Research Square

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Microbial and Molecular Differences According To The Location of Head and Neck Cancers

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Abstract

Background

Microbiome has been shown to substantially contribute to some cancers. However, the diagnostic implications of microbiome in head and neck squamous cell carcinoma (HNSCC) remain unknown. Here, we report for the first time, the molecular difference in the microbiome of oral and non-oral HNSCC.

Methods

Primary data was downloaded from the Kraken-TCGA dataset. The molecular differences in the microbiome of oral and non-oral HNSCC were identified using the linear discriminant analysis effect size method. Using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUST) and ANOVA-like differential expression (ALDEx2), we predicted bacterial metabolic contributions of oral rich and non-oral rich bacteria, common rich bacteria in two groups and their pathways. A Correlation analysis was performed between RNA expression data and common bacteria data and protein-protein interaction (PPI) analysis was performed using correlated genes. Finally, to find out unique microbial signatures, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene ontology (GO) analysis using the PPI results.

Results

The common microbiomes in oral and non-oral cancers were *Fusobacterium, Treponema*, and *Selenomonas* and *Clostridium* and *Massilia*, respectively. We found unique microbial signatures that positively and negatively correlated with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in oral cancer and negatively correlated KEGG pathways in non-oral cancer. In oral cancer, positively correlated genes were mostly found in bacterial infection pathways, while negative correlated genes were involved in HTLV-I infection, signal transduction, cell adhesion, and cancer-associated pathway. In non-oral cancer, positively correlated genes did not show any significant results, and negatively correlated genes showed results from focal adhesion pathway and regulation of actin cytoskeleton pathway.

Conclusions

These results could help in understanding the underlying biological mechanisms of the microbiome of oral and non-oral HNSCC. Microbiome-based oncology diagnostic tool warrants further exploration.

Background

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with 890,000 new cases and 450,000 deaths in 2018[1, 2].HNSCC accounts for about 6% of all cancers and 1–2% of deaths due to neoplastic diseases [3–5]. Cigarette smoking, tobacco use, and positive human papillomavirus status are well-known risk factors for HNSCC[6], with approximately three-quarters of HNSCC cases attributed to cigarette smoking and tobacco use[7, 8]. HNSCC is a heterogeneous disease and tumours are distinguished based on

location. HNSCC originates from the epithelial cells in the laryngeal and oropharynx, lips, mouth, nasopharynx, or larynx.

Trillions of microbes have evolved and continue to live on and within human beings[9]. Numerous studies have suggested a link between the microbiota, which exist in various organs (e.g., gut and placenta) and pathological conditions such as neurologic diseases, metabolic disorders, and cancers[10–13]. With the development of omics technologies, such as metagenomics, transcriptomics, and proteomics, substantial evidence has been accumulated regarding the relationship of microorganisms and various diseases, including cancers[14].

The gut microbiome has been associated with various disorders, especially malignant tumours. The gut microbiome is involved in biological processes, including modulating the metabolic phenotype, regulating epithelial development, and influencing innate immunity[15]. Chronic diseases such as obesity, inflammatory bowel disease, diabetes mellitus, metabolic syndrome, atherosclerosis, alcoholic liver disease, non-alcoholic fatty liver disease, cirrhosis, and hepatocellular carcinoma are associated with the human microbiome[16]. Several studies have demonstrated that gut microbiome dysbiosis is associated with tumourigenesis and/or tumour growth across cancer types, including colon, hepatocellular carcinoma, gastric, and breast[10, 15]. Moreover, the gut microbiome has been demonstrated to play a key role in the response to cancer therapy, such as chemotherapy, immune checkpoint blockade, and stem cell transplant[10]. For immune checkpoint blockade response, differential gut microbiome signatures exist in patients who respond to immune checkpoint blockade treatment[17–19].

Although intratumoral microbiota has not been studied as much as the gut microbiota, the importance of microbiota in tumours is increasing, with studies showing that it affects the response to cancer treatment[10, 20–23]. Intratumoral bacteria, which are metabolically active, can alter the chemical structure of anti-cancer drugs[24, 25]. In addition, *Fusobacterium nucleatum* in colorectal tumour promotes resistance to chemotherapy through modulation of autophagy[26]. HNSCC, especially oral squamous cell carcinoma (OSCC), is the most prevalent and commonly studied cancer associated with bacterial infection, and is the most common malignancy of the head and neck worldwide[27]. Two prominent oral pathogens, *Porphyromonas gingivalis*, and *F. nucleatum* have been reported to promote tumour progression in mice[28]. Periodontitis is an infectious disease causing chronic inflammation in the oral cavity[29, 30]. Periodontitis has been linked to various cancers, including oesophageal and oropharyngeal cancers[27]. Several studies have found that the risk of developing OSCC may increase with periodontal disease[31, 32], and periodontal disease increases the risk of oral cancer even after adjusting for significant risk factors[33, 34]. Herein, we investigated the underlying molecular differences of the microbiome of oral cancer and non-oral HNSCC.

