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ORIGINAL ARTICLE

Streptococcus anginosus infection in oral cancer and its infection route

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OBJECTIVE: To elucidate a possible involvement of *Streptococcus anginosus* in oral cancer, we assessed the frequency of *S. anginosus* infection in oral cancer tissues, and investigated its infection route.

MATERIALS AND METHOD: The tissue specimens were obtained from 46 oral cancer and three precancerous leukoplakia subjects. Frequency of *S. anginosus* infection was assessed by a species-specific polymerase chain reaction (PCR) assay. The genotype of the clinical isolates taken from cancer tissue and dental plaque samples was analyzed using pulsed-field gel electrophoresis (PFGE).

RESULTS: *S. anginosus* DNA was frequently detected in squamous cell carcinoma (19/42), but not in other types of cancer (lymphoma and rhabdomyosarcoma) or leukoplakia samples. A subject-based analysis revealed that *S. anginosus* was solely detected in dental plaque and not in saliva from all 19 *S. anginosus*-positive squamous cell carcinoma cases. Further, the genotype of *S. anginosus* isolated from cancer tissue was identical to that from dental plaque of the same patients.

CONCLUSION: Infection of *S. anginosus* could occur frequently in oral squamous cell carcinoma and that dental plaque could be a dominant reservoir of the *S. anginosus*.

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Keywords: *Streptococcus anginosus*; oral cancer; PCR; PFGE

Introduction

Streptococcus anginosus, one of the oral viridans streptococci, is a normal flora preferentially found in dental plaque (Hamada and Slade, 1980). Although the organism is generally considered to have a low pathogenicity, it can cause serious purulent abscesses in various body sites (Gossling, 1988; Ruoff, 1988; Whitworth, 1990; Willcox, 1995; Kitada and Inoue, 1996), and subacute infective endocarditis (Fisher and Russell, 1993; Willcox, 1995). Furthermore, Sasaki *et al* (1995a, 1998), using a polymerase chain reaction (PCR) assay with *S. anginosus*-specific primers, reported that the *S. anginosus* genome DNA sequence was frequently detected in samples from surgical specimens of most esophageal and some gastric cancers; however, it was rarely found in matched non-cancerous tissues. Other investigators have also reported the association of *S. anginosus* infection in head and neck cancer tissues (Tateda *et al*, 2000; Shiga *et al*, 2001). Therefore, *S. anginosus* may have a significant role in the carcinogenic process of these human cancers, similar to *Helicobacter pylori* (Correa, 2003). However, the origin of *S. anginosus* as well as its infection route to cancer tissues in individual patients remains to be elucidated.

Carcinogenesis may be actively induced in living organisms by a variety of different agents, most of which are involved with direct or indirect actions that damage cellular DNA. Further, DNA damage caused by free radicals is one of the major etiologic mechanisms of carcinogenesis (Pitot and Dragan, 1991). Several antigens/components of microorganisms, including lipopolysaccharide (LPS), peptidoglycans, lipoteichoic acids and carbohydrate antigens, can trigger macrophages to produce nitric oxide (NO) (Granger and Lehninger, 1982; Drapier and Hibbs, 1986; Wilson *et al*, 1996; Lamarque *et al*, 1998). Although NO has been implicated in macrophage-mediated cytotoxicity against various pathogens and may play a role in persistent or latent infections, its overproduction, induced by such bacterial antigens/components, may cause damage to host tissues and cellular DNA (Hahm *et al*, 1998; Kendall *et al*, 2001; Batista *et al*, 2002). In fact, high levels of inducible nitric oxide synthase (iNOS) expression have been observed in lung (Daiz *et al*, 1994), stomach (Koh *et al*, 1999), and colon (Radomiski *et al*, 1991; Ambs *et al*, 1998) cancer tissues.

We previously reported a novel bioactive antigen (SAA) that induces NO synthesis by murine peritoneal exudate cells (PEC) from a culture supernatant of *S. anginosus* (Sasaki *et al*, 2001). Furthermore, SAA induced cyclooxygenase-2 (COX-2) mRNA expression by PEC (Sasaki *et al*, 1995b), the overexpression of

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which has also been observed in cancer tissues (Zang *et al*, 1998; Doi *et al*, 1999; Marrogi *et al*, 2000). It has also been reported that upregulation of the inducible species of both NOS and COX-2 could be associated with a risk of cancer (Giardiello *et al*, 1993; Iwasaki *et al*, 1997; Ambs *et al*, 1999; Jadeski and Lala, 1999). Thus, it is most likely that *S. anginosus* infection could lead to increased rates of DNA damage by the induction of NO and COX-2 synthesis, resulting in carcinogenesis of the infected tissues.

