# Frequent and preferential infection of *Treponema denticola*, *Streptococcus mitis*, and *Streptococcus anginosus* in esophageal cancers

**Michihiro Narikiyo,[1,3] Chikako Tanabe,[1] Yukishige Yamada,[3] Hiroyasu Igaki,[2] Yuji Tachimori,[2] Hoichi Kato,[2] Manabu Muto,[1] Ruggero Montesano,[4] Hiromi Sakamoto,[1] Yoshiyuki Nakajima[3] and Hiroki Sasaki[1,5]**

[1]Genetics Division, National Cancer Center Research Institute and [2]Department of Surgery, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045; [3]Department of Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522; and [4]International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon, France

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**Multiple cancers frequently occur in the upper digestive tract. One possible explanation is that specific bacterial infection stimulates the normal epithelium to initiate inflammation and/or promotes carcinogenesis. This study was undertaken to determine which bacterial species is predominantly associated with esophageal cancer. We examined the bacterial diversity in this type of cancer and in the saliva from healthy people by using a culture-independent molecular method. Here we report the preferential and frequent infection of the oral periodontopathic spirochete *Treponema denticola* (*T. denticola*), *Streptococcus mitis* (*S. mitis*), and *Streptococus anginosus* (*S. anginosus*) in esophageal cancer from different regions of the world, and we also describe the induction of inflammatory cytokines by infection of *S. anginosus* and *S. mitis*. Our present data suggest that these three bacteria could have significant roles in the carcinogenic process of many cases of esophageal cancer by causing inflammation and by promoting the carcinogenic process, and that eradication of these three bacteria may decrease the risk of recurrence. (Cancer Sci 2004; 95: 569–574)**

Bacterial and viral infections are important factors in cancer development. *Helicobacter pylori* (*H. pylori*) is reportedly associated with gastritis, gastric atrophy, and gastric cancer,[1–3] and the presence of microorganisms has recently been investigated in several kinds of human cancers, while *Streptococcus anginosus* (*S. anginosus*) DNA fragments were frequently found in DNA samples from esophageal cancer tissues, gastric cancer tissues, and dysplasia of the esophagus,[4,5] suggesting that *S. anginosus* infection occurs at an early stage of esophageal cancer and is related to esophageal and gastric carcinogenesis. *S. anginosus* is classified as an oral bacterium and can be isolated from several parts of the body, such as the oral cavity, gastrointestinal tract, and genitourinary tract. It is often associated with pyogenic infections, including endocarditis.[6–8] *S. anginosus* DNA has also been found in head and neck squamous cell carcinomas,[9] but much less frequently in non-cancerous tissues of the esophagus, and is not present in colon, lung, bladder, renal, and cervical cancer tissues.[5] This suggests that *S. anginosus* DNA is associated with cancers in the upper digestive tract, although the involvement of *S. anginosus* infection in the carcinogenic process has not been clarified.

It is generally accepted that the upper digestive tract is a region in which multiple primary cancers occur at a high rate. Squamous cell carcinoma of the oral cavity is often accompanied with other squamous cell carcinomas of the digestive tract, such as oropharyngeal cancers or esophageal cancers.[10,11] The high incidence of multiple carcinomas in this region is often explained by the concept of field cancerization, 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 epidemiological studies have indicated some possible etiological factors, such as alcohol use.[12,13] One possible explanation is that specific bacterial infection stimulates the normal epithelium to initiate inflammation and/or promotes carcinogenesis. We investigated the diversity of bacteria, including *Streptococcus*, in esophageal cancer by sequence analysis of many PCR products obtained by using universal primers, which can amplify a portion of the 16S rRNA gene of all streptococcal species reported. Here we report the preferential infection of *Treponema denticola* (*T. denticola*), *Streptococcus mitis* (*S. mitis*), *and S. anginosus* in esophageal cancers from different regions of the world, and the induction of inflammatory cytokines by infection of *S. mitis* and *S. anginosus*.

## Materials and Methods

**Tissues and saliva.** Esophageal carcinoma tissues, the corresponding normal tissues, and saliva were obtained from patients at the National Cancer Center Hospital (Tokyo). All of the surgical specimens were stained with Lugol's solution followed by several washings with PBS to remove surface adherent bacteria, then frozen immediately in liquid nitrogen and stored at −80°C until use. Written informed consent was obtained from all the patients.

