



Influence of Pre-treatment Saliva Microbial Diversity and Composition on Nasopharyngeal Carcinoma Prognosis

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Background: The human microbiome has been reported to mediate the response to anticancer therapies. However, research about the influence of the oral microbiome on nasopharyngeal carcinoma (NPC) survival is lacking. We aimed to explore the effect of oral microbiota on NPC prognosis.

Methods: Four hundred eighty-two population-based NPC cases in southern China between 2010 and 2013 were followed for survival, and their saliva samples were profiled using 16s rRNA sequencing. We analyzed associations of the oral microbiome diversity with mortality from all causes and NPC.

Results: Within- and between-community diversities of saliva were associated with mortality with an average of 5.29 years follow-up. Lower Faith's phylogenetic diversity was related to higher all-cause mortality [adjusted hazard ratio (aHR), 1.52 (95% confidence interval (CI), 1.06–2.17)] and NPC-specific mortality [aHR, 1.57 (95% CI, 1.07–2.29)], compared with medium diversity, but higher phylogenetic diversity was not protective. The third principal coordinate (PC3) identified from principal coordinates analysis (PCoA) on Bray–Curtis distance was marginally associated with reduced all-cause mortality [aHR, 0.85 (95% CI, 0.73–1.00)], as was the first principal coordinate (PC1) from PCoA on weighted UniFrac [aHR, 0.86 (95% CI, 0.74–1.00)], but neither was

associated with NPC-specific mortality. PC3 from robust principal components analysis was associated with lower all-cause and NPC-specific mortalities, with HRs of 0.72 (95% CI, 0.61–0.85) and 0.71 (95% CI, 0.60–0.85), respectively.

Conclusions: Oral microbiome may be an explanatory factor for NPC prognosis. Lower within-community diversity was associated with higher mortality, and certain measures of between-community diversity were related to mortality. Specifically, candidate bacteria were not related to mortality, suggesting that observed associations may be due to global patterns rather than particular pathogens.

Keywords: nasopharyngeal carcinoma, 16S rRNA sequencing, oral microbiome, diversity, prognosis

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a rare cancer in most regions of the world, but high-incidence areas are found in regions of Southeast Asia, North Africa, and the Arctic. The highest rates in the world are reported from southern China due to EBV strain variation and genetic and environmental factors (Plummer et al., 2012; Chang et al., 2021).

Due to the high radio sensitivity of the tumor, the principal treatment radiotherapy yields good survival rates for early-stage disease. The majority of cases, however, presents at an advanced stage, and local failure and distant metastases are frequent (Chen et al., 2019). Studies attempting to identify predictors of NPC prognosis, including genomic (Dai et al., 2016; Tang et al., 2018) and proteomic (You et al., 2019) biomarkers for host and Epstein–Barr virus (EBV)-related factors (Network NCC, 2020), have yielded inconclusive results.

Recent studies have reported that microbial diversity is associated with survival outcomes in patients resected for pancreatic adenocarcinoma (Riquelme et al., 2019), colorectal cancer (Nakatsu et al., 2018), and lung cancer (Peters et al., 2019). The oral ecosystem is related to side effects of radiotherapy, such as severe oral mucositis, which can lead to discontinuation of radiotherapy or chemotherapy (Vera-Llonch et al., 2006). Some bacterial taxa may also be directly associated with response to chemotherapy or radiotherapy (Xu et al., 2014; Geller et al., 2017). We recently reported that changes in the nasopharyngeal microbiome among NPC patients are associated with short-term response to radiotherapy or chemoradiotherapy (Huang et al., 2020). Thus, we hypothesized that global community structure, intracommunity diversity, and taxonomic composition of the oral microbiome might influence NPC prognosis.

To test this hypothesis, we conducted an exploratory population-based study, using saliva bacterial profiles based on 16S rRNA sequencing, to identify associations between oral microbiome characteristics and mortality among NPC patients in southern China (Ye et al., 2017).

Abbreviations: NPC, nasopharyngeal carcinoma; EBV, Epstein–Barr virus; ASVs, amplicon sequences variants; Faith's PD, Faith's phylogenetic diversity; BMI, Body Mass Index; FDR, false discovery rate; PCoA, principal coordinates analysis; PCs, principal coordinates; RPCA, robust Aitchison principal component analysis.

METHODS

Study Population

Population-based NPC cases were enrolled from the Wuzhou region of Guangxi Autonomous Region, China, as one subset of a large case–control study (Ye et al., 2017). Wuzhou was selected for this study due to its relatively high incidence of NPC and low rate of residential mobility. All newly diagnosed cases in Wuzhou were identified through a rapid case ascertainment system involving a network of physicians who diagnosed and/or treated NPC at hospitals in the study area (Ye et al., 2017). Among 792 incident NPC cases identified in Wuzhou between 2010 and 2013, 689 (87%) participated in our cohort (Ye et al., 2017). We excluded 89 cases that rejected providing saliva samples and 58 cases whose saliva DNA were extracted by different method. After excluding three cases that failed library preparation and removing samples with fewer than 1,000 sequences per sample or ambiguous sequencing identifiers, 532 cases had valid sequencing results. We further excluded five cases with an ambiguous diagnosis, one duplicated case, and 32 former smokers [due to the heterogeneity of smoking patterns, as described in detail in our previous paper (Debelius et al., 2020)], leaving 482 (70% of 689 enrolled cases) in the final analysis (**Supplementary Figure S1**).

Microbiome Assay

At the time of the study interview, unstimulated saliva samples (2–4 ml) were collected into 50-ml falcon tubes with a Tris-EDTA buffer (Quinque et al., 2006) (**Supplementary Material S1**). The median time interval between diagnosis and sample collection was 1 day; 85% of samples were collected within 30 days after diagnosis, most before treatment initiation.