In the present study, we assessed the frequency of the presence of *S. anginosus* DNA in 46 oral cancer and three precancerous leukoplakia tissue specimens from 49 patients, and in dental plaque and saliva samples from 42 of those with squamous cell carcinoma. Further, we investigated the genotype of *S. anginosus* clinical isolates taken from cancer tissue samples as well as that from dental plaque of the same patients using pulsed-field gel electrophoresis (PFGE).

Materials and methods

Tissue specimens and DNA preparation

A total of 49 patients (25 males, 24 females; mean age, 69.6 ± 1.8 years) with oral cancer (42 squamous cell carcinoma, two lymphoma, two rhabdomyosarcoma) or precancerous leukoplakia took part in the present study after giving informed consent. The tissue specimens were obtained at the time of biopsy or surgery at Iwate Medical University Dental Hospital and immediately placed in sterile phosphate-buffered saline (PBS, pH 7.4) on ice, then treated with 0.15% trypsin at 37°C for 3 min and washed extensively with ice-cold PBS by vortexing to remove the adherent bacteria. Genomic DNA was purified from the tissue specimens using a FirstPrep FP120 and a FirstDNA Kit (Q biogene Inc., Carlsbad, CA, USA) according to the manufacturer's instructions.

As a control experiment, mouse tongue tissues (20 mg) from C57BL/6N mice (8 weeks old; CLEA Japan Co., Osaka, Japan) were soaked in 1 ml of an *S. anginosus* suspension (0 to 10^8 CFU of *S. anginosus* NCTC 10713 in PBS) at 37°C for 1 min to assess the level of contamination of *S. anginosus* from saliva. The mouse samples were then treated with trypsin and washed extensively with ice-cold PBS by vortexing as described above. Genomic DNA was also purified as described above.

Dental plaque and saliva samples and DNA preparations

Dental plaque and mixed saliva samples from the 42 squamous cell carcinoma patients were taken with sterile instruments just before collection of the cancer tissue specimens. The dental plaque samples were immediately placed in 1 ml of sterile PBS on ice. These dental plaque suspensions and 1 ml of the saliva samples were then centrifuged at 12 000 *g* for 1 min in a microfuge and the bacterial cells were pelleted. DNA preparations were prepared from the precipitates using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Oral streptococcal strains and S. anginosus *clinical isolates*

The following oral streptococcal strains were used in this study; *S. anginosus* NCTC 10713, *S. intermedius* GAI 1157, *S. constellatus* ATCC 27823, *S. sanguis* ATCC 10556, *S. mitis* ATCC 9811, *S. gordonii* ATCC 10558, *S. salivarius* ATCC 7073, *S. mutans* ATCC 25175, *S. sobrinus* 6715 and *S. oralis* ATCC 10557.

In order to isolate the *S. anginosus* strains, cancer tissue samples collected on swabs and dental plaque samples were suspended in PBS and centrifuged. The precipitates were cultured on BHI agar plates (Difco Laboratories, Detroit, MI, USA) supplemented with 5% rabbit blood. After anaerobic incubation at 37°C for 24 h, *S. anginosus* was identified from the colonies on the blood agar plates on the basis of colonial morphology, hemolysis reactions, Gram staining and key biochemical tests, including fermentation of mannitol, sorbitol and other carbohydrates, as well as β-glucosidase production (Ruoff *et al*, 2003). To confirm the results, a PCR assay using *S. anginosus*-specific primers was performed as described below.