**PCR amplification of various bacterial 16S ribosomal DNAs (rDNAs).** For amplification of a portion of the 16S rRNA gene of many oral bacteria from tumor DNA samples, PCR was performed in a total of 50 μl of a PCR mixture containing 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 3 m*M* $MgCl_2$, 0.001% gelatin, 100 μ*M* of each primer, 1 m*M* (each) dATP, dCTP, dGTP, and dTTP, and 5 units of *ExTaq* DNA polymerase. The cycling conditions were 35 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. The last cycle had an additional extension at 72°C for 10 min. The sequences of the primers having cloning sites for restriction enzymes, *Xba*I and *Eco*RI, are: 5′-GCTCTAGAGAACGGGTGAGTAACGCGTAGGT-3′ for Ust1X, and 5′-GGAATTCCACTCACGCGGCGTTGCTCGGTC-3′ for Ust2E. A portion of the 16S rRNA from nucleotide positions 1 to 312 in *S anginosus* 16S rRNA sequence can be amplified by these primers.

**Cloning and sequence analysis of the PCR products.** The PCR products obtained were digested with *Xba*I and *Eco*RI, and inserted into a pBluscript II vector (Stratagene). The more than 200 recombinant DNAs, which were amplified with PCR using universal primers, were inserted into the vector. More than 10 clones for each specimen were recovered and sequenced with an Applied Biosystems 310 DNA sequencer, and the sequences were compared with DNA databases. A species was determined

[5]To whom correspondence should be addressed.
E-mail: hksasaki@gan2.res.ncc.go.jp

when its sequence showed greater than 95% homology to the bacterial sequence.

**PCR using primers specific to *S. anginosus* or *T. denticola* 16S rDNA.** Specific primers for amplification of 16S rDNA of *S. anginosus* were previously designed.[6] Sequences of the primers were as follows: forward primer, St1 (5′-GAACGGGTGAGTAACGCGTAGGTA-3′), and reverse primer, SAS4 (5′-CGTAGCTTGCTACACCATAGA-3′). PCR for amplification of 16S rDNA of *S. anginosus* was performed using Takara *ExTaq* (TaKaRa Corp., Shiga, Japan) in a total volume of 50 µl containing 100 µ*M* of each primer and 50 ng of template DNA. The thermal cycling conditions were 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 68°C for 1 min. The last cycle had an additional extension at 68°C for 10 min. For *T. denticola* detection, sequences of primers were as follows: forward primer, 5′-TAATACCGAATGTGCTCATTTACAT-3′, and reverse primer, 5′-CAAAGAAGCATTCCCTCTCTTCTT-3′. PCR was performed using TaKaRa *ExTaq* (TaKaRa Corp.) in a total volume of 50 µl containing 100 µ*M* of each primer and 50 ng of template DNA. The thermal cycling conditions were 36 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The last cycle had an additional extension at 72°C for 10 min.

**Northern blot and slot blot analyses.** Total RNA was prepared from esophageal cancer cell line TE6 and esophageal cancer tissues using an ISOGEN kit (Nippon Gene, Toyama, Japan) according to the procedure recommended by the supplier. Ten micrograms of total RNA was electrophoresed on 1% agarose/formaldehyde gel and transferred to a NitroPlus membrane (Micron Separations, Inc., Westboro, MA). Hybridization was carried out in 50% formamide, 5× (five times concentrated) standard saline citrate (SSC) (1× SSC: 150 m*M* NaCl, 15 m*M* sodium citrate), 5× (five times concentrated) Denhardt's reagent, 5 m*M* EDTA, 0.1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 100 µg/ml denatured salmon sperm DNA at 42°C for 14–16 h. For slot blot analysis, 1 µg of total RNA was transferred to a membrane filter by using a filtration manifold. The filter was hybridized with radiolabeled probes under the same conditions as described above. The cDNA fragments for the probes were prepared by RT-PCR. All probes were labeled with [α-$^{32}$P]dCTP. The filter was washed twice in 0.1× SSC and 0.1% SDS at room temperature and twice at 65°C, and exposed to Kodak XAR film at −70°C.

