACHVGGGTWTCTAAT-3') by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The ampliconswerepurified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega, USA). Purified amplicons were pooled in equimolar and paired-end sequenced (2 ×300) on an Illumina MiSeq platform (Illumina, San Diego,USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Microbial analysis

The data were analyzed on the free online platform of Majorbio Cloud Platform (www.majorbio.com). The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH. Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE (version 7.1, http://drive5.com/uparse/), and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the 16S rRNA database (Silva SSU128) using confidence threshold of

0.7. OTUs with a number of sequences <0.005% of the total number of sequences were removed from the OTU table. After filtering, an average of 45,390 reads per sample was obtained (min: 29,987 max: 81,902). In addition, rarefaction was performed on the OTU table to prevent methodological artefacts arising from varying sequencing depth. α-Diversity was measured by species richness and diversity from the rarefied OTU table. β-Diversity was estimated by computing Bray-Curtis and was visualized with principal co-ordinates analysis. In efforts to dissect possible species for OTUs, we performed MegaBLAST search to align the reads of OTUs against reference sequences in the National Center for Biotechnology Information (NCBI) 16S rRNA database.

Statistical analysis

All statistical analyses were performed on the free online platform of Majorbio Cloud Platform (www.majorbio.com).Wilcoxon rank-sum test and ANOSIM was used for intragroup difference of a-diversity simpson index and PCoA analysis, respectively. And for the comparison of continuous variables, Mann-Whitney U test (Kruskal-Wallis test for more than two groups) was used. For correlation analysis, Spearman's rank test was performed. The relative abundance was arcsine square root transformed. To evaluate the discriminatory ability of the significant genus, operating characteristic curves (receiving operational curve, ROC) were constructed and area under curve (AUC) was calculated.

Results

Tumor microbiome communities are significantly associated with tumor invasion in resected PTC patients

A total of 80 subjects with PTC were recruited. The demographic and clinical characteristics of PTC were shown in Table 1. Bacterial DNA was extracted from surgically resected PTC tumor and taxonomic profiling via 16S rRNA gene sequencing was performed. The tumor microbial diversity wasused to comparesurgically resected patients who were in differentclinicalstage. The tumor microbial diversity was measured using different methodologies (Sobs,Shannon and Simpson indices), α-diversity of tumor microbiota was significantly lowerin PTC of patients in T1/T2 as compared with that of ones in T3/T4, respectively, calculated byShannon andSimpson index (p=0.0309, p=0.0088 respectively Wilcoxon rank-sum test; Figure 1 A),which indicated lower microbiota diversity in T1/T2 PTC patients. The microbiota richness was measured by numbers of observed OTUs (Sobsindices), no significant differences were found between different clinical stages. To extend the understanding of the role of microbiome diversity, we aimed to detect whether phylogenetic relationships existed between the bacterial communities enriched in PTC milieu of group in different stages. Beta-diversity were used to generate a principal coordinate analysis (PCoA) using Bray-Curtis metric distances. (Figure 1 C, D). A overlap of OTUs from different groups was revealed, suggesting that the tumor microbial communities exhibited phylogenetic closeness.

Considering the relationship between PTC intra-tumoral bacterial diversity and clinical stages, the general landscape of the tumor microbiome composition was assessed (Figure 2).The enrichment for specific

bacterial communities at the phylum taxonomic level was revealed (supplementary Figure S1). At the genus level, *Pseudomonas* was considered as the clearadvantage bacteria, whereas *Rhodococcus*, *Ralstonia*, *Acinetobacter* and *Sphingomonas* also present the predominate in PTC intra-tumoral bacterial composition.We next sought to determine if there were differences in the tumor microbiome composition between PTC patients in stage T1, T2, T3 and T4, which revealed the presence of similar communities in PTC patients at different stages (Figure 2 B, C). But obvious difference of the percent of community abundance were also observed (Figure 2 D E).

