Section A-Research paper

"ROLE OF FUSOBACTERIUM NUCLEATUM ASSOCIATED FadA,KRT$_7$CCL20 EXPRESSIONS IN THE PROGRESSION OF ORAL SQUAMOUS CELL CARCINOMA"

Department(s) and institution(s)

***Dr. Nanda kumar H (Corresponding Author)**

*Post graduate student,

Department of Oral Pathology and Microbiology, SRMIST, SRM Dental College, Ramapuram, Chennai - 600 089, Tamil Nadu, India

dr.nandakumar1997@gmail.com

Dr. Thayalan Dineshkumar

Associate professor, Department of Oral Pathology and Microbiology, SRMIST, SRM Dental College, Ramapuram, Chennai - 600 089, Tamil Nadu, India

drtdineshkumar@gmail.com

Dr.Krishnan Rajkumar

Professor and Head, Department of Oral Pathology and Microbiology, SRMIST, SRM Dental College, Ramapuram, Chennai - 600 089, Tamil Nadu, India

drrajkumar22163@gmail.com

Dr.A.Ramesh kumar

Professor, Department of Oral Pathology and Microbiology, SRMIST, SRM Dental College, Ramapuram, Chennai - 600 089, Tamil Nadu, India

arkjayamdj@gmail.com

Dr.Bose Divya

Senior lecturer,Department of Oral Pathology and Microbiology, SRMIST, SRM Dental College, Ramapuram, Chennai - 600 089, Tamil Nadu, India

divyab.diffy@gmail.com

Running title- "Role of fusobacterium nucleatum in the progression of oral squamous cell carcinoma"

Abstract

Background: - It has recently been discovered that there is a correlation between bacteria and carcinogenesis. Fusobacterium nucleatum is frequently detected in oral cancer tissues. Since, studies have only been performed with regard to F. nucleatum in colorectal and oesophageal

cancers, we propose to evaluate the role of this F. Nucleatum in the progression of Oral Squamous Cell Carcinoma.

Aim: - To identify the role of F. Nucleatum in the progression of oral squamous cell carcinoma.

Materials and Methods: -A total number of 14 patients,6 samples from patients with OSCC (Study group),6 normal tissue from patients who have undergone procedures like frenectomy and Crown lengthening (Control group) and 2 SCC samples as a positive control one from oesophagus and another from Sigmoid colon were included in this study. Quantification of F. Nucleatum and expressions like Fad A gene,KRT7 Gene,CCL20 were amplified using Real Time PCR.

Statistical analysis: -Statistical analysis of the data was done using SPSS Software version 21.0. Normal distribution was tested by Kolmogorov-Smirnov and Shapiro-Wilks test. Mann–Whitney U test was performed between OSCC patients (study group) and the normal tissues (control group). If the p value obtained was <0.05, it was considered to be statistically significant.

Results: -Fusobacterium nucleatum Copy Number was significantly increased in OSCC samples. The mean Fad A, KRT7 and CCL20value was greater in subjects with OSCC compared to Normal subjects. However, the difference was significant statistically only for KRT7 and CCL20.

Conclusion: -Fusobacterium species have been suggested to be associated with cell adhesion, tumorigenesis, epithelial mesenchymal transition, inflammasomes, cell cycle, etc. in oral cancer.

Key words: -F. nucleatum, Oral squamous cell carcinoma, Fad A, KRT7, CCL20

Text of the article

Introduction

Oral cancer, particularly oral squamous cell carcinoma (OSCC), is a serious health issue in many nations, and it is the leading cause of death from oral disorders [(1)]. Oral cancer poses a serious threat to life and has a poor prognosis, despite the development of medications that can increase survival and quality of life[(2)]. The etiology of OSCC is multifactorial with use of tobacco being one of the most important risk factors. Non-smoking, non-drinking patients (NSND) who present with OSCC have been identified in the literature as a distinct and emerging subgroup. Currently, 13–35% of the OSCC population is comprised of NSND individuals.Chronic inflammation brought on by infections has been proposed as one of the significant causes of cancer. Recently, commensal microorganisms have been linked to the development of cancer. An effective screening program requires early and precise diagnosis and treatment of patients with OSCC.The lack of rapid, accurate diagnostic techniques in low-income areas is a major obstacle to early treatment of oral cancer[(3)].