## Results

**The diversity of the bacterial flora in esophageal cancer tissues.** We previously reported that *S. anginosus* DNA was found in most cases of esophageal cancer and in some cases of gastric cancer.[5] However, the possible presence of bacteria other than *S. anginosus* in these two types of cancers remains unknown, and the previous studies contained no data on cancers from different regions of the world. It is well known that the 16S rRNA sequence of various organisms can be found in public nucleotide databases, and variation among 16S rRNA sequences is useful for classifying bacterial species. More than 30 *Streptococcus* species have been well classified on the basis of sequence variations.[14, 15] The bacterial 16S rRNA gene consists of three regions: conserved, variable, and hypervariable. It has been previously estimated with culture-based techniques and with a culture-independent molecular method that about 500 species of bacteria inhabit the human oral cavity.[15] We designed two primers that would amplify the sequences from the majority of oral bacteria, including all *Streptococcus* species, from the two conserved regions, which are located at both sides of the hypervariable region. However, these two primers Ust1X and Ust2E can not amplify the 16S rRNA gene sequence of *Neisseria* species that may potentially be involved in alcohol-related esophageal carcinogenesis.[16] The sequences of cloned 16S rDNA inserts can be used to determine species identity or closest relatives by comparison with sequences of known species, except *Neisseria*, thereby allowing us to investigate the diversity of the bacterial flora in esophageal cancer tissue by utilizing the sequence data of many PCR products with these primers.

By 35 cycles of PCR using the primers Ust1X and Ust2E, a 310 bp rDNA fragment was amplified in 20 esophageal cancer tissues and the corresponding non-cancerous tissues. One hundred clones obtained from 20 esophageal cancer tissues, the corresponding normal tissues and the saliva mix from 20 healthy volunteers were sequenced, and we determined species identity or closest relatives with greater than 95% homology by comparison with the sequences of known species. The diversity of the bacterial flora in esophageal cancers and in the saliva from the healthy people, whose ages ranged from 45 to 69 years, with a mean of 57.8 years—which is almost the same as that of the cancer patients examined—is shown in Fig. 1. In the saliva from the healthy people, 43% of the clones were *S. mitis*, 13% of the clones were *S.* sanguinis, 10% were *S. parasanguis*, 7% were *S. infantis*, *S. australis*, and *S. constellatus*, 3% were *S. cristatus*, and 10% were unknown *Streptococcus* species. The diversity of the bacterial flora in the saliva from esophageal cancer patients was comparable to that from the above healthy people, but both *S. anginosus* DNA and *T. denticola* DNA were detectable by PCR using specific primers (data not shown). These results suggest that both bacteria are quite minor in the saliva from healthy people and or from esophageal cancer patients. In esophageal cancer tissues, 45% of the clones were *T. denticola*, 25% of the clones were *S. mitis*, 12% were *S.*

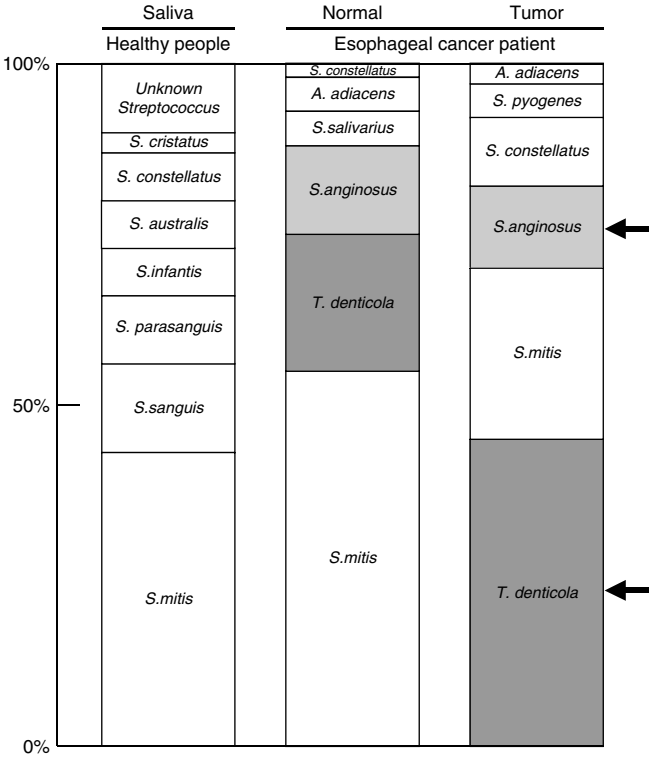


**Fig. 1.** Examination of the bacterial diversity in esophageal cancers and in the saliva from healthy people by using a culture-independent molecular method. The diversity of the bacterial flora in the esophageal cancer tissues (Tumor), non-cancerous portions (Normal), and the saliva from healthy people was examined by sequencing analysis of clones of the PCR products with the primers Ust1X and Ust2E.