To further investigate the specific changes of microbiota in tumors of PTC patients of different stages, the relative abundance of taxa was assessed. At the genus level, three genera including *Pseudomonas* (p=0.0017), *Rhodococcus*(p=0.02644), *Sphingomonas*(p=0.0073), displayed different abundance among different stages (Figure 2F and Figure S2 A). *Pseudomonas*spp the most abundant genus in all the groups, was higher in tumorsof PTC patients in stage T1 and T2, when compared to T3 or T4, respectively(p=0.0049 p=0.0138, p=0.010, p=0.0028, respectively. Figure 2F).*Rhodococcus*were also significantly higher in PTC patients in T1 compared to that in T3 (p=0.0032, Figure 2F), and *Sphingomonas*were more abudant in T1 and T2compared to T3, respectively (p=0.0001, p=0.0005, respectively. Figure 2F). Then we used these three genera with higher abundance in T1 and T2 to run area under curve (AUC)-receiver operator characteristic (ROC) analysis. The combination of these 3 taxa (*Pseudomonas, Rhodococcus, Sphingomonas*) resulted in an AUC of 0.82 in the T1_2 and T3_4 (Figure 2 G), while an AUC of 0.9 in the T1 andT4 (supplementary Figure S2 B), which showed the potential ability of the 3-genera microbiome signature to discriminate and influence the PTCtumor invasion status.

The intra-tumor microbial dysbiosis was related to thyroid function

We investigated the effects of the thyroid hormoneson intra-tumor microbiota in PTC patients, which indicated the thyroid function. The association between the thyroid related hormones(FT4, T4、FT3、T3、THS) and microbial abundance were performed by Spearman correlation test. The level of different kind of thyroid hormoneswas related to different microbial genus (Fig 3). Positive relationship was found between FT4 and Neisseria and norank_f__norank_o__Chloroplast, FT3 and Treponema, while negative relationship were showed between FT4 and Klebsiella, T4 and Klebsiella and Escherichia-Shigella, T3 and Granulicatella, TSH and norank_f__norank_o__Clostridia_UCG-014 and Prevotella, respectively. However, all these microbia had relative less richness in PTC intra tumor.

The associationbetweenintra-tumor microbiota and autoimmune thyroid diseases (AITD)-related antibodieswas also investigated, for that the AITD affect the entire metabolism of the human body, and the immunestatus may also contribute to the PTC progression. Autoantibodies against thyroid peroxidase (TPO) and thyroglobulin (TG), charactering the Hashimoto's thyroiditis (HT), and autoantibodies against thyroid stimulating receptors (TSHR), a marker for Graves' disease(GD), were involved here.The relationship between microbial abundance and the level of these three autoantibodies, were assessed (Figure 3 B).The data of level of anti-TSHR antibody, anti-TG antibody and anti-TPO was modulated, as that if the data was <0.3 was set as 0.2, <10 was set as 9, <5 was set as 4.Negative

correlation between the level of anti-TSHRand Klebsiella and Burkholderia-Caballeronia-Paraburkholderia was found. Positive correlation between the level of anti-TG and Sphingomonas, Rhodococcus, Ralstonia, Brevundimonaswas found, while Anaerococcus and Akkermansia were found to be negatively related to the level of anti-TG. Nine genera including UCG-002, *Streptococcus, Parvimonas, Akkermansia, Bacteroides, Haemophilus, Selenomonas, Prevotella* and *Bifidobacterium* showed negative relationship with the level of anti-TPO.

Associations between clinical variables and intra-tumor microbiota

We assessed for potential contributors to microbial diversity, including clinic-pathological features, gender and age. The association of gender bias and intra-tumor microbiome diversity was found in PTC patients. (Fig 4). Higher diversity in the microbiome were observed in females (Fig 4 A), which supposed to have a higher PTC incidence.Of note, *Rhodococcus*,*Ralstonia*, *Chryseobacterium* and *Burkholderia-Caballeronia-Paraburkholderia* were more abundant in female PTC patients than male patients. (uncorrected p=0.0413, 0.0092 and 0.0275, 0.0008, respectively, Wilcoxon rank-sum test, Fig 4 C). Remarkably, the gender-associated genera were the same PTC T1/T2-enriched genera *Rhodococcus*. And higher PTC microbiome diversity might show worse association with PTC. However, there was no significant difference in patients at different age or lymphatic metastasis status with respect to α-diversity, β-diversity (supplementary Fig S3).

