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Research article

Clinical relationships between the intratumoral microbiome and risk factors for head and neck cancer

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ABSTRACT

A bioinformatic analysis is a promising approach to understand the relationship between the vast tumor microbiome and cancer development. In the present study, we studied the relationships between the intratumoral microbiome and classical clinical risk factors using bioinformatics analysis of the Cancer Genome Atlas (TCGA) and the Cancer Microbiome Atlas (TCMA) datasets. We used TCMA database and investigated the abundance of microbes at the genus level in solid normal tissue (n = 22) and the primary tumors of patients with head and neck squamous cell carcinoma (HNSCC) (n = 154) and identified three major tumor microbiomes, *Fusobacterium*, *Prevotella*, and *Streptococcus*. The tissue level of *Fusobacterium* was higher in primary tumors than in solid normal tissue. However, univariate and multivariate analyses of these 3 microbes showed no significant effects on patient survival. We then extracted 43, 55, or 59 genes that were differentially expressed between the over and under the median groups for *Fusobacterium*, *Prevotella*, or *Streptococcus* using the criteria of >2.5, >1.5, or >2.0 fold and $p < 0.05$ in the Mann-Whitney *U* test. The results of a pathway analysis revealed the association of *Fusobacterium*- and *Streptococcus*-related genes with the IL-17 signaling pathway and *Staphylococcus aureus* infection, while *Prevotella*-associated pathways were not extracted. A protein-protein interaction analysis revealed a dense network in the order of *Fusobacterium*, *Streptococcus*, and *Prevotella*. An investigation of the relationships between the intratumoral microbiome and classical clinical risk factors showed that high levels of *Fusobacterium* were associated with a good prognosis in the absence of alcohol consumption and smoking, while high levels of *Streptococcus* were associated with a poor prognosis in the absence of alcohol consumption. In conclusion, intratumoral *Fusobacterium* and *Streptococcus* may affect the prognosis of patients with HNSCC, and their effects on HNSCC are modulated by the impact of drinking and smoking.

Abbreviations: HNC, head and neck cancer; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; TCGA, The Cancer Genome Atlas; TCMA, The Cancer Microbiome Atlas.

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1. Introduction

Head and neck cancer (HNC) is a malignant tumor that develops in the paranasal sinuses, nasal cavity, oral cavity, pharynx, salivary glands, and larynx [1]. It is the seventh most prevalent cancer worldwide [2] and more than 90 % of cases of head and neck squamous cell carcinoma (HNSCC) originate from squamous cells of the mucous membrane [3]. The 5-year survival rate of HNC is <50 % [4,5]. Smoking, the consumption of alcohol, and human papillomavirus (HPV) infection are important risk or carcinogenic factors for HNSCC [6,7]. Furthermore, studies on oral bacteria on tumor surfaces and in saliva have shown that several types of bacteria are associated with cancer [8], which suggests that the oral microbiome residing in the oral cavity and pharynx is another risk factor for HNSCC. Indeed, oral commensal bacteria from the genera *Streptococcus, Rothia, Fusobacterium, Haemophilus,* and *Prevotella* are frequently

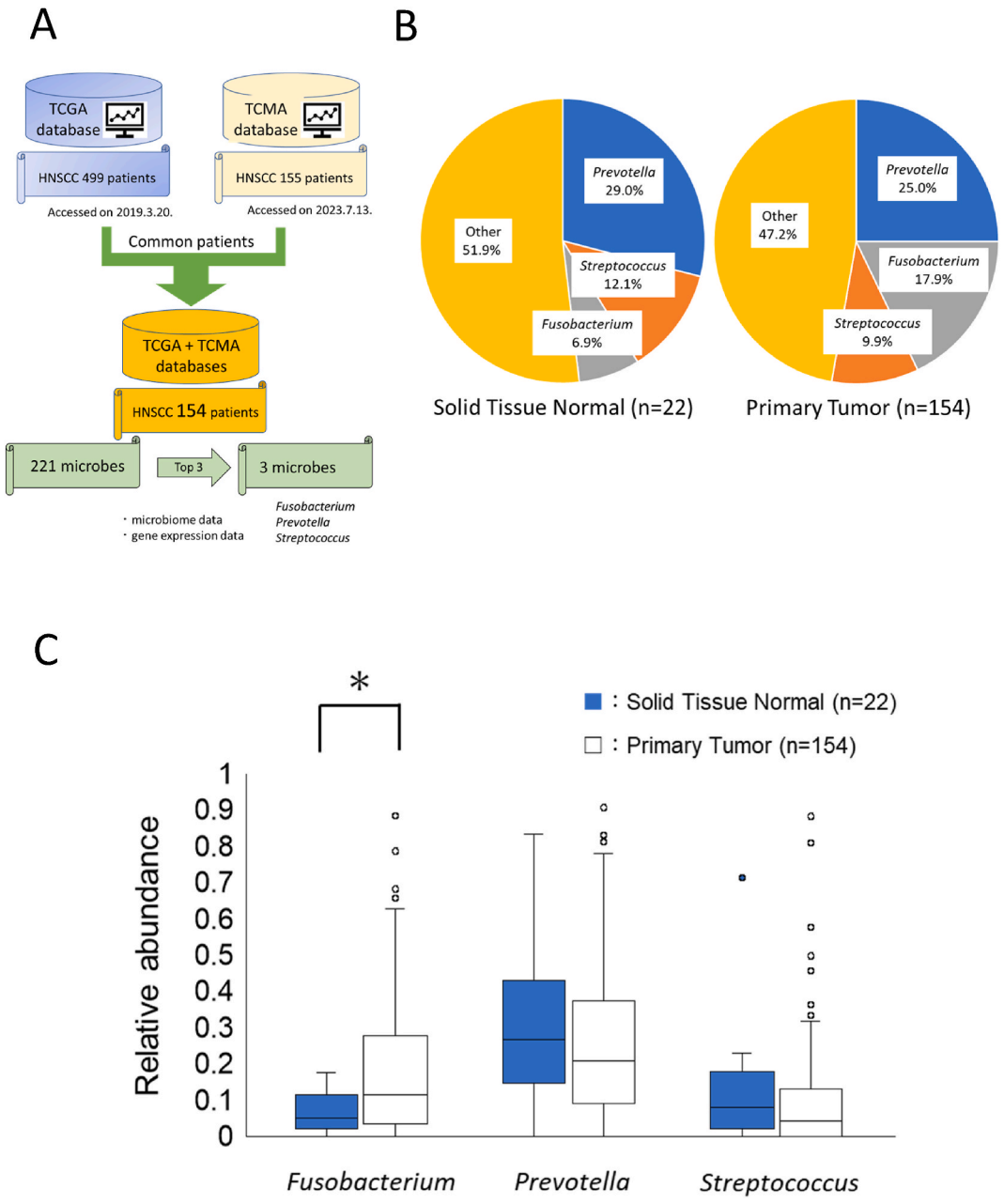


Fig. 1. Abundance of three major microorganisms in solid normal tissue and primary tumors of HNSCC patients (A) The extraction schedule for patients in both the TCGA and TCMA databases. (B) Abundance of *Fusobacterium*, *Prevotella*, and *Streptococcus*, the 3 top microbes at the genus level. (C) Comparison of the top 3 microbes at the genus level in solid normal tissue and the primary tumors of HNSCC patients. Differences were considered to be significant at $p < 0.05$.

identified in HNSCC [9–11].

Although bacteria were detected in tumors in the 19th century, its implication has not been examined in detail since then [12,13]. Due to recent advances in omics analyses and various technologies, a relationship between cancer and the microbiome has been reported for the majority of cancers, including colorectal cancer, skin cancer, breast cancer, bone cancer, cervical cancer, esophageal cancer, prostate cancer, stomach cancer, kidney cancer, lung cancer, and HNC, and this is a rapidly developing field [13,14]. The intratumoral microbiome enters the tumor site via hematogenous spread from the mouth, gut, or tumors [13]. However, HNSCC, particularly oral and pharyngeal cancers, are in close proximity to the oral cavity, and are always exposed to the oral microbiome. Therefore, the bacterial abundance of tumor-associated oral microorganisms in HNSCC is expected to be higher than in other carcinomas and most strongly affects components of the tumor microenvironment (TME) [15].

The Cancer Genome Atlas (TCGA) is a large-scale cancer genome project that was started in 2006 in the United States and has comprehensively analyzed genome methylation and gene and protein expression aberrations in more than 20 cancer types [16–18]. TCGA reported a comprehensive genomic characterization of HNC in 2015 [19]. Many omics analyses using TCGA database have been performed on HNC, and we also conducted a transcriptome analysis of gene expression induced by starvation in HNSCC in relation to prognosis and *Porphyromonas gingivalis*-infected cells and demonstrated the potential of PLAU as a prognostic biomarker using this database [20,21]. In contrast to conventional studies using mucosal swabs and saliva, a microbiome study on a tumor tissue and/or database is a relatively new research field. The Cancer Microbiome Atlas (TCMA) database, a curated and decontaminated collection of the microbial compositions of oropharyngeal, esophageal, gastrointestinal, and colorectal tissues, has been published, which allows for analyses of the pan-cancerous relationship between the microbiome and tumorigenesis [22]. It has promoted research on the intratumor microbiome of HNSCC, including a comprehensive analysis of the intratumor microbiome, the essential involvement of *Fusobacterium* in the immune microenvironment under inflammatory conditions, the clinical correlation between the intratumor oral microbiome and oral squamous cell carcinoma, and potential novel microbial markers, such as intratumoral *Leptotrichia* [15,23–27]. A previous study that extracted RNA sequencing data from TCGA investigated the relationships between the bacterial and fungal landscapes of HNSCC and HPV infection, smoking, and drinking habits [7]. We also reported that TCGA-HNSCC patients with sub-median levels of *Leptotrichia* in the intratumoral microbiome had a poorer prognosis [25].