We used 16s rRNA to sequence microbial gene fragments with 341F/805R V3–V4 primers. Protocols for DNA extraction, PCR, and sequencing are described thoroughly in our previous publication (Debelius et al., 2020) and in the supplementary methods (**Supplementary Material S2**). Two blank controls (nuclease-free water) and one single-organism control (*E. coli* positive) were included in each batch. Sequencing was performed at Beijing Genome Institute on an Illumina MiSeq using a 2 × 300 bp paired-end strategy.

Sequencing results were processed using QIIME2, as previously described (Debelius et al., 2020). To summarize,

sample sequences were demultiplexed, adaptors were trimmed, and paired-end sequences were joined and loaded into QIIME2 (November 2018 release). Subsequently, deblur (v.1.0.4; q2-deblur) workflow was applied to denoise and generate amplicon sequences variants (ASVs) with the default parameters after quality filtering (q2 quality filter). Next, a phylogenetic tree was constructed *via* q2 fragment insertion into Greengenes (August 2013) 99% identity tree backbone; ASVs were assigned taxonomy with a naive Bayesian classifier against a pre-trained reference (q2 feature classifier).

ASVs were identified by the first letter of the lowest clearly assigned taxonomic level, the first five letters of their lowest taxonomic assignment, and the first six characters of a MD5 hash of the sequencing.

Exposure Metrics

Our main exposures were alpha and beta diversity. Alpha diversity was measured by Faith's phylogenetic diversity (Faith's PD), observed ASVs, and Shannon diversity index. Observed ASVs provide a measure of microbiome richness, i.e., the number of different sequence variants in the sample. Faith's PD provides a measure of richness, weighted by the phylogeny (i.e., shared evolutionary history between organisms). Shannon diversity index provides a measure of richness and abundance (i.e., the count of each sequence variant in the sample). Alpha diversity was expressed as a continuous variable or categorized into tertiles (low, medium, and high diversity).

Beta diversity was measured by Bray–Curtis distance, unweighted UniFrac distance, and weighted UniFrac distance using q2 diversity in QIIME2 (Bolyen et al., 2019) after being rarefied to 6,500 sequences. Bray–Curtis considers dissimilarities on relative abundance; weighted UniFrac focuses on relative abundance and phylogeny, with emphasis on abundant microbiomes; and unweighted UniFrac considers presence/absence and phylogeny, with emphasis on rarer organisms.

Outcome

All-cause mortality and NPC-specific mortality were modeled as outcomes. All cases were followed up for vital status, date of death, and cause of death as of December 31, 2018 through the linkages to Wuzhou Cancer Registry, the Total Population Registry, and the Chinese Centres for Disease Control and Prevention, and by conducting in-person visits and telephone calls with village doctors and contacting local funeral parlours. We verified any ambiguous underlying causes of death by obtaining medical records from hospitals and village doctors. Among the deceased cases, all were classified as having a known cause of death except for one case, for whom we assumed that the cause of death was NPC.

Covariates

Covariates of all NPC cases, including cancer stage, treatment regimen, body mass index (BMI) before treatment, radiotherapy technique, and nasopharyngeal radiation dose, were extracted from medical charts in 15 hospitals in Guangdong and Guangxi Provinces. Two oncologists assisted by five medical students reviewed the medical records. Cancer stage was re-classified

according to the 7th AJCC version (Edge and Compton, 2010) by re-examining imaging reports. One senior oncologist checked a random sample of reports to confirm accuracy.

Potential confounders considered were tobacco use (current, former, or never) (Ouyang et al., 2013; Wu et al., 2016; Spakowicz et al., 2020), missing and filled teeth (classified as the sum of missing and filled teeth after age 20 years: 0, 1, 2, 3–5, or 6+) (Belstrom et al., 2018; Cetindag et al., 2019), teeth brushing frequency ($\leq 1/\text{day}$ and $\geq 2/\text{day}$), BMI (< 18.5 , $18.5\text{--}22.9$, $23.0\text{--}27.5$, or $> 27.5 \text{ kg/m}^2$) (Lin et al., 2015; Maruvada et al., 2017), and alcohol use (never, former, or current) (Capurso and Lahner, 2017; Chen et al., 2016). We also included residential region (He et al., 2018) and saliva sampling season (Amato et al., 2015) as covariates, since they might affect the oral microbiome, and we included cancer stage, treatment regimen (Network NCC, 2020), education, radiotherapy technique, calendar year of diagnosis, radiotherapy dose, and radiotherapy technique as potential predictors of mortality.

STATISTICAL ANALYSIS

Univariate associations with all-cause mortality (i.e., overall survival) and NPC-specific mortality (i.e., disease-specific survival) were visualized using Kaplan–Meier curves.

We used univariate Cox proportional hazards regression, with years since diagnosis as the time scale, to evaluate associations between covariates and all-cause or NPC-specific mortality. Covariates with $p < 0.05$ were included in multivariate Cox models. Although radiotherapy dose and radiotherapy technique were significant mortality predictors, they were excluded due to high collinearity with other included covariates, resulting in severe variance inflation.

All Cox models took time since diagnosis as underlying timescale. We tested the proportional hazards assumption based on Schoenfeld's residuals and found no violations. We used likelihood ratio tests to test for interactions. All reported p -values are two-sided. We used SAS 9.4 for data management and R 4.0.3 for statistical analysis.

