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Salivary *CCL20* Level as a Biomarker for Oral Squamous Cell Carcinoma

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Abstract. *Background/Aim: This study investigated the utility of C-C motif chemokine ligand 20 (CCL20) expression in saliva as a biomarker for oral squamous cell carcinoma (OSCC) and also examined the associated microbiome. Materials and Methods: The study group included patients with OSCC or oral potentially malignant disorder (OPMD), and healthy volunteers (HVs). microarray and qRT-PCR were used to compare salivary CCL20 expression levels among groups. Data on CCL20 levels in oral cancer tissues and normal tissues were retrieved from a public database and examined. Furthermore, next-generation sequencing was used to investigate the salivary microbiome. Results: A significant increase in the expression level of CCL20 was observed in both OSCC tissues and saliva from patients with oral cancer. Fusobacterium was identified as the predominant bacteria in OSCC and correlated with CCL20 expression level. OSCC screening based on salivary CCL20 expression enabled successful differentiation between patients with OSCC and HVs. Conclusion: CCL20 expression may be a useful biomarker for OSCC.*

Oral malignant tumors account for approximately 2% of all cancer; among them, the most common histological type is oral squamous cell carcinoma (OSCC), representing 90% of oral cancers (1, 2). Although the 5-year survival rate for OSCC in early stage is estimated to be 90% (3), at approximately 30%, advanced OSCC is associated with a poor prognosis, (4). Furthermore, while surgical resection is often the first-choice treatment, postoperative oral tissue loss in locally advanced cancer causes severe dysfunctions, such as compromised swallowing and articulation, resulting in a marked reduction in a patient's quality of life (5). Therefore, early diagnosis in early-stage OSCC is important because of its involvement in minimizing surgical invasion and improving prognosis. Oral cancer can be diagnosed relatively easily *via* oral examination. However, some cases are overlooked and only identified at an advanced stage of disease (3, 6, 7). This may be due to the asymptomatic nature of early-stage disease or to the lack of adequate routine examination by healthcare practitioners (8, 9). Therefore, there is a need to develop simple and reliable screening methods that can be used in general dental clinics. This has incentivized a shift from physical examination towards the analysis of salivary RNA expression and the characterization of the salivary microbiome for the identification of diagnostic biomarkers for OSCC (10-15). Indeed, salivary constituents reflect different physiological and disease states of the human body (11, 12, 16), and the microbial profile of saliva is similar to that of soft tissues within the oral cavity (17, 18). Therefore, we hypothesized that by comparing the salivary microbiome between those with OSCC and those with non-OSCC, we would be able to identify novel oral bacterial biomarkers for disease diagnosis.

In recent years, several studies have found that periodontal pathogens can contribute to the development of cancer (19-24). Periodontal pathogens are components of the microbiota colonizing the oral cavity, where they can attach to epithelial cells, fibroblasts, endothelial cells, and other host cells, as well as extracellular matrix proteins, *via Fusobacterium* adhesin A (FadA) and lipopolysaccharides (LPS) (25-27). Adhesion

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Key Words: Biomarker, saliva, oral squamous cell carcinoma, OSCC, Fusobacterium, microbiome, C-C motif chemokine ligand 20, CCL20.

Table I. *Characteristics of the study populations*.

Clinicopathological characteristic	Subgroup	OSCC (n=48), n (%)	OPMD (n=35), n (%)	HVs (n=50), n (%)
Gender	Male	35 (73)	19 (54)	27 (54)
	Female	13 (27)	16 (46)	23 (46)
Age	≥60 Years	38 (79)	11 (31)	24 (48)
	<60 Years	10 (21)	24 (69)	26 (52)
Alcohol	Yes	12 (25)	17 (49)	8 (16)
	No	36 (75)	18 (51)	42 (84)
Cigarette smoking	Yes	10 (21)	3 (9)	4 (8)
	No	38 (79)	32 (91)	46 (92)
Lesion site	Tongue	17 (35)	10 (29)	-
	Gingiva	20 (42)	12 (34)	-
	Floor of mouth	6 (13)	0 (0)	-
	Buccal mucosa	5 (10)	4 (11)	-
	Multiple sites	0 (0)	9 (26)	-
Stage	I+II	27 (56)	-	-
	III+IV	21 (44)	-	-
T Class	T1, T2	32 (67)	-	-
	T3, T4	16 (33)	-	-
N Status	Node-positive	12 (25)	-	-
	Node-negative	36 (75)	-	-
Distant metastasis	Yes	0 (0)	-	-
	No	48 (100)	-	-

OSCC: Oral squamous cell carcinoma, OPMD: oral potentially malignant disorders, HVs: healthy volunteers. Samples were histologically classified using the seventh edition of the UICC staging system for oral cancer (37).

through FadA and LPS is recognized by toll-like receptors (TLR2 and TLR4) (28). Thereafter, the TLR2/TLR4/MYD88 pathway is activated in response to fusobacteria, leading to the activation of nuclear factor-κB, which in turn promotes carcinogenesis by causing inflammation and DNA damage *via* the up-regulation of cytokines such as tumor necrosis factor α and interleukin-1β as well as C-C chemokines (28-32). C-C Chemokines contribute to cancer progression and metastasis as critical mediators within the tumor microenvironment (33-35). In particular, the gene for C-C motif chemokine ligand 20 (*CCL20*), encoding a 96-amino acid precursor protein, is located on chromosome 2q33-37 (36). In oral cancer cell lines, *CCL20* expression is frequently increased by LPS, and has been reported to be related to the proliferation and invasion of OSCC cells, thereby contributing to cancer progression (37).