Although many studies have recently been published on the association between HNSCC and intratumor bacteria [15,23–27], the findings obtained are not always consistent, even for the same bacterial species, and at this stage no conclusions can be drawn on how the intra-tumor microbiome may act. Therefore, the relationship between the major intratumoral microbiota of HNSCC and the impact of its classical prognostic factors on patient survival were examined herein, and differentially expressed genes in tumor cells associated with infections by the major microbes were also investigated. The present results suggest the impact of intratumoral *Fusobacterium* and *Streptococcus* on the prognosis of HNSCC in relation with the consumption of alcohol and smoking.

2. Materials and methods

2.1. Data collection from TCGA and TCMA databases

RNA-Seq count data (HTSeq version) on TCGA-HNSCC (499 primary tumor samples and 45 solid normal tissue samples) were obtained from the GDC Data Portal [28] (accessed on March 20, 2019.) with Subio Platform [29] software ver 1.24.5859 (Subio Inc. Aichi, Japan). The intratumor microbiome compositions of 177 TCMA-HNSCC samples (155 primary tumor samples and 22 solid normal tissue samples) at the genus level were collected from TCMA database [30] (accessed on 13 July 2023.). A total of 154 patients in both the TCGA and TCMA datasets were examined (Fig. 1A).

2.2. Filtering of TCMA genus microbes

Microbes were filtered using Subio Platform [29] software ver 1.24.5859. From the downloaded TCMA data, it was the 221 microbes at the genus level, but there were too many to analyze, so there were still 48 microbes at 0.01 or less, so 18 microbes were extracted by reducing it to 0.1 or less. Eighteen microbes remained, and we focused on three using mean relative abundance. The rates of the other microbes were summed and labeled as “Other.”

2.3. Kaplan-Meier survival analysis and Cox proportional hazards model

TCGA-HNSCC 154 primary tumor samples were divided into two groups for each of the 18 filter-passed microbes: a rate over and under the median. Kaplan-Meier survival curves were generated using Subio Platform software to compare the results for groups above and below the median for each microorganism. Interactions with known risk factors were confirmed using a Kaplan-Meier curve and the Log-rank test with gene expression as a factor and stratification by known risk factors. A Cox proportional hazards model that included significant interaction terms was constructed to confirm the effects of interactions after adjustments for confounding factors. We examined the relationships between classical prognostic factors, namely, the consumption of alcohol (yes: $n = 110$, no: $n = 40$), smoking (yes (cigarettes 2.82 ± 1.86/day): $n = 85$, no: $n = 69$), the HPV status (positive: $n = 42$, negative: $n = 111$), sex (male: $n = 112$, female: $n = 42$), lymph node metastasis (yes: $n = 79$, no: $n = 72$), and tumor size (T1-2: $n = 61$, T3-4: $n = 92$), and the abundance of each microbe.

2.4. Extraction of genes differentially expressed between over and under the median groups for Fusobacterium, Prevotella, and Streptococcus

The RNA-Seq data processing method were performed according to our previous study [25]. The Subio Platform (https://www.subioplatform.com/info_technical/293) was used for normalization and preprocessing of the RNA-Seq data. In this platform, low signal cut-off processing is a measure to align the lower limit of count with the lower end of the signal range to prevent falsely detecting differentially expressed genes (DEGs) due to the measurement values in the noise range. It is not recommended to set a constant cutoff value for all data. To prevent false detection of DEGs due to measurements within the noise range, the cut-off values of 50 and 32 were set in this study.

RNA-Seq count data at the 90th percentile were normalized, non-zero counts less than 50 were replaced with 50, and 0 as the low signal cut-off was replaced with 32. Normalized counts were converted to log2 ratios against the average of solid normal tissue samples. Genes with counts that were too low (count <50 in all samples) or too stable (log2 ratios between −1 and 1 in all samples) were excluded.

2.5. Functional pathway and protein-protein interaction (PPI) analyses

For selected genes, the Database for Annotation, Visualization, and Integrated Discovery (DAVID) server was used to examine the molecular pathways of gene ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. GO enrichment was performed at three main levels: cellular components (CC), biological processes (BP), and molecular functions (MF). Based on the STRING online database (https://string-db.org/accessed on 29 February 2024.), a PPI network was constructed using these genes. Next, we visualized the most important modules in the PPI network.

2.6. Statistical analysis

Data were plotted in boxplots, and comparisons between two groups were performed using the Student's *t*-test with Microsoft Excel (Microsoft, Redmond, WA, USA) according to our previous study [25]. The effects of *Fusobacterium*, *Prevotella*, and *Streptococcus* population rates (Under the median vs. Over the median) on all-cause mortality within 5 years were assessed using a Cox proportional hazards model analysis, with *Fusobacterium*, *Prevotella*, and *Streptococcus* population rates as the independent variable. Multivariate models were constructed adjusting for known risk factors, including age, gender, HPV status, alcohol intake, smoking (number of cigarettes per day), and the M, N, and T stages, which were also used as independent variables. Double log plots were generated to confirm the proportional hazard nature of *Fusobacterium*, *Prevotella*, and *Streptococcus* population rates. Selected known risk factors shall not include those with significant collinearilty. SPSS version 24.0 for Windows (IBM Japan, Tokyo, Japan) was used for statistical analyses. P-values were two-tailed and values < 0.05 indicated a significant difference.

3. Results

3.1. Microbiome profiling of top 3 microbes at the genus level

A total of 154 patients in the TCGA and TCMA datasets were selected and we planned to select major microbes from the 221 microbes defined at the TCMA gene level (Fig. 1A). The top 3 microbes in primary tumors were *Prevotella* (25.0 %), *Fusobacterium* (17.9 %), and *Streptococcus* (9.9 %) (Fig. 1B). For other, the results were *Actinomyces* (2.1 %), *Aggregatibacter* (0.7 %), *Alloprevotella* (1.9 %), *Campylobacter* (2.0 %), *Capnocytophaga* (4.8 %), *Granulicatella* (0.4 %), *Haemophilus* (4.0 %), *Lactobacillus* (1.0 %), *Leptotrichia* (1.7 %), *Mycoplasma* (0.6 %), *Neisseria* (2.3 %), *Porphyromonas* (2.3 %), *Rothia* (1.0 %), *Treponema* (4.9 %), and *Veillonella* (3.4 %) (Fig. 1B). The most population rates of *Prevotella*, *Streptococcus*, and *Fusobacterium* in normal tissue were 29.0, 12.1, and 6.9 %, respectively (Fig. 1B). We then investigated differences in the population rates of *Fusobacterium*, *Prevotella*, and *Streptococcus* at the genus level between solid normal tissue (n = 22) and the primary tumors (n = 154) of HNSCC patients. Differences were observed in the tissue microbiome profiles of *Fusobacterium* between solid normal tissue and primary tumors (Fig. 1C), with higher abundance in tumors than in solid normal tissue.

3.2. Cox regression analysis of relationships of top 3 microbes at the genus level and classical prognostic factors affecting survival in TCGA-HNSCC patients

The top 3 microbes and classical risk factors, including sex, HPV, smoking, drinking, age, and TNM stage as independent variables, using supplementary material (Table S1) were subjected to univariate and multivariate analyses (Cox proportional hazard model) of all-cause mortality within 5 years. The population rates of *Fusobacterium*, *Prevotella*, and *Streptococcus* at the genus level were divided into two groups: a rate over and under the median. Double log plots were performed and the results confirmed that the population rates of *Fusobacterium*, *Prevotella*, and *Streptococcus* were proportional hazards. In the univariate analysis, *Fusobacterium* Over the median (vs. Under the median) was HR = 0.722, 95 % CI = 0.442–1.178, $p = 0.192$. *Prevotella* Over the median (vs. Under the median) was HR = 1.589, 95 % CI = 0.971–2.600, $p = 0.065$. *Streptococcus* Over the median (vs. Under the median) was HR = 1.037, 95 % CI = 0.637–1.689, $p = 0.883$ (Table 1). In addition, the multivariate analysis showed that *Fusobacterium* Over the median (vs. Under the median) was HR = 0.884, 95 % CI = 0.497–1.574, $p = 0.676$. *Prevotella* Over the median (vs. Under the median) was HR = 1.720, 95 %

CI = 0.991–2.987, $p = 0.054$. *Streptococcus* Over the median (vs. Under the median) was HR = 0.988, 95 % CI = 0.573–1.706, $p = 0.996$. These results indicate that the abundance of these three bacteria was not associated with the prognosis of the TCGA-HNC patients examined.

3.3. Extraction of genes differentially expressed between over and under the median groups for Fusobacterium, Prevotella, and Streptococcus

To establish whether intratumoral *Fusobacterium*, *Prevotella*, and *Streptococcus* affect gene expression in HNSCC cells, genes with expression levels that were higher or lower in tumors than in solid normal tissue were analyzed. We divided the population rates of *Fusobacterium*, *Prevotella*, and *Streptococcus* at the genus level into the following two groups: a rate over and under the median. To facilitate our functional pathway and PPI analyses, the appropriate number of genes to be extracted was approximately 50. When the fold change was examined at >1.5, >2.0, and >2.5 and $p < 0.05$ in the Mann-Whitney *U* test, the extracted genes were 503, 117, and 43 for *Fusobacterium*, 55, 4, and 0 for *Prevotella*, and 381, 59, and 14 for *Streptococcus*, respectively. By using the criteria of >2.5, >1.5, or >2.0 fold and $p < 0.05$ in the Mann-Whitney *U* test, 43, 55, or 59 genes were extracted, respectively, determining approximately 50 up- and down-regulated genes in tumor cells between the over and under the median groups (Fig. 2A–C). Heat maps also showed 43, 55, and 59 genes that were differentially expressed between the over and under the median groups (Fig. 2D–F), the patterns of which differed for each of the genes extracted for *Fusobacterium*, *Prevotella*, and *Streptococcus* (Fig. 2D–F).