*anginosus*, 10% were *S. constellatus*, 5% were *S. pyogenes*, and 3% were *Abiotrophia adiacens*. In the corresponding normal tissues, 55% of the clones were *S. mitis*; 20% of the clones were *T. denticola*, 13% were *S. anginosus*, 5% were *S. salivarius and Abiotrophia adiacens*, and 2% were *S. constellatus*. These results indicate that *T. denticola, S. mitis*, and *S. anginosus* preferentially infected the normal mucosa of the esophagus as well as esophageal cancer tissues.

**PCR with *S. anginosus*- and *T. denticola*-specific primers in esophageal cancer tissues from other countries.** We successfully designed specific PCR primers for detecting *S. anginosus* and *T. denticola* 16S rRNA genes, but were unable to design a specific primer for *S. mitis* 16S rRNA gene detection, and, therefore, we performed PCR using specific primers for detecting *S. anginosus* and *T. denticola* with 58 independent esophageal cancer tissues from Japan, 4 from China, 2 from France, and 5 from Italy. In this study, 40 (69%) out of 58 Japanese esophageal cancer patients showed positive for *S. anginosus* (Fig. 2A). *T. denticola* was detected in 22 (38%) out of 58 Japanese patients (Fig. 2A). In the other countries (Fig. 2B), *S. anginosus* was detected in 10 (91%) out of 11 (3 of 4 from China, all 7 from France and Italy), and *T. denticola* was detected in 5 (46%) out of 11 (1 of 4 from China, 2 of 2 from France, and 2 of 5 from Italy). These results demonstrate that the frequent presence of *S. anginosus* and *T. denticola* in esophageal cancer is not unique to Japan.

The PCR product of total *Streptococci* including *S. mitis*, which is amplified with Ust1X and Ust2E primers was detected in 53 (91%) out of 58 Japanese esophageal cancer patients and in 7 (63%) out of 11 samples of the other countries. Among 18

