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Presence of *Streptococcus* DNA Sequence in Surgical Specimens of Gastric Cancer

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In Southern blot analysis using *Mycoplasma* 16S ribosomal DNA (rDNA) as a probe, positive signals were detected in DNA samples from surgical specimens of gastric cancers. The DNA that hybridized to *Mycoplasma* 16S rDNA was eluted from the gel, cloned and sequenced. The cloned sequence was identical to 16S rDNA of *Streptococcus anginosus*. In Southern blot analysis with the *S. anginosus* 16S rDNA fragment as a new probe, positive signals were detected in 9 (20%) out of 43 cases of gastric cancer.

Key words: Gastric cancer — *Streptococcus* — *Mycoplasma*

It is well established now that multiple genetic alterations, including those caused by viral integration, occur during development of cancer, including gastric cancer.[1)] Gastric cancer is one of the most frequently occurring cancers, not only in Japan, but also worldwide. While infection of *Helicobacter pylori* is thought to be associated with gastric cancer,[2–10)] the role of other bacterial infections is still unknown. We have been searching for other microorganisms associated with gastric cancer by the modified in-gel competitive DNA reassociation method, which is useful for isolation of viral DNA from cancer tissue.[11)]

By this in-gel competitive DNA reassociation method, *Mycoplasma hyorhinis* was frequently identified in cancer cell lines (our unpublished observation). Subsequently, *M. hyorhinis* was frequently detected not only in cancer cell lines, but also in human gastric cancer tissues by polymerase chain reaction (PCR) using the *Mycoplasma* detection system (our unpublished observation). By Southern blot analysis with 16S rDNA of *M. hyorhinis* as a probe, positive signals were detected in some DNA samples from gastric cancer tissues, but not in those from the corresponding normal tissues. From one of the DNA samples, we cloned a DNA fragment which hybridized with *M. hyorhinis* 16S rDNA, and sequence analysis showed that the DNA fragment was not derived from the 16S rRNA gene of *M. hyorhinis*, but from that of *Streptococcus anginosus*. Here we report for the first time the presence of *S. anginosus* or its related DNA sequences in surgical specimens from gastric cancer. Nine (20%) out of 43 cases of human gastric cancer contained sequences showing homology to a portion of the 16S rRNA gene of *S. anginosus*.

DNAs of gastric adenocarcinomas and corresponding normal tissues from 43 patients were extracted by means of the conventional phenol procedure. Ten μg of *Eco*RI-digested DNA per lane was loaded onto 1% agarose gel, blotted onto a nylon membrane filter, Hybond N plus (Amersham), and hybridized with a ^{32}P-labeled DNA probe at 42°C in 5×SSC/0.1% SDS/50% formamide for 12 h. Filters were washed three times in 0.1×SSC/0.1% SDS at 65°C, and exposed to X-ray films at −70°C. A cDNA containing nucleotides from 1 to 269 of the 16S rRNA gene of *M. hyorhinis* and an approximately 1.7 kb *Eco*RI-fragment containing 16S rRNA gene of *S. anginosus* were used as probes for Southern blot analysis. From an agarose gel, *Eco*RI-digested DNA fragments from 1.6 kb to 2.0 kb, containing a sequence that hybridized with cDNA corresponding to 16S ribosomal RNA of *M. hyorhinis*, were extracted and then ligated to λgt10 vector. A total of 4×10^4 plaques were screened with a cDNA probe corresponding to 16S ribosomal RNA of *M. hyorhinis*. Four positive clones were isolated. By restriction enzyme mapping of the DNA inserts of the four clones, it was shown that all four clones contained DNA fragments derived from the same sequence. The insert DNA was subcloned into pUC18 vector, and sequenced by an automated sequencing apparatus (Model 373A, Applied Biosystems) by using vector primers.

Using *Mycoplasma* generic primers, MCGpF1,2 and MCGpR1,2 (Takara), which enabled us to amplify the 16S–23S spacer region in the rRNA operons of 12 species of *Mycoplasmas*,[12)] PCR products were obtained in 11 (48%) out of 23 DNA samples from surgical specimens of human gastric cancers. Sequencing of 8 of these 11 PCR products revealed the presence of *M. hyorhinis* rRNA gene in 4 of them (unpublished observation). Southern blot analysis with cDNA of *M. hyorhinis* 16S rRNA was performed on *Eco*RI-digested DNAs from

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surgical specimens of gastric cancers. Positive signals were detected in 4 (15%) out of 27 gastric cancers tested. Representative Southern blot data are shown in Fig. 1. Positive bands were found in DNAs from gastric cancer tissues, but not in corresponding normal tissues. In case 3261, the sizes of major bands were about 1.7, 4.4 and 4.5 kb. *Eco*RI-digested DNA fragments with sizes ranging from 1.6 kb to 2.0 kb were extracted from an agarose gel, and a DNA library was constructed. After screening 4×10^4 plaques with cDNA corresponding to 16S rRNA of *M. hyorhinis*, four positive clones were isolated. By restriction enzyme mapping of the four DNA inserts, it was shown that all four clones contained the same 1.7 kb DNA fragment. The insert DNA was subcloned into pUC18 vector, and sequenced. As shown in Fig. 2, the cloned DNA had 99% homology to cDNA corresponding to the 16S rRNA of *S. anginosus*, and 69% homology to 16S rRNA of *M. hyorhinis*. In Southern blot analysis with the DNA corresponding to a portion of the 16S rRNA gene of *S. anginosus* as a new probe, positive bands were detected in 9 (20%) out of 43 gastric cancer tissues. Representative data are shown in Fig. 3. In all of the positive cases, more than four bands were detected, and the sizes of the bands varied from sample to sample.