Therefore, in this study we examined whether *CCL20* expression might be used as an early diagnostic marker of OSCC and investigated the relationship between *CCL20* expression and the abundance of fusobacteria in the context of OSCC.

Materials and Methods

Patients and specimens. This study was conducted on 48 patients with OSCC, 35 with oral potentially malignant disorder (OPMD), and 50 healthy volunteers (HVs) who visited the Department of Maxillofacial Surgery at Aichi Gakuin University Dental Hospital or the Japanese Red Cross Nagoya Daiichi Hospital between December 2015 and March 2020. Individuals with a history of antibiotic intake in the previous 3 months or a disease/condition known to modify oral microbial composition, such as diabetes, pregnancy, or contraceptive pill intake, were excluded. Patients with OSCC had undergone primary surgical treatment, including tumor resection, neck dissection, and primary reconstruction with vascular microsurgery. Patients with OPMD included 31 with oral leukoplakia and four with oral lichen planus without dysplasia. HVs were hospital staff and their families. More detailed information on the research subjects is reported in Table I. Samples were histologically classified using the seventh edition of the Union for International Cancer Control staging system for oral cancer(37).

This study was approved by the Aichi Gakuin University ethics committee (approval number: 66, 74) and the Japanese Red Cross Nagoya Daiichi Hospital Ethics Committee (approval number: 2015-113). The study was carried out in accordance with the Declaration of Helsinki. All patients and HVs agreed to the use of their samples for the purposes of the current research.

Saliva collection. Two types of saliva samples were obtained for DNA and RNA extraction. For DNA isolation, saliva collection was performed as the patient first woke up, prior to any intake of food and drinks. Saliva samples were collected by the spitting method, and the patient was instructed to collect 2 ml of saliva within 15 min. For RNA isolation, samples were collected in the morning in order to avoid biochemical changes in saliva. Patients were prohibited from drinking water or eating food 1 hour prior to sampling in order to prevent changes in salivary enzyme levels.

Using these samples, the following analyses were performed (Figure 1).

Table II. *Primers and annealing temperatures*.

Primer	Experiment	Type	Sequence (5'-3')	Annealing temperature
CCL20	qRT-PCR	Forward	CCTCTGCGGCGAATC AGAAG	60˚C
		Reverse	CTGCCGTGTGAAGCCCACAA	
GAPDH	qRT-PCR	Forward	TTAGCACCCCTGGCCAAGGT	60˚C
		Reverse	GGCCATCCACAGTCTTCTGG	
1st 515f-806r	NGS	Forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCT GTGCCAGCMGCCGCGGTAA	50˚C
		Reverse	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT GGACTACHVGGGTWTCTAAT	
2nd 515f-806r	NGS	Forward	AATGATACGGCGACCACCGAGATCTACAC-Index2-ACACTCTTTCCCTACACGACGC	60˚C
		Reverse	CAAGCAGAAGACGGCATACGAGAT-Index1-GTGACTGGAGTTCAGACGTGTG	

CCL20: C-C Motif chemokine ligand 20; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR: quantitative real-time reverse-transcription polymerase chain reaction; NGS: next-generation sequencing.

Extraction of bacterial DNA. The extraction of total bacterial DNA from saliva samples was carried out using the Oragene® DNA Self-Collection kit (DNA Genotek Inc., Ontario, Canada), according to the manufacturer's protocol. In brief, the collected material underwent lysis with a purifying buffer provided in the kit for protein precipitation, followed by an ice bath, and DNA precipitation with 100% ethanol. The DNA was rehydrated in 100 μl of LoTE buffer (10 mmol L-1 Tris hydrochloride, 1 mmol L-1 ethylenediaminetetraacetic acid buffer, pH 8) and stored at −20˚C.

Total RNA extraction. Recovery of total RNA from saliva was performed according to the protocol of the Oragene® RNA Self-Collection kit (DNA Genotek Inc.) (38). mRNA was then reverse transcribed into cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany).

Microarray analysis. Microarray analysis of clinical samples was performed using Gene Expression Hybridization Kit (Agilent Technologies, Santa Clara, CA, USA) as previously described (39). Data were processed using the Agilent Feature Extraction software and normalized using a 75th percentile shift.