F. nucleatum is a known oral bacterium which is involved in the formation of dental plaque. Interestingly, F.nucleatum is frequently detected in oral cancer tissues. Moreover, it has been proposed that the detection of F.nucleatum is correlated with the clinical stage of oral cancer [(4)]. Although the detailed mechanism of carcinogenesis is still unclear, Fusobacterium species have been suggested to be associated with cell adhesion, tumorigenesis, epithelial mesenchymal transition, inflammasomes, cell cycle, etc. in oral cancer[(5)].

Several genetic studies have been done in the past to find its association in colorectal and gastoesophageal cancers. In the current study we have seen the expression of Fusobacterium adhesion A (FadA),Keratin 7 (KRT_7) and C-C Motif chemokine ligand 20 (CCL20) which is said to play an important role in tumor progression, promotemetastasis, cell proliferation and cell migration respectively.Since genetic studies have only been performed with regard to F. nucleatum in colorectal and oesophageal cancers This study aims to evaluate these factors to identify the role of F. nucleatum this in the progression of OSCC.

Materials and Methods

The study was approved by the Institutional review board and ethical committee of SRM Dental College, Ramapuram [IRB NO: SRMDC/IRB/2020/MDS/No.602]. A total of 14 patients between 2020-2022 were enrolled in the study. The details of the study were explained to all patients and written informed consent was obtained from all the participants before entering into the study. They were categorized into three major groups: Normal/Healthy controls(n=6), Tissue samples from OSCC patients (n=6) and Tissue samples from Oesophageal/Colorectal Cancer (n=6). None of the patients in the current study had a history of tobacco or alcohol usage.**(Figure 1)**

Detailed case history was taken, after complete intra and extra oral examination, tissue biopsy was obtained from each participant in all the three groups.

The obtained biopsy tissue is then cut into two separate specimens, one is stored in formalin for confirmation of the diagnosis through histopathological examination, the other specimen which is taken for our study is stored in Eppendorf Tube containing RNA Later. The study samples are then stores in the deep freezer at –80˚C.A small portion of the sample was subjected for absolute quantification of fusobacterium nucleatum using real time PCR by SYBER Green Chemistry after DNA extraction. While the other portion of the samples were subjected to mRNA extraction, followed by cDNA conversion to check the expressions of FadA, KRT7 and CCL20. hRNaseP was used as an endogenous Control for KRT7 & CCL20 & Fun gyrA was used as an endogenous Control for Fad A to normalize the data.

For Fusobacterium nucleatum Copy Number detection, DNA was isolated from all the sample using Maverick Nucleic acid Extraction Kit, Cat No.EC00001A (Mylab, India). Absolute quantification was done using Real Time PCR by SYBR Green Chemistry

For expression study RNA was extracted from 14 samples (6 OSCC, 6 Controls and two colon cancer tissue) using Maverick Nucleic acid Extraction Kit, Cat No.EC00001A (Mylab, India).cDNA from each sample was synthesized using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific USA).Real-Time PCR using SYBR Green Chemistry-Relative expression were analyzed using cDNA of specific targets **(Fad gene, KRT-7,** and **CCL20)** and control (**hRNaseP**) gene using TB Green® Premix Ex Taq™II (Tli RNaseH Plus) PCR Kit (Takara, Japan).

The reactions were carried out in duplicates for each sample and expressions and the mean Ct were obtained for all groups**(Figure 2)**.