3.4. Functional and PPI analyses of Fusobacterium-, Prevotella-, and Streptococcus-related genes

To investigate the mechanisms associated with these microbes, functional and PPI analyses of differentially expressed genes were performed. Biological properties and potential signaling pathways were examined using GO terms and KEGG pathway analyses. The following enriched terms were common to *Fusobacterium* and *Streptococcus* in the GO enrichment analysis: antimicrobial humoral immune response mediated by antimicrobial peptide, cellular response to UV-A, cornification, epidermis development, extracellular matrix disassembly, intermediate filament organization, keratinocyte differentiation, keratinization, positive regulation of antibacterial peptide production, and proteolysis. Only epidermis development was common to *Fusobacterium*, *Prevotella*, and *Streptococcus* (Fig. 3A–C). The KEGG analysis revealed that *Fusobacterium* and *Streptococcus*-related genes correlated with the IL-17 signaling pathway and *Staphylococcus aureus* infection, while pathways associated with *Prevotella* were not extracted (Fig. 3D and E). The PPI analysis showed that these genes formed a dense network; density decreased in the order of *Fusobacterium*, *Streptococcus*, and *Prevotella* (Fig. 4A–C). Extracted genes densely related to *Fusobacterium* were CNFN, CRCT1, DEFB4A, DSG1, IL36G, IL36RN, KLK5, KLK7, KLK8, KLK10, KRT1, KRT14, KRT16, KRT6B, KRT6C, KRT75, KRTDAP, LCE3D, PI3, S100A12, S100A7, S100A7A, SBSN, SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR2D, SPRR2E, SPRR2F, SPRR2G, and TGM1. Extracted genes densely related to *Streptococcus* were CASP14, CRCT1, DEFB4A, DSC1, DSG1, FOXA1, KLK5, KLK7, KLK8, KRT1, KRT14, KRT15, KRT19, KRT6C, KRT75, KRTDAP, LCE3D, LCE3E, NTRK2, NTS, PI3, S100A7, S100A7A, SBSN, SPRR2B, and SPRR2G. The network formation of up-regulated genes (color-coded red) was characteristic in *Fusobacterium*, (Fig. 4A), while that of down-regulated genes (color-coded blue) was observed in *Streptococcus* (Fig. 4C).

Table 1
Univariate and multivariate analyses of all-cause mortality within 5 years of top 3 selected microbial species in TCGA-HNSCC patients.

	Univariate					Multivariate				
	HR	95 % CI			P-value	HR	95 % CI			P-value
*Fusobacterium*_Over (vs. Under)	0.722	0.442	–	1.178	0.192	0.884	0.497	,	1.574	0.676
*Prevotella*_Over (vs. Under)	1.589	0.971	–	2.600	0.065	1.720	0.991	,	2.987	0.054
*Streptococcus*_Over (vs. Under)	1.037	0.637	–	1.689	0.883	0.988	0.573	,	1.706	0.966
Age (per 1 year)	1.000	0.979	–	1.021	0.993	1.009	0.984	,	1.033	0.492
Sex_male (vs. female)	0.825	0.491	–	1.388	0.469	0.805	0.434	,	1.494	0.491
HPV status_Positive (vs. Negative)	0.693	0.384	–	1.253	0.225	0.515	0.263	,	1.008	0.053
Alcohol_history_Yes (vs. No)	1.302	0.736	–	2.304	0.365	1.350	0.667	,	2.733	0.404
Cigarettes per day_>0 (vs. 0)	1.308	0.797	–	2.146	0.288	1.366	0.780	,	2.393	0.275
M stage_m1 (vs. m0)	8.793	1.166	–	66.316	**0.035**	11.016	1.206	,	100.589	**0.033**
N stage (Continuous variable per 1)	1.093	0.966	–	1.236	0.158					
N stage (Category)										
Lymph node metastasis no	1.000	ref				1.000		ref		
Lymph node metastasis yes	1.388	0.842	,	2.289	0.198	1.482	0.853	,	2.576	0.163
T stage (Category)										
T1-2	1.000		ref			1.000		ref		
≥T3	1.250	0.748	,	2.092	0.394	1.276	0.707	,	2.305	0.419

HR: hazard ratio; 95 % CI: 95 % confidence interval; ref: reference value; Over: Over the median; Under: Under the median. Bold type indicates $p < 0.05$.

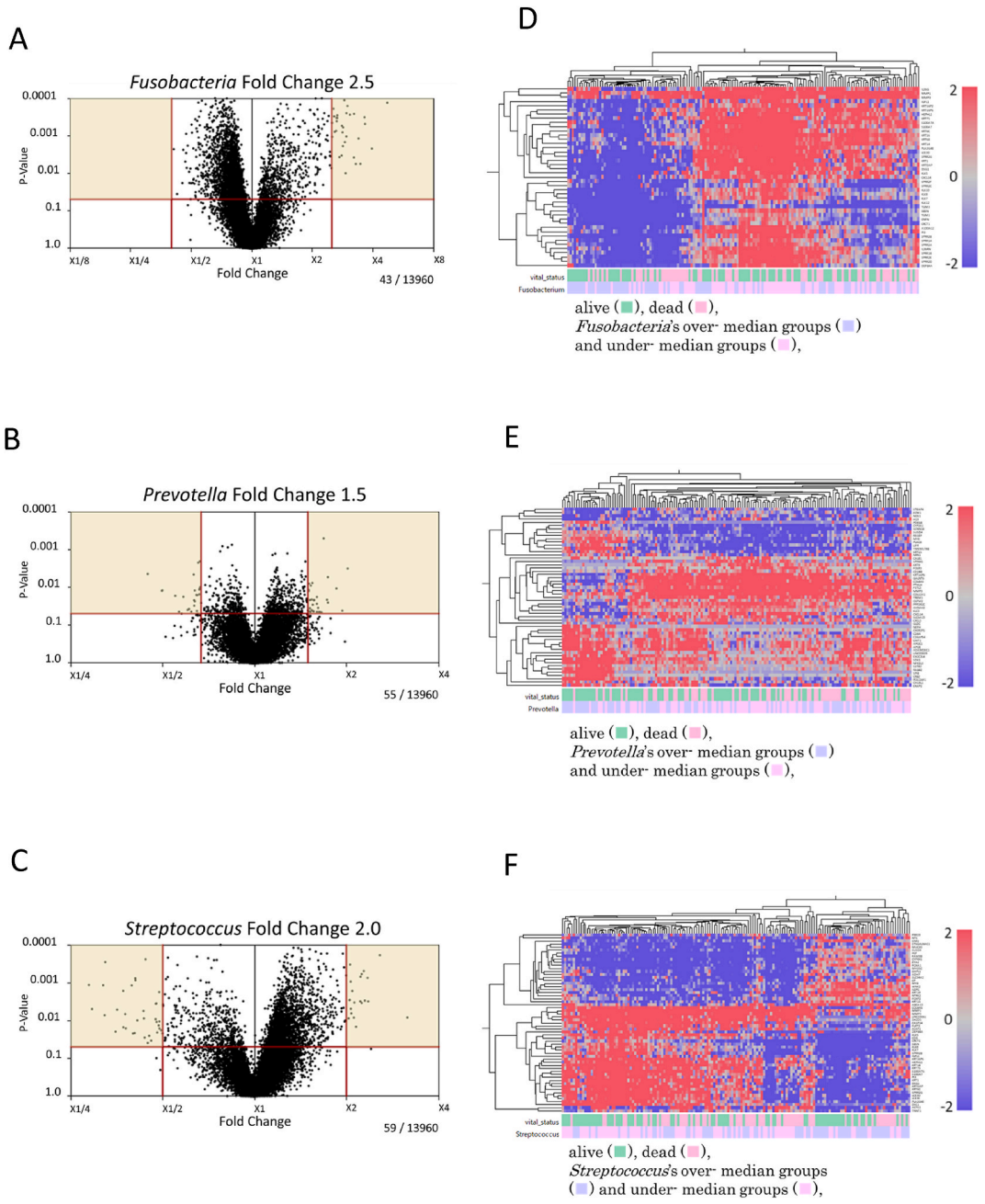


Fig. 2. Extraction of genes related to *Fusobacterium*, *Prevotella*, and *Streptococcus* (A) Forty-three genes that were differentially expressed between the over and under the median groups for *Fusobacterium* were extracted using the criteria of >2.5 fold and $p < 0.05$ in the Mann-Whitney *U* test. (B) Fifty-five genes that were differentially expressed between the over and under the median groups for *Prevotella* were extracted using the criteria of >1.5 fold and $p < 0.05$ in the Mann-Whitney *U* test. (C) Fifty-nine genes that were differentially expressed between the over and under the median groups for *Streptococcus* were extracted using the criteria of >2.0 fold and $p < 0.05$ in the Mann-Whitney *U* test. Heat maps and the hierarchical clustering of extracted genes related to *Fusobacterium* (D), *Prevotella* (E), and *Streptococcus* (F). The vital status of TCGA-HNSCC patients and the rate of each microbe are coded as follows: alive ( ), dead ( ), over the median ( ), and under the median ( ).

