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Predominant presence of *Streptococcus anginosus* in the saliva of alcoholics

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Morita E, Narikiyo M, Yokoyama A, Yano A, Kamoi K, Yoshikawa E, Yamaguchi T, Igaki H, Tachimori Y, Kato H, Saito D, Hanada N, Sasaki H. Predominant presence of Streptococcus anginosus *in the saliva of alcoholics.*
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Chronic alcohol consumption is known to be a major risk factor for cancers of the upper aerodigestive tract. The incidence of esophageal cancer (4.4%) in alcoholics is reported to be much higher than that in the Japanese population as a whole (0.0001%). This suggests the presence of specific factors in chronic alcohol consumption-related carcinogenesis. Recently, data showing a significant correlation between *Streptococcus anginosus* and carcinogenesis in the upper aerodigestive tract have been reported. In this study, the ratio of *S. anginosus* to oral bacteria in the saliva of 38 alcoholic patients was investigated to determine if there is an association between alcoholic patients and *S. anginosus* infection. The level of *S. anginosus* in the saliva from 22 healthy people, 41 esophageal cancer patients, 32 gastritis patients, and 24 periodontitis patients was also investigated and compared to the level in alcoholic patients. In the saliva from esophageal cancer patients, the level of *S. anginosus* was not significantly different from that of healthy people. The levels of *S. anginosus* in periodontitis and gastritis patients were also similar. In alcoholics, however, there was an extremely high level of *S. anginosus*, suggesting that they, rather than healthy people and general esophageal cancer patients, have a high risk for *S. anginosus* infection.

Key words: alcoholis; esophageal cancer; saliva; *Streptococcus anginosus*

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Alcoholism is a serious social and medical problem. Epidemiologic data have identified chronic alcohol consumption as a major risk factor for cancers of the upper aerodigestive tract (12). For example, of 1000 Japanese alcoholics, a total of 53 patients (5.3%) had histologically confirmed cancer: 36 esophageal cancer, 17 gastric cancer, 9 oropharyngolaryngeal cancer, and 8 esophageal-cancer patients with gastric cancer and/or oropharyngolaryngeal cancer (22). The incidence of esophageal cancer (44 of 1000, or 4.4%) in alcoholics is much higher than that in the Japanese population as a whole (0.0001%).

Recently, data showing a significant correlation between *Streptococcus anginosus* and carcinogenesis in the upper aerodigestive tract have accumulated (8, 11, 13–15, 17). *S. anginosus* is one of the oral streptococci isolated from the oral cavity, gastrointestinal tract and other parts of the body (18). It has been associated with various pyogenic infections including cardiac, abdominal, skin and central nervous system infections. *S. anginosus* DNA sequences were frequently found in DNA samples from esophageal cancer tissues, whereas they were much less frequently found in DNA samples from colon, lung, bladder, renal, cervical, and oral cancer tissues (14). In addition, it was reported that *S. anginosus* is invasive, and cytokine induction by *S. anginosus* infection to esophageal cancer cell lines has been confirmed (11). Therefore, *S. anginosus* is considered to be associated with esophageal cancer development, although the cause and effect relationship between *S. anginosus* and esophageal cancer is still ambiguous.

In the present study, to examine whether alcoholics have a higher risk for *S. anginosus* infection, the ratio of *S. anginosus* to oral bacteria was investigated in the saliva of 38 alcoholic patients. The levels of *S. anginosus* in the saliva of 22 healthy people, 41 esophageal cancer patients, 32 gastritis patients, and 24 periodontitis patients were compared with that for alcoholics, and the level in esophageal cancer tissues was quantified as a positive control.