PCR assay

The *S. anginosus*-specific primers used in this study were: 5′-GAACGGGTGAGTAACGCGTAGGTA-3′, and 5′-AAGCATCTAACATGTGTTACATAC-3′ (Sasaki *et al*, 1998). Further, a ubiquitous primer set that matches almost all bacterial 16S rRNA genes was used as a positive control (5′-GAACGGGTGAGTAACGC GTAGGTA-3′ and 5′-CTACGCATTTCACCGCTAC ACATG-3′). The PCR assay was performed as previously described (Ohara-Nemoto *et al*, 1997, 2002; Kimura *et al*, 2002). Briefly, template DNA (50 ng) was added to a 10-μl PCR reaction mixture containing 1 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 0.2 mM of dNTP and 0.8 μM of each primer. PCR amplification was performed in a thermal cycler (GeneAmp PCR System 9600; Applied Biosystems) with the cycling parameters set as follows. An initial denaturation at 95°C was performed for 15 min; and then 35 cycles consisting of 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s were conducted, followed by a final extension step at 72°C for 4 min. The PCR products were subjected to electrophoresis on a 1.8% agarose gel. Bands in the gel were visualized with ethidium bromide (1 μg ml^{-1}), and photographed under UV illumination.

In some experiments, to assess the specificity and sensitivity of the PCR assay, purified genomic DNA samples from nine other oral streptococcal species, *S. intermedius*, *S. constellatus*, *S. sanguis*, *S. mitis*, *S. gordonii*, *S. salivarius*, *S. mutans*, *S. sobrinus* and *S. oralis*, were mixed with or without *S. anginosus* DNA (0.05 to 500 pg each), and then PCR amplification was performed as described above.

PFGE

Streptococcus anginosus strains were isolated from three patients with squamous cell carcinoma, by swab from the cancer tissues and from dental plaque. These

S. anginosus isolates were subcultured in Todd Hewitt broth (BBL Microbiology System, Cockeysville, MD, USA) at 37°C for 24 h. After washing, the bacterial cells were embedded in low-melting-point agarose and lysed with a lysis buffer [6 mM Tris-HCl (pH 8.0), 100 mM ethylenediaminetetraacetic acid (EDTA), 1 M NaCl, 0.5% Briji 58, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine, lysostaphin (20 μg ml^{-1}) and lysozyme (0.5 mg ml^{-1})]. The lysis buffer was replaced with a proteolysis buffer [1% sodium lauryl sarcosine, 0.25 M EDTA (pH 8.0), and 100 μg ml^{-1} of proteinase K (Sigma Chemical Co., St Louis, MO, USA)] and incubation with gentle shaking at 52°C was performed for 6 h. After inactivation with 0.1 mM of phenylmethylsulfonyl fluoride (Sigma) for 1 h, the DNA blocks were washed in 0.1X TE [1 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA] at 4°C for 30 min. The DNA specimens were then digested with 2 U of restriction endonuclease *Sma*I (New England Biolabs, Boston, MA, USA) at 25°C for 4 h. DNA fragments were separated by PFGE (Gene Navigator; Amersham Pharmacia Bioteck, Buckinghamshire, UK) through 1.1% agarose (Wako Pure Chem. Ind., Osaka, Japan) at a field strength of 6 V cm^{-1} for 14 h at 10°C, with the pulse time increased from 5 to 40 s.

Results

Specificity and sensitivity of the PCR

Using 5 pg of *S. anginosus* DNA and the *S. anginosus*-specific primers enabled detectable amplification of a 103-bp DNA fragment, which was the expected size of the PCR product (Figure 1a). Our preliminary experiments revealed that 5 pg of DNA was equivalent to approximately 10^3 CFU of *S. anginosus* (data not shown). The *S. anginosus*-specific PCR assay showed no false positive result in the mixed cultures with nine other oral streptococcal species (*S. intermedius*, *S. constellatus*, *S. sanguis*, *S. mitis*, *S. gordonii*, *S. salivarius*, *S. mutans*, *S. sobrinus* and *S. oralis*) (Figure 1b).

In the mixed DNA samples from murine tongue tissues (20 mg) and *S. anginosus*, the lower limit for detection of *S. anginosus* was estimated to 10^3 to 10^4 CFU (Figure 2a). Although the tissue samples were treated with trypsin and washed extensively with ice-cold PBS by vortexing to remove adherent bacteria, it is still possible that *S. anginosus* in saliva contaminated the tissue samples during the biopsy procedure. To estimate bacteria carryover, the murine tongue tissue samples were soaked in an *S. anginosus* suspension (0 to 10^8 CFU ml^{-1}) at 37°C for 1 min before the level of *S. anginosus* was assessed with the PCR assay. Our results indicated that a 1-min flash soaking of the tissue specimens, even with a markedly higher density of *S. anginosus* (up to 10^6 CFU), did not affect the PCR results (Figure 2b).