Discussion

The role of the PTC intra-tumor microbiome composition was explored in this study. Overall substantial abundance of microbiome in PTC tumors from all patients were detected. We found that PTC patients with advanced lesion (T3 or T4) had significantly higher tumor bacteria diversity than the patients with relative mild lesion (T1/T2). Furthermore, the T1/T2 and T3/T4 groups each had a distinctive tumor microbiome signature with specific bacterial genusthat may indicate the PTC tumorinvasion status.

Recent studies have shown that the intra-tumor microbiome composition confer effect on the patient outcomes[18] and response to cancer therapies[6].Our finding suggested that the PTC tumor microbiome diversity and composition can influence the tumor invasion. We found higher alpha-diversity in the tumor microbiome of PTC patientsof group in stage T3 and T4, importantlywe identified asignature of three tumor bacterial taxa (*Pseudomonas, Rhodococcus, Sphingomonas*) highly predictive of PTC tumor invasion status. These data suggest that PTC tumor microbial host factors, independent of the genomic composition of the tumor, may determine tumor behavior and patient outcomes.

Most of the bacterial communities found in the tumoral milieu are present commonly in the gut microbiome[19], suggesting that potentially bacterial translocation from the gut to the tumor of other site might be occurring. The cross-talk betweenpancreatic adenocarcinoma (PDAC)microbiome composition and gut microbiome has been demonstrated by Riquelme et al[18].Fourphyla of bacteria predominate in the PTC intra-tumor, including *Proteobacteria, Actinomycetes*, *Firmicutes* and *Bacteroides*,which has also been reported as main strains in gut[20]. However, different main dominant strains of intra-tumor

microbiome were found at genus level in PTC when the abundance was set as more than 0.01, compared with gut microbiome, for instance that *Pseudomonas, Rhodococcus, Ralstonia, Acinetobacter, Sphingomonas and Brevundimonas* were only found in PTC tumor but not in gut as reported before[21], even in the gut of TC patients [22].This may account for the change of the environment where the microbiome lives, but the translocation of bacterial from gut to PTC tumor still need further explored.

It is known that thyroid hormone could indicate the thyroid function, and TSH and FT3 levels are higher in thyroid cancer cases.The gut microbes influence thyroid hormone levels by regulating iodine uptake, degradation, and enterohepatic cycling[23]. The intratumor microbes have also been proved to be related to the thyroid hormone here. It has reported that the intra-tumoral bacteria are metabolically active; bacteria often found within tumors can alter the chemical structure of common chemotherapeutic agents, changing their activityand thus their effective local concentration[24]. And the environment formed by the thyroid hormone also affect the microbe abundance. Therefore, the interaction of intra-tumoral bacteria and the thyroid hormone levels may be a contributor to the tumor progression and invasion.

The effect of the tumor microbiota on the Autoimmune Thyroid Diseases (AITDs) -related antibodies were also observed here. The gut microbiota has been proved to be involved in HT and GD[25], which are both AITDsand major causes of hypothyroidism and hyperthyroidism, respectively.However, its role in the AITD response is not entirely clear. Studies in humans reported a higher abundance of *Prevotellaceae* and *Pasteurellaceae* in Graves' disease patients[26]. An decrease in *Bifidobacteria* and *Lactobacillaceae* were also reported in hyperthyroid patients[27].A significant increase in the *Bacteroides* species and a decrease in *Bifidobacterium* in stool samples of patients with HT were observed[28],whereas the abundance levels of *Bacteroides* genera were decreased in HT patients were also reported[29]. *Prevotellaceae,Bacteroides*and *Bifidobacteria*were also showed negative relationship with the level of anti-TPO in this study,which further indicated their immunoregulatory impact.Previous studies have showed that *Lactobacillus* spp. and *Bifidobacterium* spp. may induce antibodies cross reacting with thyroperoxidase and thyroglobulin, owing to molecular mimicry [30]. However, their role in HT and GD remains to be explored. Furthermore, the mechanism that the tumor microbiota modulates the immune system or reverse may improve or impair the immune response against the tumor, and then affect the PTC tumor behavior and outcome.