3.5. The relationship between the abundance of top 3 major microorganisms and classical risk factors in primary tumors of TCGA-HNSCC patients, and relationships between taxonomic prognostic factors associated with Fusobacterium, Prevotella, and Streptococcus and survival

We initially examined the relationships between classical prognostic factors, namely, the consumption of alcohol, smoking, the HPV status, sex, lymph node metastasis, and tumor size, and the abundance of *Fusobacterium* (Fig. 5A), *Prevotella* (Fig. 5B), and

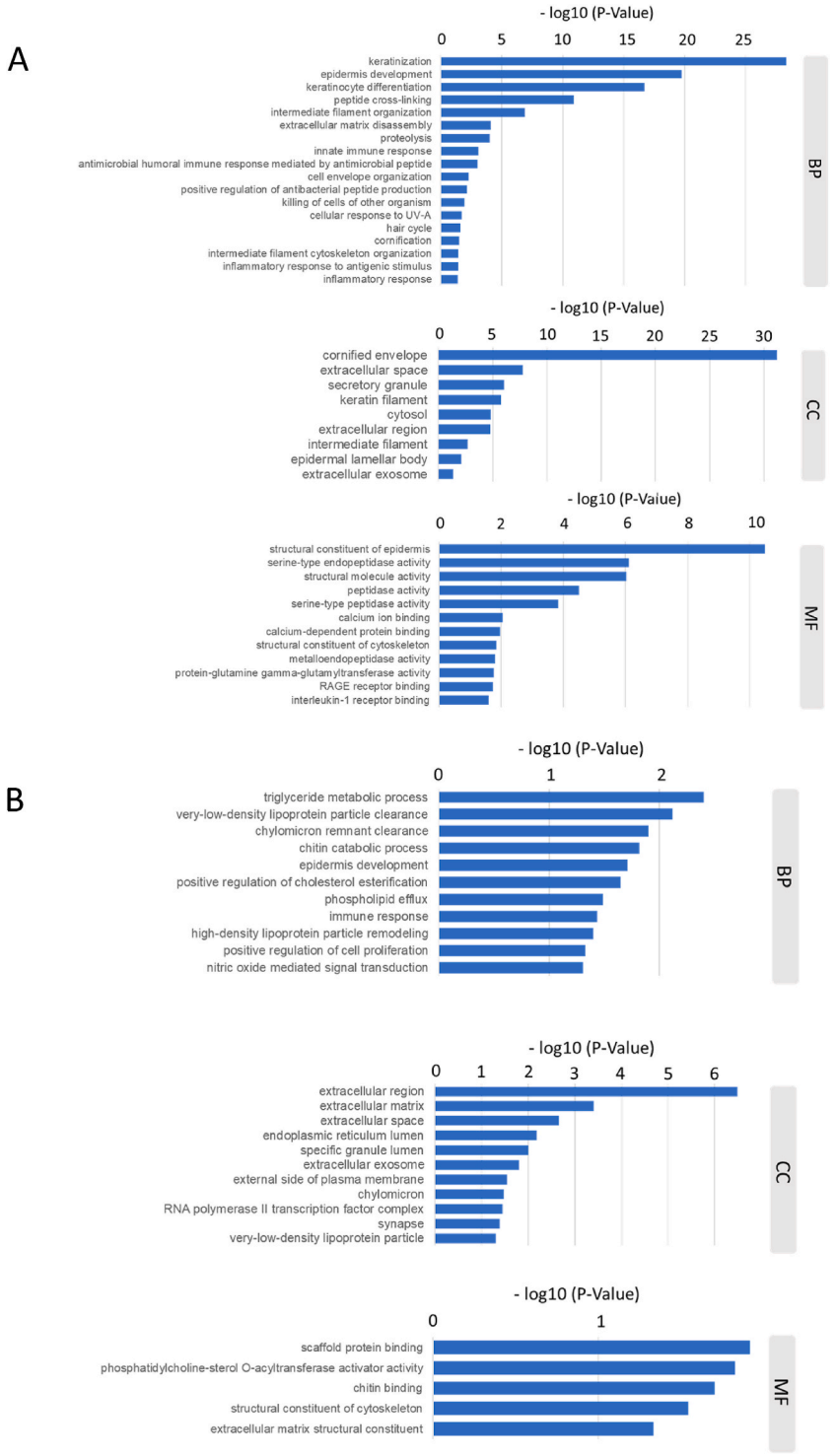


Fig. 3. Functional analyses of *Fusobacterium*-, *Prevotella*-, and *Streptococcus*-related genes (A) GO terms identified in a GO enrichment analysis of 43 genes that were differentially expressed between the over and under the median groups for *Fusobacterium* and extracted using the criteria of >2.5 fold and $p < 0.05$ in the Mann-Whitney *U* test are shown. (B) GO terms identified in a GO enrichment analysis of 55 genes that were differentially expressed between the over and under the median groups for *Prevotella* and extracted using the criteria of >1.5 fold and $p < 0.05$ in the Mann-Whitney *U* test are shown. (C) GO terms identified in a GO enrichment analysis of 59 genes that were differentially expressed between the over and under the median groups for *Streptococcus* and extracted using the criteria of >2.0 fold and $p < 0.05$ in the Mann-Whitney *U* test are shown. BP, biological process; CC, cellular composition; MF, molecular function. (D) Molecular pathways identified in a KEGG pathway enrichment analysis of

43 extracted genes related to *Fusobacterium* are shown. (E) Molecular pathways identified in a KEGG pathway enrichment analysis of 59 extracted genes related to *Streptococcus* are shown.

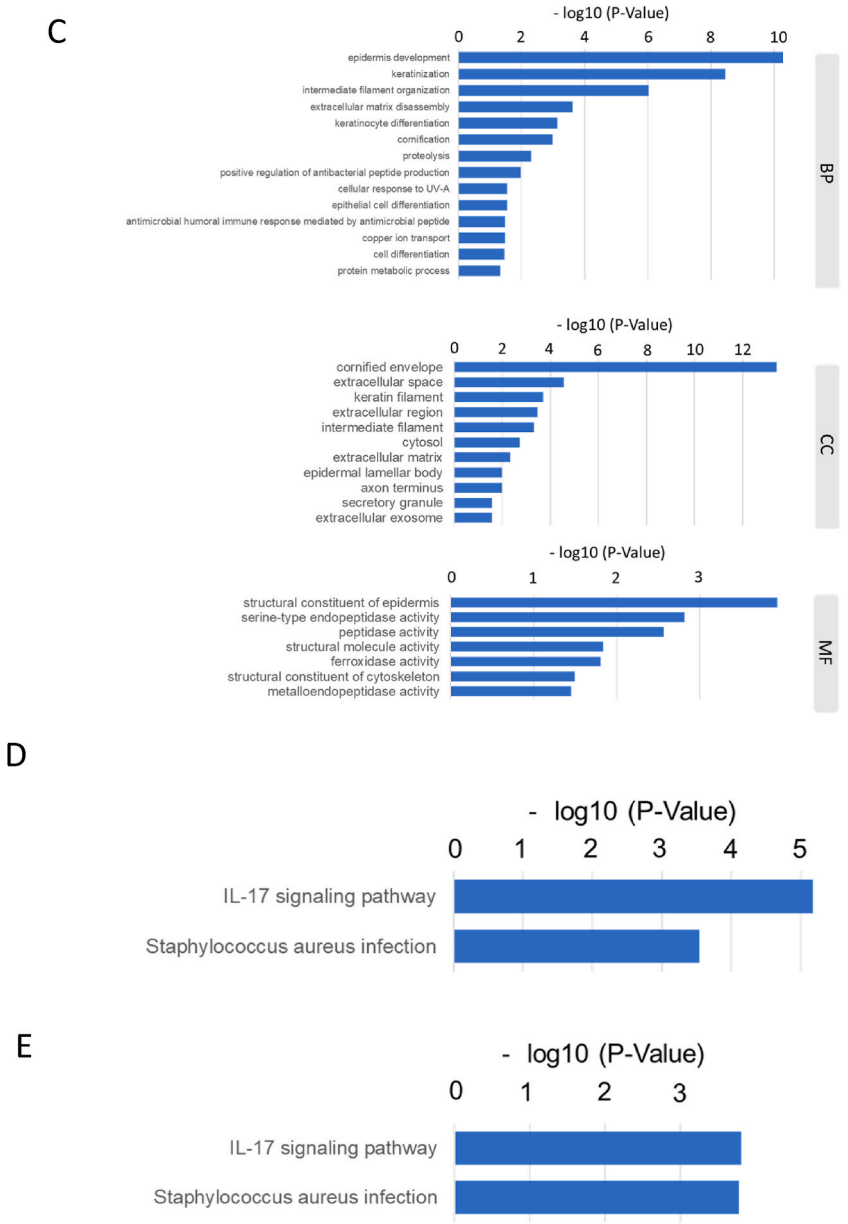


Fig. 3. (*continued*).

Streptococcus (Fig. 5C). The majority of classical prognostic factors were not associated with the abundance of microbes; however, the abundance of *Prevotella* at the genus level was associated with the effects of alcohol consumption.

We then investigated whether classical risk factors affected the survival curve of HNSCC patients in relation to each bacterium using the generalized Wilcoxon and Log-rank tests. Regarding *Fusobacterium*, the survival probability of patients with abundant bacteria and no history of alcohol consumption was increased, while no significant difference was observed in the survival curve of patients with a history of alcohol consumption (Fig. 6A–a, b). Similar results were obtained in the generalized Wilcoxon test and Log-rank test for patients without a history of smoking, neck lymph node metastasis, HPV infection, and females (Fig. 6A–d, -f, h, j). Survival probability was not significantly affected by other factors, regardless of whether patients were over or under the *Fusobacterium* mean. Regarding *Prevotella,* survival probability was not markedly affected by classical prognostic factors (Fig. S1). On the other hand, when survival probability in relation to the abundance of *Streptococcus* was examined, the survival probability was decreased for patients with abundant *Streptococcus* and no history of alcohol consumption (Fig. 6B–b). No significant difference was observed when survival probability was compared in patients with a history of alcohol consumption (Fig. 6B–a).