Methods

Microbiome datasets & TCGA RNA-sequencing datasets

We downloaded Kraken-TCGA-Raw-Data (n=17625) from microbial count datasets[35] for this study. Primary tumours were selected from HNSCC of microbiome data, classified into RNA and WGS, and combined with TCGA clinical information to separate oral and non-oral subtype. RNA-expression sequencing and clinical data sets of 546 HNSCC samples were downloaded from the Broad GDAC Firehose [36] on 20 Feb 2020. The samples were categorised based on the site of occurrence as either oral cancer (alveolar ridge, base of the tongue, buccal

mucosa, floor of the mouth, hard palate, oral cavity, oral tongue) or non-oral cancer (hypopharyngeal, larynx, lips, oropharynx, tonsil). Preprocessing was used with the R program (version 4.0.3)[37].

Linear discriminant analysis effect size (LEfSe)

To identify significantly different bacteria (as biomarkers) between the two groups at the genus level, taxa summaries were reformatted and inputted into LEfSe via the Huttenhower Lab Galaxy Server[38]. The LDA values of oral and non-oral HNSCC microbiome data of RNA and DNA were obtained. Then, we obtained common bacteria of RNA and DNA with the threshold on the logarithmic LDA score for discriminative features of 2.0. In the settings of LEfSe, the Kruskal-Wallis sum-rank test ($\alpha = 0.05$) was used to detect taxa with significant differential abundance. We used the LDA method to estimate the effect size of the abundant genus level[38].

Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUST) and ANOVA-like differential expression (ALDEx2)

The name of the common bacteria was changed to ID of Greengenes (97% taxonomy) (version 13.5) (http://greengenes.lbl.gov) and used as an input file. PICRUSt was performed using the Galaxy web application, which was used to predict bacterial metabolic contributions of oral rich and non-oral rich bacteria, respectively[39]. To filter the results of the PICRUSts, we merged results of oral rich and non-oral rich bacteria, and used the ALDEx2[40] to obtain top five pathways with a p-value of 0.05 or less.

Correlation analysis

A correlation analysis was performed with respect to the RNA expression data and common bacteria data of oral and non-oral HNSCC. Using the Spearman correlation test, genes with oral/non-oral correlation coefficients $r > 0.15$ and $r < -0.15$ were obtained. Significance levels were considered at $P < 0.05$.

Protein-protein interaction (PPI) analysis & Hub gene

PPI analysis of correlated genes was performed using the plug-in Search Tool for the Retrieval of Interacting Genes (STRING) app (version 1.5.1)[41]. The results of the analysis were imported into Cytoscape (version 3.8.2)[42] to establish a network model. The plug-in app cytohubba (version 0.1)[43] in Cytoscape was downloaded and installed. The top ten scores of the degree algorithm were taken as the criteria to screen out the hub genes with high connectivity in the gene expression network.

KEGG pathway and gene ontology (GO)

KEGG pathway and GO analysis were performed on the DAVID website[44] with the genes in the node table resulting from the PPI. Then, the genetic symbol was transferred to entrezID using the org.Hs.eg.db (version 3.12.0) package[45] with the same input file from the PPI for subsequent analysis. The results of enhanced GO entries and KEGG were visualised as path point plots using clusterProfiler (version 3.18.1), ggplot (version 3.3.5), and Enrichplot2 (version 1.10.2) packages. GO and KEGG analysed the used data with statistically significant false discovery rates < 0.05.

Results

Characterisation of unique microbial signatures of oral and non-oral HNSCC

To evaluate the unique microbial signatures of oral and non-oral HNSCC, we analysed Kraken-TCGA (The Cancer Genome Atlas) data sets using the linear discriminant analysis (LDA) method. We divided 691 HNSCC samples into 172 DNA whole genome sequencing (WGS) data and 519 RNA sequencing data (Fig. 1). Next, we analysed RNA sequencing as subtypes divided into 338 oral cancer and 181 non-oral cancer. DNA WGS data were also analysed as 124 oral and 48 non-oral subtypes. Medical information related to these samples is described in Table 1.

Table 1
Patient's characteristics

RNA		TCGA-HNSCC ($N = 519$)		
		Oral (338)	Non-oral (181)	P-value
Age	< 55	94	56	0.5172
	≥ 55	244	125	
Gender	Female	105	31	0.005043**
	Male	233	150	
Clinical Stage	I	19	8	0.005159**
	II	56	18	
	III	63	18	
	IVA	161	92	
	IVB	7	5	
	IVC	0	1	
	Not available	32	39	
Race	American Indian or Alaska native	1	1	0.01246*
	Asian	10	1	
	Black or African American	22	26	
	White	295	148	
	Not available	10	5	
DNA		TCGA-HNSCC ($N = 172$)		
		Oral (124)	Non-oral (48)	P-value
Age	< 55	40	20	0.3256
	≥ 55	84	28	
Gender	Female	42	5	0.003688**
	Male	82	43	
Clinical Stage	I	8	3	0.005159**