In conclusion, the PTC tumor microbiome composition and their effect on tumor invasion has been present in this study. The tumor microbiome may interact with the thyroid hormone and autoimmune antibodies to regulate the tumor microenvironment, which may contribute toward the tumor invasion. In addition, high microbiome diversity was observed in female PTC patients and PTC patients in T3/T4, which suggested the microbiome character in the patients with aggressive traits, such as high PTC incidence or advanced PTC tumor.

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Table

Table 1 Clinical Characteristics of PTC cohort Patients

Patients Characteristics	
Gender	
Male	19
female	61
Age(years)	
Median	43.314.92
Range	11-76
Stage	
T1	35
T2	29
T3	10
T4	6
Lymphatic metastasis	
Yes	57
No	23
Adjuvant therapy (Chemo-radiation)	
Yes	0
No	80

Figures

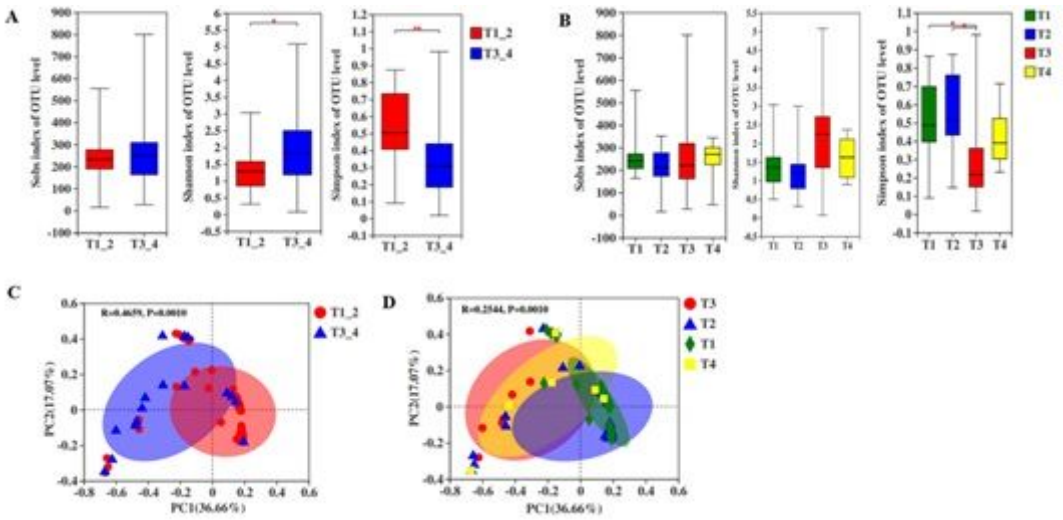

Figure 1

Changes of global intra-tumor microbiota in PTC patients in different clinical stages. (A) α-Diversity at OUT level (estimated by Sobs and Shannon Simpson estimator) in PTC patients of group T1_2 and T_4; (B) α-Diversity at OUT level (estimated by Sobs and Shannon Simpson estimator) in PTC patients of clinical stage T1, T2, T3 and T4; (*p<0.05, **p<0.01); (C) Principal coordinate analysis (PCoA) score plots based on Bray-Curtis distance at OUT level in PTC patients of group T1_2 and T3_4. (D) Principal coordinate analysis (PCoA) score plots based on Bray-Curtis distance at OUT level in PTC patients of clinical stage T1, T2, T3 and T4; Wilcoxon rank-sum test and ANOSIM was used for intragroup difference of a-diversity and PCoA analysis, respectively.