Real-time quantitative reverse transcription PCR (qRT-PCR). The expression levels of *CCL20* in clinical samples were determined *via* qRT-PCR analysis using specific primers (Table II) as described previously (39). mRNA levels were normalized using glyceraldehyde-3-phosphate dehydrogenase as the internal control. Experiments were performed in triplicate.

Bioinformatic analysis of gene expression. The expression of *CCL20* in normal (n=26) and oral cancer samples (n=401) was analyzed using the gene expression database of normal and tumor tissues (GENT2) (40). Gene expression data were downloaded from the National Center for Biotechnology Information Gene Expression Omnibus public repository using the U133Plus2 (GPL570) platform.

DNA isolation and next-generation sequencing (NGS) of bacterial 16S rRNA genes. A DNA library was produced through amplification of purified DNA samples with a first (515F/806R) and a second primer set (Table II). In particular, these amplicons were constructed by two-step PCR using unique barcode primers targeting the V4 region of the bacterial 16S rRNA gene, and sequenced using an Illumina MiSeq pyrosequencing platform (Illumina, San Diego, CA, USA) as described previously (41). Taxonomic assignment was performed against the 16S rRNA gene reference sequences present in the Human Oral Microbiome database. It was possible to identify most sequences at the phylotype/species level. Sequences with less than 97% identity were classified at the genus level and not at the species level. Since the absolute amount of bacteria present in a sample cannot be evaluated by NGS (42), the abundance of bacteria was evaluated based on relative abundance. OSCC and non-OSCC group libraries were constructed by clonal analysis.

Diversity analysis and linear discriminant analysis of effect size. To analyze the diversity of the bacterial flora in saliva samples, linear discriminant analysis of effect size (LefSe analysis) (42) was used to determine the characteristic bacteria for each group. Genera and species with a linear discriminant analysis score ≥2.4 were extracted and used as candidate OSCC-associated bacteria.

Statistical analysis. Qualitative variables were compared between two groups using the chi-squared test, and quantitative variables were compared using the Mann–Whitney test. The differential expression of each marker was used to construct receiver operating characteristic (ROC) curves. The area under the ROC curve was calculated by numerical integration. A value of $p<0.05$ was considered to denote statistically significant results. All statistical analyses were performed using the R software (The R Foundation for Statistical Computing) on EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan).

Results

Microarray analysis. We randomly selected saliva samples from four patients with OSCC, four patients with OPMD, and four HVs for comparison of gene expression by microarray analysis. We found that *CCL20* was up-regulated

