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Possible Translocation of Periodontal Pathogens into the Lymph Nodes Draining the Oral Cavity

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Numerous publications have reported the presence of periodontopathogenic bacteria in peripheral and central vascular lesions. However, it is unclear how this bacterial translocation occurs. The objective of this study was to investigate whether periodontopathic bacteria are translocated to lymph nodes proximal to the oral cavity. Obtaining lymph node samples is not ethically feasible unless they are excised as part of the surgical management of patients with cancer. This study analyzed formalin-fixed and paraffin-embedded lymph nodes, histologically negative for cancer cell invasion, that were excised from 66 patients with histories of head and neck cancer. Real-time PCR was performed to amplify the 16S ribosomal DNA fragments from *Porphyromonas gingivalis*, *Treponema denticola*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, and *Prevotella intermedia*. The relationship between bacterial detection and cancer severity, gender, and the use of anti-cancer therapy was examined by Fisher's exact test. *P. gingivalis*, *T. forsythia*, and *P. intermedia* were present in 17%, 8%, and 8% of the samples of submandibular and submental lymph nodes, respectively. There were no significant relationships between bacterial detection and the cancer disease status, patient gender or use of anticancer therapy. According to these data, it appears that the translocation of periodontopathic bacteria may occur via lymphatic drainage, irrespective of the cancer disease status, gender or anticancer therapy.

Keywords: submandibular/submental lymph nodes, periodontopathic bacteria, bacterial translocation, real-time PCR

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Introduction

Periodontitis is a bacterially-induced, localized chronic inflammatory disease that destroys the bone and connective tissues supporting the teeth. Bacterial accumulation is necessary for the onset and progression of periodontitis (Kornman *et al.*, 1997; Friedewald *et al.*, 2009), and bacterial infection is often followed by host-mediated soft tissue destruction caused by hyperactivated or primed leukocytes, and the subsequent release of cytokines, eicosanoids, and matrix metalloproteinases, which leads to clinically significant connective tissue and bone destruction (Pihlstrom *et al.*, 2005). When biofilm formed on the tooth surface is undisturbed, ecological changes occur (Mustapha *et al.*, 2007), which facilitate the emergence of anaerobic or facultatively anaerobic, Gram-negative bacterial species, such as *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, and *Prevotella intermedia.* These bacteria are considered to be pathogenic, as they activate numerous immunoinflammatory responses in the host and cause disruptions in the mechanisms of bacterial clearance.

In recent years, numerous publications have reported an association between periodontitis and atherosclerotic cardiovascular disease. Periodontal disease has been reported as a risk factor or a marker independent of traditional coronary artery disease risk factors (Humphrey *et al.*, 2008; Dorn *et al.*, 2010), and it has been established that periodontal disease is an important risk factor for all forms of cerebrovascular disease (Pussinen *et al.*, 2007), particularly non-hemorrhagic stroke (Wu *et al.*, 2000). A few studies have reported a link between peripheral arterial disease and periodontitis (Bloemenkamp *et al.*, 2002; Chen *et al.*, 2007, 2008, 2009). However, so far no clear mechanism linking these atherosclerotic cardiovascular diseases and periodontitis has been established, although two biologically plausible mechanisms have been described: (1) moderate to severe periodontitis increases the level of systemic inflammation, and is associated with increased systemic inflammatory markers (Tonetti *et al.*, 2007; Paraskevas *et al.*, 2008; Nakajima *et al.*, 2010) and (2) in patients with untreated periodontitis, the population of gram-negative bacteria inhabiting the periodontal pocket may vary from 10^8 to 10^{12}, and predominantly pathogenic bacterial species such as *P. gingivalis, A. actinomycetemcomitans*, *T. forsythia*, and *P. intermedia* have been found close to the ulcerated epithelium of periodontal pockets and also in atheromas (Haraszthy *et al.*, 2000; Lau *et al.*, 2004; Gaetti-Jardim *et al.*, 2009). However, it is unclear how the translocation of these bacteria occurs. A few reports have discussed transient bacteremia (Kinane *et al.*, 2005; Elkaim *et al.*, 2008), but the influence of transient bacteremia is of short duration.

It has also been reported that bacteria such as *Helicobacter pylori* from the gastrointestinal tract are translocated to gastric lymph nodes, despite their non-invasive characteristics (Ito *et al.*, 2008), but it is unknown whether a similar phenomenon occurs with periodontal pathogens.

The main objective of this study was to investigate whether periodontopathic bacteria are translocated to the lymph nodes that drain the oral cavity.

Material and Methods

Sample selection

Study group: Obtaining lymph node samples is not ethically feasible unless they are excised as part of the surgical management and/or for further histological investigation of lymph node metastasis in patients with cancer. Therefore, lymph nodes that were negative for cancer cell invasion, but that were excised as part of the treatment and/or investigation of cancer patients, were used in this study. Ethical clearance was obtained from the Ethics Committee of Tokyo Medical and Dental University. Written informed consent was obtained from each patient after explaining the purpose of the study. In Experiment 1, we used the submandibular and submental lymph nodes. Other available oral tissue sections, including the gingiva or oral mucosa, were also analyzed to identify the periodontal bacterial profiles in each patient at the time of surgical management (44 samples). In patients whose gingival sections were unavailable, we used tissue sections with oral mucosa for the detection of bacteria, since there was a possibility that the epithelium that comes in contact with the saliva may have retained some bacteria during the tissue fixation process. In Experiment 2, available superficial and deep cervical lymph nodes from patients who showed positive results for submandibular and submental lymph nodes were analyzed. Patient information was retrieved by searching the patient database of the Human Pathology Department. Using the obtained information, formalin-fixed and paraffin-embedded samples were collected from the Human Pathology Department archive of patient samples. From the selected samples, lymph nodes with cancer cell invasion were excluded after a histological examination. A total of 66 patients with head and neck carcinoma were thus eligible to be included in this study, and had an age range of 24 to 80 years (mean±SD was 59±11 years).