* P < 0.05 ** P < 0.01 *** P < 0.001

Chi-squared test was done for age, gender and Fisher's exact-test was done for clinical stage, race

HNSCC, head and neck squamous cell carcinoma

	II	23	3	
	III	19	7	
	IVA	59	17	
	IVB	1	2	
	IVC	0	0	
	Not available	14	16	
Race	American Indian or Alaska native	0	0	0.2463
	Asian	2	0	
	Black or African American	6	6	
	White	114	42	
	Not available	2	0	
* P < 0.05 ** P < 0.01 *** P < 0.001				
Chi-squared test was done for age, gender and Fisher's exact-test was done for clinical stage, race				
HNSCC, head and neck squamous cell carcinoma				

Investigation of the common microbiome of oral and non-oral HNSCC

The common microbiomes of oral and non-oral HNSCC are shown in Fig. 2. The common microbiomes in oral HNSCC were *Fusobacterium, Treponema*, and *Selenomonas*, and the common microbiomes in non-oral HNSCC were *Clostridium* and *Massilia*, as determined by the linear discriminant analysis effect size (LEfSe) method (Fig. 2). The distribution of count data for common microbiome subtypes is depicted in Fig. 2. Microbiome with higher levels of distribution in oral HNSCC were *Selenomonas, Fusobacterium*, and *Treponema*, and microbiome with higher levels of distribution in non-oral HNSCC were *Clostridium* and *Massilia* (Fig. 2).

Microbial Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and protein network of oral and non-oral HNSCC

We analysed the molecular mechanism of the microbiome of oral and non-oral HNSCC using KEGG pathway analysis and protein network analysis (Fig. 3, Table 1, and Table 2). We found unique microbial signatures that positively correlated KEGG pathways in oral HNSCC, negatively correlated KEGG pathways in oral HNSCC, and negatively correlated KEGG pathways in non-oral HNSCC (Fig. 3). In oral HNSCC, positively correlated genes were

mostly found in bacterial infection pathways, and the genes involved in HTLV-I infection, signal transduction, cell adhesion, and cancer-associated pathway were negatively correlated with the disease. In non-oral cancer, positively correlated genes did not show any significant results, and negatively correlated genes showed results from focal adhesion and regulation of actin cytoskeleton. In addition, we conducted a pathway and gene expression analysis using microbial data of subtypes from each oral and non-oral HNSCC (Table 2 and Supplementary Table 1). Rich microbiome within non-oral cancer was found to be associated with biosynthesis and metabolism of glycan, transport, catabolism, and biosynthesis of other secondary metabolites. Rich microbiome within oral cancer was involved in the biodegradation and metabolism of xenobiotics, neurodegenerative diseases, and the circulatory system. We showed the KEGG pathway of protein-protein interaction (PPI) node table genes by correlating mRNA expression data with the microbiome (Table 3). The results of the phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analysis are shown in Supplementary Fig. 1. The dots represent the differences in the abundance of oral vs non-oral HNSCC KEGG pathways. Further, we analysed the hub genes of oral and non-oral HNSCC using the Cytohubba method in Cytoscape software and identified positive and negative expression related to them (Supplementary Table 2).

Table 2
Results of PICRUSt KEGG pathway enrichment analysis

	LEVEL 1	LEVEL 2	LEVEL 3	rab.win.NO	rab.win.O	diff.btw
Oral rich bacteria	Metabolism	Xenobiotics Biodegradation and Metabolism	Caprolactam degradation	1.6747495	8.430766	6.279402
	Human Diseases	Neurodegenerative Diseases	Huntington's disease	2.4877375	8.382426	5.413706
	Organismal Systems	Circulatory System	Cardiac muscle contraction	2.907211	8.378313	5.100822
	Organismal Systems	Circulatory System	Mineral absorption	2.8999521	8.38446	5.017036
	Human Diseases	Neurodegenerative Diseases	Parkinson's disease	2.9033953	8.351826	5.003632
Non-oral rich bacteria	Metabolism	Glycan Biosynthesis and Metabolism	Other glycan degradation	10.85589	-0.53392	-11.3316
	Metabolism	Glycan Biosynthesis and Metabolism	Glycosphingolipid biosynthesis – globo series	10.81923	0.339303	-10.5042
	Cellular Processes	Transport and Catabolism	Lysosome	10.830371	0.335549	-10.4971
	Metabolism	Glycan Biosynthesis and Metabolism	Glycosaminoglycan degradation	10.836729	0.609203	-10.0124
	Metabolism	Biosynthesis of Other Secondary Metabolites	Flavone and flavonol biosynthesis	10.838202	1.609486	-9.10254

BH < 0.05 compared to the oral and non-oral (ALDEx2), BH,Benjamini-Hochberg

diff.btw cut off > abs(5)

rab.win.NO: a vector containing the median clr value for each feature in non-oral, clr, centred log-ratio

rab.win.O: a vector containing the median clr value for each feature in oral

diff.btw: a vector containing the per-feature median difference between condition non-oral and oral

PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states; KEGG, Kyoto Encyclopedia of Genes and Genomes

Table 3
DAVID gene-annotation enrichment analysis of KEGG pathway

	ID	KEGG pathway	count	P-value	FDR	Genes
Positively correlated genes in oral cancer	hsa05130	Pathogenic Escherichia coli infection	6	1.65E-04	0.015558519	*TUBA1C, TUBB6, ARPC1A, ARPC5L, TUBB4B, TUBA4A*
	hsa05132	Salmonella infection	7	1.97E-04	0.015558519	*IL1A, KLC3, CXCL8, IL1B, ARPC1A, ARPC5L, IL18*
Negatively correlated genes in oral cancer	hsa04070	Phosphatidylinositol signalling system	11	6.56E-04	0.024540845	*PRKCB, INPP5D, PI4KA, ITPR1, MTMR8, PIK3R3, PLCE1, PIP5K1B, PIK3R1, PIK3CG, PIK3R5*
	hsa04360	Axon guidance	12	0.001414405	0.035531246	*ROBO2, SEMA5B, NRP1, SEMA3D, SEMA3G, NTN4, UNC5C, SLIT3, SLIT2, PLXNC1, NTN1, EPHA3*
	hsa04514	Cell adhesion molecules (CAMs)	14	3.09E-04	0.019256786	*NLGN3, SELPLG, VCAM1, ITGB2, NRXN3, PTPRM, ITGAL, SELP, SPN, PTPRC, PDCD1, NCAM2, TIGIT, NEO1*
	hsa04666	Fc gamma R-mediated phagocytosis	12	3.63E-05	0.006794491	*HCK, SCIN, PTPRC, PRKCB, INPP5D, WAS, PIK3R3, PIP5K1B, PIK3R1, VAV1, PIK3CG, PIK3R5*

KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate

ID	KEGG pathway	count	P-value	FDR	Genes
hsa04810	Regulation of actin cytoskeleton	16	0.001520053	0.035531246	*ITGB2, WAS, PIK3R3, PIK3R1, ITGAL, IQGAP2, PIK3CG, VAV1, PIK3R5, FGF7, SCIN, ITGA11, FGF18, PIP5K1B, NCKAP1L, PPP1R12B*
hsa04960	Aldosterone-regulated sodium reabsorption	7	9.58E-04	0.02984755	*PRKCB, INSR, PIK3R3, PIK3R1, PIK3CG, NR3C2, PIK3R5*
hsa05144	Malaria	8	5.75E-04	0.024540845	*SELP, VCAM1, LRP1, TGFB3, ITGB2, TLR9, ITGAL, THBS4*
hsa05166	HTLV-I infection	20	2.00E-04	0.018711974	*FZD1, MAP3K3, NRP1, EGR2, SMAD4, SPI1, VCAM1, TGFB3, FZD7, ITGB2, PIK3R3, PIK3R1, ITGAL, ELK1, PIK3CG, PIK3R5, IL2RB, LTA, MAP3K14, PRKACB*
hsa05205	Proteoglycans in cancer	15	0.002607227	0.048755147	*FZD1, PRKCB, FZD7, PTCH1, ITPR1, PIK3R3, PIK3R1, HSPG2, ELK1, PIK3CG, PIK3R5, HCLS1, PLCE1, PPP1R12B, PRKACB*
hsa05210	Colorectal cancer	8	0.002372152	0.048755147	*SMAD4, TGFB3, TCF7, LEF1, PIK3R3, PIK3R1, PIK3CG, PIK3R5*

KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate

	ID	KEGG pathway	count	P-value	FDR	Genes
Negatively correlated genes in non-oral cancer	hsa04510	Focal adhesion	7	8.31E-04	0.045276517	*ACTN3, ACTN1, ITGB3, ITGAV, FLNB, ITGB6, TLN1*
	hsa04810	Regulation of actin cytoskeleton	8	1.26E-04	0.01373759	*ENAH, ACTN3, ACTN1, ITGB3, F2R, MSN, ITGAV, ITGB6*
KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate						

Discussion

The microbiome plays an important role in the human host and participates in the development of a wide variety of diseases, such as cancer[9]. The tumor microbiome is associated with a chronic inflammatory state and modulates the initiation and development of various cancers, such as lung, breast, colon, gastric, pancreatic, cholangiocarcinoma, ovarian, and prostate cancers[10, 20–23, 46–48]. In colorectal cancer (CRC), transplant of stool containing the tumor microbiome from patients with CRC can induce polyp formation[49, 50]. Moreover, some bacterial species (*F. nucleatum)* can stimulate an inflammatory state that can promote carcinogenesis via increased production of reactive oxygen species[51], induction of proinflammatory toxins[52, 53], and suppression of anti-tumor immune functions[54, 55]. In this study, we identified for the first time, the tumor microbiome in oral and non-oral HNSCC.