Based on the obtained C_T values the fold difference was calculated using comparative Ct value by the **delta-delta C_T** ($\Delta\Delta C_T$) method, also known as the $2^{-\Delta\Delta CT}$ or **Livak** method and those fold changes were taken for statistical analysis.

Results

Fusobacterium nucleatum copy number real time PCR results:

Fusobacterium nucleatum Copy Number was significantly increased in OSCC samples when compared to samples of the other two groups The count was three to four times greater when compared to the F. nucleatum count in the normal oral mucosa.

Fusobacterium nucleatum count in each sample is depicted in (table 1)

Table 1. *Fusobacterium nucleatum* count in each sample

Conditions	Fn Count
OSCC1	88,316
OSCC2	1000
OSCC3	3400
OSCC4	2,448
OSCC5	2150
OSCC6	5,846
NT1	3,055
NT2	1510
NT3	3,239
NT4	4840

NT5	600
NT6	200
SCC-G1	1900
SCC-G2	2000

Expression study - real-time pcr based relative quantification

Expression of Fad A, KRT7 &CCL20between healthy tissue and OSCC tissues

Comparing the expressions of Fad A,KRT7 & CCL20between healthy group tissue and OSCC tissues, it was observed that the expression levels were significantly increased in OSCC samples in comparison to healthy controls.

Ct Mean, Fold Change is depicted in (table 2)

Table 2: Ct Mean, Fold Change in study samples

S. NO	Condition	Sample Name	Ct Mean hRNaseP (Endogenous Control)	Ct Mean Fun gyrA (Endogenous Control)	Ct Mean (Target Gene)			Fad A Fold Change FC =2^-ΔΔCt	KRT7 Fold Change FC =2^-ΔΔCt	CCL20 Fold Change FC =2^-ΔΔCt
			hRNaseP	Fun gyrA gene	Fad A	KRT7	CCL20	FC=2^-ΔΔCt	FC=2^-ΔΔCt	FC=2^-ΔΔCt
1	Diseased	OSCC1	23.34	18.24	17.96	28.46	19.80	4.00	0.02	7.59
2	Diseased	OSCC2	28.21	32.71	34.75	29.81	27.50	0.80	0.29	1.06
3	Diseased	OSCC3	29.02	30.73	29.60	34.88	29.62	7.24	0.02	0.43
4	Diseased	OSCC4	29.47	23.77	25.68	35.32	38.39	0.88	0.02	0.00
5	Diseased	OSCC5	24.29	27.68	25.66	22.78	21.31	13.38	2.49	5.20
6	Diseased	OSCC6	31.58	22.37	24.67	0.00	33.31	0.67	NE	0.20
			hRNaseP	Fun gyrA gene	Fad A	KRT7	CCL20	FC=2^-ΔΔCt	FC=2^-ΔΔCt	FC=2^-ΔΔCt
1	Normal	NT1	23.78	23.43	0	0.00	31.79	NE	NE	0.00
2	Normal	NT2	24.03	28.24	28.92	0.00	35.58	2.06	NE	0.00
3	Normal	NT3	22.45	23.35	24.66	34.53	28.99	1.33	0.00	0.01
4	Normal	NT4	22.12	26.37	26.59	28.28	24.82	2.82	0.01	0.10
5	Normal	NT5	28.38	33.51	34.43	34.88	31.48	1.74	0.01	0.08
6	Normal	NT6	28.43	22.12	28.28	34.89	31.26	0.01	0.01	0.09

			hRNaseP	Fun gyrA gene	Fad A	KRT7	CCL20	FC=2^-ΔΔCt	FC=2^-ΔΔCt	FC=2^-ΔΔCt
1	Positive Control	G1	26.42	32.17	34.84	26.24	25.26			
2	Positive Control	G2	28.86	31.82	32.59	28.65	28.79			
		Calibrator	**27.64**	32.00	**33.72**	**27.44**	**27.03**	1.00	1.00	1.00

Statistical analysis

Statistical analysis of the data was done using SPSS Software version 21.0. The descriptive statistics such as mean and standard deviation were calculated for individual groups. The obtained data were analysed for normality. Normal distribution was tested by Kolmogorov-Smirnov and Shapiro-Wilks test. The value of the K-S test statistic (D) is .30091.The *p*-value is .00215. This provides good evidence that the data is not normally distributed and therefore non-parametric tests are applied to analyse the data

Mannwitney u test was done to compare the difference in gene expression between different groups. All p-values were 2-sided; $p \leq 0.05$ was considered statistically significant.