Control group: As the control group, regional lymph node samples that were negative for cancer cell invasion from patients with cancer in the lungs (18 patients), stomach (20 patients), and colon (20 patients) were used, since it was not possible to obtain lymph nodes from healthy subjects. The age of patients included in the control group ranged from 43 to 93 years (mean±SD: 68±10 years).

Primer and probe preparation

During the process of tissue fixation and paraffin embedding, genomic DNA becomes fragmented. Thus, the amplicon sizes of DNA in formalin-fixed paraffin wax-embedded tissues vary from 100–150 bp. Therefore, newly designed primers and probes that are shorter than conventional primers and probes were used to amplify the 16S ribosomal DNA fragments of *P. gingivalis*, *T. denticola*, *A. actinomycetemcomitans*, *T. forsythia*, and *P. intermedia* (Sanz *et al.*, 2004). Probes were labeled with 6-carboxyfluorescein on the 5′-end and 6-carboxytetramethylrhodamine (TAMRA) on the 3′-end. The primers and probes used are listed in Table 1.

Preparation of standard curves

The sensitivity and reproducibility of the TaqMan Polymerase Chain Reaction (PCR) were evaluated with eight dilutions of a 10-fold dilution series for each species of bacterial DNA. The real-time PCR mixtures contained a total volume of 50 µl, which included 5 µl of template DNA, 100 nM concentrations of each forward and reverse primer, 40 nM probe, and Absolute QPCR ROX (500 nM) Mix (ABgene, UK). Amplification and detection were performed using a detection system (ABI PRIZM Sequence Detection System; Applied Biosystems, USA) under conditions of 95°C for 5 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. The DNA concentrations are expressed as the number of bacterial genomes with 1.25×10^{10} Da per genome used in the conversion. Negative controls without bacterial DNA were included in every PCR.

Confirmation of primer and probe specificity

The specificity and absence of cross-reactivity was confirmed using DNA extracted from different bacteria commonly present in the human body as commensal and pathogenic bacteria (Table 2). Triplicates of 0.02 ng/µl DNA extracted from each bacterial species were used for the TaqMan anal-

Table 1. Primers and probe

Bacteria	Primers and probes	
P. gingivalis	F: GTTCAGCCTGCCGTTGAAAC 20 bp R: ACCGCTACACCACGAATTCC 20 bp	Probe: CTTGAGTTCAGCGGCGGCAGG 21 bp
T. forsythia	F: CGACGGAGAGTGAGAGCTTTCT 22 bp R: GCGCTCGTTATGGCACTTAAG 21 bp	Probe: CGTCTATGTAGGTGCTGCATGGTTGTCG 28 bp
T. denticola	F: AACGGTAAGGGAGAGCTTGCT 21 bp R: ATATTTCTACTAGCTATCCCCATCTTCAG 29 bp	Probe: TCCCCTAGAGTGGCGGACTGGTGA 24 bp
A. actinomycetemcomitans	F: GTCATCATGGCCCTTACGAGTAG 23 bp R: CCCCATCGCTGGTTGGT 17 bp	Probe: ACACGTGCTACAATGGCGTATACAGAGGGT 30 bp
P. intermedia	F: CCGCCTAATACCCGATGTTG 20 bp R: CCCATCCTCCACCGATGA 18 bp	Probe: CACATATGGCATCTGACGTGGACCAAA 27 bp

ysis with each set of primers and probes.

Analysis of samples using real-time PCR

Two sections (3 μm) were cut from each formalin-fixed paraffin-embedded block and placed in 1.5-ml centrifuge tubes. To prevent sample-to-sample contamination, a different microtome blade was used for each sample. Paraffin sections were deparaffinized and rehydrated with xylene and alcohol, respectively. Recovered tissue pellets were used for DNA extraction of bacteria with a QIAamp DNA mini kit according to the manufacturer's instructions (QIAGEN, Germany) after suspension in ATL buffer (QIAGEN) and overnight digestion with proteinase-K (QIAGEN) at 54°C. TaqMan PCR was performed to amplify fragments of 16s ribosomal DNA of the above mentioned periodontal pathogens and the same real time PCR protocol was followed. The number of bacterial genomes in tissue samples was estimated using an internal standard curve for each bacterial species, prepared with three replicates of four concentrations (2,000,000, 200,000, 20,000 and 2,000 genomic copies) in each assay. Negative controls without bacterial DNA were included in every reaction. The total number of bacteria in a single 3 μm-thick tissue section was obtained by multiplying the assay result by 40.