Alpha Diversity Analysis

The Kruskal–Wallis rank-sum test was used to explore contributors of alpha diversity after z -normalization. $p < 0.05$ was considered the threshold inclusion in multivariate Cox proportional hazards regression models together with significant explanatory mortality predictors. We included age at diagnosis, sex, tobacco use, BMI before treatment, cancer stage, treatment regimen, alcohol use, the number of missing or filled teeth, sequence running number, residential community, and saliva sampling season (**Supplementary Table S1**). Stratified analyses were conducted by age at diagnosis.

Beta Diversity Analysis

Beta diversity was compared using Adonis *via* the R vegan package (version 2.5.7), adjusted for age, sex, and sequencing running number, with 999 permutations. Covariates with false

discovery rate (FDR)-adjusted $p < 0.05$ were included in multivariate Cox models together with mortality predictors. It included age at diagnosis, sex, sequencing running number, tobacco use, diagnosis calendar year, the number of missing or filled tooth, cancer stage, treatment regimen, saliva sampling season, BMI before treatments, alcohol use, diagnosis calendar year, and residential community, with or without Faith's PD (**Supplementary Figure S2**).

Principal coordinates analysis (PCoA) was visualized using Emperor in QIIME2. We took the top three principal coordinates (PCs), corresponding to microbiome pattern, as covariates (after z-normalization) for inclusion in adjusted Cox regression models and tested the three PCs jointly by using likelihood ratio tests to compare nested models with and without the PCs (Plantinga et al., 2017).

Robust Aitchison Principal-Component Analysis

To account for the sparse compositional nature of microbiome data and the large proportion of zero values (Morton et al., 2017), we applied Aitchison principal-component analysis (RPCA) to describe and visualize beta diversity. RPCA provides a solution to managing the zero-inflation problem *via* matrix completion while preserving feature abundance information to enable identification of taxa that drive the differences among sample groups. The two main procedures in RPCA are transformation (i.e., robust centered log ratio transformation of feature absolute abundance to approximate a normal distribution) and matrix completion (i.e., treating all zero values as missing and building a model to handle the missing data using matrix completion) (Martino et al., 2019). The RPCA, generated using *DEICODE* in QIIME2, reflects the evenness of community (Martino et al., 2019) (code provided in **Supplementary Material S3**). The RPCA metrics and biplots were visualized using Emperor in QIIME2, importing the raw and unrarefied feature tables. Sample loading of PCs as candidates of beta diversity evenness was applied in Cox models as a continuous or ordinal (teriled) variable.

To assess the relationship between ASV abundance and mortality, we used multivariate Cox models (adjusted for the same covariates as in the beta diversity analysis) to test selected ASVs. Because multiple differential abundance analysis might result in high false-positive rates (Hawinkel et al., 2019), we selected only the ASVs with top 10 and bottom 10 feature loadings of PC3 from RPCA. Meanwhile, the additive log ratio transformation of relative abundance was applied, using the top 10 ASVs as a reference frame to reveal the compositional nature of microbiome data (Morton et al., 2019). FDR-adjusted p -values were used.

Sensitivity Analysis

To confirm the robustness of our results, we performed three sets of sensitivity analyses: first, testing alpha diversity as a continuous variable; second, including 32 former smokers in both the alpha and beta diversity analysis; and third, excluding 55 cases whose saliva samples were collected during or after treatment.

RESULTS

Characteristics of the Patients

There were 482 NPC patients included in the analysis (**Table 1**). Their mean age at diagnosis was 48 years, 71% were male, and 93% were diagnosed at stage III or IV. In terms of NPC risk factors, 78% had < 9 years of education, 66% brushed their teeth no more than once per day, and 52% were current smokers. Mean (\pm SD) follow-up time was 5.29 (\pm 2.07) years; one case was lost to follow-up at 51 months.

Low Alpha Diversity Was Associated With Poorer Overall Survival And Disease-Specific Survival

Overall survival and NPC-specific survival throughout the study period were considerably and consistently poorest among patients with relatively low Faith's PD, based on Kaplan–Meier curves (**Figure 1**).

Compared with the medium diversity group based on Faith's PD, patients with lower Faith's PD (i.e., lower within-community richness and phylogenetic diversity) had significantly higher all-cause and NPC-specific mortality in both crude and multivariate-adjusted models [adjusted HRs: 1.52 (95% CI, 1.06–2.17) and 1.57 (95% CI, 1.07–2.29)] (**Table 2**). Observed ASVs had a similar pattern of association with all-cause and NPC-specific mortality, although associations were statistically and marginally nonsignificant, whereas Shannon diversity was not associated with either outcome (**Table 2**; **Supplementary Figure S3**).

In subgroup analysis by age, associations with Faith's PD and observed ASVs appeared to be stronger among cases of older age (> 50 years) vs. younger cases (**Supplementary Figure S4**); however, interactions were statistically nonsignificant.

Beta Diversity Was Associated With Overall Survival

Because beta diversity was affected by alpha diversity (Debelius et al., 2020), we also controlled for alpha diversity in multivariate models for beta diversity and mortality. Comparing nested models (**Supplementary Table S2**) with and without PCs from PCoA, nested models of PC3 (Bray–Curtis distance) and PC1 (weighted UniFrac distance) were at least marginally significantly associated with all-cause mortality but not with NPC-specific mortality. The adjusted HR for all-cause mortality in association with normalized PC3 from Bray–Curtis distance was 0.85 (95% CI, 0.73–1.00, $p < 0.05$) and that for PC1 from weighted UniFrac distance was 0.86 (95% CI, 0.74–1.00, $p > 0.05$) (**Supplementary Figure S5**). The other two PCs from Bray–Curtis distance and the other two PCs from weighted UniFrac, however, were not significantly associated with all-cause mortality, and none were significantly associated with NPC-specific mortality.