Mean, Std. Deviation, Median, Mann–Whitney U test (p-Value) is depicted in table (3).

Table 3: Mann–Whitney U test was performed for Fad A, KRT7& CCL20between Group I (normal tissues) and Group II (OSCC patients)

Parameter	Group	N	Mean	Std. Deviation	Median	IQR	p-Value
Fad A	**Normal**	6	1.326	1.1342	1.535	6.12	0.378
	OSCC	6	4.4950	5.06113	2.44000	8.01	
KRT7	**Normal**	6	.0050	.00548	.00500	0.01	0.026
	OSCC	6	.4733	.99410	.02000	0.83	
CCL20	**Normal**	6	.0467	.04803	.04500	0.09	0.041
	OSCC	6	2.4133	3.19536	0.74500	5.65	

On comparing the data obtained for Fad A, KRT7 and CCL20 between healthy tissue and OSCC tissues

- The mean Fad A value is greater in subjects with OSCC compared to Normal subjects. However, the difference was not significant statistically.
- The mean KRT7&CCL20value is greater in subjects with OSCC compared to Normal subjects. The difference was significant statistically.

Thus, the above mentioned statistical data analysis between healthy controls and the study group suggests that the expression of Fad A, KRT7 and CCL20 were significantly increased in all study groups when compared to healthy controls.

Discussion

OSCC is the most common type of HNC that has been reported, and it is currently a major problem around the world. Tobacco, alcohol and betel quid with or without added tobacco are the three main risk factors in the complex etiology of OSCC [(6)].Non-smoking, non-drinking patients (NSND) who present with OSCC have been identified in the literature as a distinct and emerging subgroup[(7)]. One of the principle causes of (NSND) is thought to be chronic inflammation brought on by infections. It has recently been discovered that there is a correlation between bacteria and carcinogenesis[(8)].It is yet unclear how bacteria may contribute to the development of oral cancer. The possibility that oral commensal bacteria are involved in the pathogenesis of OSCC has raised concerns.

Studies have shown that Fusobacterium species present in the oral cavity play a crucial role for the development of Colorectal and Oesophageal cancer. According to Abed et al F. nucleatum was found in both CRC and oral saliva samples, with 75% of those samples containing the same strain. Thus,F. nucleatum which may have existed in the oral cavity, is frequently found in CRC samples and is involved in malignant behaviour. Hence we would like to postulate the same hypothesis that,F. nucleatum might also have an important role in the development of Oral squamous cell carcinoma [(9)].

In the current study, we found out that F. nucleatum was significantly increased in the tissue samples of OSCC patients. The count was three to four times greater when compared to the F. nucleatum count in the normal oral mucosa. We have also taken one upper esophageal & colorectal cancer tissue as a positive control for our study which also showed elevated levels.

The ability of F. nucleatum to adhere to pathogenic organisms and cells is its most significant characteristic[(10)]. So first we evaluated Fusobacterium adhesion A (FadA), a fimbrial

adhesin protein, which is required for attachment and invasion of host cells, it plays an important role in tumor progression. This unique virulence factor FadA could bind to E-cadherin and activate β-catenin, which promoted the growth of tumour cells.It possesses a secreted form that has been demonstrated to increase the expression of Wnt gene and the β-catenin pathway. Both are important developmental pathways, that when dysregulated are leading to cause carcinogenesis. The present Expression Study - Real-Time PCR based Relative Quantification- The Mean Fad A value is greater in subjects with OSCC compared to Normal subjects. Statistically, the difference was not significant.