Statistical analysis

The relationships between the presence of bacteria in the oral cavity and bacterial detection in the submental/submandibular lymph nodes, patient gender, as well as the relationship between bacterial detection and factors related to cancer severity (other than cancer cell differentiation), were examined by Fisher's exact test for a 2×2 table. The relationship between bacterial detection in submental/submandibular lymph nodes and the presence or absence of

Table 2. Specificity and cross-reactivity of the primers and probes (mean±SD of the number of bacterial genomic copies)

Bacterial species	*P. g.* Primer and Probe	*T. f.* Primer and Probe	*A. a.* Primer and Probe	*T. d.* Primer and Probe	*P. i.* Primer and Probe
Aggregatibacter actinomycetemcomitans ATCC 43718	0	0	24015 ± 3562	0	0
Tannerella forsythia ATCC 43037	0	15524 ± 867	0	0	0
Treponema denticola ATCC 33520	0	0	0	33172 ± 1495	0
Porphyromonas gingivalis ATCC 53978	19661 ± 5094	0	0	0	0
Prevotella intermedia ATCC 25611	0	0	0	0	16645 ± 1348
Actinomyces viscosus ATCC 19246	0	0	0	0	0
Actinomyces naeslundii ATCC 12104	0	0	0	0	0
Bacteroides fragilis ATCC 25285	0	0	0	0	0
Bacteroides levii ATCC 29147	0	0	0	0	0
Bacteroides vulgatus ATCC 8482	0	0	0	0	0
Enterococcus faecalis ATCC 29212	0	0	0	0	0
Escherichia coli ATCC 25922	0	0	0	0	0
Fusobacteruim nucleatum ATCC 25586	0	0	0	0	0
Haemophilus aphrophilus ATCC 33389	0	0	0	0	0
Haemophilus influenzae ATCC 19418	0	0	0	0	0
Haemophilus parainfluenzae ATCC 33392	0	0	0	0	0
Helicobacter pylori ATCC 43504	0	0	0	0	0
Mycobacterium avium ATCC 25291	0	0	0	0	0
Mycobacterium fortuitum ATCC 6841	0	0	0	0	0
Mycobacterium kansaii ATCC 12478	0	0	0	0	0
Mycobacterium marinum ATCC 927	0	0	0	0	0
Mycobacterium paratuberculosis JATA48-01	0	0	0	0	0
Mycobacterium tuberculosis ATCC 25177	0	0	0	0	0
Peptostreptococcus micros ATCC 33270	0	0	0	0	0
Prevotella denticola ATCC 33185	0	0	0	0	0
Prevotella nigrescens ATCC 33563	0	0	0	0	0
Propionibacterium acnes ATCC 6919	0	0	0	0	0
Propionibacterium avidum ATCC 25577	0	0	0	0	0
Propionibacterium granulosum ATCC 25564	0	0	0	0	0
Pseudomonas aeruginosa ATCC 27853	0	0	0	0	0
Staphylococcus epidermidis ATCC 12228	0	0	0	0	0
Streptococcus pyogenes JRS4	0	0	0	0	0
Streptococcus sanguis ATCC 10556	0	0	0	0	0
Veillonella parvula ATCC 35184	0	0	0	0	0

Abbreviations: *P. g.*, *P. gingivalis*; *T. f.*, *T. forsythia*; *A. a.*, *A. actinomycetemcomitans*; *T. d.*, *T. denticola*; *P. i.*, *P. intermedia*

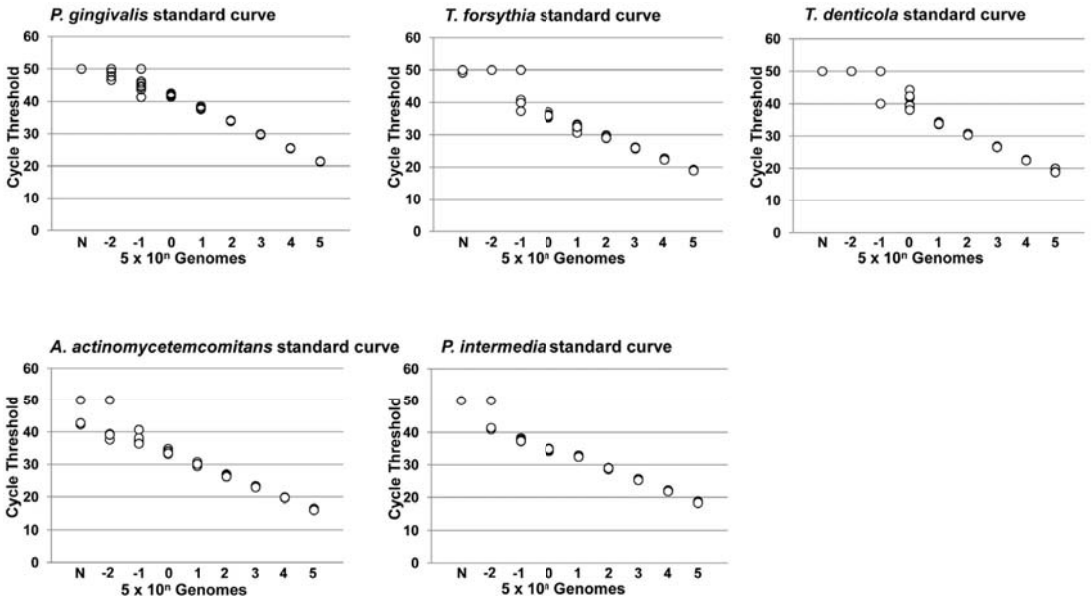

Fig. 1. Standard curves for the real-time (TaqMan) PCR for bacterial DNA. Eight samples of a given amount of bacterial DNA (from 5×10^{5} to 5×10^{-2} genomes per PCR) were amplified by 50 cycles of TaqMan PCR. The cycle threshold for each sample was calculated. Some data points overlap. Abbreviations: N, negative control; n, exponent of 10.

anticancer therapy and detection of bacteria in lymph nodes in relation to periodontal risk factors (smoking and patients' ages) were also examined using a similar method. The relationship between bacterial detection and cancer cell differentiation was examined by Fisher's exact test for a 2×3 table. A two-sided P value<0.05 was considered to be statistically significant. All analyses were performed using the SAS statistical software package (Version 9.1; SAS Institute, USA).