Material and methods

DNA samples

Paraffin-stimulated saliva samples were collected for 3 min from 38 alcoholics aged 42–68 years (mean 56.3 years) at the National Hospital Organization Kurihama Alcoholism Center (Yokosuka, Japan), from 41 esophageal cancer patients aged 46–69 years (mean 59.8 years) at the National Cancer Center Hospital (Tokyo, Japan), from 32 gastritis patients aged 41–68 years (mean 58.2 years) at the National Cancer Center Hospital, from 24 periodontitis patients aged 44–69 years (mean 55.5 years) at the Nippon Dental University Hospital (Tokyo, Japan), and from 22 healthy volunteers aged 41–66 years (mean 56.1 years). All of the alcoholics met the DMS-III-R criteria for alcohol dependence (1). Saliva of alcoholics was collected the first day they were admitted to the Center's detoxification unit. The study with cancer and alcoholic patients was approved by our institutional review boards. Written informed consent was obtained from all the patients and healthy volunteers. The 41 esophageal cancer patients, 32 gastritis patients and 24 periodontitis patients showed no signs of alcoholism and no esophageal cancer. The samples were centrifuged, frozen and stored at − 80°C until use. Fifteen esophageal carcinoma tissues were obtained from patients at the National Cancer Center Hospital. The surgical specimens were stained with Lugol's solution, washed with phosphate-buffered saline (PBS) several times to remove surface-adherent bacteria, then frozen and stored at − 80°C until use. Genomic DNA was isolated from the saliva and tissue samples by a standard phenol-chloroform method as described previously (8).

Real-time polymerase chain reaction (PCR)

Quantification of *S. anginosus*, *Streptococcus constellatus*, and *Streptococcus intermedius* was performed by real-time PCR using SYBR green chemistry as described previously (9). The sequences of the primers are 5′-CTAATACATGCAAGTAGG-3′ and 5′-CAAGCATCTAACATGTGTTAC-3′ for *S. anginosus*. For *S. constellatus* and *S. intermedius*, the consensus sequences 5′-CACCGTAGTTTACTACACCGTATT-3′ and 5′-CTACCATGCAGTAAATGTTC-3′ were used as primers to quantify DNA of two species simultaneously. For comparison with many other oral bacteria, real-time PCR with primers 5′-GAACGGGGTGAGTAACGCGTAGGT-3′ and 5′-CACTCACGCGGCGTTGCTCGGTC-3′ was performed. Ten ng of genomic DNA was used as a template for each quantification experiment. Differences in the quantity of the *S. anginosus* levels in saliva and tumor samples were analyzed statistically using the Mann–Whitney *U*-test.

Results

Since a high frequency of *S. anginosus* in esophageal cancer tissues has been reported, it was expected that *S. anginosus* would be predominantly present in the saliva from the esophageal cancer patients, and quantification of *S. anginosus* DNA and oral bacterial DNA by our previously established real-time PCR method (9) did indeed demonstrate a very high level of *S. anginosus* in the esophageal cancer tissues (Table 1). However, the levels in saliva from esophageal cancer patients was only slightly higher than those from healthy people (Table 1, Fig. 1). The average ratios of *S. anginosus* to oral bacteria in the saliva from esophageal cancer patients and from healthy people were 1.6×10^{-2} (1.6%) and 1.1×10^{-2} (1.1%), respectively. The maximum value, 6.4×10^{-2} (6.4%), of the *S. anginosus* level in the saliva from esophageal cancer patients was slightly higher than that from healthy people; 5.5×10^{-2} (5.5%), but this difference was not significant ($P < 0.01$). Both the average levels and the maximum levels of *S. anginosus* in the saliva from periodontitis patients and gastritis patients were lower than or comparable to those from healthy people (Table 1, Fig. 1). Accordingly, the level of *S. anginosus* showed no increment in the saliva from periodontitis patients, gastritis patients or esophageal cancer patients compared with healthy people.

Compared to the healthy people, periodontitis patients, gastritis patients, and esophageal cancer patients, the *S. anginosus* level in the saliva of alcoholics was much higher: 5 times on average (Table 1). Nine of 38 alcoholics (38%) exhibited an *S. anginosus* level more than 5×10^{-2}

Table 1. The average ratio of *S. anginosus* and *S. constellatus* (*S. intermedius*) to *Streptococcus* in saliva and esophageal cancer tissues