We systematically selected five microbiomes as unique microbial signatures of oral and non-oral HNSCC. Microbiomes with higher levels of distribution in oral HNSCC were *Selenomonas*, *Fusobacterium*, and *Treponema*, while microbiomes with higher levels of distribution in non-oral HNSC were *Clostridium* and *Massilia*. Further, we demonstrated the molecular mechanisms for positively correlated KEGG pathways in oral HNSCC, negatively correlated KEGG pathways in oral HNSCC, and negatively correlated KEGG pathways in non-oral cancer from HNSCCs (Fig. 4).

The prevention and treatment of diseases by targeting the microbiome have been widely investigated[27]. Modulation of the microbiome may also contribute to the treatment of cancer[56]. Cancer therapy requires an intact commensal microbiome that mediates the therapy effects by modulating functions of myeloid-derived suppressor cells in the tumor microenvironment[21, 56, 57]. Some studies have shown the deleterious effects of antibiotics on the treatment of cancer[10, 58]. Patients with metastatic renal cell carcinoma or non-small-cell lung cancer had significantly worse survival outcomes if they received antibiotics just before or just after the initiation of treatment with immune checkpoint blockade[59]. In addition, patients who received anti-Gram-positive antibiotics along with cyclophosphamide for chronic lymphocytic leukemia or cisplatin for relapsed lymphoma had a lower overall response rate[52, 60]. These microbiomes may confer susceptibility to certain cancers, either through a direct effect by the local presence within the tumor microenvironment or via the systemic impact of the microbiome from a distant location, such as the gut and the skin[61].

Conclusions

Taken together, stress conditions, such as diet, antigen exposure, medications, and stress are important factors that contributing to the state of health and also affect the microbiome[35]. This field is young, and we are left with many unanswered questions - especially regarding the mechanism of action as well as the group of bacterial species that are most important in mediating antitumor effects. Multifaceted strategies are needed to modulate precision medicine and treat disease. Efforts are currently underway to enhance therapeutic responses and/or abrogate treatment-associated toxicity chemotherapeutic agents via modulation of the microbiome. We represent the molecular difference in the microbiome of oral and non-oral cancers in this study. These results could help to understand the underlying biological mechanisms of the microbiome of oral and non-oral cancers. Microbiome-based oncology diagnostic tool warrants further exploration.

Abbreviations

ALDEx2
ANOVA-like differential expression tool for high-throughput sequencing data
CRC
colorectal cancer
GO
Gene Ontology
HNSCC
head and neck squamous cell carcinoma
HTLV-I
Human T-cell lymphotropic virus type 1
KEGG
Kyoto Encyclopedia of Genes and Genomes
LDA
linear discriminant analysis
LDA
Linear Discriminant Analysis
LEfSe
linear discriminant analysis effect size
OSCC
Oral squamous cell carcinoma
PICRUSt
phylogenetic investigation of communities by reconstruction of unobserved states
PPI
Protein-protein interaction
STRING
Search Tool for the Retrieval of Interacting Genes/Proteins
TCGA
The Cancer Genome Atlas
WGS
whole genome sequencing

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Not applicable.

Data availability statement

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

Authors' contribution

DL and YHK initiated the study and guided the work. YKK and EJK collected, normalised, and interpreted the data. YY, JK, SYW, HSC, MK, KJ, HSK, and HRP analysed the experimental data. All authors wrote the manuscript with input from all co-authors.

Conflict of interest statement

The authors declare that they have no competing interests.