Results

The sensitivity and reproducibility of the TaqMan PCR primers and probes are shown in Fig. 1. The results were reproducible for 5 or more genomes per reaction for *T. denticola*, *A. actinomycetemcomitans*, *T. forsythia*, and *P. intermedia*. For *P. gingivalis*, the results were reproducible for 50 or more genomes per reaction. The genomes of *T. denticola*, *A. actinomycetemcomitans*, *T. forsythia*, and *P. intermedia* were not detected in the negative controls, but fewer than 50 genomes were detected in some of the negative controls for *P. gingivalis*. Therefore, for *P. gingivalis*, the average of the background values were subtracted from the raw data to obtain the final results, and for other bacterial species, the background values were always less than one genomic copy. Samples with more than one genomic copy (after subtraction) in the final results for *P. gingivalis* were considered to be positive. For other bacterial species, samples with more than one genomic copy in the raw data were considered to be positive.

Study group

The real-time PCR data of patients with positive results are shown in Table 3 for each oral tissue section, and the submandibular, submental and cervical lymph nodes. Out of the available oral samples from 44 patients, *P. gingivalis* was detected in 11, *T. forsythia* in 7, *T. denticola* in 11, and *P. intermedia* in 17. *A. actinomycetemcomitans* was not detected in any of the oral tissue sections.

The results for the bacterial detection in submandibular and submental lymph nodes out of the 66 available cases were as follows (Table 4): *P. gingivalis* was detected in 11 (17%) patients, *T. forsythia* was detected in 5 (8%) patients and *P. intermedia* was detected in 5 (8%) patients. Based on the location of lymph nodes, the results were as follows: in submandibular lymph nodes, *P. gingivalis* was detected in 5 (8%) patients, *T. forsythia* in 4 (6%) patients, and *P. intermedia* in 5 (8%) patients. In the submental lymph nodes, *P. gingivalis* was detected in 7 (11%) patients, *T. forsythia* in 1 (2%) patient and *P. intermedia* in 2 (3%) patients. Two of the patients were positive for all three bacteria, and one patient was positive for both *P. gingivalis* and *T. forsythia*. All of the other patients were positive for a single species of bacteria.

From all of the patients who were positive for bacterial detection, superficial and deep cervical lymph nodes were available for only 10 patients. Of these 10 patients, *P. gingivalis* was detected in 4, *T. forsythia* in 2 and *P. intermedia* in 4. Of the cervical lymph nodes, bacteria were detected in the superior internal jugular nodes, mid-internal jugular nodes, inferior internal jugular nodes, spinal accessory nodes, and supraclavicular nodes. Neither *T. denticola* nor *A. actinomycetemcomitans* were detected in the submandibular, submental, superficial or deep cervical lymph nodes.

Control group

The real-time PCR analyses of the regional lymph nodes that

Table 3. Real-time PCR data for the patients with positive results for oral tissues, submandibular, submental and other cervical lymph nodes