	Ratio to *Streptococcus*	
Source	*S. anginosus*	*S. constellatus* (*S. intermedius*)
Saliva of healthy people	$1.1 \times 10^{-2} \pm 1.6 \times 10^{-2}$	$1.0 \times 10^{-3} \pm 2.4 \times 10^{-3}$
Saliva of periodontitis patients	$0.6 \times 10^{-2} \pm 1.0 \times 10^{-2}$	$1.1 \times 10^{-3} \pm 1.2 \times 10^{-3}$
Saliva of gastritis patients	$0.7 \times 10^{-2} \pm 0.1 \times 10^{-2}$	$1.7 \times 10^{-3} \pm 2.8 \times 10^{-3}$
Saliva of esophageal cancer patients	$1.6 \times 10^{-2} \pm 1.8 \times 10^{-2}$	$1.2 \times 10^{-3} \pm 3.0 \times 10^{-3}$
Saliva of alcoholics	$5.9 \times 10^{-2} \pm 2.0 \times 10^{-2}$	$2.1 \times 10^{-3} \pm 3.9 \times 10^{-3}$
Esophageal cancer tissues	$3.0 \times 10^{-2} \pm 7.0 \times 10^{-2}$	$2.7 \times 10^{-3} \pm 4.7 \times 10^{-3}$

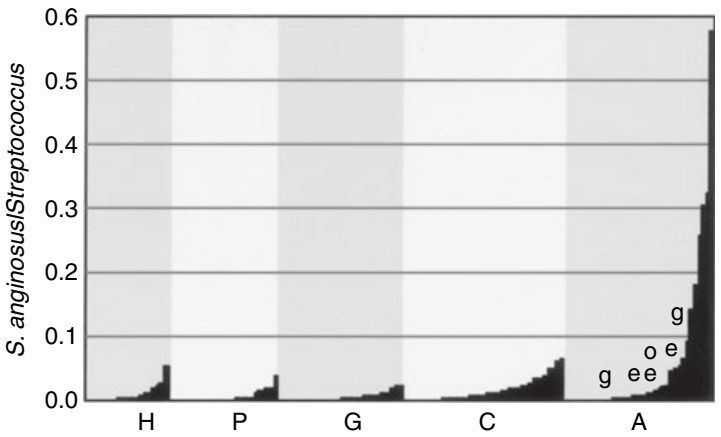

Fig. 1. The ratios of *S. anginosus* to total *Streptococci* in saliva from healthy people, periodontitis patients, gastritis patients, esophageal cancer patients and alcoholics. Obtained values are expressed in a bar graph arranged from low to high in each group. H, healthy people. P, periodontitis patients. G, gastritis patients. C, esophageal cancer patients. A, alcoholics. e above a bar, an alcoholic patient with esophageal cancer; g above a bar, an alcoholic patient with gastric cancer; o above a bar, an alcoholic patient with oropharyngeal cancer.

(> 5%) (Table 1, Fig. 1). The maximum ratio of *S. anginosus* in the alcoholic patients' saliva samples was 58×10^{-2} (58%), which is a level comparable to that in esophageal cancer tissues (Table 1, Fig. 1). On the other hand, the average levels of *S. constellatus* (*S. intermedius*) in the saliva from the healthy people, periodontitis patients, gastritis patients, esophageal cancer patients, and alcoholics were not very different, although the alcoholics exhibited the highest value. In addition, none of the alcoholics exhibited an extremely high level of *S. constellatus* (*S. intermedius*) in the saliva (data not shown).

In the alcoholic patients, the *S. anginosus* levels decreased rather than increased with age (13% at 40–49 years, 5% at 50–59 years, and 2.8% at 60–69 -years)(Table 2). Therefore, it was concluded that *S. anginosus* increased specifically in the saliva of alcoholic patients regardless of age.

Discussion

It is generally accepted that the upper aerodigestive tract is a region in which multiple primary cancers occur at a high rate. Squamous cell carcinoma of the oral cavity is often accompanied by other squamous cell carcinomas of the digestive tract, such as oropharyngeal cancers or esophageal cancers (2, 3). The high incidence of multiple carcinomas in this region is often explained by the concept of field cancerization (16), which is based on the hypothesis that exposure to carcinogenic agents leads to independent carcinogenesis in epithelial cells at different sites in this region. Although little is known about this hypothetical etiology, many epidemiologic studies have indicated some possible etiologic factors, such as alcohol use (4, 6). Multiple mechanisms are thought to be involved in alcohol-associated cancer development. Recently, evidence that acetaldehyde is predominantly responsible for alcohol-related carcinogenesis has accumulated. Acetaldehyde is the first metabolite from ethanol by alcohol dehydrogenase produced by mucosal and cellular alcohol dehydrogenase, and is also formed in a high concentration in saliva by oral bacteria (5, 10). Aldehyde dehydrogenase-2 (ALDH2) is a key enzyme for the elimination of acetaldehyde, an established animal carcinogen generated by alcohol metabolism. In the presence of ALDH2*****2, a mutant allele that is prevalent in East Asia but rare in Western countries, this enzyme is inactive, leading to excessive accumulation of acetaldehyde. A positive association between this inactive form of ALDH2 and multiple-field cancerization in the upper aerodigestive tract has been demonstrated among Japanese alcoholic patients (20, 21, 23).