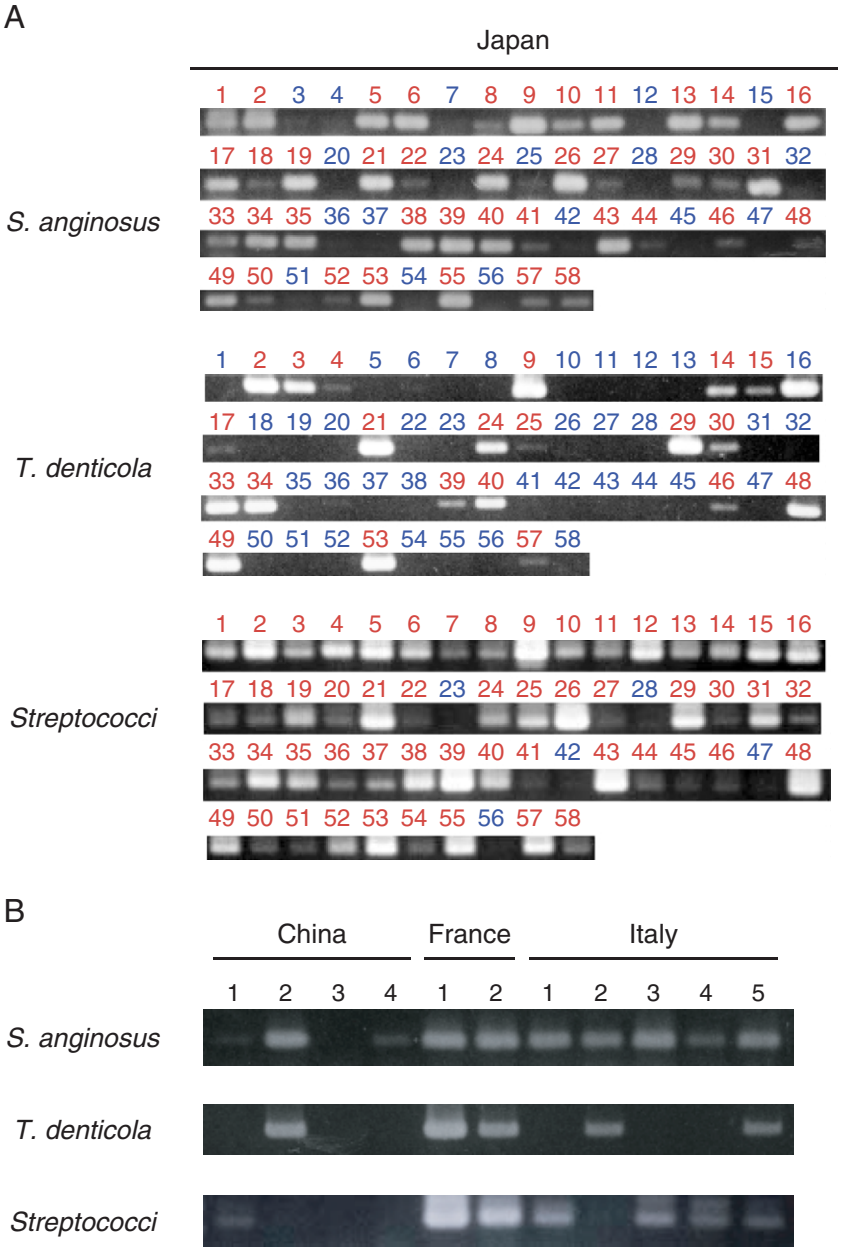


**Fig. 2.** PCR with *S. anginosus*- and *T. denticola*-specific primers in esophageal cancer tissues. Detection of *S. anginosus* DNA and *T. denticola* DNA in 58 independent esophageal cancers from Japan (A) and from China, France, and Italy (B). PCR with Ust1X and Ust2E for detecting total *Streptococci* including *S. mitis* was also performed as a control experiment. Cases positive for *S. anginosus* or *T. denticola* are shown in red; case negative or minimally positive for *S. anginosus* or *T. denticola* are shown in blue.

*S. anginosus*-negative Japanese cases, 13 (72%) were positive for total *Streptococci*. Among 36 *T. denticola*-negative Japanese cases, 31 (82%) were positive for total *Streptococci*. Among 5 *Streptococci*-negative Japanese cases, no case was positive for both *S. anginosus* and *T. denticola*. These data indicate that *S. mitis* infection is most frequent in esophageal cancer.

**Induction of inflammatory cytokines by infection of *S. anginosus* and S. *mitis* in esophageal cancer.** Inflammatory cytokines, including IL-6 and IL-8, and CD54 are induced by *H. pylori* infection of tumor cells or normal epithelial cells,[17, 18] suggesting that this bacterial infection stimulates the normal epithelium to initiate inflammation and neutrophil recruitment and activation. To investigate cytokine production from esophageal epithelial cells after infection of *Streptococci* or *T. denticola*, we tried to isolate these two bacteria from esophageal cancer tissues. Many strains of *S. anginosus* were established from the esophageal cancer tissues of 95 independent patients, while *T. denticola* was difficult to isolate from these samples without special culture systems because it is an anaerobic bacterium. All of the 10 *S. anginosus* strains examined were able to adhere to all of the 3 esophageal epithelial cell lines used (data not shown). A representative result of adhesion of an isolated *S. anginosus* strain to an esophageal epithelial cell line (TE 6) after 2 h of incubation is shown in Fig. 3A. Uninfected or *S. anginosus*-infected TE 6 cells were harvested at 6 h post-infection and then assessed for mRNA expression of two CXC-chemokine genes, *IL-8* and *GRO*$\alpha$. Northern blot data showed induced mRNA expression of both genes after infection with our isolated *S. anginosus* strain, as well as a standard strain (ATCC8787) (Fig. 3B). Importantly, *S. mitis* and *S. salivarius*, which are abundant bacteria in the saliva, also induced *IL-8* and *GRO*$\alpha$ expressions.

Gram's staining and Giemsa staining of esophageal cancer tissue sections showed invasion of the gram-positive streptococci including *Streptococci* (Fig. 4A). *Streptococci* frequently show intracellular localization in host/target cells. However, Gram's staining indicated that *Streptococci* seem to be located in the intercellular space. *Streptococci* were visualized on the cell surface by Geimsa staining, although this staining alone can not show intracellular location of *Streptococci* in cancer cells. In esophageal cancer tissues, 4 CXC chemokine genes (*IL-8, GRO*$\alpha$*, GRO*$\gamma$, and *IP-10*) as well as 2 CC chemokine genes (*MIP1*$\beta$ and *RANTES*) were expressed more highly than in the non-cancerous tissues (Fig. 4B). The mRNA levels of a C chemokine gene, *LPTN*, were found to be similar in cancer tissues and normal tissues (Fig. 4B). These results are consistent with a higher content of *Streptococci* in the cancer tissues compared with the normal tissues, and also suggest that recruitment and activation of both neutrophils and monocytes could be stimulated during cancer progression by the above 4 CXC chemokines and 2 CC chemokines.