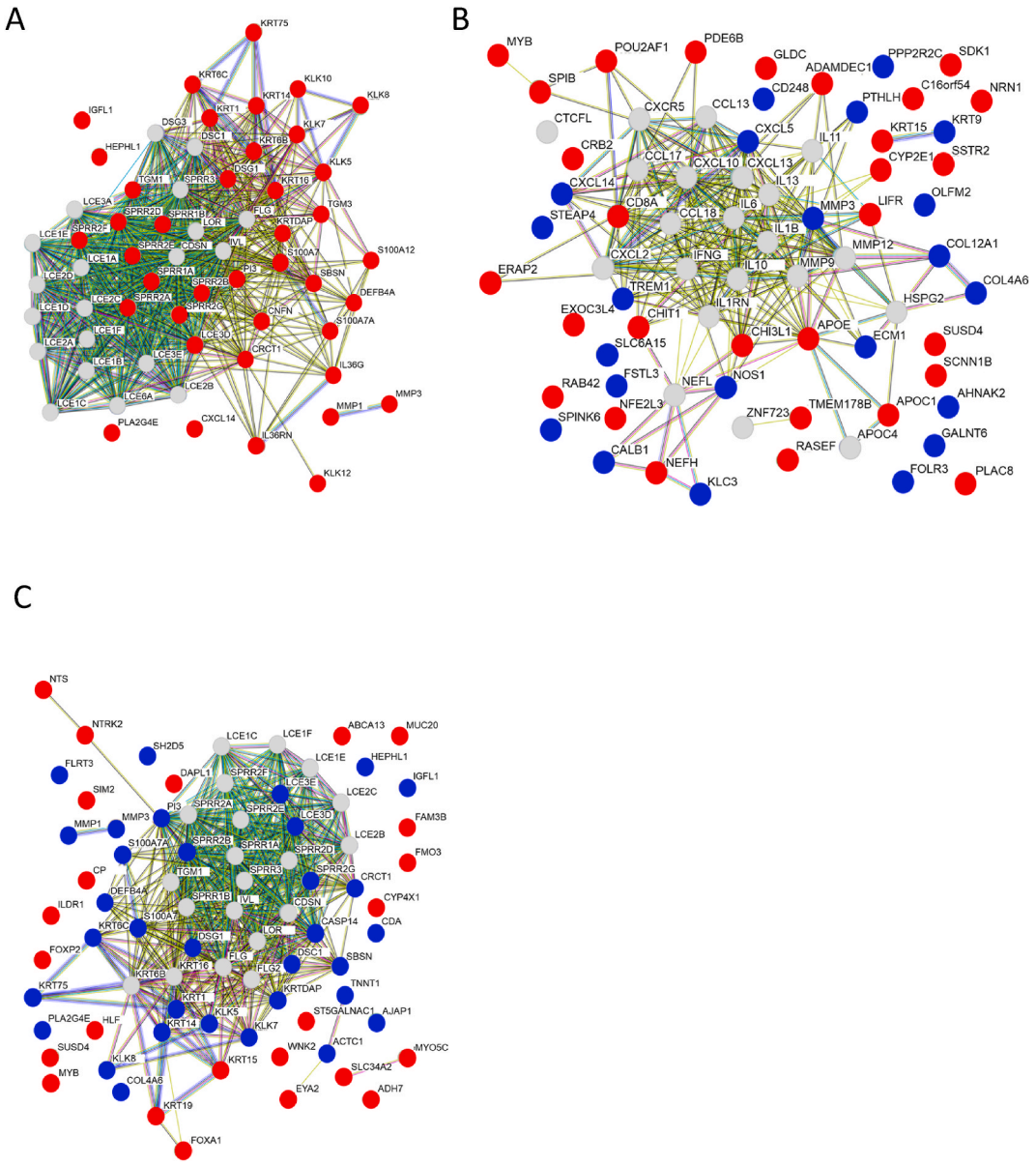


Fig. 4. Protein–protein interaction analyses of *Fusobacterium-, Prevotella-,* and *Streptococcus*-related genes (A) Proteins encoded by 43 extracted genes related to *Fusobacterium* were subjected to a PPI network analysis. (B) Proteins encoded by 55 extracted genes related to *Prevotella* were subjected to a PPI network analysis. (C) Proteins encoded by 59 extracted genes related to *Streptococcus* were subjected to a PPI network analysis. Genes with fold changes (over/under the median) > upper limit (2.5, 1.5, or 2.0), and lower limit (0.4, 0.66, or 0.5) are coded in red (■) and blue (■), respectively. Gray (■) was used for all other cases. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Tables 2 and 3 show the results of a multivariate analysis of these interaction terms with adjustments for confounding factors. The hazard ratio (HR) for *Fusobacterium* in the group of patients with no history of alcohol consumption was 0.280, with a P- value of 0.018. Similarly, the HR for *Fusobacterium* in the group of patients with no smoking history was 0.367, with a P value of 0.017 (Table 2). These results indicate that mortality was reduced in patients with an abundance of *Fusobacterium* in their tumors and no history of alcohol consumption or smoking. In the interaction analysis with drinking or smoking and *Fusobacterium*, the interaction terms *Fusobacterium* × Alcohol history and *Fusobacterium* × Cigarettes per day were significant at $p = 0.029$ and $p = 0.015$, respectively.

The interaction analysis with alcohol consumption identified the abundance of *Streptococcus* as a significant factor affecting all-cause mortality in patients with a history of alcohol consumption (HR = 2.960 [95%CI: 1.019–7.904], $p = 0.030$), and the interaction term *Streptococcus* × Alcohol history was significant ($p = 0.025$).

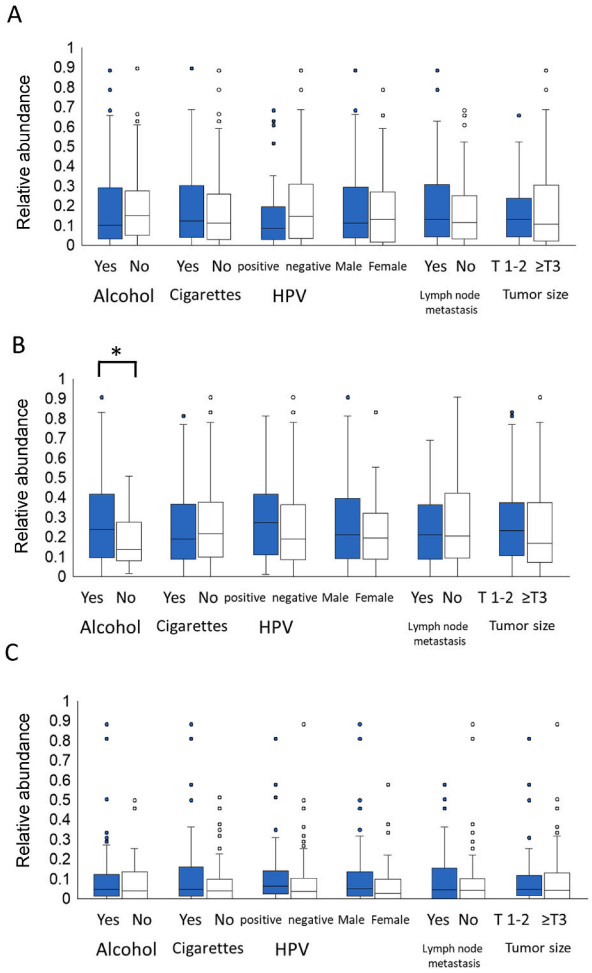


Fig. 5. Relationship between the abundance of 3 major microorganisms in primary tumors and classical risk factors. Comparison of the abundance of *Fusobacterium* (A), *Prevotella* (B), and *Streptococcus* (C) at the genus level in consideration of classical prognostic factors, such as alcohol consumption, smoking, the HPV status, sex, lymph node metastasis, and tumor sizes. Differences were considered to be significant at $p < 0.05$.

4. Discussion

We herein used TCMA database, examined intratumoral microbiomes, and found that the well-known oral resident bacteria, *Fusobacterium*, *Prevotella*, and *Streptococcus* at the genus level accounted for approximately 50 % of the population in all intratumoral microbiomes. In addition, *Actinomyces*, *Aggregatibacter*, *Alloprevotella*, *Campylobacter*, *Capnocytophaga*, *Granulicatella*, *Haemophilus*, *Lactobacillus*, *Leptotrichia*, *Mycoplasma*, *Neisseria*, *Porphyromonas*, *Rothia*, *Treponema*, and *Veillonella* each accounted for over 0.1 % of detected, together accounting for approximately 36 % of the total, of which *Leptoplechia* was associated with prognosis in our previous study [25]. We then focused on these key bacteria by examining their abundance in HNSCC tissues, their interactions with other HNC prognostic factors, and their impact on the survival of HNSCC patients.

Among the three bacteria examined, only *Fusobacterium* was more abundant in tumors than in solid normal tissue. Furthermore, when the effects of the presence of each bacterium in tumors on patient survival was examined, HR slightly decreased for *Fusobacterium* and increased for *Prevotella* and *Streptococcus*; however, no obvious bacterial effect was observed. These results are inconsistent with those of Li et al. [27], showing the lower survival probability of oral cancer patients in the *Fusobacterium*-rich group. We also examined interactions between the major bacteria in the microbiome and known risk factors for HNCCC, namely, smoking, alcohol consumption, HPV infection, and the T, N, and M stages; however, only the M stage was significant.

To detect bacteria-induced changes in the expression of genes in HNSCC tumor tissue, a KEGG pathway analysis was performed and revealed that *Fusobacterium* and *Streptococcus* were associated with the IL-17 signaling pathway and *S. aureus* infection. The tight network between genes that were up-regulated with *Fusobacterium* infection and those that were down-regulated with *Streptococcus* infection provides insights into the functional effects of these bacteria.

The roles of *Fusobacterium* and *Streptococcus* in patient survival were herein examined in relation to classical risk factors and by a multivariate analysis. The results obtained showed that HNSCC patients with high *Fusobacterium* levels and no history of alcohol

consumption or smoking had a lower HR than those with low *Fusobacterium* levels (Fig. 6A–b, d) (Table 2). Similar results were not obtained for patients with a history of alcohol consumption and smoking. This supports the contribution of *Fusobacterium* to the favorable prognosis of patients and that alcohol and smoking may eliminate the beneficial impact of this microorganism on patient outcomes. In contrast, patients with HNSCC without a history of alcohol consumption had a poorer prognosis with a higher level of intratumoral *Streptococcus*. Collectively, these results suggest that the prognosis of HNSCC is modulated by the combined effects of the intratumoral microbiome and classical risk factors.