Robust Aitchison Principal-Component Analysis

Given that Bray–Curtis and weighted UniFrac reflect relative abundance, we applied RPCA, which takes abundance into

TABLE 1 | Characteristics of NPC cases and univariate associations between covariates and mortality.

Characteristics	Total n(%) ^a	Deaths n(%) ^a	All-cause HRs (95%CI)	Deaths from NPC n(%) ^a	NPC-specific HRs (95%CI)
Number of cases	482 (100.0)	210 (43.6)		181 (37.6)	
Mean follow-up yeas (SD)	5.29 (2.07)				
Mean age at cancer diagnosis (SD)	48.45 (10.55)		1.03 (1.01,1.04)		1.02 (1.00,1.03)
Sex					
Male	342 (71.0)	165 (78.6)	ref	144 (79.6)	ref
Female	140 (29.0)	45 (21.4)	0.58 (0.42,0.81)	37 (20.4)	0.55 (0.38,0.79)
Residential community					
Wuzhou	108 (22.4)	41 (19.5)	ref	36 (19.9)	ref
Cangwu	115 (23.9)	51 (24.3)	1.26 (0.83,1.89)	46 (25.4)	1.29 (0.83,1.99)
Cenxi	165 (34.2)	79 (37.6)	1.45 (0.99,2.11)	63 (34.8)	1.31 (0.87,1.97)
Tengxian	94 (19.5)	39 (18.6)	1.17 (0.76,1.82)	36 (19.9)	1.23 (0.78,1.96)
Educational attainment					
≤ 6 years	204 (42.3)	95 (45.2)	ref	75 (41.4)	ref
7-9 years	170 (35.3)	77 (36.7)	0.96 (0.71,1.30)	70 (38.7)	1.11 (0.80,1.54)
≥ 10 years	108 (22.4)	38 (18.1)	0.72 (0.49,1.04)	36 (19.9)	0.86 (0.58,1.28)
Tobacco use					
Never	230 (47.7)	83 (39.5)	ref	67 (37.0)	ref
Current	252 (52.3)	127 (60.5)	1.56 (1.18,2.06)	114 (63.0)	1.73 (1.28,2.34)
Diagnosis calendar year					
2011	224 (46.5)	115 (54.8)	ref	96 (53.0)	ref
2012	149 (30.9)	55 (26.2)	0.75 (0.54,1.04)	53 (29.3)	0.87 (0.62,1.23)
2013	109 (22.6)	40 (19.0)	0.88 (0.61,1.26)	32 (17.7)	0.83 (0.55,1.25)
Season of saliva sampling					
Winter	114 (23.7)	51 (24.3)	ref	43 (23.8)	ref
Spring	141 (29.3)	60 (28.6)	0.98 (0.67,1.42)	50 (27.6)	0.97 (0.64,1.46)
Summer	101 (21.0)	45 (21.4)	1.03 (0.69,1.54)	40 (22.1)	1.09 (0.71,1.68)
Autumn	126 (26.1)	54 (25.7)	0.99 (0.67,1.45)	48 (26.5)	1.04 (0.69,1.58)
Tooth brushing frequency					
≤ 1/day	316 (65.6)	140 (66.7)	ref	119 (65.7)	ref
≥ 2/day	166 (34.4)	70 (33.3)	0.89 (0.67,1.18)	62 (34.3)	0.93 (0.68,1.26)
Missing or filled teeth					
0	209 (43.4)	77 (36.7)	ref	70 (38.7)	ref
1	61 (12.7)	25 (11.9)	1.15 (0.74,1.81)	22 (12.2)	1.12 (0.69,1.80)
2	52 (10.8)	21 (10.0)	1.13 (0.70,1.83)	16 (8.8)	0.95 (0.55,1.63)
3-5	79 (16.4)	39 (18.6)	1.48 (1.00,2.17)	36 (19.9)	1.49 (1.00,2.23)
6+	81 (16.8)	48 (22.9)	1.87 (1.31,2.69)	37 (20.4)	1.58 (1.06,2.35)
Cancer stage					
I-II	35 (7.3)	5 (2.4)	ref	4 (2.2)	ref
III	209 (43.4)	65 (31.0)	2.51 (1.01,6.23)	54 (29.8)	2.60 (0.94,7.19)
IV	238 (49.4)	140 (66.7)	6.06 (2.48,14.81)	123 (68.0)	6.61 (2.44,17.92)
Treatment regimen					
CCRT	250 (51.9)	103 (49.0)	ref	88 (48.6)	ref
CCRT+ICT/ACT	150 (31.1)	66 (31.4)	1.14 (0.84,1.56)	60 (33.1)	1.21 (0.87,1.69)
RT only	60 (12.4)	28 (13.3)	1.22 (0.80,1.85)	20 (11.0)	1.02 (0.63,1.66)
No RT	22 (4.6)	13 (6.2)	2.01 (1.12,3.57)	13 (7.2)	2.33 (1.30,4.17)
BMI before treatment					
Normal Weight	257 (53.3)	130 (61.9)	ref	114 (63.0)	ref
Underweight	62 (12.9)	26 (12.4)	0.78 (0.51,1.19)	23 (12.7)	0.79 (0.50,1.24)
Overweight	93 (19.3)	36 (17.1)	0.65 (0.45,0.95)	29 (16.0)	0.60 (0.40,0.90)
Obese	70 (14.5)	18 (8.6)	0.43 (0.26,0.70)	15 (8.3)	0.41 (0.24,0.70)
History of alcohol use					
Never	330 (68.5)	134 (63.8)	ref	114 (63.0)	ref
Former	20 (4.1)	13 (6.2)	1.97 (1.11,3.48)	10 (5.5)	1.77 (0.93,3.39)
Current	132 (27.4)	63 (30.0)	1.24 (0.92,1.68)	57 (31.5)	1.32 (0.96,1.82)
Radiotherapy technique					
No radiotherapy	22 (4.6)	13 (6.2)	ref	13 (7.2)	ref
2DRT/3DRT	266 (55.2)	141 (67.1)	0.67 (0.38,1.19)	119 (65.7)	0.57 (0.32,1.02)
IMRT	194 (40.2)	56 (26.7)	0.36 (0.19,0.65)	49 (27.1)	0.31 (0.17,0.58)
Nasopharyngeal radiation dose					
< 70 Gy	144 (30.1)	41 (19.8)	ref	38 (21.2)	ref
≥ 70 Gy	312 (65.3)	153 (73.9)	1.73 (1.22,2.44)	128 (71.5)	1.56 (1.09,2.24)
No radiotherapy	22 (4.6)	13 (6.3)	2.83 (1.52,5.29)	13 (7.3)	3.03 (1.61,5.69)