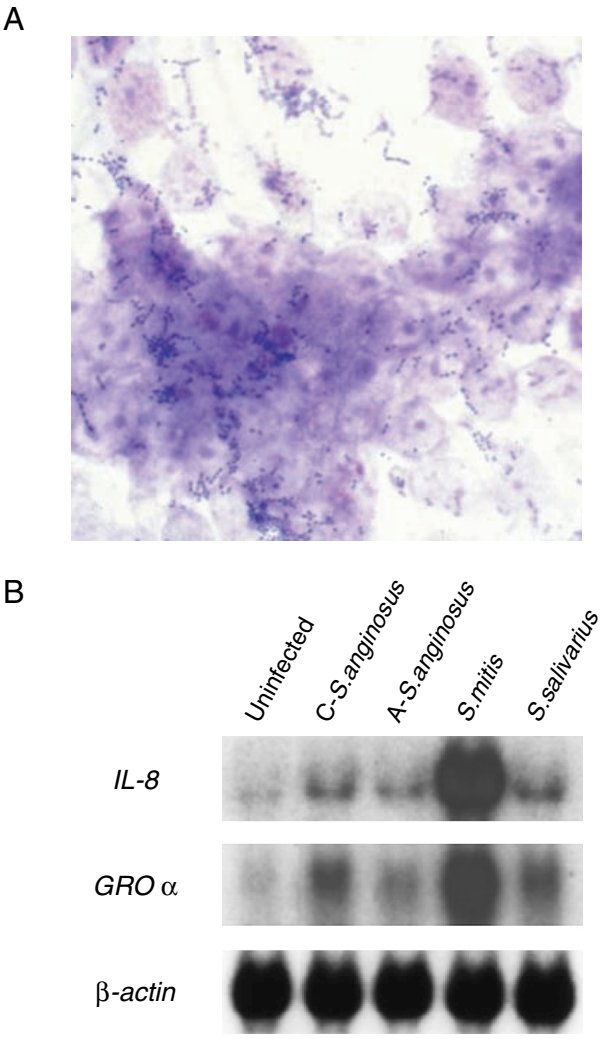


**Fig. 3.** Induction of *IL-8* and *GRO*$\alpha$ mRNAs in esophageal cancer cells after *S. anginosus* infection *in vitro*. (A) *S. anginosus* adhered to TE 6 esophageal cancer cells after incubation for 2 h *in vitro*. (B) Northern blot data showing induction of *IL-8* and *GRO*$\alpha$ mRNAs after infection of three *Streptococcus species*. A-*S. anginosus*, a standard strain (ATCC8787); C-*S. anginosus*, our isolated-*S. anginosus* strain from cancer tissue; *S. mitis* (our isolated strain), and *S. salivarius* (our isolated strain).

## Discussion

Epithelial tissues in the oral cavity and the esophagus are exposed to similar kinds of oral bacteria through the saliva. Therefore, we expected that each bacterium would be present to the same degree in the saliva as in esophageal cancer tissues. In accordance with this expectation, the most abundant species (*S. mitis*) in the saliva was also found in esophageal cancer. However, comparison of the bacterial diversities in the saliva from the healthy volunteers, cancer tissues, and normal tissues (Fig. 1) showed that *T. denticola* and *S. anginosus*, which are minor bacteria in the saliva, preferentially infect the normal mucosa of the esophagus, as well as to esophageal cancer tissues. *Streptococcus* is known to be an invasive bacterium, which targets host fibronectin in its adhesion to and invasion of host cells[19] and invades by CD44-mediated cell signaling.[20] In accordance with this character, *Streptococci* were found to be located in the middle or deep parts of tumors rather than in the surface or ulcerated portions of the cancer tissues (ref. 21 and Fig. 4A). Although the amounts of total *Streptococci* DNA in non-cancerous tissue were less than those of the cancer tissue when Ust1X and Ust2E primers were used (ref. 22 and data not shown), cytokine induction by *Streptococci* infection (Figs. 3 and 4) might stimulate not only the differentiated surface epithelium, but also the dividing cells (basal and suprabasal cells) in the esophagus to initiate inflammation, and might result in their transition to dysplasia, thereby promoting carcinogenesis. In a rat model experimental system, administration of either *Streptococcus bovis* or of antigens from this bacterium promotes the progression of preneoplastic lesions through the increased formation of hyperproliferative aberrant colonic crypts and increased IL-8 production in the colonic mucosa.[23] This report suggests that *Streptococci* could be involved in carcinogenesis of the digestive tract.