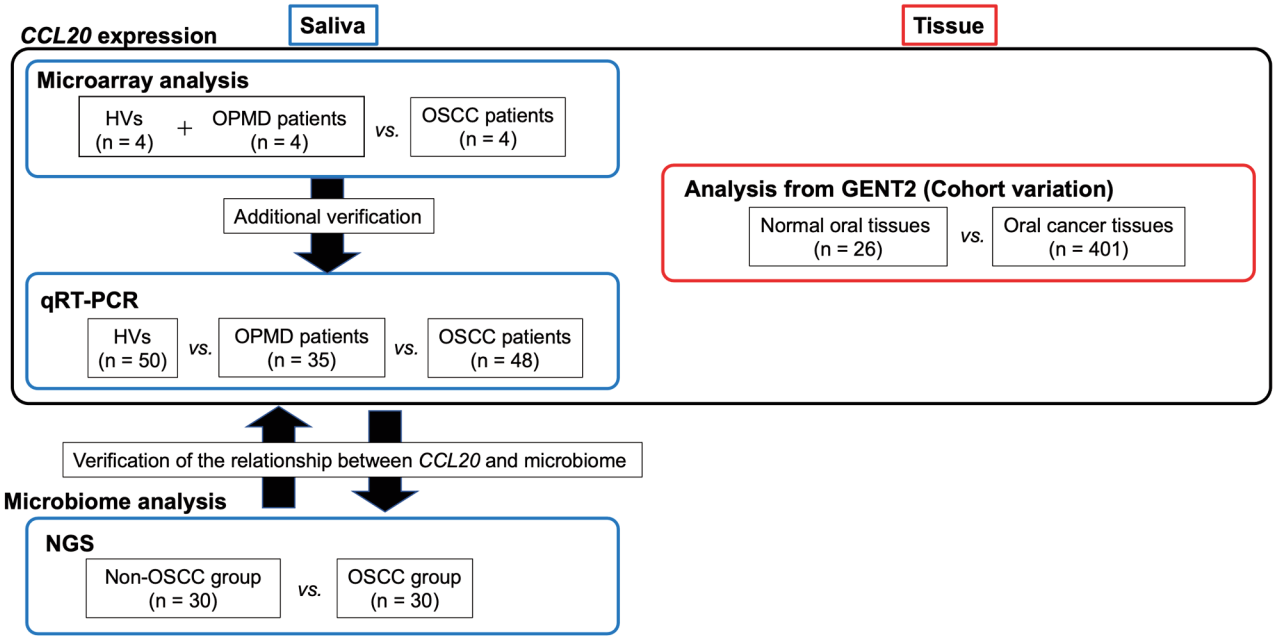

Figure 1. *Sample collection and study flowchart. A small number of cases were randomly selected, and microarray analysis was performed. For saliva samples, the expression level of C-C motif chemokine ligand 20 (CCL20) was compared between patients with oral squamous cell carcinoma (OSCC) and the group of healthy volunteers (HVs) and patients with oral potentially malignant disorder (OPMD). To confirm observations from our cohort, we compared the expression levels of CCL20 in oral cancer tissues (n=401) and normal oral tissues (n=26) from the Gene Expression database of Normal and Tumor tissues (GENT2) datasets. For additional verification, quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) and sequencing analyses were performed in samples from patients with OSCC (n=48) or OPMD (n=35), and HVs (n=50). In the search for bacteria associated with OSCC and CCL20 expression, we grouped patients (30 randomly selected patients with OSCC) and non-OSCC individuals (20 randomly selected patients with OPMD and 10 randomly selected HVs) and explored their oral microbiome via next-generation sequencing.*

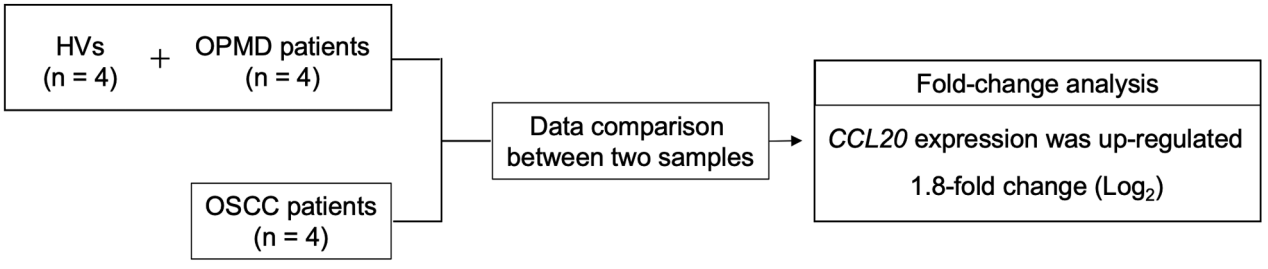

Figure 2. *Microarray analysis of saliva samples. We randomly selected four patients with oral squamous cell carcinoma (OSCC), four with oral potentially malignant disorder (OPMD), and four healthy volunteers (HVs) for comparison. The expression of C-C motif chemokine ligand 20 (CCL20) was up-regulated 1.8-fold in the OSCC group when compared to that in the OPMD and HV groups.*

1.8-fold in patients with OSCC when compared to those with OPMD and HVs (Figure 2). Thus, microarray analysis confirmed that *CCL20* mRNA was highly accumulated in the saliva of patients with OSCC.

Expression of CCL20 in oral cancer tissues. Next, we compared *CCL20* expression between oral cancer tissues and normal tissues using data from the GENT2 database. We found that *CCL20* expression was significantly higher in cancer tissues than in normal oral tissues ($p<0.001$, Figure 3A).

Therefore, we considered *CCL20* to be a potential diagnostic marker of OSCC. To further test this hypothesis, *CCL20* expression levels in saliva of patients with OSCC or OPMD, and HVs were quantified by qRT-PCR.