Case No.	Real-time PCR values of oral tissue samples					Submandibular/ submental lymph nodes	Real-time PCR values of submental and submandibular lymph node samples					Other cervical lymph nodes	Real-time PCR values of other cervical lymph node samples				
	P.g.	*T.f.*	*T.d.*	*A.a.*	*P.i.*		*P.g.*	*T.f.*	*T.d.*	*A.a.*	*P.i.*		*P.g.*	*T.f.*	*T.d.*	*A.a.*	*P.i.*
1	120	0	8120	0	40	Left submandibular	0	0	0	0	0	Not available					
2	120	0	240	0	0	Submental	0	0	0	0	0	Not available					
5	0	0	0	0	40	Right submandibular	0	0	0	0	0	Not available					
						Left submandibular	0	0	0	0	0						
						Right submental	0	0	0	0	0						
						Left submental	0	0	0	0	0						
6	40	0	0	0	40	Right submandibular	0	0	0	0	0	Not available					
						Left submandibular	0	0	0	0	0						
						Submental	0	0	0	0	0						
7	0	0	0	0	800	Right submandibular	0	0	0	0	0	Not available					
						Submental	0	0	0	0	0						
8	0	0	1320	0	4920	Submandibular	0	0	0	0	0	Not available					
						Submental	0	0	0	0	0						
10	0	0	15000	0	120	Right submandibular	0	0	0	0	720	Right superior internal jugular nodes	0	0	0	0	0
						Left submandibular	0	0	0	0	320	Right mid-internal jugular nodes	1520	0	0	0	4280
												Right inferior internal jugular nodes	0	0	0	0	3040
												Right spinal accessory nodes	0	0	0	0	640
												Right supraclavicular nodes	0	0	0	0	1240
												Left superior internal jugular nodes	0	0	0	0	4400
												Left mid internal jugular nodes	0	0	0	0	1040
												Left inferior internal jugular nodes	0	0	0	0	2040
												Left spinal accessory nodes	120	0	0	0	40
												Left supraclavicular nodes	0	0	0	0	280
12	Not available					Submandibular	0	0	0	0	0	Superior internal jugular nodes	0	0	0	0	0
						Submental	40	0	0	0	0	Mid internal jugular nodes	0	0	0	0	0
												Inferior internal jugular nodes	0	0	0	0	0
												Supraclavicular nodes	0	0	0	0	0
13	0	40	0	0	0	Right submandibular	40	0	0	0	0	Right spinal accessory nodes	40	0	0	0	0
												Right inferior internal jugular nodes	0	0	0	0	0
												Right superior internal jugular nodes	20	0	0	0	0
												Right supraclavicular nodes	0	0	0	0	0
												Left superior internal jugular nodes	0	0	0	0	0
												Left spinal accessory nodes	0	0	0	0	0
15	0	0	0	0	0	Right submandibular	0	0	0	0	0	Right mid internal jugular nodes	0	0	0	0	0
						Left submandibular	0	0	0	0	0	Right spinal accessory nodes	0	0	0	0	0
						Submental	40	0	0	0	0	Right inferior internal jugular nodes	0	0	0	0	0
												Left superior internal jugular nodes	0	0	0	0	0
21	0	0	1400	0	440	Submandibular	0	0	0	0	0	Not available					
23	0	0	0	0	46240	Submental	0	0	0	0	0	Not available					
24	0	329400	0	0	0	Right submandibular	0	0	0	0	0	Not available					
						Left submandibular	0	0	0	0	0						
						Submental	0	0	0	0	0						
26	0	320	84960	0	6360	Submandibular	0	0	0	0	0	Right superior internal jugular nodes	0	0	0	0	0
						Submental	40	0	0	0	0	Right mid internal jugular nodes	0	0	0	0	0
												Right inferior internal jugular nodes	0	0	0	0	0
												Spinal accessory nodes	0	0	0	0	0
29	0	0	4080	0	0	Submental	0	0	0	0	0	Not available					
38	0	0	0	0	40	Submandibular	40	0	0	0	0	Superior internal jugular nodes	0	0	0	0	0
												Mid internal jugular nodes	360	0	0	0	0
												Inferior internal jugular nodes	0	0	0	0	80
39	0	600	0	0	40	Submandibular	0	0	0	0	0	Not available					
						Submental	0	0	0	0	0						
40	0	0	0	0	200	Submental	0	0	0	0	0	Not available					
41	80	0	80	0	0	Right submandibular	320	0	0	0	0	Right superior internal jugular nodes	0	0	0	0	0
						Left submandibular	0	0	0	0	0	Right mid internal jugular nodes	0	0	0	0	0
												Right inferior internal jugular nodes	0	0	0	0	0
												Left superior internal jugular nodes	53	27	0	0	0
												Left mid internal jugular nodes	0	0	0	0	0

Table 3. Continued

Case No.	Real-time PCR values of oral tissue samples					Submandibular/ submental lymph nodes	Real-time PCR values of submental and submandibular lymph node samples					Other cervical lymph nodes	Real-time PCR values of other cervical lymph node samples				
	P.g.	*T.f.*	*T.d.*	*A.a.*	*P.i.*		*P.g.*	*T.f.*	*T.d.*	*A.a.*	*P.i.*		*P.g.*	*T.f.*	*T.d.*	*A.a.*	*P.i.*
42	160	0	12640	0	160	Right submental	0	0	0	0	0	Not available					
						Left submental	0	0	0	0	0						
43	1240	40	0	0	0	Right submandibular	0	0	0	0	0	Right superior internal jugular nodes	0	0	0	0	40
						Left submandibular	0	0	0	0	0	Right mid internal jugular nodes	0	0	0	0	0
						Submental	0	40	0	0	0	Left superior internal jugular nodes	0	0	0	0	0
												Left mid internal jugular nodes	0	0	0	0	0
												Left inferior internal jugular nodes	0	0	0	0	0
												Left spinal accessory nodes	0	0	0	0	0
												Left supraclavicular nodes	0	0	0	0	0
44	0	0	0	0	40	Submandibular	0	0	0	0	0	Not available					
						Submental	0	0	0	0	0						
45	0	0	200	0	0	Right submental	0	0	0	0	0	Not available					
46	40	0	0	0	0	Submental	0	0	0	0	0	Not available					
47	720	0	0	0	0	Left submandibular	0	0	0	0	0	Not available					
						Submental	0	0	0	0	0						
48	0	0	0	0	0	Submental	0	0	0	0	0	Not available					
50	40	0	0	0	0	Right submandibular	0	0	0	0	0	Right superior internal jugular nodes	0	0	0	0	0
						Left submandibular	0	0	0	0	0	Right mid internal jugular nodes	0	0	0	0	0
						Submental	40	0	0	0	0	Left superior internal jugular nodes	0	0	0	0	0
												Left mid internal jugular nodes	0	80	0	0	0
54	0	1280	84200	0	60320	Submandibular	0	0	0	0	0	Not available					
59	0	0	0	0	2000	Left submandibular	0	0	0	0	40	Right superior internal jugular nodes	0	0	0	0	1920
												Right mid internal jugular nodes	0	0	0	0	0
												Right inferior internal jugular nodes	0	0	0	0	0
												Left superior internal jugular nodes	0	0	0	0	560
												Left mid internal jugular nodes	0	0	0	0	320
												Left inferior internal jugular nodes	0	0	0	0	760
60	40	0	0	0	0	Right Submandibular	0	0	0	0	40	Not available					
						Left Submandibular	0	40	0	0	40						
						Submental	120	0	0	0	0						
61	3040	640	3760	0	48640	Right submandibular	280	100	0	0	700	Not available					
						Left submandibular	0	100	0	0	80						
						Submental	280	0	0	0	560						
63	0	0	0	0	0	Right submandibular	0	400	0	0	0	Not available					
						Left submandibular	0	0	0	0	0						
						Submental	40	0	0	0	0						
64	Not available					Submandibular	217	0	0	0	0	Not available					
						Submental	0	0	0	0	0						
65	0	0	0	0	160	Right submandibular	0	0	0	0	40	Not available					
						Left submandibular	0	0	0	0	280						
						Left submental	0	0	0	0	40						