The second genetic factor that we evaluated was KRT7 gene. Keratin 7 (KRT_7) which is also known as cytoskeletal 7/ Sarcolenin is a protein in humans which is encoded by KRT7 gene. F. nucleatum upregulates Krt_7 by activating NF- κ β pathway, which promotes cell migration and metastasis[11].A number of studies have shown the overexpression of KRT7 in cancer cells. The present Expression Study - Real-Time PCR based Relative Quantification- KRT7 was upregulated in more in OSCC samples when compared to Normal samples. The difference was significant statistically. It promoted cell growth, S-phase entry, and migration. So this could be its possible pathogenesis in the contribution of OSCC.

The third and final genetic factor that we evaluated was C-C Motif Chemokine Ligand 20(CCL20). C-C Motif chemokine ligand 20 (CCL20) is a small cytokine belonging to the CC chemokine family.It plays a crucial role in cell proliferation and cell migration[12]. CCL20 elicits its effect on its target cell by binding and activating the chemokine receptor.In the presentReal-Time PCR based Relative Quantification Study - CCL20 was upregulated in more in OSCC samples when compared to Normal samples. The difference was significant statistically.The importance of chemokines and their receptors in tumor formation and progression in OSCC has been progressively made evident in the statistics. In the current investigation, CCL20 showed to be the most elevated chemokine in Oral cancers that were F. nucleatum positive.

Conclusion

Since genetic expressions (Fad-A, KRT7, CCL20) has not been studied in OSCC patients, the present study aims to provide preliminary data on this genetic expression and therefore, the sample size in both the group was taken as 6.We had a significant difference between both the groups, keeping these values as a preliminary data upcoming studies can be done with even larger sample size to improve the chances of a clear outcome and clarify the processes through which F. nucleatum affects tumour behavior.

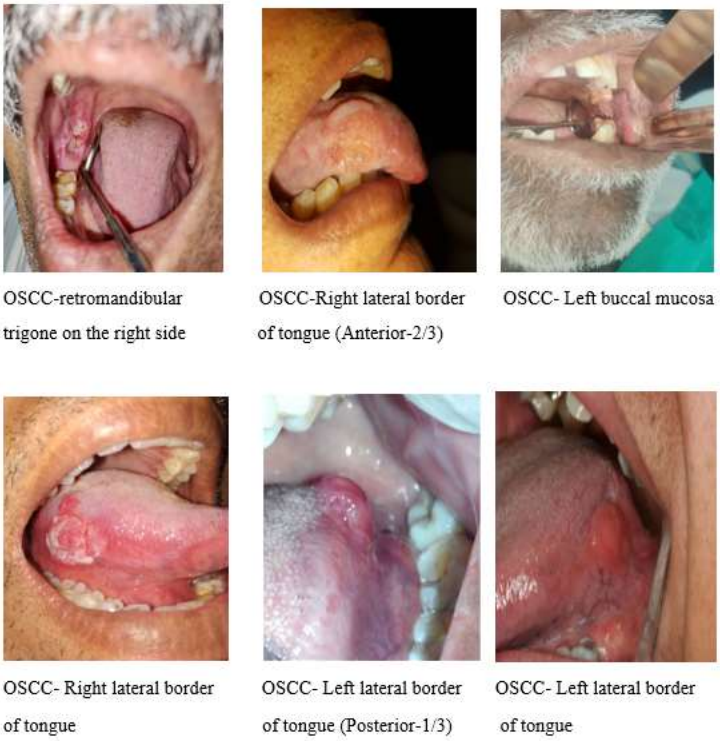

Figure 1. Clinical images of OSCC (Patients without habit)