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Figures

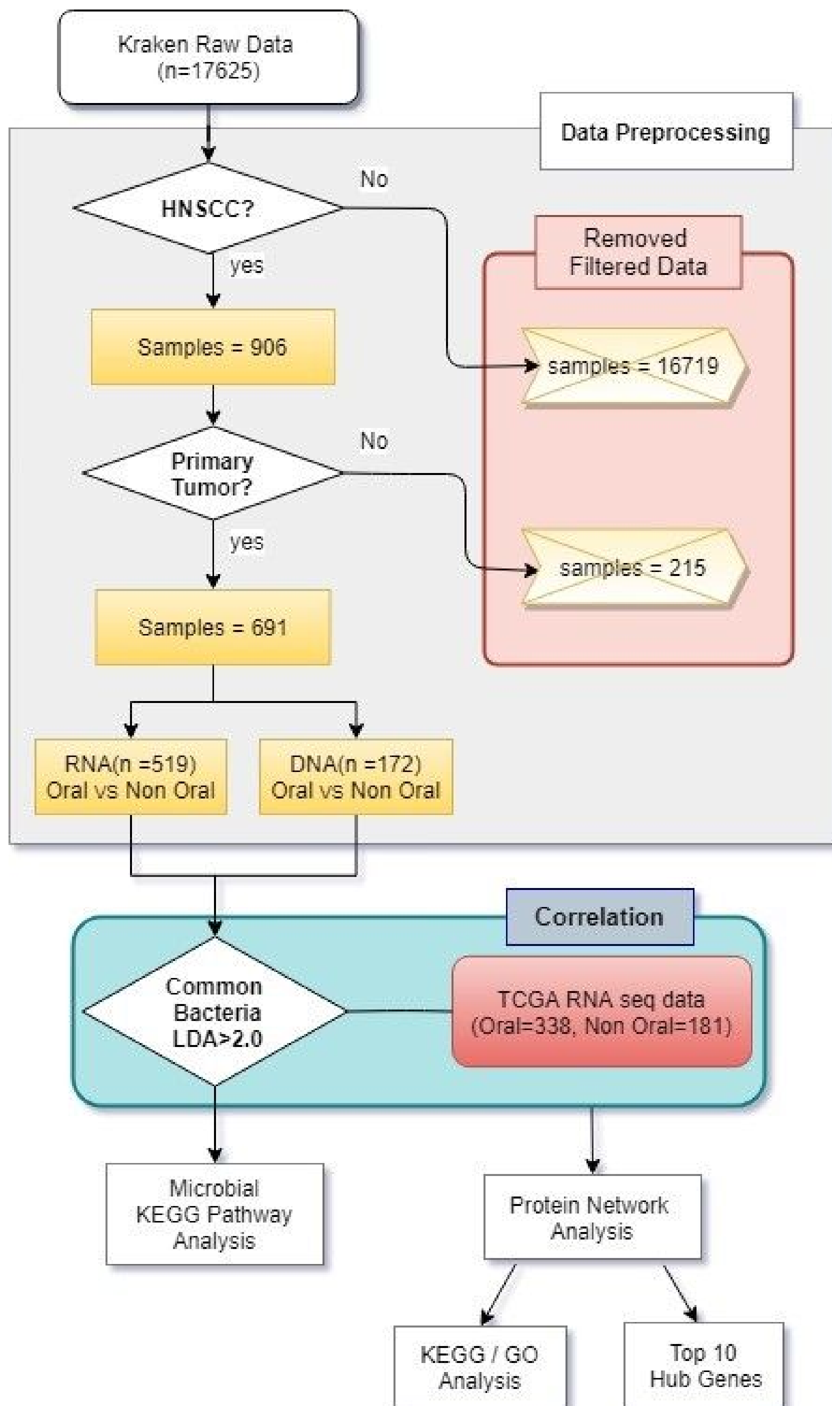

Figure 1

Pipeline flow chart throughout the study

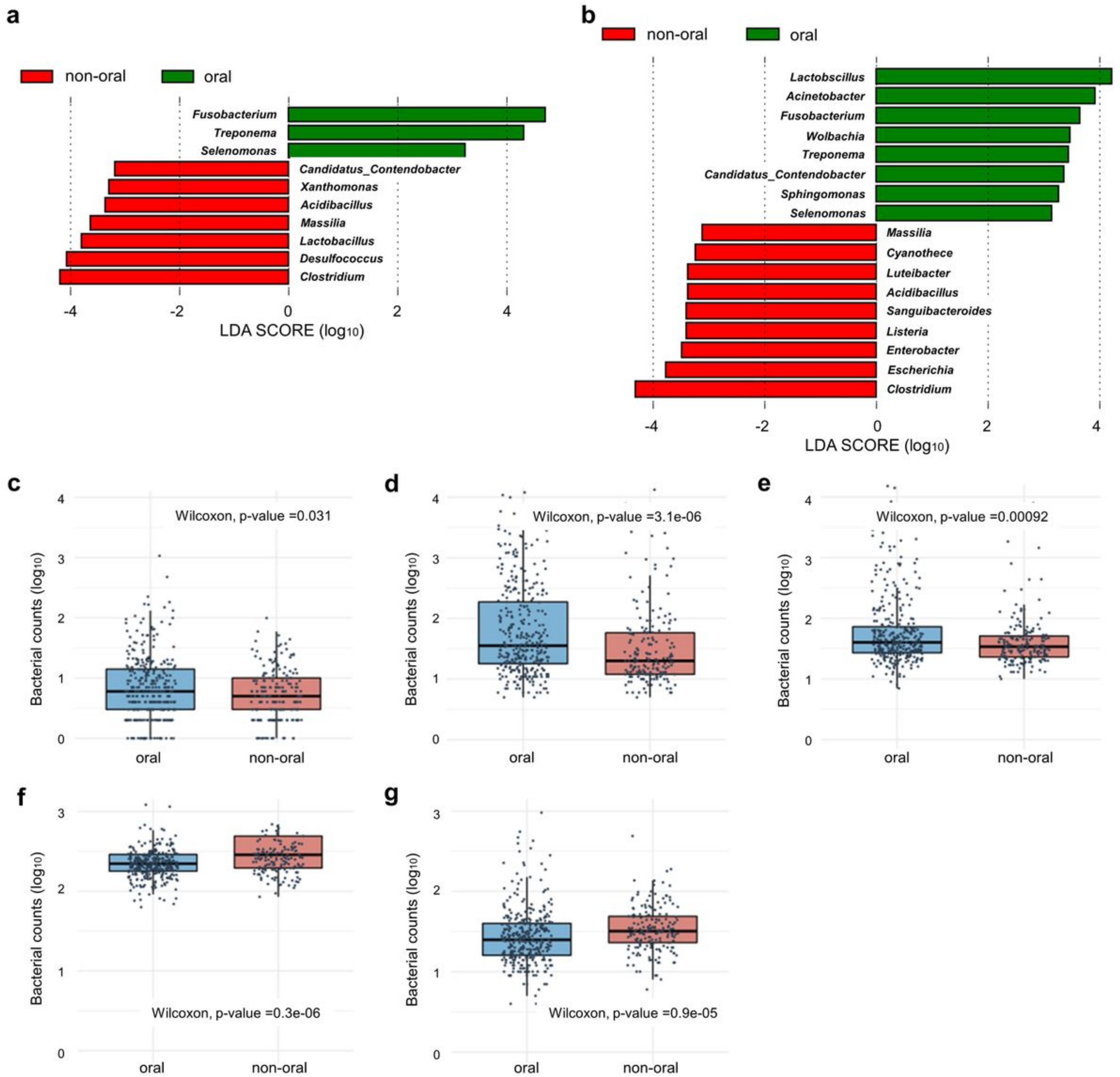

Figure 2

Linear discriminant analysis effect size (LEfSe) analyses and distribution of the microbiome by subtype LEfSe analysis of microbiome composition between oral and non-oral-associated cancers was performed on (a) bacterial DNA and (b) bacterial RNA, respectively. Bacteria species enriched in oral cancer had a positive linear discriminant analysis (LDA) score, while bacteria species enriched in non-oral cancer had a negative score. Microbiomes with higher levels of distribution in oral cancer were (c) Selenomonas, (d) Fusobacterium, and (e) Treponema. Microbiomes with higher levels of distribution in non-oral cancer were (f) Clostridium and (g) Massilia