HRs, hazard ratios; SD, standard deviation; BMI, body mass index; CCRT, concurrent chemoradiotherapy; ICT, induction chemotherapy; ACT, adjuvant chemotherapy; RT, radiotherapy; IMRT, intensity-modulated radiation therapy; 2DRT, conventional 2D radiotherapy; 3DRT, conventional 3D radiotherapy.

^aPercentages may not be 100 because of rounding.

The statistically significant hazards ratios were in bold.

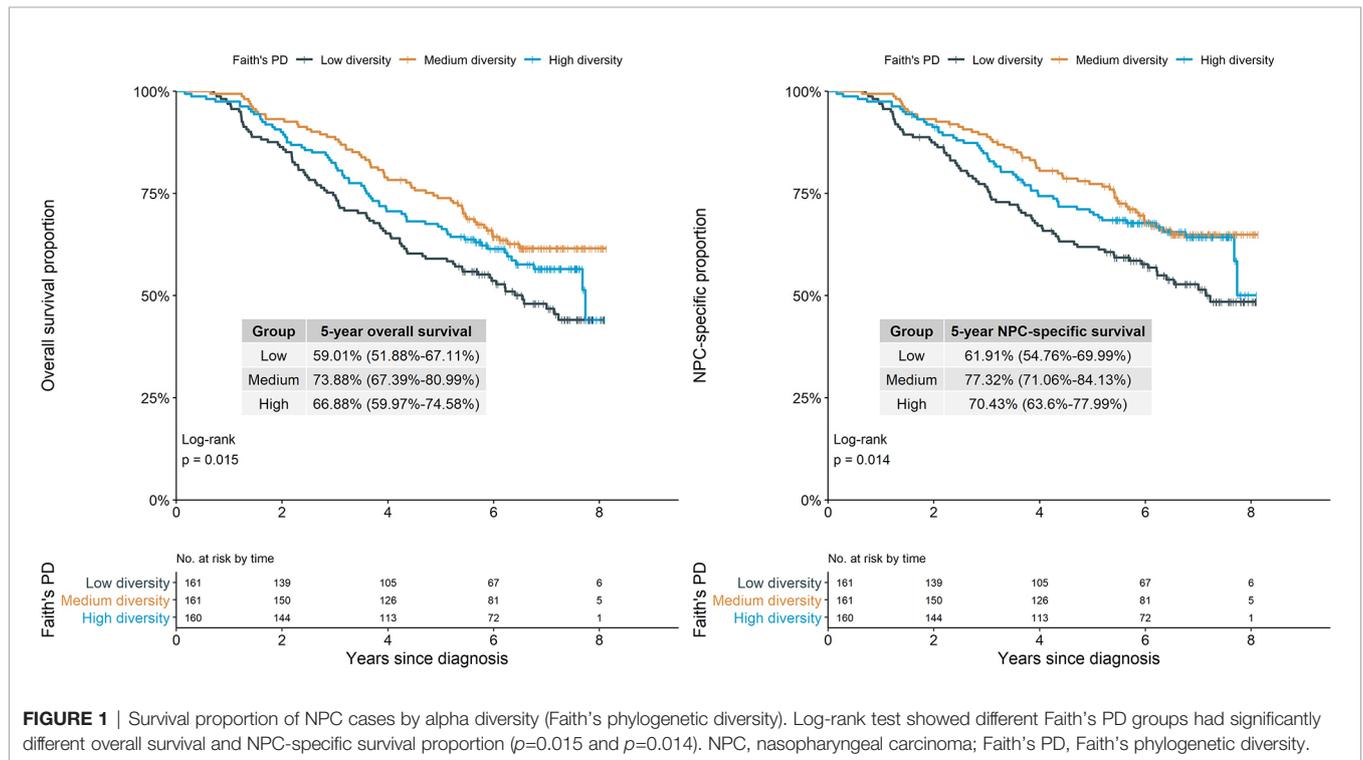


FIGURE 1 | Survival proportion of NPC cases by alpha diversity (Faith's phylogenetic diversity). Log-rank test showed different Faith's PD groups had significantly different overall survival and NPC-specific survival proportion ($p=0.015$ and $p=0.014$). NPC, nasopharyngeal carcinoma; Faith's PD, Faith's phylogenetic diversity.

account, to derive additional beta diversity metrics. Normalized PC3 was significantly associated with both all-cause mortality [adjusted HR, 0.72 (95% CI, 0.61–0.85)] and NPC-specific mortality [adjusted HR, 0.71 (95% CI, 0.60–0.85)] (**Figure 2A, B; Supplementary Table S3**), but PC1 and PC2 from RPCA were not significantly associated with either outcome. To confirm the robustness of the associations with these PCs, we categorized them into tertiles, drew Kaplan–Meier curves, and constructed multivariate Cox models, which shows that tertile 3 vs. tertile 1 of PC3 had 47% lower all-cause mortality and 51% lower NPC-specific mortality (**Figure 2C, D**), whereas associations with PC1

and PC2 remained statistically nonsignificant after adjustment (**Table 3; Supplementary Figure S6**).