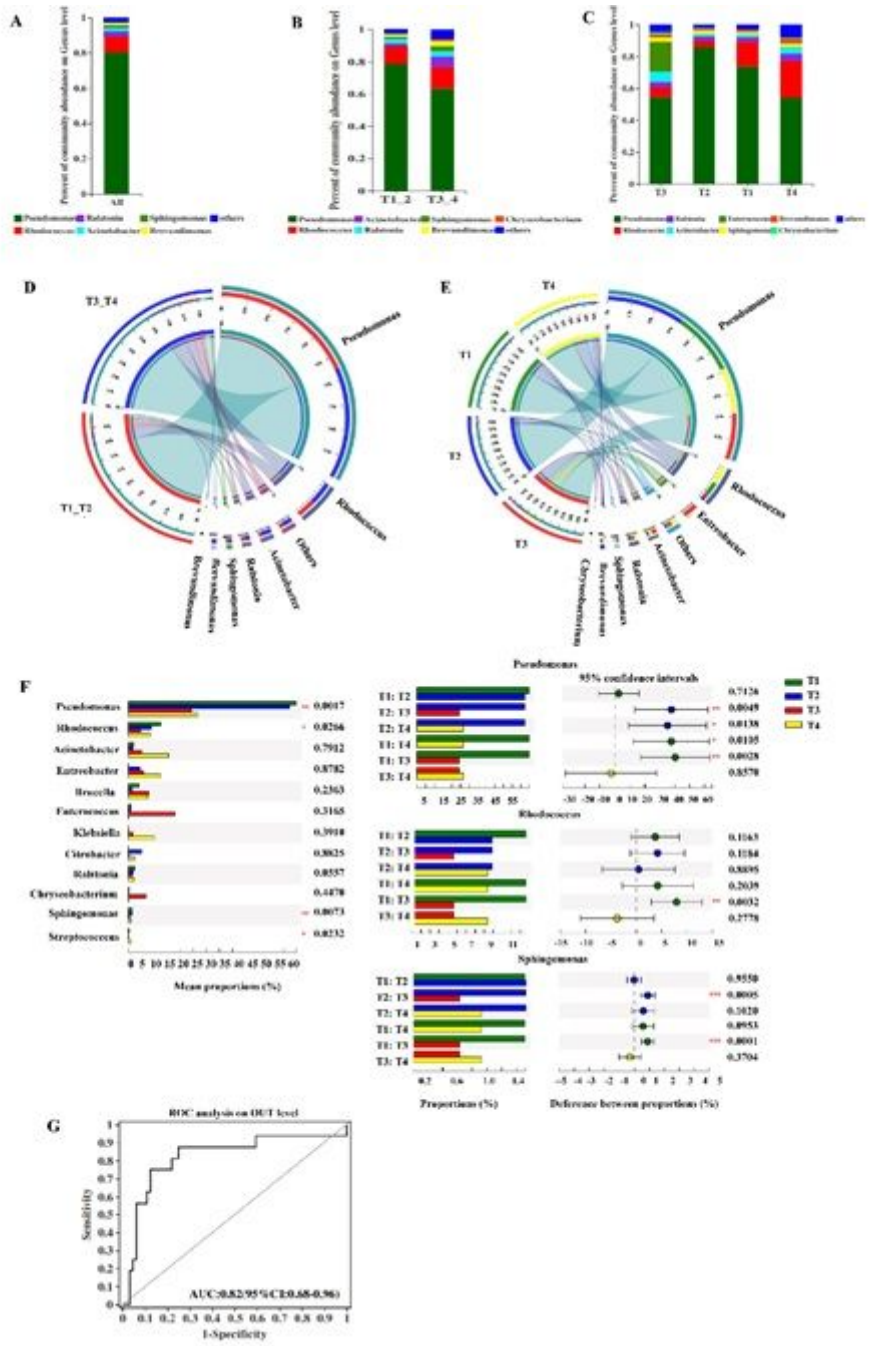

Figure 2

Tumor microbiome communities are significantly different between clinical stages. (A-C) Bar plots of the genus taxonomic levels in PTC patients. (A) Relative abundance is plotted for all PTC patients. (B) Relative abundance is plotted for PTC patients in group T1_2 and T3_4. (C) Relative abundance is plotted for PTC patients in clinical stage T1, T2, T3 and T4. (D-E) Distribution of microbial community for each group at genus level. (D) Distribution of microbial community for PTC patients of group T1_2 and T3_4 at genus level; (E) Distribution of microbial community for PTC patients of clinical stage T1, T2, T3 and T4 at genus level. The data were visualized by Circos. The width of the bars from each genus indicates the relative abundance of that genus in the group. (F) Microbiome alterations at the genus level in PTC patients of clinical stage T1, T2, T3 and T4. The relative abundance of 4 genera were significantly

different. The intragroup differences were analyzed by Kruskal-Wallis test. The difference between either two groups were analyzed by post-hoc test using Welch's uncorrected test and adjusted by FDR. The top 3 differential bacteria (genus) identified were tested individually. (G) ROC analysis of Taxa relative abundance as predictive of clinical T stage status. The top 3 differential bacteria (genus) identified were tested.