FadA	KRT7

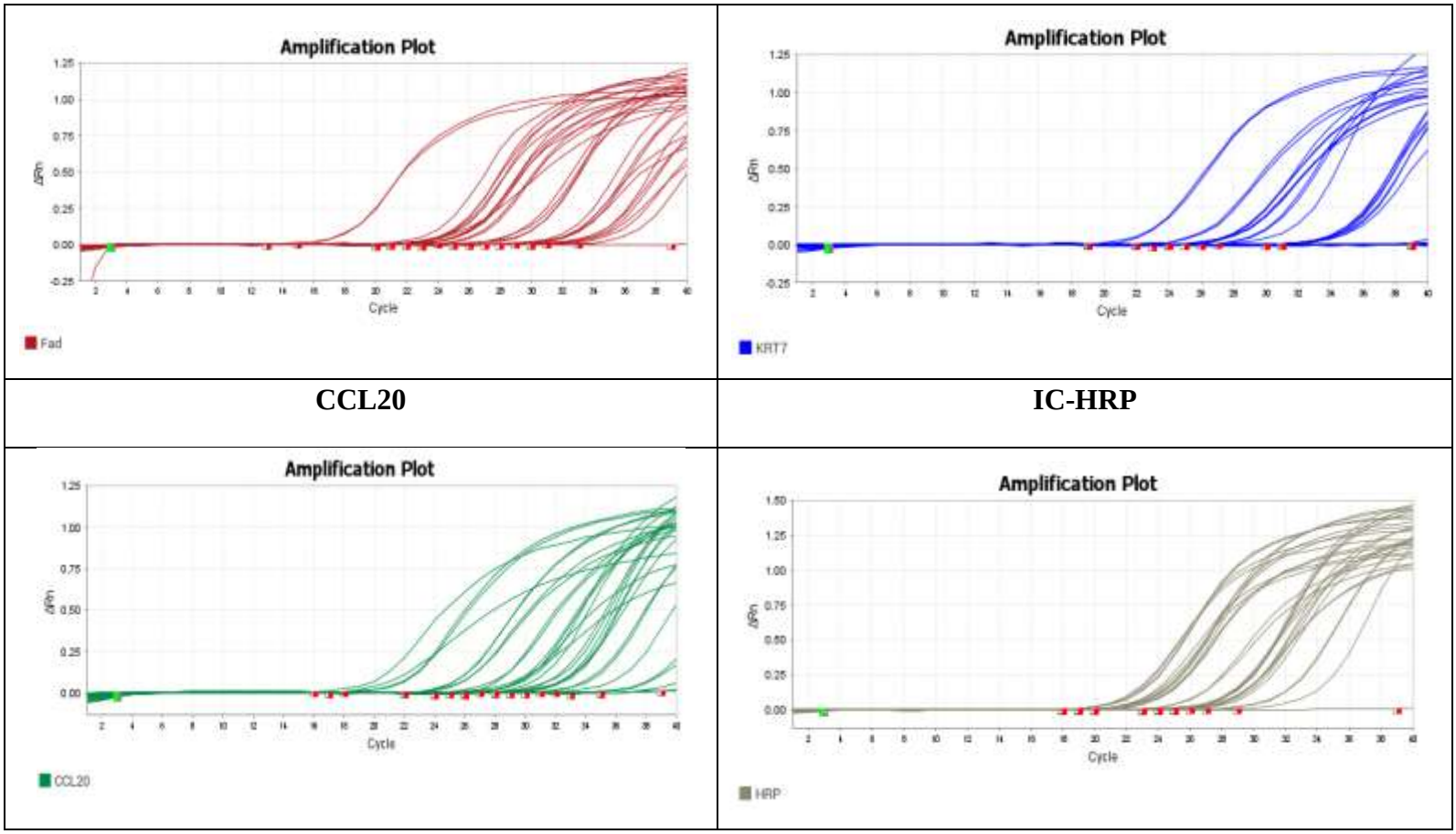

Figure 2. Compiled expressions of FadA, KRT7, CCL20, IC-HRP

Acknowledgments

Disclosure of potential conflicts of interest

The authors declare that no conflict of interests was present during the making of the study.