were negative for cancer cell invasion from patients with cancer in the lungs, stomach and colon were negative for all five species of bacteria.

Statistical analyses

Statistical significance was not observed for the differences in the existence of periodontal bacteria in available oral samples and the detection of bacteria in the submandibular and submental lymph nodes (Table 5). The *P* values were 0.13 for *P. gingivalis*, 0.05 for *T. forsythia*, 0.06 for *P. intermedia* and 0.09 for any bacteria. The relationships between patient gender and the detection of *P. gingivalis* only, *T. forsythia* only and *P. intermedia* only or detection of any bacteria (in the submental and submandibular lymph nodes) were also not significant, with *P* values of 0.19, 0.88, 0.88, and 0.21. There was also no significant relationship between the ages of patients and the detection of bacteria (*P. gingivalis* only, *T. forsythia* only and *P. intermedia* only or detection of any bacteria) in the submental and submandibular lymph nodes, since the *P* values were 0.65, 0.98, 0.75, and 0.44.

Information regarding smoking habits was available for only 30 patients. Within the available data, there was no significant relationship showing that bacteria translocation is enhanced by the smoking habits of the patients. The *P* values were 0.85 for *P. gingivalis* only, 0.89 for both *T. forsythia* only and *P. intermedia* only and 0.87 for any bacteria.

The relationship between the detection of bacteria and factors related to cancer severity was also examined, as there was a possibility that these factors play a role in bacterial translocation. The factors related to cancer severity that we

Table 4. Positive results for the study group and control group

	Real time-PCR results				
	P. g.	*T. f.*	*T. d.*	*A. a.*	*P. i.*
Study Group					
Out of the total number of patients (n=66)	11 (17%)	5 (8%)	0 (0%)	0 (0%)	5 (8%)
Submandibular lymph nodes	5 (8%)	4 (6%)	0 (0%)	0 (0%)	5 (8%)
Submental lymph nodes	7 (11%)	1 (2%)	0 (0%)	0 (0%)	2 (3%)
Control group					
Regional lymph nodes of lung cancer patients (n=18)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Regional lymph nodes of gastric cancer patients (n=20)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Regional lymph nodes of colon cancer patients (n=20)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

**one patient was positive in both the submandibular and submental lymph nodes separately for *P. gingivalis* and *P. intermedia*
**In two cases both the submandibular and submental lymph nodes were fixed together in one block

examined were the tumor size, histological classification, site of the tumor, tumor ulceration, lymphatic invasion, venous invasion and lymph node metastasis. The relationship between the detection of one bacterial species (*P. gingivalis* only/*T. forsythia* only/*P. intermedia* only) or detection of any bacteria and tumor size did not show any significance (*P* values=0.58, 0.29, 0.93, and 0.27, respectively). The *P* values for the relationships between the histological classification and detection of a single species of bacteria or detection of any bacteria were 0.59 (*P. gingivalis*), 0.57 (*T. forsythia*), 0.93 (*P. intermedia*), and 0.79. The site of the tumor in relation to the detection of a single species of bacteria also did not play any significant role in the translocation of bacteria (*P. gingivalis*=0.07, *T. forsythia*=0.18, and *P. intermedia*=0.18). However, the relationship between the site of the tumor and detection of any bacteria showed a significant relationship, with a *P* value of 0.003.

The presence of ulceration at the site of the tumor was not a factor enhancing the translocation of bacteria, since the statistical relationships between the presence of ulceration and the detection of a single species of bacteria (*P. gingivalis* only/*T. forsythia* only/*P. intermedia* only) or any bacteria

Table 5. Detection of bacteria in lymph nodes and the relationship to the existence of bacteria in the oral cavity, patient gender, periodontal risk factors, factors related to cancer severity and the use of anti-cancer therapy