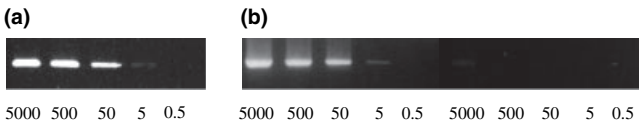

Figure 1 Specificity and sensitivity of the PCR assay using *S. anginosus*-specific primers. The *S. anginosus* template DNA (0.5–5000 pg) was added to the PCR reaction mixture containing AmpliTaq Gold, dNTP and the primers (**a**). The template DNAs from nine other oral streptococcal species, *S. intermedius*, *S. constellatus*, *S. sanguis*, *S. mitis*, *S. gordonii*, *S. salivarius*, *S. mutans*, *S. sobrinus* and *S. oralis*, were mixed with (left 5 columns) or without (right 5 columns) *S. anginosus* template DNA (0.5–5000 pg/total DNA), and added to a PCR reaction mixture containing AmpliTaq Gold, dNTP and the primers (**b**). PCR amplification was performed as described in 'Materials and Methods'

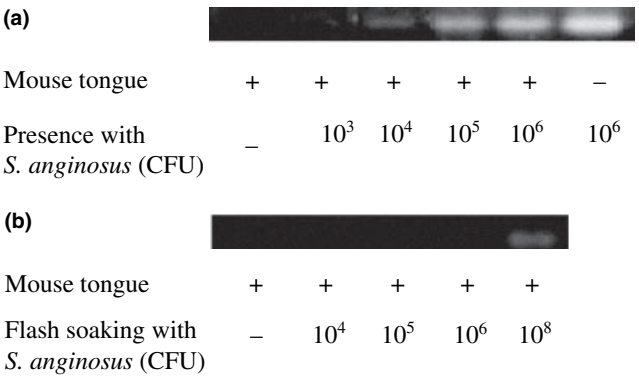

Figure 2 The PCR assay in the mixed DNA samples from tissue samples and *S. anginosus* (**a**) and the effect of the flash soaking of tissue specimens with *S. anginosus* suspensions. (**b**) The mixed DNA sample was prepared from murine tongue tissues (20 mg) and *S. anginosus* cells (0 – 10^6 CFU), and added to a PCR reaction mixture containing AmpliTaq Gold, dNTP and the primers. (**a**) The murine tongue tissues (20 mg) were soaked in *S. anginosus* suspension (0–10^8 CFU ml^{-1}) at 37°C for 1 min, and the tissue samples were treated with trypsin and washed extensively with ice-cold PBS by vortexing to remove the adherent bacteria. The DNA was then prepared, and added to a PCR reaction mixture containing AmpliTaq Gold, dNTP and the primers (**b**). PCR amplification was performed as described in 'Materials and Methods'

Detection of S. anginosus *DNA in tissue specimens*

The frequency of *S. anginosus* DNA in different types of oral cancer is summarized in Table 1. In the 46 oral cancer specimens, an *S. anginosus* DNA fragment (103 bp) was detected in nearly half of the squamous cell carcinoma cases (19/42, 45.2%); however, not in the other type of oral cancers, i.e. lymphoma and rhabdomyosarcoma, or in the three cases of precancerous leukoplakia. In the 42 oral squamous cell carcinomas, gingiva and tongue cancers were predominant (Table 2). However, the frequency of the presence of *S. anginosus* DNA was not significantly different among the

Table 1 Frequency of presence of *S. anginosus* DNA in various types of oral cancer

Types	*Number of subjects*	*S. anginosus*-positive cases (%)
Squamous cell carcinoma	42	19 (45.2)
Lymphoma	2	0
Rhabdomyosarcoma	2	0
Leukoplakia	3	0

Table 2 Frequency of presence of *S. anginosus* DNA in the 42 oral squamous cell carcinoma

	Number of subjects	*S. anginosus*-positive cases (%)
Gingiva		
Male	10	5 (50)
Female	11	7 (64)
Tongue		
Male	9	3 (33)
Female	6	3 (50)
Floor of mouth		
Male	2	0 (0)
Female	0	0 (0)
Buccal mucosa		
Male	1	0 (0)
Female	3	1 (33)

cancerous lesions. In addition, the frequency between males and females also showed no obvious difference.