Fusobacterium are Gram-negative anaerobic rod bacteria with species-specific reservoirs in the human oral cavity and

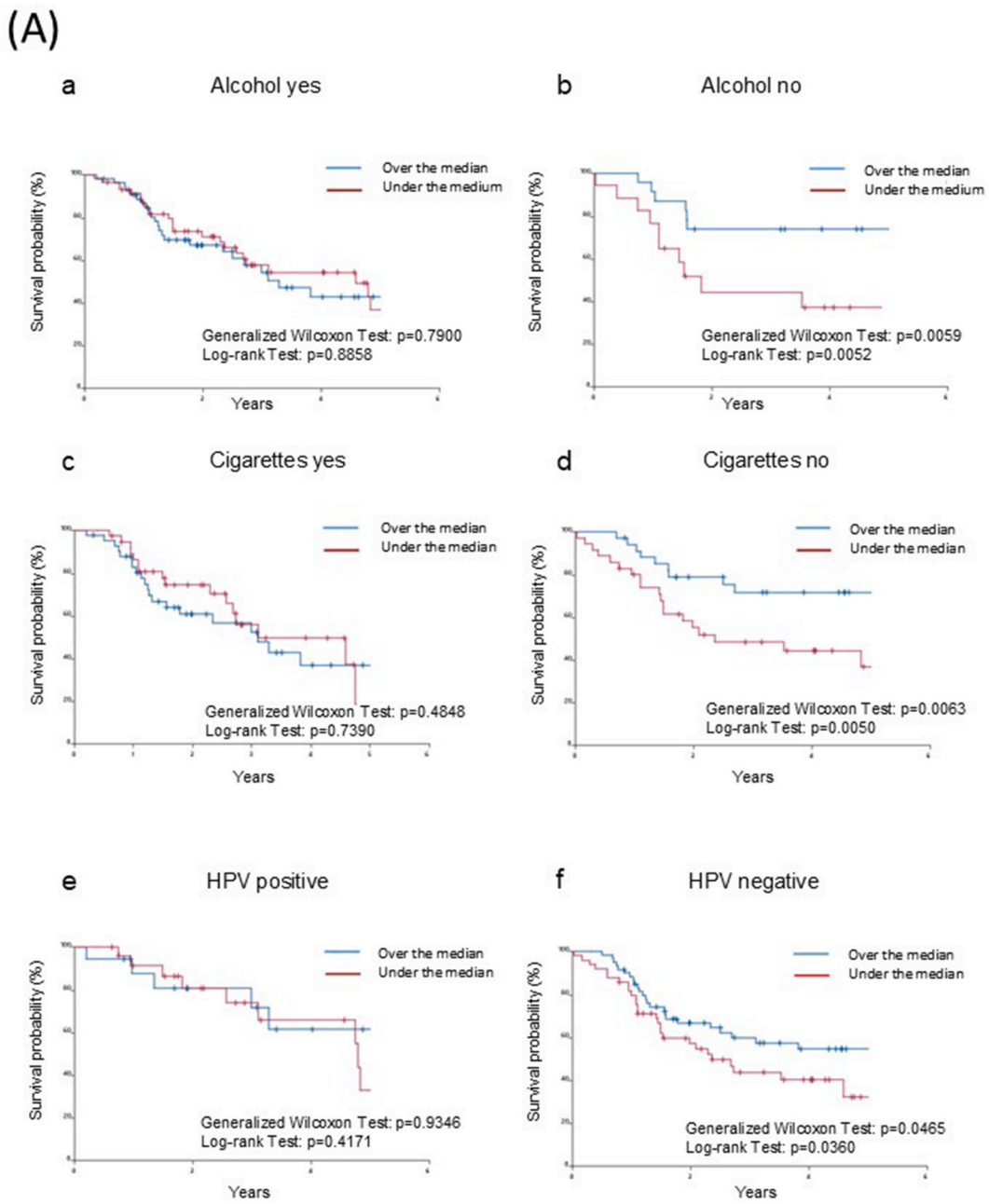


Fig. 6. Relationships between classical prognostic factors and survival rates associated with *Fusobacterium*, *Prevotella*, and *Streptococcus* in TCGA-HNSCC patients. Survival curves were recalculated based on the populations of *Fusobacterium* (A), and *Streptococcus* (B) in consideration of classical prognostic factors, such as alcohol consumption, smoking, the HPV status, sex, lymph node metastasis, and tumor size. (a) A history of alcohol consumption. (b) No history of alcohol consumption. (c) A history of smoking. (d) No history of smoking. (e) A history of HPV infection. (f) No history of HPV infection. (g) Males. (h) Females. (i) Lymph node metastasis. (j) No lymph node metastasis. (k) Tumor sizes T1-T2. (l) Tumor sizes > T3. Differences were considered to be significant at $p < 0.05$.

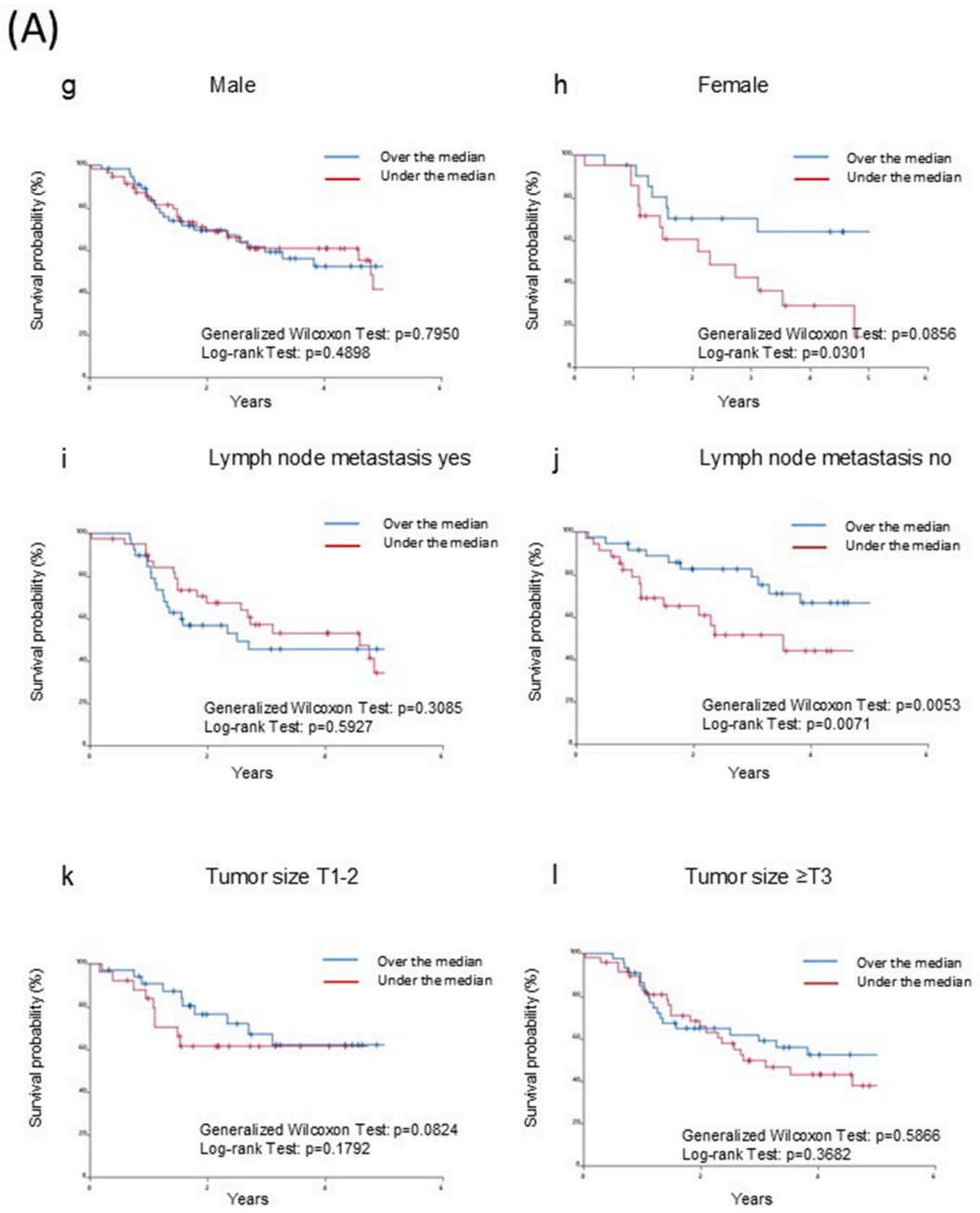
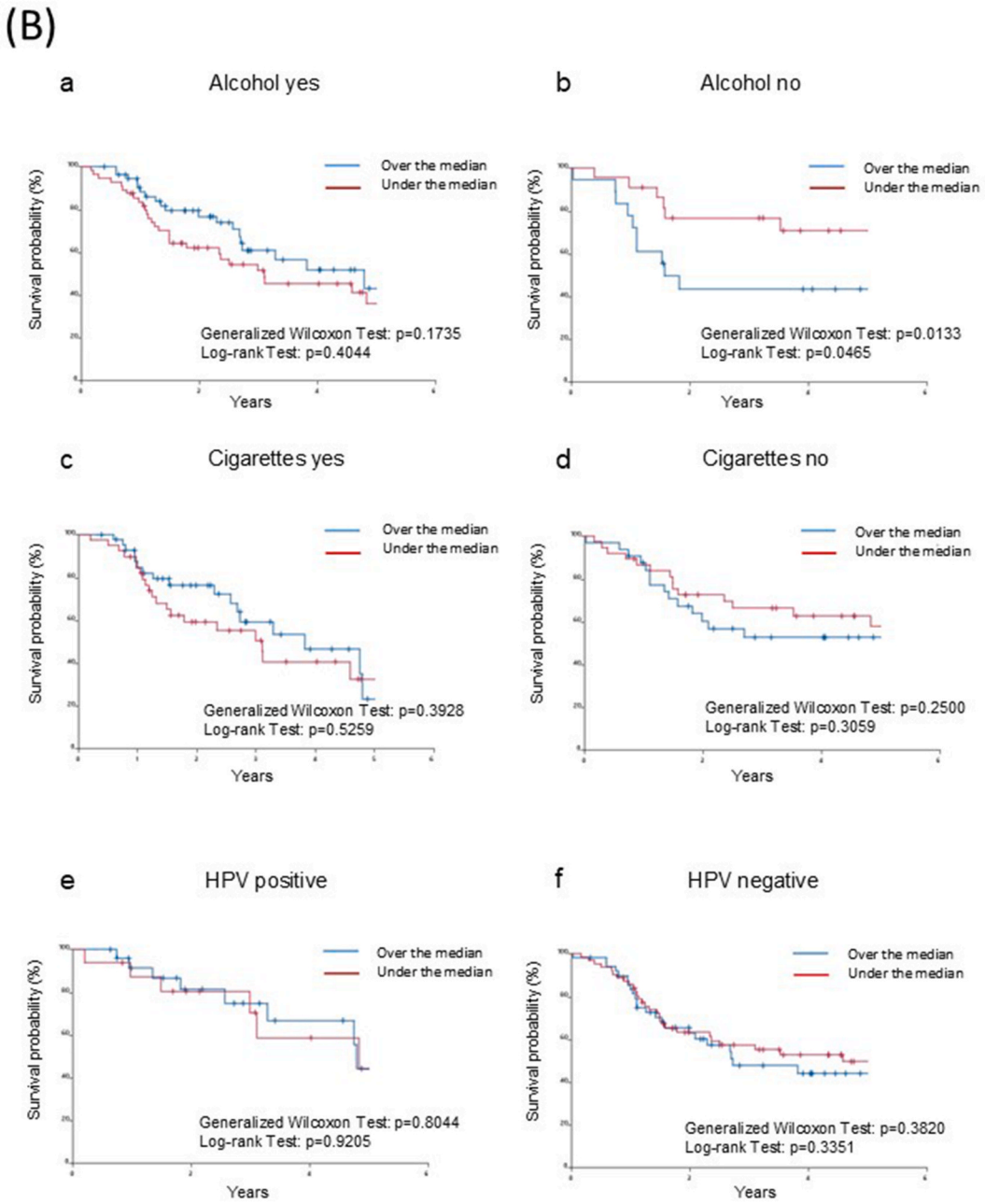