We tested for effect modification by performing stratified analysis by age at diagnosis and found stronger associations between tertile PC3 and all-cause and NPC-specific mortality for cases older than 50 years (**Supplementary Figure S4**).

To evaluate whether between-community features are associated with mortality in NPC patients, we tested the top and bottom 10 ASVs with the highest and lowest feature loadings of PC3 from RPCA (**Supplementary Figure S7; Supplementary Table S4**). None were significantly associated with all-cause or NPC mortality, except for a

TABLE 2 | Hazard ratios (HRs) for mortality of NPC cases in relation to alpha diversity, Cox regression models.

Alpha diversity	Cases (n=482)	Deaths (n=210)	All-cause HRs (95%CI)		Deaths from NPC (n=181)	NPC-specific HRs (95%CI)	
			Crude	Adjusted ^a		Crude	Adjusted ^a
Faith's PD							
Low diversity	161	83	1.62 (1.16,2.27)	1.52 (1.06,2.17)	74	1.64 (1.15,2.33)	1.57 (1.07,2.29)
Medium diversity	161	59	ref	ref	52	ref	ref
High diversity	160	68	1.24 (0.88,1.76)	1.18 (0.82,1.72)	55	1.14 (0.78,1.66)	1.10 (0.73,1.64)
Observed ASVs							
Low diversity	161	77	1.44 (1.03,2.02)	1.45 (1.01,2.10)	68	1.47 (1.02,2.10)	1.44 (0.97,2.12)
Medium diversity	161	61	ref	ref	53	ref	ref
High diversity	160	72	1.30 (0.92,1.83)	1.27 (0.88,1.84)	60	1.25 (0.86,1.80)	1.24 (0.83,1.83)
Shannon							
Low diversity	161	73	1.05 (0.75,1.45)	1.07 (0.75,1.52)	65	1.08 (0.76,1.53)	1.14 (0.78,1.66)
Medium diversity	161	71	ref	ref	61	ref	ref
High diversity	160	66	0.94 (0.67,1.31)	0.96 (0.68,1.36)	55	0.91 (0.63,1.31)	0.94 (0.64,1.37)

Faith's PD, Faith's phylogenetic diversity.

^aHRs were adjusted for age, sex, smoking history, BMI before treatment, cancer stage, treatment pattern, alcohol consumption, the number of missing or filled teeth, sequence running number, residential community and season of saliva sampling.

The statistically significant hazards ratios were in bold.