Subject-based analysis of S. anginosus *infection in cancer tissues, dental plaque and saliva*

In order to assess the infection route of *S. anginosus*, a subject-based analysis was performed in the 42 patients with squamous cell carcinoma (Table 3). In all 19 cases of *S. anginosus*-positive squamous cell carcinoma, *S. anginosus* was solely detected in dental plaque, and not in the saliva samples. The other 23 patients with *S. anginosus*-negative cancer tissues were divided into three groups; 12 with *S. anginosus*-positive dental plaque and *S. anginosus*-negative saliva, five with *S. anginosus*-positive dental plaque and saliva, and six with *S. anginosus*-negative dental plaque and saliva. In addition, use of the ubiquitous primer set provided positive PCR results in all the dental plaque and saliva samples tested.

Genotype analysis of S. anginosus *isolates*

Streptococcus anginosus strains were isolated from both cancer tissue swabs and dental plaque samples in three of the 19 patients with *S. anginosus*-positive squamous cell carcinoma. A PFGE analysis of the isolates indicated that the genotype of the tissue isolate (pattern of genome DNA digested by *Sma*I) was identical to that of the bacterial isolate in dental plaque from each individual patient, although the patterns of each of the three patients were different (Figure 3).

Table 3 Presence of *S. anginosus* DNA in cancer tissues, dental plaque and saliva from the 42 subjects with squamous cell carcinoma

Cancer tissues	*Dental plaque*	*Saliva*	*S. anginosus*-positive cases
+	+	+	0
+	+	−	19
+	−	+	0
+	−	−	0
−	+	+	5
−	+	−	12
−	−	+	0
−	−	−	6

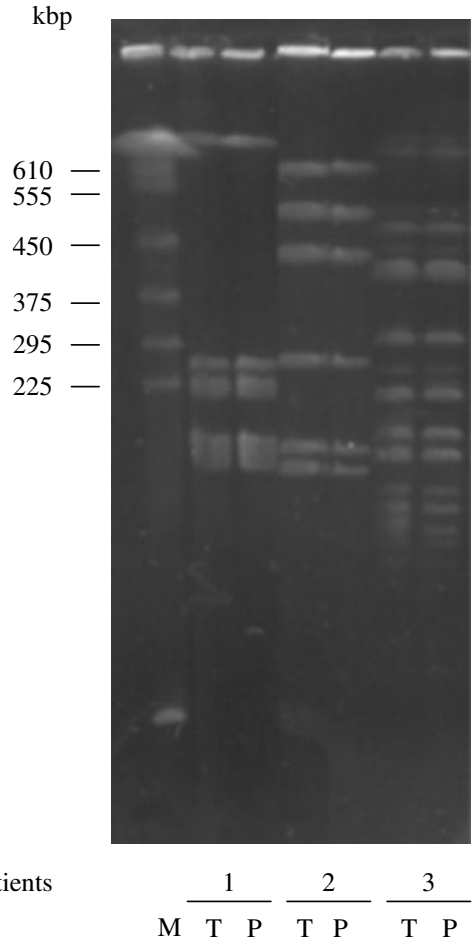

Figure 3 The PFGE analysis of the *S. anginosus* isolates from swab on the cancer tissues and dental plaque. *S. anginosus* strains were isolated from three patients with squamous cell carcinoma both from the swab on the cancer tissues and dental plaque. The PFGE was performed as described in 'Materials and Methods'. The content of each lane is as follows; M, yeast DNA-PFGE markers; T, *S. anginosus* isolates from cancer tissue swab samples from patient no. 1, 2 and 3; P, *S. anginosus* isolates from dental plaque samples from patient no. 1, 2 and 3

Discussion

It has been reported that *S. anginosus* infection is closely associated with esophageal, gastric, pharyngeal and oral cancers (Sasaki *et al*, 1998; Tateda *et al*, 2000; Shiga *et al*, 2001; Morita *et al*, 2003), however, the origin of *S. anginosus* as well as the infection route to cancer tissues has not been elucidated. In the present study, we assessed the frequency of *S. anginosus* infection in oral cancer tissues, dental plaque and saliva, and investigated the genotype of the *S. anginosus* clinical isolates using PFGE.