Legends

Table 1. *Fusobacterium nucleatum* count in each sample

Table 2: Ct Mean, Fold Change in study samples

Table 3: Mann–Whitney U test was performed for Fad A, KRT7&CCL20between Group I (normal tissues) and Group II (OSCC patients)

Figure 1. Clinical images of OSCC (Patients without habit)

Figure 2. Compiled expressions of FadA, KRT7, CCL20, IC-HRP

References

1) Li Q, Hu Y, Zhou X, Liu S, Han Q, Cheng L. Role of oral bacteria in the development of oral squamous cell carcinoma. Cancers. 2020 Sep 29;12(10):2797.
2) Krishnan R, Thayalan DK, Padmanaban R, Ramadas R, Annasamy RK, Anandan N. Association of serum and salivary tumor necrosis factor-α with histological grading in oral cancer and its role in differentiating premalignant and malignant oral disease. Asian Pacific Journal of Cancer Prevention. 2014;15(17):7141-8.
3) Dineshkumar T, Ashwini BK, Rameshkumar A, Rajashree P, Ramya R, Rajkumar K. Salivary and serum interleukin-6 levels in oral premalignant disorders and squamous cell carcinoma: diagnostic value and clinicopathologic correlations. Asian Pacific journal of cancer prevention: APJCP. 2016;17(11):4899.
4) Nithianantham S, Xu M, Yamada M, Ikegami A, Shoham M, Han YW. Crystal structure of FadA adhesin from Fusobacterium nucleatum reveals a novel oligomerization motif, the leucine chain. Journal of Biological Chemistry. 2009 Feb 6;284(6):3865-72.
5) Fujiwara N, Kitamura N, Yoshida K, Yamamoto T, Ozaki K, Kudo Y. Involvement of Fusobacterium species in oral cancer progression: a literature review including other types of cancer. International Journal of Molecular Sciences. 2020 Aug 27;21(17):6207.
6) Whitmore SE, Lamont RJ. Oral bacteria and cancer. PLoS pathogens. 2014 Mar 27;10(3): e1003933.
7) Koo K, Barrowman R, McCullough M, Iseli T, Wiesenfeld D. Non-smoking non-drinking elderly females: a clinically distinct subgroup of oral squamous cell carcinoma patients. International journal of oral and maxillofacial surgery. 2013 Aug 1;42(8):929-33.
8) Liao JB. Cancer issue: Viruses and human cancer. The Yale journal of biology and medicine. 2006 Dec;79(3-4):115.
9) Abed J, Maalouf N, Manson AL, Earl AM, Parhi L, Emgård JE, Klutstein M, Tayeb S, Almogy G, Atlan KA, Chaushu S. Colon cancer-associated Fusobacterium nucleatum may originate from the oral cavity and reach colon tumors via the circulatory system. Frontiers in cellular and infection microbiology. 2020 Aug 7;10:400.
10) Xu M, Yamada M, Li M, Liu H, Chen SG, Han YW. FadA from Fusobacterium nucleatum utilizes both secreted and nonsecreted forms for functional oligomerization for attachment and invasion of host cells. Journal of Biological Chemistry. 2007 Aug 24;282(34):25000-9.
11) Chen S, Su T, Zhang Y, Lee A, He J, Ge Q, Wang L, Si J, Zhuo W, Wang L. Fusobacterium nucleatum promotes colorectal cancer metastasis by modulating KRT7-AS/KRT7. Gut microbes. 2020 May 3;11(3):511-25.
12) Yamamura K, Baba Y, Nakagawa S, Mima K, Miyake K, Nakamura K, Sawayama H, Kinoshita K, Ishimoto T, Iwatsuki M, Sakamoto Y. Human microbiome Fusobacterium

nucleatum in esophageal cancer tissue is associated with prognosis. Clinical Cancer Research. 2016 Nov 15;22(22):5574-81.