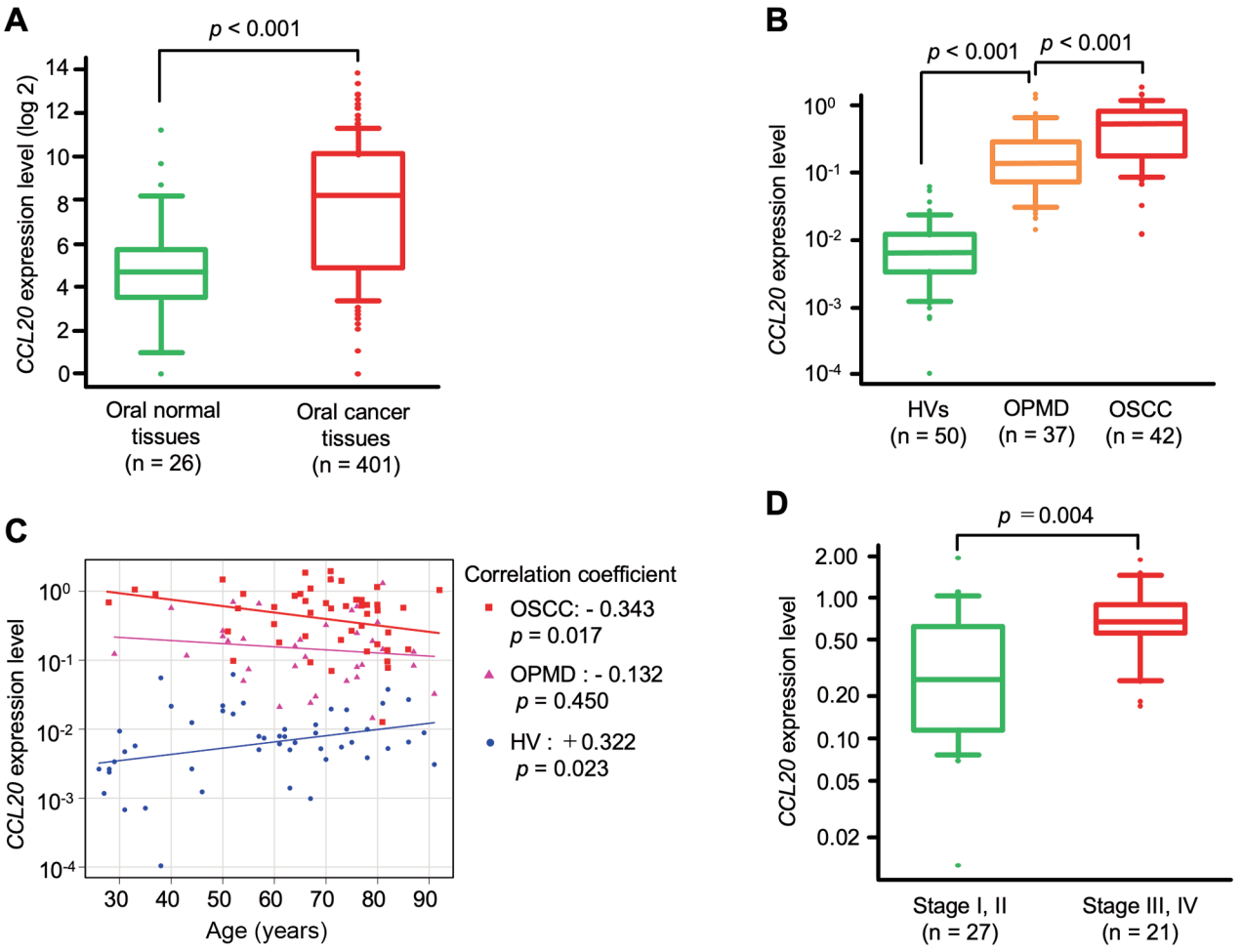

Figure 3. *Analysis of C-C motif chemokine ligand 20 (CCL20) expression in salivary and tissue samples. A: CCL20 levels in 401 oral cancer tissues and 26 normal tissues from the Gene Expression database of Normal and Tumor tissues (GENT2). B: Salivary CCL20 expression levels in 42 patients with oral squamous cell carcinoma (OSCC), 37 patients with oral potentially malignant disorder (OPMD), and 50 healthy volunteers (HVs). C: Correlation between CCL20 expression level in saliva and age. D: Comparison of CCL20 expression levels in saliva and pathological stage.*

Expression levels of CCL20 in saliva. Using qRT-PCR, we observed that *CCL20* expression in the saliva of patients with OSCC was significantly up-regulated with respect to that of HVs and patients with OPMD ($p<0.001$; Figure 3B). Moreover, *CCL20* expression was higher in patients with OPMD than in HVs ($p<0.001$).

Clinical significance of CCL20 expression level. The relationship between *CCL20* expression levels and clinical factors such as age, gender, lesion location, T class, and pathological stage was analyzed. In the OSCC group, age was negatively correlated with *CCL20* expression (correlation coefficient: –0.343, $p=0.017$), whereas in the HV group, age was positively correlated with *CCL20* expression (correlation coefficient: 0.322, $p=0.023$) (Figure 3C). Moreover, salivary *CCL20* expression was significantly higher in patients with advanced-stage OSCC (stages III and IV) than in those with early-stage disease (stages I and II) ($p=0.004$) (Figure 3D). However, the expression level of *CCL20* exhibited no significant correlation with gender, lesion location, or T class.

Next-generation sequencing. Since qRT-PCR results indicated that salivary *CCL20* expression was significantly higher in patients with OSCC than in those with OPMD and HVs, we assessed the presence of characteristic bacteria in the saliva by NGS and identified bacteria associated with salivary *CCL20* expression by LEfSe analysis.

Fusobacterium, Porphyromonas, and *Treponema* were identified as the three predominant bacterial genera in the OSCC group. Conversely, in the non-OSCC group, *Streptococcus* was identified as the predominant genus