Fig. 6. (*continued*).



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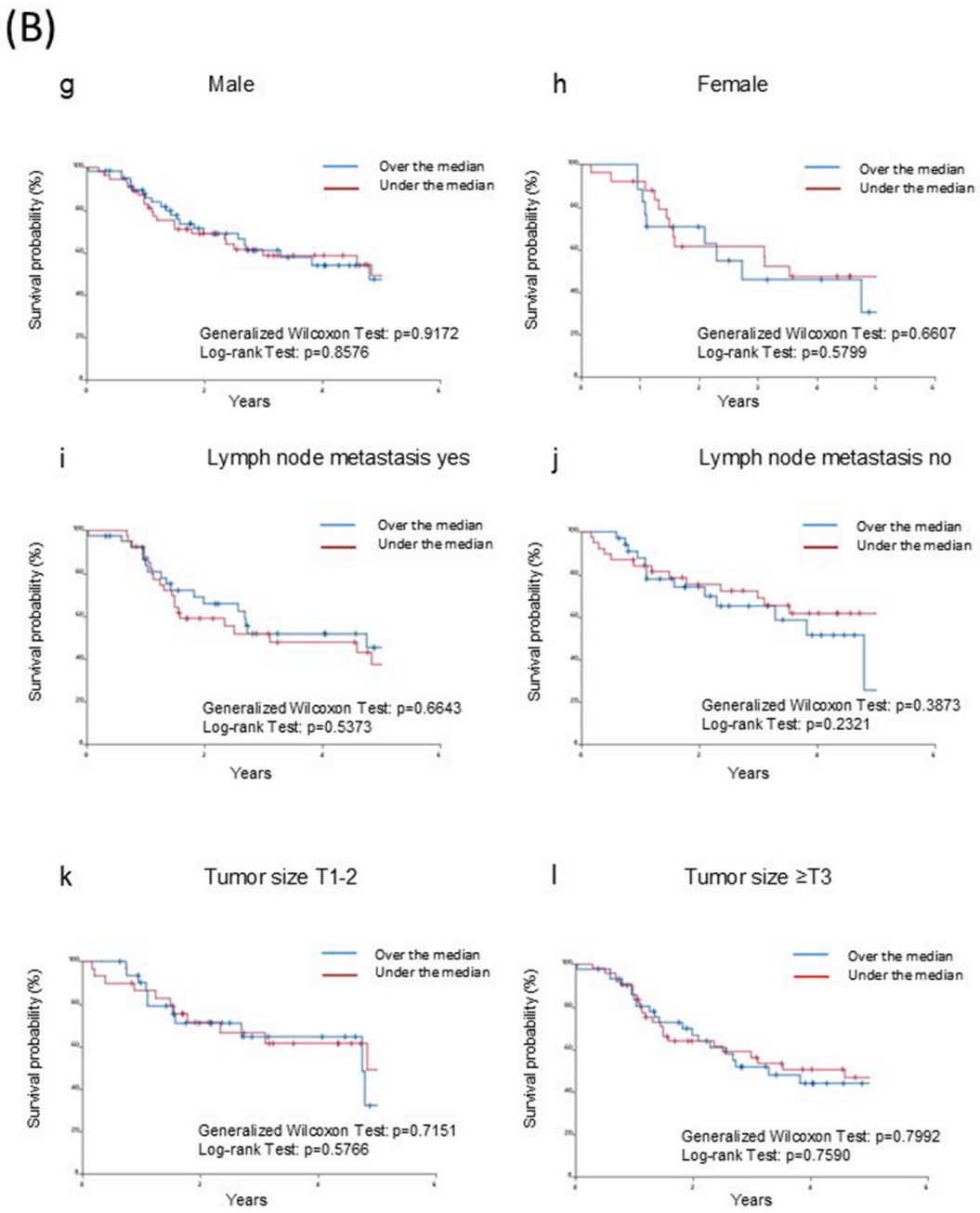

Fig. 6. (*continued*).

gastrointestinal tract [31,32]. Among them, *Fusobacterium nucleatum* has been implicated in the pathogenesis of esophageal, colorectal, gastric, pancreatic, and breast cancers [13,32]. *F. nucleatum* has been associated with tumor progression, recurrence, and a worse prognosis in patients with colon cancer [13,14,31]. It was also shown to target innate immune signaling pathways and mRNA-mediated autophagy and contributed to the acquisition of chemoresistance by colorectal cancer [33]. Furthermore, *F. nucleatum* promoted tumor progression by generating a pro-inflammatory microenvironment [34]. The short-chain fatty acid butyrate released from anaerobic bacteria was found to exert pro-tumorigenic effects by inhibiting natural killer cell functions [35]. In a study on HNSCC, Qiao et al. [15] demonstrated that *Fusobacterium* was enriched in HNSCC tissues, and this was associated with a more severe inflammatory response and poor prognosis. In studies on patients with a worse prognosis, *F. nucleatum* increased bone marrow -derived immune cells and regulatory T cells, promoted M2 polarization of macrophages, and induced tumorigenic inflammation [36,37]. The present results on the prognosis of HNSCC with *Fusobacterium* were not consistent with colon and HNSCC findings [32,38,39]. The abundance of *Fusobacterium* alone did not affect the prognosis of patients. However, the survival probability of HNSCC

Table 2
Multivariate analyses of TCGA-HNSCC patient data using the interaction mode of *Fusobacterium*.

	Multivariate				
	HR	95 % CI			P-value
With the interaction mode [*Fusobacterium* × Alcohol history]					
Alcohol_history_Yes (vs. No)	0.665	0.269	–	1.648	0.379
Cigarettes per day_>0 (vs. 0)	1.309	0.750	–	2.285	0.343
M stage_m1 (vs. m0)	13.056	1.444	–	118.062	**0.022**
N stage (Category)			–		
Lymph node metastasis no	1.000		ref		
Lymph node metastasis yes	1.596	0.903	–	2.823	0.108
T stage (Category)			–		
T1-2	1.000		ref		
≥T3	1.182	0.661	–	2.114	0.572
HPV status_Positive (vs. Negative)	0.571	0.293	–	1.113	0.100
Sex_male (vs. female)	0.881	0.475	–	1.633	0.687
Age (per 1)	1.008	0.984	–	1.034	0.507
*Fusobacterium*_Over (vs. Under) [in Alcohol history_Yes]	1.094	0.600		1.994	0.769
*Fusobacterium*_Over (vs. Under) [in Alcohol history_No]	0.280	0.098		0.804	**0.018**
Fusobacterium × Alcohol history	3.904	1.152		13.228	**0.029**
With the interaction mode [*Fusobacterium* × Cigarettes per day]					
Alcohol_history_Yes (vs. No)	1.364	0.675	–	2.755	0.387
Cigarettes per day_>0 (vs. 0)	0.694	0.335	–	1.439	0.326
M stage_m1 (vs. m0)	16.548	1.773	–	154.429	**0.014**
N stage (Category)			–		
Lymph node metastasis no	1.000		ref		
Lymph node metastasis yes	1.413	0.817	–	2.445	0.216
T stage (Category)			–		
T1-2	1.000		ref		
≥T3	1.198	0.673	–	2.133	0.539
HPV status_Positive (vs. Negative)	0.505	0.260	–	0.982	**0.044**
Sex_male (vs. female)	0.797	0.432	–	1.471	0.468
Age (per 1)	1.004	0.980	–	1.029	0.738
*Fusobacterium*_Over (vs. Under) [Cigarettes per day_>0]	1.385	0.684		2.806	0.366
*Fusobacterium*_Over (vs. Under) [Cigarettes per day_0]	0.367	0.162		0.834	**0.017**
Fusobacterium × Cigarettes per day	3.770	1.287		11.039	**0.015**
With the interaction mode [*Fusobacterium* × HPV status]					
Alcohol_history_Yes (vs. No)	1.437	0.716	–	2.884	0.307
Cigarettes per day_>0 (vs. 0)	1.239	0.710	–	2.161	0.451
M stage_m1 (vs. m0)	12.906	1.385	–	120.232	**0.025**
N stage (Category)			–		
Lymph node metastasis no	1.000		ref		
Lymph node metastasis yes	1.474	0.849	–	2.560	0.168
T stage (Category)			–		
T1-2	1.000		ref		
≥T3	1.111	0.616	–	2.004	0.727
HPV status_Positive (vs. Negative)	0.474	0.197	–	1.142	0.096
Sex_male (vs. female)	0.818	0.447	–	1.498	0.516
Age (per 1)	1.009	0.985	–	1.034	0.478
*Fusobacterium*_Over (vs. Under) [HPV status_Positive]	1.003	0.308		3.268	0.996
*Fusobacterium*_Over (vs. Under) [HPV status_Negative]	0.727	0.405		1.303	0.284
Fusobacterium × HPV status	1.380	0.368		5.168	0.633
With the interaction mode [*Fusobacterium* × N stage]					
Alcohol_history_Yes (vs. No)	1.500	0.747	–	3.011	0.254
Cigarettes per day_>0 (vs. 0)	1.219	0.702	–	2.117	0.482
M stage_m1 (vs. m0)	13.790	1.517	–	125.402	**0.020**
N stage (Category)			–		
Lymph node metastasis no	1.000		ref		
Lymph node metastasis yes	0.932	0.437	–	1.989	0.856
T stage (Category)			–		
T1-2	1.000		ref		
≥T3	1.185	0.663	–	2.117	0.566
HPV status_Positive (vs. Negative)	0.570	0.293	–	1.109	0.098
Sex_male (vs. female)	0.835	0.456	–	1.530	0.560
Age (per 1)	1.007	0.982	–	1.032	0.603
*Fusobacterium*_Over (vs. Under) [N stage_ Lymph node metastasis yes]	1.138	0.568		2.281	0.716
*Fusobacterium*_Over (vs. Under) [N stage Lymph node metastasis no]	0.469	0.210		1.050	0.066
Fusobacterium × N stage	2.426	0.837		7.032	0.103