*T. denticola* is an oral spirochete, which is thought to induce matrix destruction in the gingiva and periodontitis through its

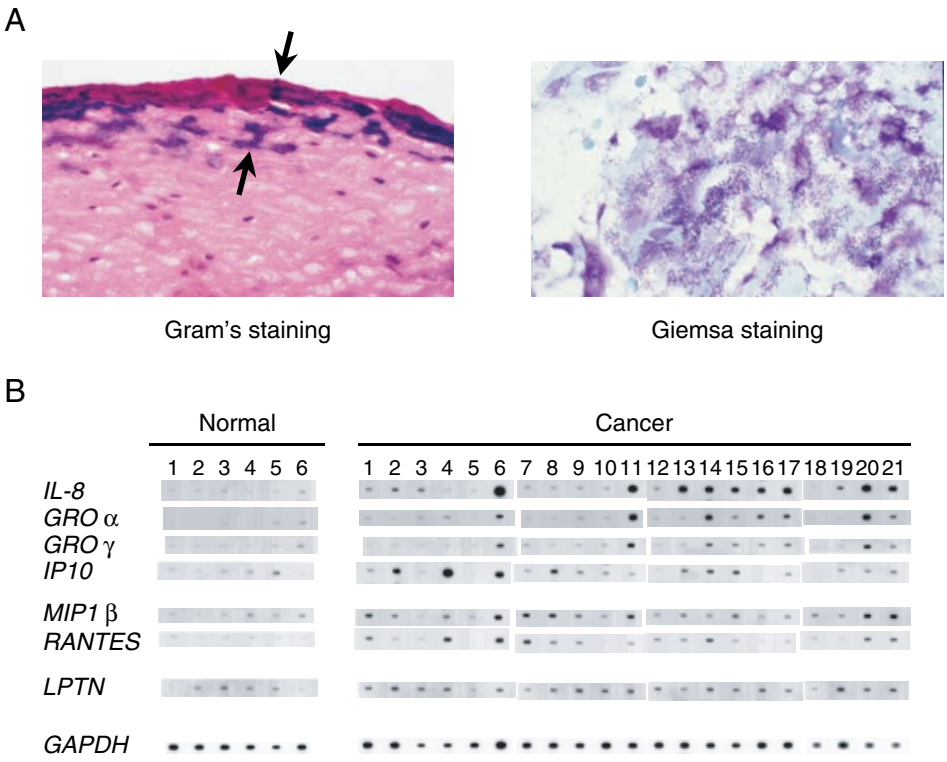


**Fig. 4.** Expression of inflammatory cytokines in esophageal cancer. (A) Gram's staining and Giemsa staining of esophageal cancer tissue sections showed invasion of gram-positive *Streptococci* including *S. anginosus*. Arrows, gram-positive *Streptococci*. (B) Slot blot data showing that *IL-8, GROα, GROγ, IP-10, MIP1β,* and *RANTES* were expressed more highly in cancer tissues than in non-cancerous tissues. *GAPDH* was used as a control.

bacterial proteases,[24] and induces matrix metalloproteinases[25] as well as IL-8[26] in the host cells. β-Defensins (HBDs) are broad-spectrum antimicrobial peptides expressed at epithelial surfaces.[27] HBD-2 has been reported to be expressed in the lung,[28] while HBD-3 and HBD-4 are expressed preferentially in the esophagus and tongue.[28, 29] Interestingly, *T. denticola* is resistant to these HBDs,[30] and some *Streptococcal* species are also resistant.[31] These facts may explain in part why two oral bacteria, *T. denticola* and *S. anginosus*, are adapted to the esophagus mucosa and cancer.

Our present data suggest that *S. anginosus, S. mitis*, and *T. denticola* could have a significant role in the carcinogenic process in most cases of esophageal cancer by causing inflammation and by promoting the carcinogenic process, and that eradication of these two bacteria may decrease the risk of recurrence.

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