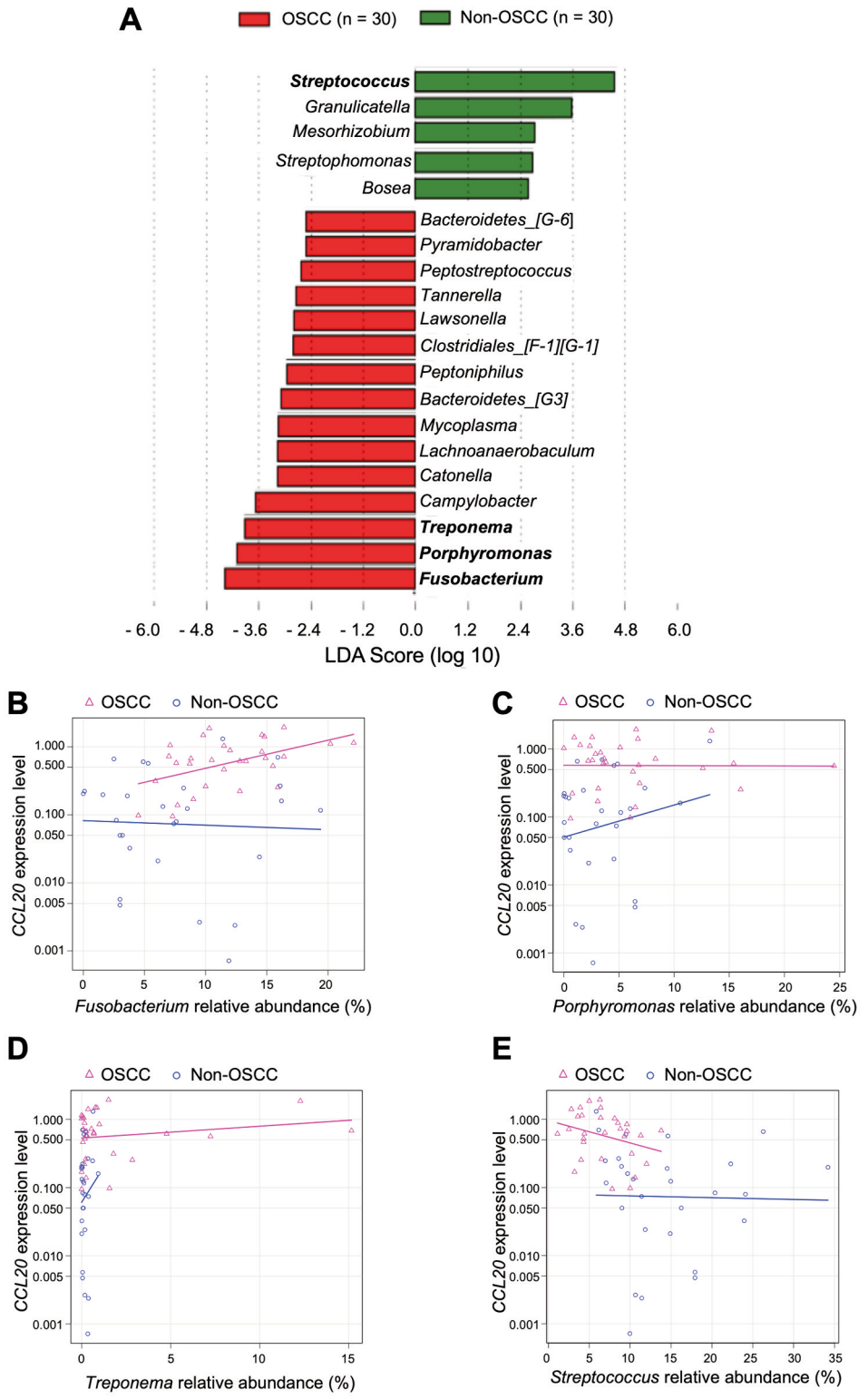

Figure 4. *Differentially enriched bacteria in saliva from the oral squamous cell carcinoma (OSCC) group when compared to the non-OSCC group. A: Linear discriminant analysis (LDA) of effect size showing bacteria observed in the OSCC and non-OSCC groups at the genus level. Correlation between CCL20 expression level and relative abundance of B: Fusobacterium (OSCC: 0.426, p=0.019; non-OSCC: −0.051, p=0.789); C: Porphyromonas (OSCC: −0.126, p=0.505; non-OSCC: 0.100, p=0.613); D: Treponema (OSCC: 0.101, p=0.594; non-OSCC: 0.073, p=0.714); and E: Streptococcus (OSCC: −0.310, p=0.095; non-OSCC: −0.275, p=0.157).*