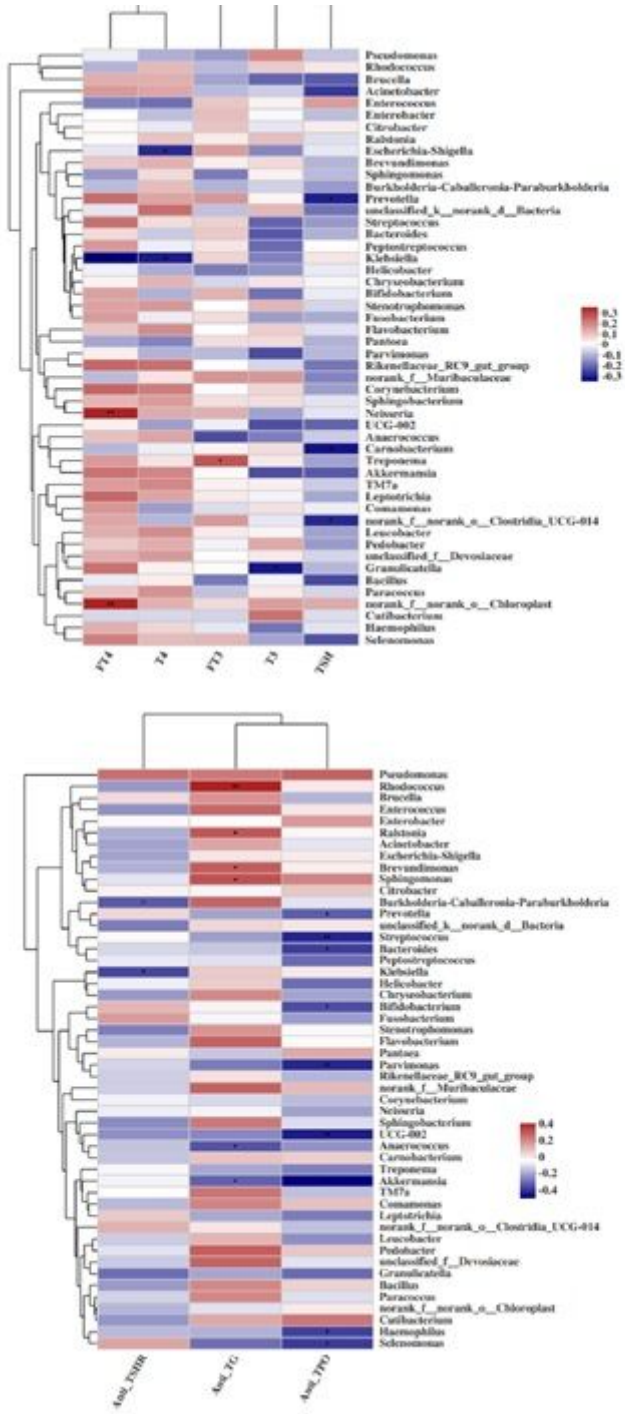

Figure 3

Heatmap of Spearman's correlation analysis between the PTC intratumor microbiome and the clinical factors including thyroid related hormones and thyroid diseases (AITD) related antibodies. *p < 0.05, **p < 0.01

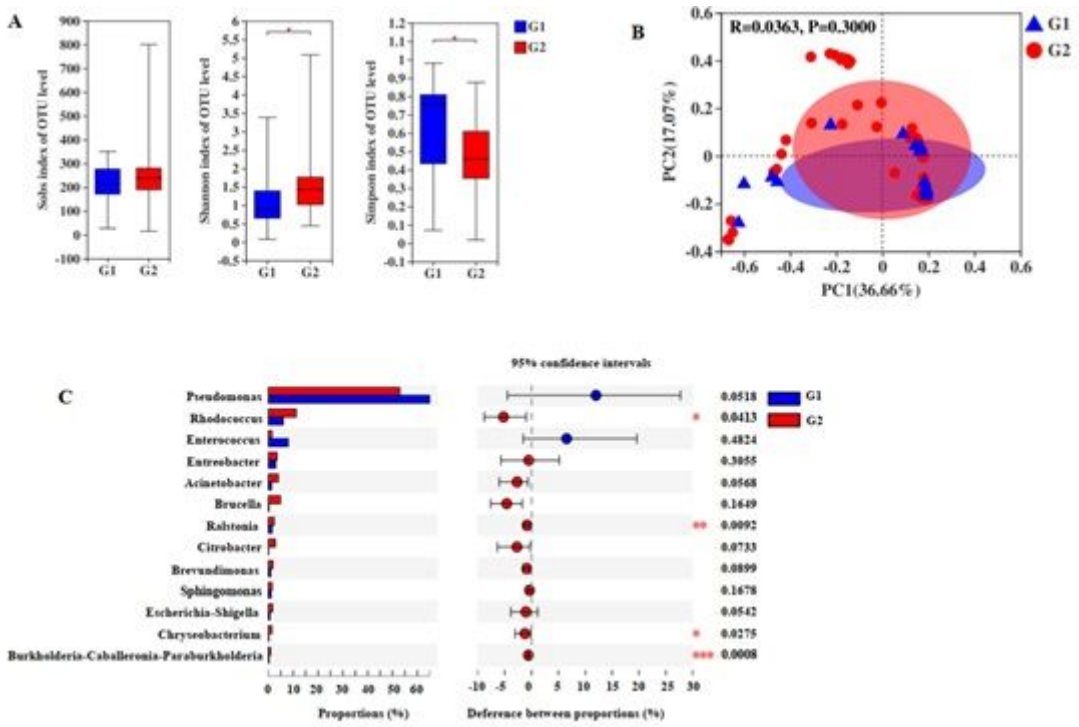

Figure 4

Changes of intra-tumor microbiota in PTC patients with different gender. (A) α-Diversity at OUT level (estimated by Sobs and Shannon Simpson estimator) in PTC patients with different gender (*p<0.05, **p<0.01); (B) Principal coordinate analysis (PCoA) score plots based on Bray-Curtis distance at OUT level in PTC patients with different gender. Wilcoxon rank-sum test and ANOSIM was used for intragroup difference of a-diversity simpson index and PCoA analysis, respectively. (C) Microbiome alterations at the genus level in PTC patients with different gender. The differences between two groups were analyzed by Wilcoxon rank-sum test. 95%CI were calculated by bootstrap.

Supplementary Files

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