	Percentage of patients	Detection in lymph nodes by real-time PCR											
		P. gingivalis			*T. forsythia*			*P. intermedia*			Any Bacteria		
		Positive (%)	Negative (%)	P value	Positive (%)	Negative (%)	P value	Positive (%)	Negative (%)	P value	Positive (%)	Negative (%)	P value
Presence of bacteria in the oral cavity													
Positive	44	6	11	0.13	3	8	0.05	6	21	0.06	17	27	0.09
Negative	23	8	42		3	53		2	38		3	20	
Sex													
Male	77	17	61	0.19	6	71	0.88	6	71	0.88	29	48	0.21
Female	23	2	21		2	21		2	21		5	18	
Age													
20 yr -60 yr	61	9	52	0.65	5	56	0.98	6	55	0.75	17	44	0.44
61 yr - 80 yr	39	8	32		3	36		3	36		8	32	
Smoking habit													
Smoker	24	5	20	0.85	3	21	0.89	3	21	0.89	8	17	0.87
Non-smoker	21	5	17		3	18		3	18		6	15	
Tumor size													
Small (*MD < 2.0 cm)~ Medium (2.0 cm ≤ MD < 4.0 cm)	59	9	50	0.58	3	56	0.29	5	55	0.93	17	42	0.27
Large (≥ 4.0 cm)	36	8	29		5	32		3	33		15	21	
Histological classification													
Squamous cell carcinoma	88	15	73	0.59	6	82	0.57	8	80	0.39	29	59	0.79
Others	12	3	9		2	11		0	12		5	8	
Site of the tumor													
Oral cavity	52	14	38	0.07	6	45	0.18	6	45	0.18	26	26	0.003
Other sites	48	5	44		2	47		2	47		8	41	
Ulceration of the tumor													
With ulceration	65	11	55	0.96	5	61	0.80	6	59	0.59	21	44	0.62
Without ulceration	29	5	24		2	27		2	27		8	21	

were all >0.05 (*P* value=0.96, 0.80, 0.59, and 0.62 respectively).

When the relationships between cancer cell differentiation and the detection of individual bacterial species (*P. gingivalis* only/*T. forsythia* only/*P. intermedia* only) were examined, no significant relationship was noted (*P*=0.14, 0.07, 0.11 respectively). However, the *P* value for the relationship between cancer cell differentiation and the presence of any bacteria was significant (*P*=0.04). The lymphatic invasion of cancer cells in relation to the detection of *P. gingivalis* only/*T. forsythia* only/*P. intermedia* or any bacteria did not show any statistically significant relationship, with *P* values of 0.10, 0.64, 0.64, and 0.06 respectively. The venous invasion of cancer cells in relation to the detection of bacteria was also not statistically significant; the *P* values were 0.95 for *P. gingivalis*, 0.45 for *T. forsythia*, 0.45 for *P. intermedia* and 0.42 for the presence of any bacteria. None of the statistical relationships proved to be significant for the relationship between lymph node metastasis and presence of bacteria, since the *P* values were 0.85 for *P. gingivalis*, 0.42 for *T. forsythia*, 0.42 for *P. intermedia* and 0.27 for any combination of bacteria.

The relationship between the use of anti-cancer therapy and detection of individual bacterial species (*P. gingivalis* only/*T. forsythia* only/*P. intermedia* only) or any bacterial species was then examined. The *P* values obtained were all >0.05 (0.72, 0.96, 0.96, and 0.86), which demonstrates that the presence or absence of anti-cancer therapy does not play a role in the translocation of bacteria from the site of cancer to lymph nodes. The relationship between the presence/absence of cancer cell invasion in other cervical lymph nodes and the presence of bacteria (*P. gingivalis* only/*T. forsythia* only/*P. intermedia* only or any bacteria) was also examined, and the *P* values obtained were 0.07, 0.44, 0.47, and 0.20. This clearly shows the absence of a significant relationship between the presence of bacteria and cancer cell invasion of other cervical lymph nodes.

Discussion

The smallest lymph vessels, the lymph capillaries, form an extensive network in the connective tissues. The walls of lymph capillaries consist of a single layer of endothelial cells. For this reason, such capillaries are difficult to identify in histological tissue sections. Lymph is absorbed from the tissue fluid through the thin walls into the lymph capillaries. From these capillaries, lymph passes into larger lymph vessels that are often present near the corresponding blood vessels. Before lymph enters the blood stream, it passes through one or more lymph nodes, where the lymph is filtered and supplied with lymphocytes. Lymph from the periodontal tissues is drained to the lymph nodes that belong to the anterior and posterior triangles of the neck region; the labial and lingual gingivae of the mandibular incisor region are drained to the submental lymph nodes, the palatal gingiva of the maxilla is drained to the deep cervical lymph nodes, and the buccal gingiva of the maxilla and the buccal and lingual gingiva in the mandibular premolar and molar regions are drained to the submandibular lymph nodes. Finally, except for the third molars and mandibular incisors, all teeth with their adjacent periodontal tissues are drained to the submandibular lymph nodes. Therefore, in this study, we decided to examine the submandibular, submental, superficial and deep cervical lymph nodes. The red complex bacteria *P. gingivalis*, *T. forsythia*, and *T. denticola* are commonly identified as being disease-related (Socransky and Haffajee, 2005; Bodet *et al.*, 2007). *P. intermedia*, an orange complex bacterium, frequently shows inter-species agglutination with *P. gingivalis,* and has often been found attached to intact epithelial cells, together with *P. gingivalis* (Gibbons, 1989). *A. actinomycetemcomitans*, which is prevalent in aggressive periodontitis, is reportedly capable of invading epithelial cells derived from the periodontal pocket or gingival sulci (Rabie *et al.*, 1988). Based on this information, we decided to investigate the presence of *P. gingivalis*, *T. denticola*, *A. actinomycetemcomitans*, *T. forsythia*, and *P. intermedia* in the lymph nodes.