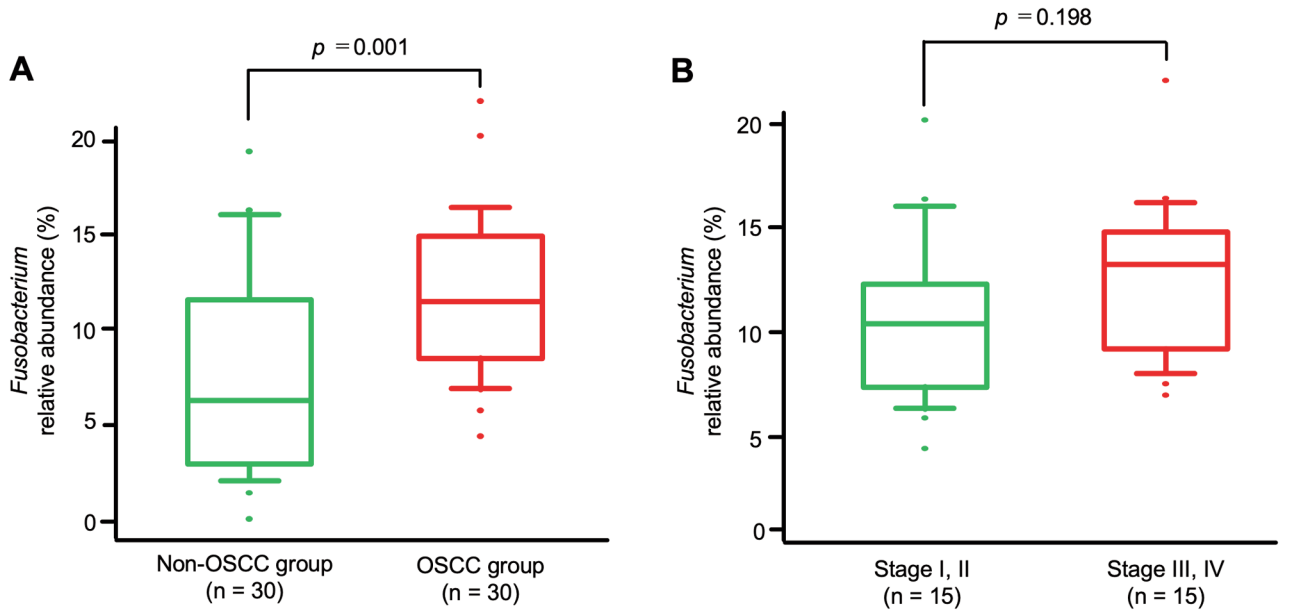

Figure 5. *Analysis of the relative abundance of the Fusobacterium genus in salivary samples. A: The detection rate of the Fusobacterium genus was significantly higher in the oral squamous cell carcinoma (OSCC) group than in the non-OSCC group (p=0.001). B: Comparison of the relative abundance of Fusobacterium in different pathological stages of OSSC The detection rate of the genus Fusobacterium was not significantly higher in patients with advanced-stage disease (stages III and IV) than in those with early-stage disease (stages I and II) (p=0.198).*

(Figure 4A). We examined correlation between *CCL20* expression level and relative abundance of these four genera. *Fusobacterium* was the only bacterial genus whose presence was positively correlated with *CCL20* expression level (Spearman's rank correlation coefficient=0.426, p=0.019) (Figure 4B-E).

Interestingly, a comparison of the relative abundance of *Fusobacterium* between the OSCC and the non-OSCC group revealed a significant enrichment of this bacterial genus in the saliva of patients with OSCC (p=0.001) (Figure 5A).

However, the abundance of *Fusobacterium* was not significantly higher in patients with advanced stage OSCC (stages III and IV) than in those at the early stage (stages I and II) of disease (p=0.198) (Figure 5B).

Diagnostic value of CCL20 expression in OSCC. In order to assess the diagnostic value of *CCL20* expression in OSCC, the area under the ROC curve for salivary *CCL20* expression levels was calculated, and a *CCL20* expression level of 0.069 was used as the cut-off value (Figure 6). Values higher than the cut-off indicated a positive result, and values below the cut-off were interpreted as a negative result. The following results were obtained: For patients with OSCC *vs*. those with OPMD and HVs, the specificity and the positive predictive value of *CCL20* were 0.983 and 0.979, respectively, demonstrating satisfactory accuracy. However, the sensitivity and the negative predictive value of the expression level of this gene were poor. Moreover, for patients with OSCC *vs*. OPMD, the negative predictive value of *CCL20* was 0.229, which was quite low. Finally, comparing patients with OSCC

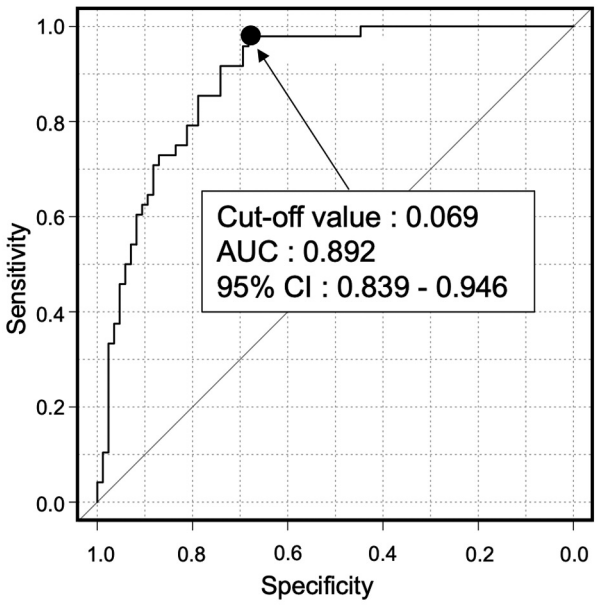

Figure 6. *Receiver operating characteristic curve for detection of oral squamous cell carcinoma by salivary C-C motif chemokine ligand 20. expression level. AUC: Area under the curve; CI: confidence interval.*