In the present study, the level of *S. anginosus* in the saliva of alcoholics was higher than that in periodontitis patients, gastritis patients, esophageal cancer patients and healthy people. On the other hand, the level of *S. constellatus* (*S. intermedius*) in the saliva was not as different in each group. These results suggest a specific increase of *S. anginosus* in the saliva of alcoholics. Although the relationship between *S. anginosus* and esophageal cancer development is not clearly elucidated, *S. anginosus* is considered to be associated with esophageal cancer. Therefore, our present results suggest that *S. anginosus* could be associated with chronic alcohol consumption-related carcinogenesis in the upper aerodigestive tract. Moreover, the frequent presence of *S. anginosus* in esophageal cancer is observed not only in Japanese people but also in Caucasians in the United States, France, and Italy (11), suggesting that the association occurs not only in Japan. *S. anginosus* is not regarded as an aldehyde-producing bacterium. Another possible explanation for alcohol-associated cancer development is that specific bacterial infection stimulates the normal epithelium to initiate inflammation and/or promotes carcinogenesis. *S. anginosus* could be the cause.

Five (13.2%) of the 38 alcoholic patients investigated in this study had cancers in the upper aerodigestive tract, three (7.9%) patients had esophageal cancer, two (5.3%) had gastric cancer, and one (2.6%), who had esophageal cancer, also had oropharyngeal cancer. However, alcoholic patients with the highest value of *S. anginosus* in the saliva did not always have esophageal cancer (Fig. 1). To elucidate this discrepancy, it is noted that esophageal endoscopy with iodine staining always showed the frequent presence of dysplasia (20–30%) in the alcoholics (unpublished observation). Therefore, a follow-up study, which is often prevented by the alcoholics' attitude, is very important to correctly address the association between the high level of *S. anginosus* and esophageal cancer in alcoholics.

We recently reported *S. anginosus* levels in oral bacteria in the saliva from 65 healthy people aged 25–70 years (9). In this report, the average ratio of *S. anginosus* increased with age (0.38% at 25–49 years, 1.12% at 50–69 years, and 2.02% at 70 years). Moreover, 25% of the healthy people 50–69 years of age exhibited more than 2% *S. anginosus* in oral bacteria, whereas only 1.7% of the healthy people aged 25–49 years did so. An age-dependent increment of the *S. anginosus* level in the saliva from healthy people was also observed in Bangladesh (unpublished observation). Loss of an age-related increment in the saliva *S. anginosus* levels of alcoholics suggests that an oral immune response of younger alcoholics seems to mimic that of elderly people. This may be caused by a quite unbalanced intake of food; for example, a lower intake of green and yellow vegetables and fruit, which is one of the risks for esophageal cancer (19). Moreover, in general, alcoholics are known to often neglect the care of the oral cavity, which results in periodontitis. In this regard, it is noted that one study reported an increased *S. anginosus* level in the saliva of severe periodontitis patients (7).

To our knowledge, no microbial analysis of the saliva in alcoholics has yet been done. This study contains significant results promoting a comprehensive study in a large number of alcoholics. A large-scale epidemiologic study should be the next challenge.

Table 2. The average ratio of *S. anginosus* and *S. constellatus* (*S. intermedius*) to *Streptococcus* in saliva of alcoholics in different age groups

Age group	Average age	Ratio to *Streptococcus*	
		S. anginosus	*S. intermedius*
40–49	45.8	$13.0 \times 10^{-2} \pm 19.0 \times 10^{-2}$	$3.2 \times 10^{-3} \pm 5.2 \times 10^{-3}$
50–59	55.2	$5.0 \times 10^{-2} \pm 8.4 \times 10^{-2}$	$1.7 \times 10^{-3} \pm 2.8 \times 10^{-3}$
60–69	62.8	$2.8 \times 10^{-2} \pm 5.2 \times 10^{-2}$	$1.8 \times 10^{-3} \pm 3.5 \times 10^{-3}$

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