The present study confirms that the periodontal pathogens *P. gingivalis*, *T. forsythia*, and *P. intermedia* are translocated to the submandibular and submental lymph nodes, which are the most proximal to the oral cavity. In addition, by investigating the cervical lymph nodes, we determined that, from the submandibular and submental lymph nodes, the bacteria are further translocated to more distal lymph nodes (in relation to the oral cavity) further down the neck. Only a few of the patients were positive for a combination of bacterial species. From the statistical analysis, it is clear that the presence of bacteria in lymph nodes is independent of gender and anticancer therapy. All of the other statistical analyses, except for the relationships between the detection of any bacteria and site of the tumor/cancer cell differentiation, showed the absence of a significant relationship between the cancer disease status and translocation of bacteria. Further investigations are necessary to clarify whether the site of the tumor and cancer cell differentiation play a significant role in the translocation of bacteria. The absence of periodontapathic bacteria in the regional lymph nodes of patients with cancer in the stomach, lungs, and colon supports the fact that systemic effects arising due to cancer do not enhance the translocation of periodontal bacteria.

Due to the difficulties in obtaining fresh oral samples in this study, we could not analyze saliva or plaque to detect periodontal pathogens. However, we used available oral tissue samples to identify whether there was a significant relationship between the existence of these bacteria in oral tissues and their detection in lymph nodes. Our data showed an absence of any correlation between the presence of these bacteria in the oral cavity and lymph nodes. This result might have been due to the low number of samples or as a result of the type of samples examined (such as tissue sections with oral mucosa, which retain low numbers of bacteria). Since the patient data and samples were analyzed to retrospectively to include all the possible patients within a duration of 10 years, it was not possible to record the status of patients' periodontal health. To overcome this deficit, we looked at their risk factors for periodontal disease, such as the age and smoking habits of patients, and found that there was no significant relationship between the detection of bacteria in the submandibular and submental lymph nodes and the age/smoking status of the patients. These factors need further

investigation to identify whether they actually play a role in the enhancement of bacterial translocation. In addition, the virulence of certain bacterial strains within one species could play a role in the process of translocation and intracellular survival, since not all of the lymph nodes were positive in the patients who were positive in their oral tissue samples.

Periodontal bacteria have been detected in a number of distal extra-oral sites, such as atherosclerotic arterial lesions. However, the pathway of periodontal bacterial translocation is not understood. In untreated periodontal pockets with very high populations of bacteria, the capture of pathogens by macrophages and drainage via lymph flow from the periodontal tissues is possible, but this has yet to be confirmed by histological studies. This proposed mechanism suggests that, after the pathogens are drained to the proximal submandibular and submental lymph nodes, they may drain down the neck along the superficial and deep cervical lymph nodes, which drain to the jugular trunk. On the right side of the body, the jugular trunk drains to the right lymph trunk, while on the left, the left jugular trunk drains to the thoracic duct. Both the thoracic duct and right lymph trunk drain into venous flow at the angle between the internal jugular vein and the subclavian vein. After macrophages with pathogenic bacteria reach the venous flow, the distribution of periodontal bacteria into the arterial system via the heart is possible after the venous blood is drained into the heart. Further studies are necessary to confirm this hypothesis, as it is beyond the scope of this study.

In the oral cavity, *P. gingivalis* and *T. forsythia* have been shown to co-colonize in subgingival biofilm as late colonizers in the red complex group. As noted previously, *P. intermedia* has frequently been found attached to intact epithelial cells, together with *P. gingivalis* (Gibbons, 1989). This inter-species agglutination may lead to clustering effects during translocation that result in the detection of *P. gingivalis, T. forsythia* and *P. intermedia* in the same patient. However, *A. actinomycetemcomitans* and *T. denticola* were not detected in any of the samples in our study. *A. actinomycetemcomitans* has frequently been reported in younger patients with aggressive periodontitis. The average age range of patients recruited for this study was 59±11 years, which could be the reason for the absence of *A. actinomycetemcomitans*. *T. denticola* has been shown to express major outer sheath protein (Msp), which can diminish the chemotactic and phagocytic abilities of neutrophils through modification of their cytoskeleton (Puthengady Thomas *et al.*, 2006). This may be a factor contributing to the absence of these bacteria in the lymph nodes despite their presence in the oral tissue samples.

The identification of the pathway of periodontopathic bacterial translocation has several clinical implications. Higher populations of bacteria in periodontal pockets can result in higher numbers of bacteria being translocated into the systemic circulation, which can enhance the release of chronic inflammatory mediators. Such enhanced chronic inflammation may lead to poor treatment responses in both type I (Karjalainen *et al.*, 1994) and type II diabetic patients. It is clear that periodontal treatment and good oral hygiene practices lead to reductions in bacterial populations and healing of periodontal pockets. At the same time, the number of translocated bacteria and systemic inflammatory load may be reduced, thereby improving the patient response to treatment.

From the present investigations, we conclude that bacterial translocation is possible via lymphatic drainage of the oral cavity. Nevertheless, further investigations are necessary to identify the cell types and the mechanisms of translocation of periodontopathic bacteria into the systemic circulation.

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