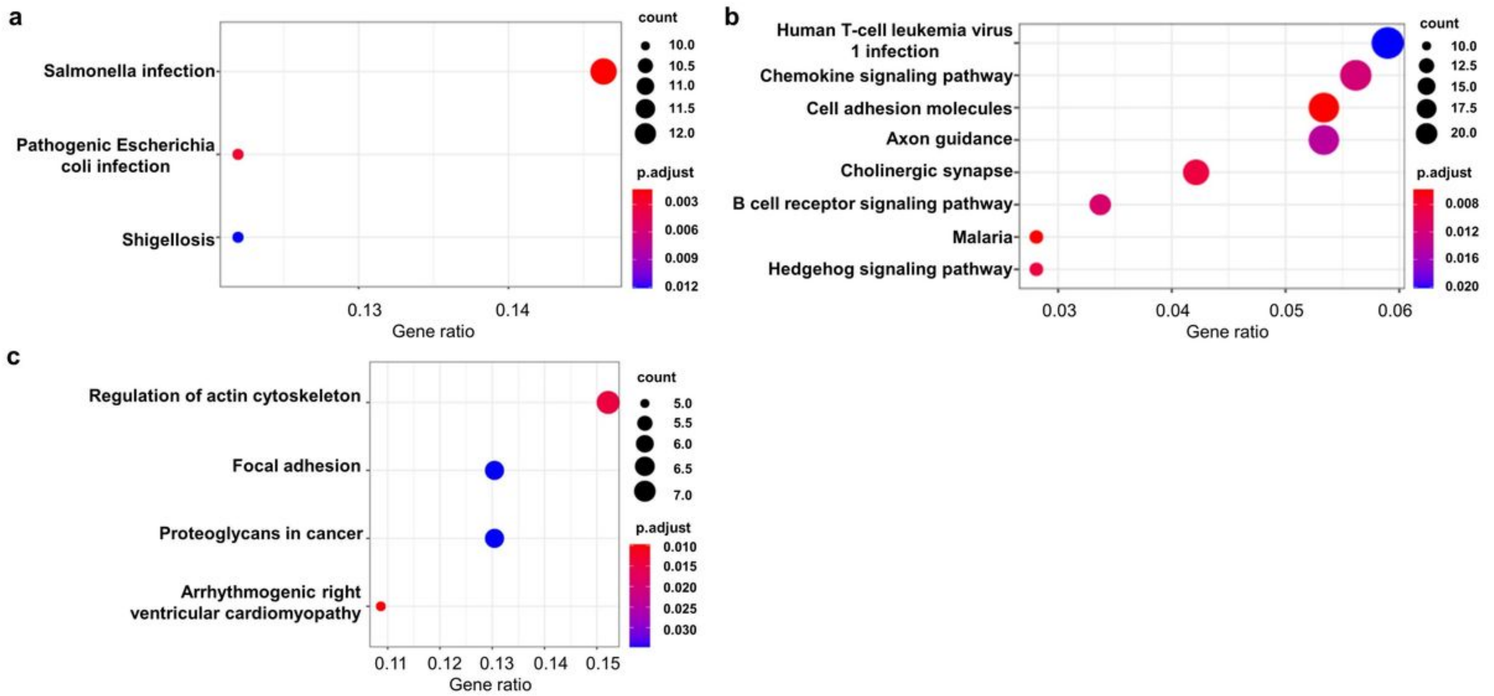

Figure 3

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. (a) Significantly enriched KEGG pathways of the positively correlated genes in oral cancer. (b) Significantly enriched KEGG pathways of the negatively correlated genes in oral cancer. (c) Significantly enriched KEGG pathways of the negatively correlated genes in non-oral cancer. The left Y-axis shows the KEGG pathway. The X-axis shows the gene ratio.

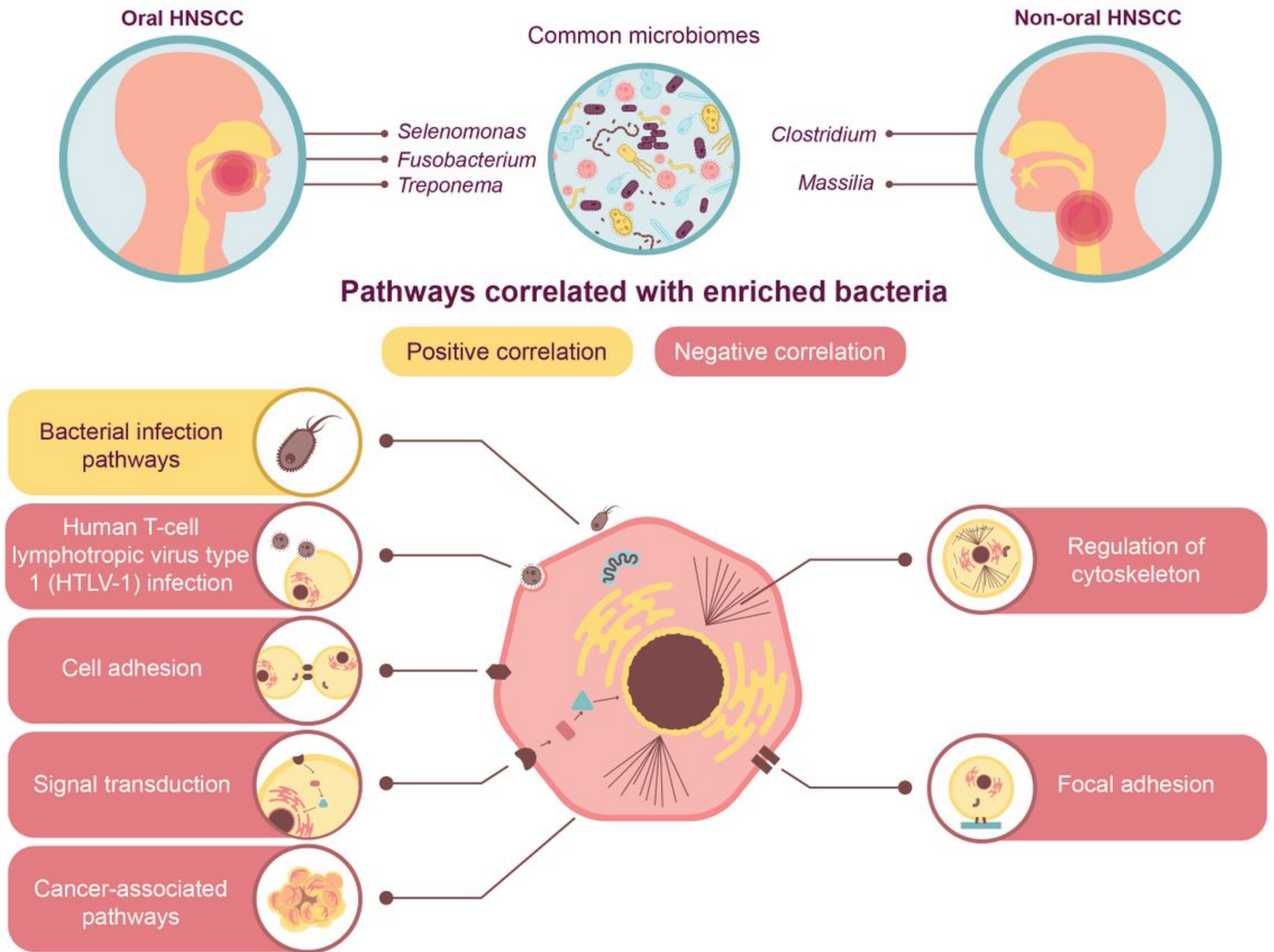

Figure 4

Graphical summary of this study Our study showed differences in the positive and negative KEGG pathways of the oral cancer-associated bacteria Selenomonas, Fusobacterium, and Treponema and the non-oral cancer-associated bacteria Clostridium and Massilia.

Supplementary Files

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