HR: hazard ratio; 95 % CI: 95 % confidence interval; ref: reference value; Over: Over the median; Under: Under the median.
Analyses were performed with adjustments for background factors (age, sex, alcohol use, smoking, and the TNM stage). Bold type indicates $p < 0.05$.

Table 3
Multivariate analyses of TCGA-HNSCC patient data using the interaction mode of *Streptococcus*.

	Multivariate				
	HR	95 % CI			P-value
With the interaction mode [*Streptococcus* × Alcohol history]					
Alcohol_history_Yes (vs. No)	2.960	1.109	–	7.904	**0.030**
Cigarettes per day_>0 (vs. 0)	1.146	0.660	–	1.990	0.628
M stage_m1 (vs. m0)	18.551	1.974	–	174.364	**0.011**
N stage (Category)			–		
Lymph node metastasis no	1.000		ref		
Lymph node metastasis yes	1.415	0.808	–	2.479	0.225
T stage (Category)			–		
T1-2	1.000		ref		
≥T3	1.182	0.661	–	2.113	0.573
HPV status_Positive (vs. Negative)	0.580	0.299	–	1.127	0.108
Sex_male (vs. female)	0.837	0.458	–	1.532	0.565
Age (per 1)	1.015	0.990	–	1.040	0.252
*Streptococcus*_Over (vs. Under) [in Alcohol history_Yes]	0.721	0.395		1.317	0.287
*Streptococcus*_Over (vs. Under) [in Alcohol history_No]	2.953	1.021		8.540	**0.046**
Streptococcus × Alcohol history	0.244	0.071		0.838	**0.025**

HR: hazard ratio; 95 % CI: 95 % confidence interval; ref: reference value; Over: Over the median; Under: Under the median.
Analyses were performed with adjustments for background factors (age, sex, alcohol use, smoking, and the TNM stage). Bold type indicates $p < 0.05$.

patients was improved in patients harboring high abundance of *Fusobacterium* but without a history of alcohol consumption or smoking. Although these results were opposite to the findings obtained on the prognosis of colorectal cancer, similar outcomes have been reported. Neuzillet et al. [40] examined HNSCC patients and found that *F. nucleatum* positivity was associated with older age, lower alcohol intake, combined alcohol and tobacco use, and less frequent lymph node metastasis. Furthermore, the recurrence rate was slightly lower and survival was significantly longer. They also found that high loads of *F. nucleatum* were associated with low levels of TLR4 and M2 macrophages. Chen et al. [41] examined HNSCC tumor tissue samples and found that the enrichment of *F. nucleatum* in HNSCC tumor tissue correlated with longer cancer survival and lower recurrence rates. Using TCMA database, Yeo et al. [26] showed that a high *Fusobacterium* signature and the enrichment of *F. nucleatum* in HNSCC tumor tissue was associated with better overall survival. They suggested that butyrate produced by bacteria decreased tumor cell growth and invasion, while increasing $CD8^+$ T cell-mediated anti-tumor responses [42,43].

The possibility that data may differ depending on the method of analysis is not simply be due to a single bacterium, but also the bacterial flora, such as a combination of bacteria or the presence or absence of mutations that may affect the impact of a bacterium.

In the KEGG pathway analysis, the pathways associated with *Fusobacterium* and *Streptococcus* were the IL-17 signaling pathway and *S. aureus* infection. The IL-17 family is a subset of cytokines consisting of IL-17A-F, which plays an important role in acute and chronic inflammatory responses [44–47]. Signaling of IL-17 family members through their corresponding receptors activates downstream pathways, including NF-kappaB, MAPKs, and C/EBPs, to induce expression of antimicrobial peptides, cytokines, and chemokines [44–47]. Locally induced cytokines, including IL-6, IL-8, IL-17, TNF-α, and COX-2, in the tumor environment may promote tumorigenesis in colorectal cancer [48–50]. Th17, an effector subset of $CD4^+$ helper T cells, was identified based on its ability to produce IL-17A [51]. Th17 cells have been suggested to play a dual role in tumorigenesis, promoting tumors by generating angiogenic factors, but paradoxically inhibiting tumor development by producing IL-17 and IFN-γ [52]. Therefore, the relationship between intratumoral *Fusobacterium* and IL-17 signaling does not necessarily indicate a facilitative role for IL-17 in the development of HNSCC.

Streptococcus has been implicated in the pathogenesis of lung cancer, gastric cancer, and melanoma [13]. Furthermore, *Streptococcus mutans*, a major pathogen of dental caries, was recently reported to promote breast cancer cell metastasis to the lungs *in vivo* and promote tumor progression in oral squamous cell carcinoma [53,54]. Consistent with these findings, the present results showed that the abundance of *Streptococcus* in tumor tissues was associated with a poor prognosis in HNSCC patients with no history of alcohol consumption (Fig. 6B–b) (Table 3). However, this was not the case for patients with a history of alcohol consumption. The survival probability of patients with high *Streptococcus* levels was lower than that of patients with low *Streptococcus* levels, suggesting a role for alcohol in the tumor-promoting effects of bacteria. The current study focused on the top 3 microbes, but future research is needed as other microbes that are detected at more than 0.1 % account for 36 % of the total.

Smoking and alcohol consumption both cause DNA damage and impair DNA repair [6]. Alcohol toxicity is induced both directly by ethanol and indirectly by alcohol metabolites, including reactive oxygen species generated during the biotransformation of ethanol involving CYP2E1 [55–57]. Multiple components of tobacco smoke have carcinogenic potency; large amounts of N-nitrosamines are present in tobacco smoke [58]. In a large human study on alcohol consumption and the oral microbiome, the diversity of the oral microbiota and the overall bacterial profile were found to differ between heavy drinkers and non-drinkers [59]. Alcohol consumption correlated with the abundance of *Fusobacterium* in colorectal cancer [60]. Chakladar et al. [7] characterized HPV, smoking, and alcohol consumption in HNSCC patients. Smokers and non-smokers had a similar number of dysregulated microbes that were excessively abundant, while heavy drinkers had an underabundance of dysregulated microbes. Synergistic effects between these risk factors have been proposed.

Diet, medication, prebiotics, postbiotics, probiotics, and antibiotics all have the potential to change the tumor microbiome [14].

Cancer chemotherapy also changes the microbiome, and modifications to the body's microbiome and intra-tumor microbiome may be advantageous for one treatment modality, but detrimental for another [14,61,62]. As intratumoral *Fusobacterium* and *Streptococcus* may affect the prognosis of patients with HNSCC related in drinking and smoking, changing the tumor microbiome may improve prognosis. For example, treatment with antibiotics may be considered. It is difficult to eliminate only these specific bacteria and whether their elimination would be of benefit in total, or whether supplementation with specific bacteria might be useful, but these need to be explored further. In addition, further research is needed, as numbers are limited and large prospective studies will be required to prove this.

It is critical to recognize the limitations of the present study, such as the number of patients included and the tumor location (mainly tongue, larynx, tonsil), as well as the need for further research to address these limitations. In addition, these results were only confirmed in this population, and future work is needed to confirm their generalizability to other populations. Although many recent studies have investigated the intratumor microbiota, the findings obtained have not always been consistent. Therefore, further research is warranted.

5. Conclusion

The relationships between the microbiome in tumors and the following factors were analyzed. Analysis of the relationship with classical clinical risk factors such as alcohol consumption, smoking, HPV status, gender, lymph node metastasis, and tumor size, showed that high levels of *Fusobacterium* were associated with a better prognosis in patients without a history of alcohol consumption and smoking. On the other hand, high levels of *Streptococcus* were associated with a poor prognosis in patients with no history of alcohol consumption. Intratumoral *Fusobacterium* and *Streptococcus* may affect the prognosis of HNSCC patients, and their effects on HNC are modulated by the effects of the clinical risk factors, drinking, and smoking.

CRediT authorship contribution statement

Masakazu Hamada: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kyoko Nishiyama:** Writing – review & editing, Methodology, Investigation, Funding acquisition. **Ryota Nomura:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Tatsuya Akitomo:** Writing – review & editing, Methodology. **Chieko Mitsuhata:** Writing – review & editing, Methodology. **Yoshiaki Yura:** Writing – review & editing, Writing – original draft, Supervision, Methodology. **Kazuhiko Nakano:** Writing – review & editing, Supervision, Methodology. **Michiyo Matsumoto-Nakano:** Writing – review & editing, Supervision, Methodology. **Narikazu Uzawa:** Writing – review & editing, Supervision, Methodology. **Hiroaki Inaba:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization.

Institutional review board statement

Not applicable.

Ethics approval and consent to participate

Not applicable.

Data availability

Data are available from the corresponding author upon reasonable request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39284.

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