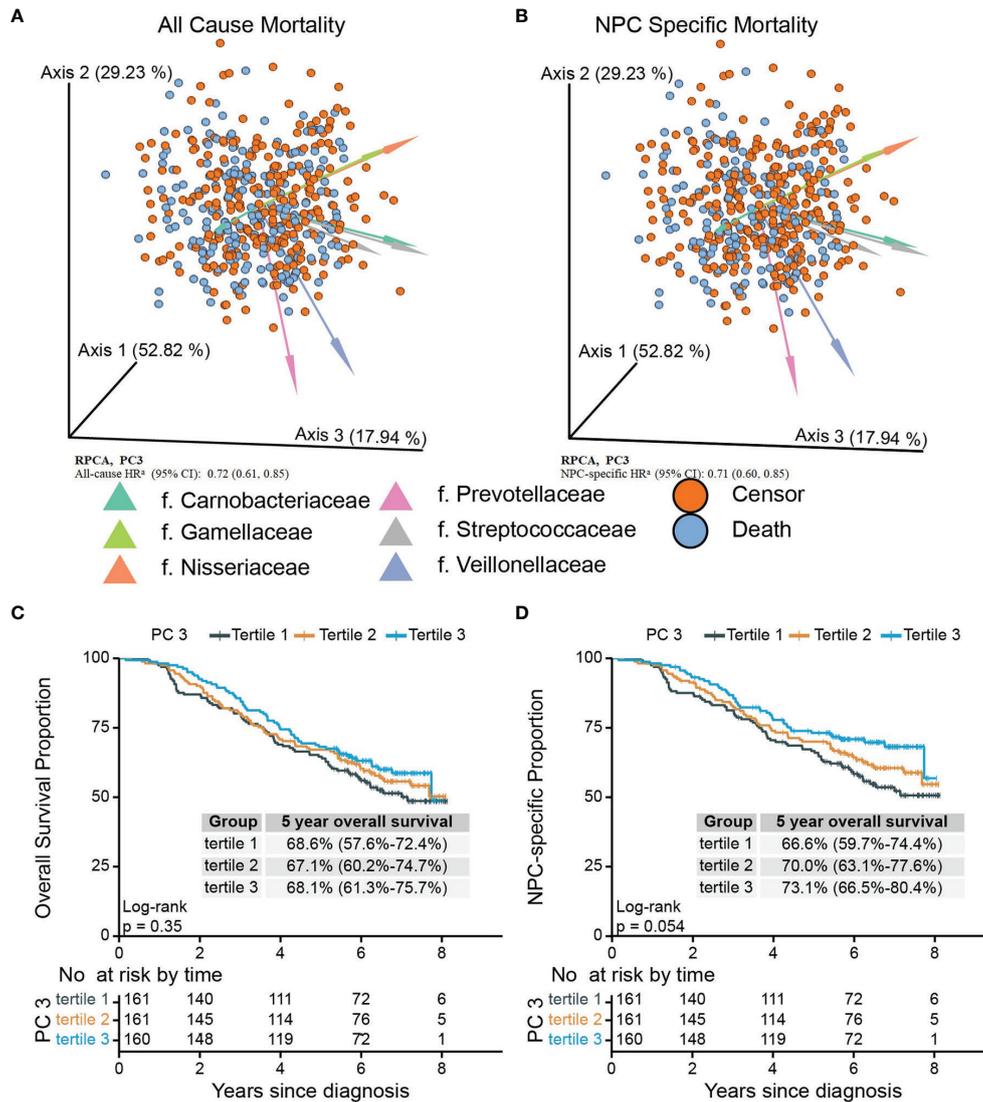


FIGURE 2 | Biplots of RPCA by survival status (A) and NPC-specific survival status (B) and Kaplan–Meier curves of overall (C) and NPC-specific survival (D) proportion between tertiled PC3 groups generated from RPCA. PC3 of RPCA were significant mortality predictor (A, B) in Cox model with all-cause HR of 0.72 (95% CI, 0.61–0.85) and NPC-specific HR of 0.71 (95% CI, 0.60–0.85). Arrows in (A) and (B) were top 8 taxa influencing the principal component axis. Axis1, axis2, and axis3 were equal to PC1, PC2, and PC3. The axes were labeled with the variation proportion that PCs explain. Sample loadings PC3 were z-normalized in Cox models. ^aHRs were adjusted for age at diagnosis, sex, sequencing running number, tobacco use, the number of missing or filled tooth, cancer stage, treatment pattern, saliva sampling season, BMI before treatments, alcohol use, diagnosis calendar year, and residential community and Faith’s PD. PC3 were z-normalized. RPCA, robust Aitchison principal-component analysis; NPC, nasopharyngeal carcinoma; Faith’s PD, Faith’s phylogenetic diversity.

single modest association between a *Streptococcus* ASV (gStrep.05312a) and all-cause mortality that was nonsignificant after FDR adjustment ($p=0.018$ and $FDR=0.368$).

Sensitivity Analysis

When alpha diversity was classified as a continuous variable, higher Faith’s PD, but not the other two measures of alpha diversity, was significantly associated with lower NPC-specific mortality (HR, 0.85; 95% CI, 0.73–1.00), but not all-cause mortality. When we included 32 former smokers or excluded 55 cases with saliva samples during or after treatment in the

analysis, results were not meaningfully changed (Other Supporting Materials).

DISCUSSION

The goal of this study was to explore whether the oral microbiome affects mortality among NPC patients in an endemic area. Our results showed that some measures of lower enriched and phylogenetic within-community diversity were related to higher overall and disease-specific mortality, and some measures of

TABLE 3 | Hazard ratios (HRs) for mortality of NPC cases in relation to tertiled PCs from RPCA, Cox regression models.

PCs ^a	Cases (n=482)	Deaths (n=210)	All-cause HRs		Deaths of NP (n=181)	NPC-specific HRs		
			Crude	Adjusted ^b		Crude	Adjusted ^b	
PC1								
tertile 1	161	63	ref	ref	51	ref	ref	
tertile 2	161	81	1.44 (1.04,2.00)	1.21 (0.84,1.74)	73	1.60 (1.12,2.29)	1.31 (0.89,1.94)	
tertile 3	160	66	1.13 (0.80,1.59)	1.30 (0.88,1.93)	57	1.20 (0.82,1.75)	1.32 (0.86,2.02)	
PC2								
tertile 1	161	69	ref	ref	64	ref	ref	
tertile 2	161	75	1.15 (0.83,1.60)	1.07 (0.75,1.54)	61	1.01 (0.71,1.43)	1.01 (0.68,1.48)	
tertile 3	160	66	0.99 (0.71,1.39)	1.14 (0.78,1.67)	56	0.90 (0.63,1.29)	1.02 (0.68,1.52)	
PC3								
tertile 1	161	77	ref	ref	72	ref	ref	
tertile 2	161	70	0.88 (0.64,1.22)	0.66 (0.46,0.96)	61	0.82 (0.59,1.16)	0.68 (0.46,1.00)	
tertile 3	160	63	0.78 (0.56,1.09)	0.53 (0.36,0.80)	48	0.64 (0.44,0.92)	0.49 (0.32,0.76)	

RPCA, robust Aitchison principal-component analysis; PC, principle component.

^aSample loading of PCs were grouped into three tertiles.

^bAdjusted for age at diagnosis, sex, sequencing running number, tobacco use, the number of missing or filled tooth, cancer stage, BMI before treatments, alcohol use, diagnosis calendar year, treatment pattern, saliva sampling season, residential community and Faith's phylogenetic diversity.

The statistically significant hazards ratios were in bold.

between-community diversity and composition were associated with overall mortality. However, results were not consistent, as several measures of alpha and beta diversities were not associated with either overall or NPC-specific mortality.

It is widely believed that a diverse and balanced microbiome plays an essential role in human oral mucosal immune function. The normal commensal microbiota can protect hosts from colonization by exogenous pathogens and overgrowth by indigenous pathobionts. An imbalance between altered commensal microbiota and immune regulation could increase the risk of developing and exacerbating disease (Tomkovich and Jobin, 2016). Interestingly, our stratified results suggest that the impact of diversity on mortality may be more pronounced among elder patients. Previous research reported that older patients with less richness diversity are more prone to treatment complications and lower immunological adaptability (Zawadzki et al., 2017).