Table III. *The accuracy of the screening test when OSCC is determined based on the expression level of CCL20 in saliva.*

Statistic, value (95% CI)	OSCC *vs*. OPMD and HVs	OSCC *vs*. OPMD	OSCC *vs*. HVs
Sensitivity	0.635 (0.515-0.744)	0.635 (0.515-0.744)	1.000 (0.889-1.000)
Specificity	0.983 (0.909-1.000)	0.889 (0.518-0.997)	0.980 (0.896-1.000)
Positive predictive value	0.979 (0.889-0.999)	0.979 (0.889-0.999)	0.979 (0.889-0.999)
Negative predictive value	0.682 (0.572-0.779)	0.229 (0.104-0.401)	1.000 (0.896-1.000)

vs. HVs revealed a specificity of 0.980 and a positive predictive value of 0.979 for *CCL20*, indicating satisfactory accuracy. Furthermore, the sensitivity and negative predictive value of expression level of this gene were also found to be quite high (Table III).

Discussion

Recent clinical studies have reported that the presence of oral fusobacteria is associated with the development and progression of cancer (19, 20, 27, 44-46). For example, predominantly poor prognosis was recorded in patients with esophageal cancer whose cancer tissue was colonized by *Fusobacterium*. Moreover, *CCL20* was highly expressed in esophageal carcinoma tissues colonized by *Fusobacterium* (19, 20). We hypothesized that the bacteria of the genus *Fusobacterium* in these reports were derived from saliva. Therefore, we evaluated the relationship between *CCL20* expression in saliva and the salivary microbiome.

In this study, we used saliva samples and demonstrated that *CCL20* was highly expressed in patients with OSCC by microarray analysis and qRT-PCR. Furthermore, by analyzing *CCL20* expression in the GENT2 database, we confirmed that it is also highly expressed in OSCC tissues. In addition, salivary *CCL20* expression in patients with advanced-stage OSCC was increased compared with that in patients with early-stage OSCC, suggesting that *CCL20* expression might be involved in the development and progression of OSCC.

Since CCL20 is a chemokine, it might be affected by various bacteria (47). For this reason, we performed LEfSe analysis of the NGS results. *Streptococcus* was detected as the most characteristic bacteria in the non-OSCC group, and *Fusobacterium*, *Porphyromonas*, and *Treponema* were the top three characteristic bacteria in the OSCC group. Next, we evaluated the relationship between *CCL20* expression in saliva and the abundance of each genus. In the OSCC group, only *Fusobacterium* showed a positive correlation with *CCL20* expression. However, it is unclear whether OSCC is due to the increase in *Fusobacterium* in saliva or whether the increase of *Fusobacterium* is due to the emergence of OSCC.

These results suggest the potential for OSCC diagnostic screening based on the expression level of *CCL20*, and we examined the accuracy of OSCC screening using this marker. Such OSCC screening might accurately differentiate patients with OSCC from those with HV, with a sensitivity and specificity of approximately 1.00. However, the ability of this gene to differentiate patients with OSCC from those with OPMD was not satisfactory.

This study had some limitations. Indeed, a previous report on OSCC showed that the amount of *Fusobacterium* detected in mouthwash increases with disease progression (48), which our results are not consistent with. In fact, in our study, the detection rate of *Fusobacterium* did not differ based on progression by stage. However, the median detection rate of *Fusobacterium* was higher in advanced-stage disease than in early-stage. Thus, it is possible that a larger sample size would have yielded similar results to those of previous reports. Furthermore, we did not distinguish between oral leukoplakia and oral lichen planus without dysplasia within OPMD cases due to the limited sample size. Therefore, further studies with larger sample size are warranted for more detailed subgroup analysis. Finally, an OSCC screening test based on salivary *CCL20* might accurately differentiate patients with OSCC from HVs. However, the ability of this gene to differentiate patients with OSCC from those with OPMD was not satisfactory. Nevertheless, the current results suggest that salivary *CCL20* expression might be a potential biomarker for OSCC screening.

Conclusion

OSCC screening using salivary *CCL20* expression can be performed using the cut-off value identified in the current work. Future research should also determine whether fusobacteria-induced cytokine/chemokine signaling, including CCL20 signaling, selectively modulates inflammation and immunosuppression within the tumor microenvironment, in turn promoting OSCC growth and progression.

Conflicts of Interest

None to be declared.

Authors' Contributions

SU and SN conceived the study concept and design, analyzed data and wrote the article. SU, MG, KH, SH, MI, MT, IO, KS, TN, and SN contributed to data acquisition and interpretation. SU contributed to statistical analysis. SU, MG, and SN revised the draft. All Authors read and approved the final version of the article.

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