Streptococcus spp. comprise the majority of bacteria found in the oral cavity, though the predominant species are significantly different in the various sites, and dental plaque is one of the major habitats of *S. anginosus* (Hamada and Slade, 1980). Therefore, we first examined the detection level of *S. anginosus* in bacterial samples mixed with other oral streptococcal species. The results indicated that 5 pg of *S. anginosus* DNA was detectable in both single and mixed cultures with nine other oral

streptococcal species (Figure 1). In the present study, the tissue samples were treated with trypsin and washed extensively with ice-cold PBS by vortexing to remove adherent bacteria, and our preliminary study revealed the number of *S. anginosus* organisms in saliva was quite low ($<10^5$ CFU ml^{-1}), although it is possible that *S. anginosus* in saliva contaminated the tissue samples during the biopsy procedure. Thus, we assessed the carry-over of bacteria in murine tongue tissues soaked in an *S. anginosus* suspension, and found that flash soaking of the tissue specimens, even with a markedly higher density of *S. anginosus* (up to 10^6 CFU), did not affect the PCR results. As bacterial adherence occurs immediately, the present results strongly suggest that the carry-over of *S. anginosus* from saliva and/or loosely adhered *S. anginosus* to the cancer specimens were negligible (Figure 2b).

In the 49 oral cancerous and precancerous tissue specimens tested, most of the oral cancers (42/46) were squamous cell carcinoma (Table 1), which is in agreement with previous studies (Tateda *et al*, 2000; Iype *et al*, 2001; Shiga *et al*, 2001). *S. anginosus* DNA was exclusively and frequently detected in the squamous cell carcinoma specimens (19/42) (Table 1). Therefore, it is likely that *S. anginosus* infection is closely associated with oral cancers, especially squamous cell carcinoma. This finding is in accordance with the results of Tateda *et al* (2000) and Shiga *et al* (2001), who reported the frequency of *S. anginosus* infection in head and neck squamous carcinoma specimens (33% to 40%). Recently, Morita *et al* (2003) studied *S. anginosus* infection in both oral and esophageal cancer specimens, and reported that the frequency in oral cancer was lower than that in the esophageal cancer (13% *vs* 44%).

Our subject-based analysis of *S. anginosus* infection in 42 squamous cell carcinoma specimens revealed that *S. anginosus* was solely detected in dental plaque and not saliva in all 19 *S. anginosus*-positive squamous cell carcinoma cases, suggesting that *S. anginosus* infection was derived from the dental plaque of each individual (Table 3). Further, PFGE analysis showed that the genotype of *S. anginosus* isolated from the cancer tissue samples was identical to that from the dental plaque of the same patients, with distinguishable individual genotypes (Figure 3). These results strongly suggested that the *S. anginosus* infection in cancer tissues was derived from the dental plaque of each individual, implying that dental plaque is a dominant reservoir of the 'pathogenic' *S. anginosus*, at least in the present 19 subjects with *S. anginosus*-positive cancer tissues.

We previously reported that SAA, a novel bioactive antigen of *S. anginosus*, induces NO synthesis and COX-2 mRNA expression by murine PEC (Sasaki *et al*, 1995b; Sasaki *et al*, 2001). Therefore, it is possible that, in some cases of oral squamous cell carcinoma, *S. anginosus* in dental plaque could infect to oral mucosa and the infection may lead to increased rates of DNA damage by the over-synthesis of NO and/or COX-2, resulting in carcinogenesis of the infected tissues.

The present results with *S. anginosus*-specific PCR assay indicated that infection of *S. anginosus* occurs frequently in oral squamous cell carcinoma. Moreover, this is the first report to indicate the infection route of *S. anginosus* to oral cancer tissues. Although the development of the cancer is not simply ascribable to *S. anginosus* infection, the present findings may provide further understanding of the events in bacteria-related cartinogenesis, especially in those related to *S. anginosus*. However, further studies are obviously required to elucidate the precise role(s) of infection of *S. anginosus* in the etiologic processes of carcinogenesis and the involvement of other 'pathogenic' microbes in oral cancer, since *S. anginosus* was not detected in 23 of 42 tissue specimens from squamous cell carcinoma patients.

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