Few studies have looked at the relationship between microbiome and long-term cancer prognosis, and to our knowledge, none of these evaluated NPC. Our previous study (Huang et al., 2021) investigated the association between the nasopharyngeal microbiome and short-term response to chemotherapy and radiotherapy, with results suggesting differences in microbial diversity between late and early NPC responders. Hou et al. (2018) found that two microbe candidate taxa in the retropharyngeal wall were significantly related to the progression of oral mucositis, which is a common adverse effect of radiotherapy; this complication could result in discontinuation of chemotherapy or radiotherapy and malnutrition, leading to poorer prognosis among NPC patients. Other studies (Zhu et al., 2017) have reported that as oral mucosal lesions progressed from mild to severe mucositis, their bacterial UniFrac distances from healthy controls increased. Previous results (Xu et al., 2014) also indicated that oral or nasopharyngeal microbes may influence the response to therapy and side effects of radiotherapy among NPC cases. Our findings augment these prior results by suggesting that saliva microbiome diversity might have an important influence on long-term prognosis.

Results from microbiome-based survival studies of other cancers may also be relevant for comparison. Our finding was in line with

results showing improved recurrence- and disease-free survival in association with higher richness and evenness of diversity in normal tissue from non-small cell lung cancer patients (Peters et al., 2019). Another study found longer overall survival in association with higher alpha diversity in the tumor tissue from pancreatic adenocarcinoma patients (Riquelme et al., 2019). Among 55 cervical cancer cases, Sims et al. (2020) found that Shannon diversity index for the baseline fecal microbiome was an independent predictor of overall survival and relapse-free survival after chemoradiation treatment. Finally, a meta-analysis (Yang et al., 2020) revealed that antibiotic administration, which decreases microbiome richness, was correlated with poorer overall survival in solid cancer patients, although this observed association could be biased due to confounding by indication (i.e., underlying infection).

Our results showed that the association between mortality and UniFrac diminished after adjustment for alpha diversity. These results suggest that the association of microbiome richness with mortality is more likely to be driven by within-community diversity, whereas the association with between-community diversity is driven mainly by the evenness of beta diversity. Although some previous studies of other cancers (Mitsuhashi et al., 2015; Yamamura et al., 2016; Yamamura et al., 2019) identified certain candidate microbes as predictors of cancer mortality, we found no prominent associations with specific organism. Our results, therefore, suggest that future research focus on community level oral dysbiosis, rather than focusing on interventions with specific organisms.

The strengths of our study include its prospective design, making it the first long-term prospective investigation of the relationship between the oral microbiome and NPC prognosis. Second, the cases are population-based, making results generalizable to NPC in endemic Guangxi Autonomous Region in southern China. Third, by using complementary metrics and broad views of community, we were better positioned to characterize true relationship between microbes and outcomes. Fourth, we had low loss to follow-up (0.2%), thereby minimizing the risk of selection bias. Finally, we collected extensive information on potential confounders, enabling us to adjust for other known and potential prognostic factors.

Limitations include the exploratory nature of the analysis, with numerous statistical tests and the potential for false-positive significant findings. Second, we lacked information on the use of antibiotics and anti-inflammatory therapy before sample collection, which could affect measures of microbiome diversity and survival outcomes. Previous work has suggested that the oral microbiome is more robust to antibiotics than the fecal microbiome (Zaura et al., 2015); however, more research is needed in this area to fully understand the dynamics of the relationship and for full extrapolation to our work. Third, there were 55 cases whose saliva samples were collected during or after treatment. We used the interview date as proxy of sampling date, and in practice, sampling date was 1 or 2 days before interview date. To check the influence of this limitation, we did the sensitivity analysis excluding these 55 cases. Forty-nine of these 55 samples were collected more than 200 days after treatment started (**Figure A in Other Supporting Materials**). The sensitivity analysis' results remained largely unchanged. Moreover, we have not known the survival outcome when we collected samples and did the sequence of microbiome. The bias in sampling time point was undifferentiated and only drove the results to null direction. Fourth, we did not get the nasopharyngeal samples considering the feasibility of sampling in a population-based context. Finally, our results may not be generalizable outside of southern China, given that the oral microbiome community is strongly determined by dietary pattern and geographic region. More research is needed to assess the generalizability of these results to other populations.

In summary, our study revealed that some measures of oral microbiome diversity are associated with long-term mortality among NPC patients. In particular, lower within-community diversity was associated with poorer mortality, especially among elder cases. Underlying mechanisms, especially the role of immune status, could reveal ways to ameliorate the generally poor prognosis of advanced NPC. We tended to believe that microbiome was the risk factor because most samples were collected before treatment, and we have adjusted lots of confounders and also performed sensitivity analysis, which can increase the evidence of causality. We acknowledge the limitations and lack of external validation and cannot demonstrate directly and strongly that oral microbiome could be biomarkers. However, our results might inspire new ideas on microbiome. We will further extend and validate our results in the future.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ebi.ac.uk/ena>, PRJEB37445.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Karolinska Institutet, Wuzhou Red Cross Hospital.

The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The study was raised by WY, H-OA, GH and YZ, ZZ, YC, YD, RF, JD, LY, EC, WY, ZZ and YC refined the design. LL, YC, YL and YZ contributed to sample collection and management. TH, XZ and XX were responsible for the lab work. YD, RF, MX, YC and ZZ collected follow-up information and reviewed medical records. YD, JD, LY and RF performed the statistical analysis. Bioinformatic analysis was performed by JD and YD. YD wrote and revised the manuscript. EC provided editing. All were supervised and coordinated by WY. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2022.831409/full#supplementary-material>

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Conflict of Interest: Author EC was employed by Exponent, Inc., Center for Health Sciences.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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