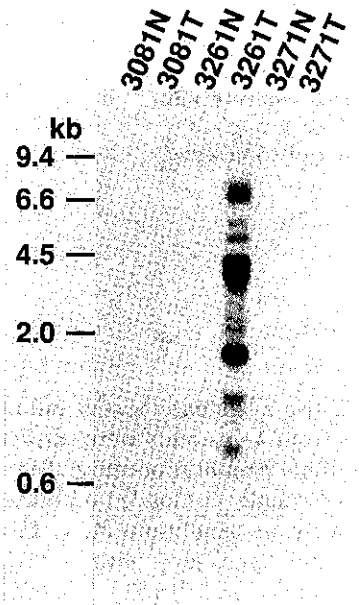

Fig. 1. Southern blot analysis of DNA from gastric cancer tissues (T) and the corresponding normal tissues (N). The case number is given at the top. Ten μg of *Eco*RI digested DNA was hybridized with 16S rDNA probe of *M. hyorhinis*.

Although the incidence of gastric cancer has declined worldwide in recent decades, gastric cancer is still one of the leading causes of cancer-death in Japan.[13,14] Several risk factors for gastric carcinogenesis, such as salt, nitrates, and low intake of fresh vegetables and β-carotene, have been reported.[15,16] Recently, an association has been reported between *H. pylori* infection and gastric

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S.anginosus: GAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAAGGAAGAACGAGTGTGAGAATGGAA 371
             ************************************************************
  1.7kb DNA: GAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAAGGAAGAACGAGTGTGAGAATGGAA

             AGTTCATACTGTGACGGTACTTAACCAGAAAGGGACGGCTNACTACGTGCCAGCAGCCGC 431
             ******* ******************************** ******************
             AGTTCATCCTGTGACGGTACTTAACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGC

             GGTAATACGTAGGTCCCNAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGG 491
             ***************** ****************************************
             GGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGG

             TTAGAAAAGTCTGAAGTGAAAGGCAGTGGCTCAACCATTGTAGGCTTTGGAAACTGTTTA 551
             ************************************************************
             TTAGAAAAGTCTGAAGTGAAAGGCAGTGGCTCAACCATTGTAGGCTTTGGAAACTGTTTA

             ACTTGAGTGCAGAAGGGGAGAGTGAATTC 580
             *****************************
             ACTTGAGTGCAGAAGGGGAGAGTGAATTC
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Fig. 2. Comparison of the nucleotide sequences of the cloned 1.7 kb DNA with the 16S rRNA gene of *S. anginosus*. Nucleotide sequences from 312 to 580 of the 16S rRNA gene of *S. anginosus* are shown in the upper section, and partial sequences of the cloned 1.7 kb DNA fragment hybridized with 16S rDNA of *M. hyorhinis*, in the lower section. Identical nucleotides are indicated by asterisks, and undetermined nucleotides, by an underlined N. The *Eco*RI recognition site is underlined.

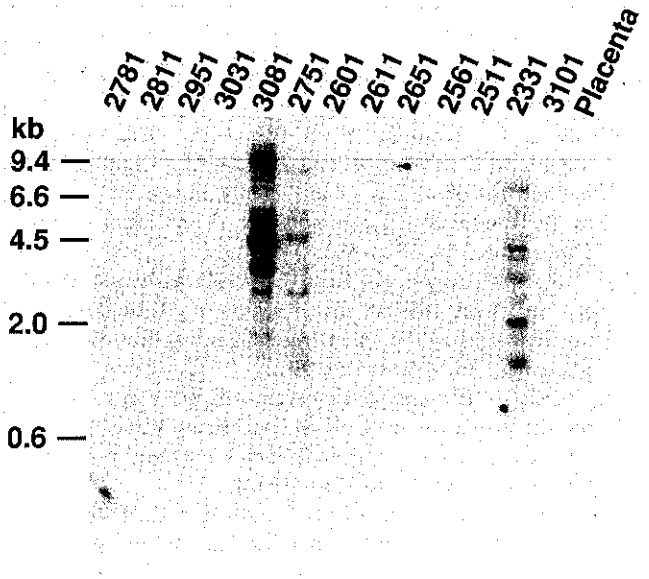

Fig. 3. Southern blot analysis of DNA from gastric cancer tissues. The case number is given at the top. Ten μg of *Eco*RI digested DNA was hybridized with 16S rDNA probe of *S. anginosus*.

cancer in several prospective and cross-sectional studies.[2-10] Although the involvement of infection with bacteria other than *H. pylori* in gastric cancer is unknown, it can not be excluded. We therefore searched for other microorganisms in gastric cancer. *Mycoplasma* infection was detected in 11 (48%) out of 23 DNA samples of gastric cancer patients by PCR amplification using *Mycoplasma*-specific primers. Sequence analysis of the PCR products showed that the most frequently found *Mycoplasma* was *M. hyorhinis*. The possibility of frequent association of *Mycoplasma* infection with gastric cancer was indicated by the Southern blot data with *M. hyorhinis* 16S rDNA (Fig. 1). However, a homology search of DNA data bases revealed that the DNA fragment used as a probe can hybridize with 16S rDNA sequences of other species of *Mycoplasmas* and with those of some bacterias. In case 3081, which gave positive bands on the Southern blot analysis, PCR amplification of the *Mycoplasma* sequence was unsuccessful. Secondly, strong bands were observed in case 3261 by Southern blot analysis with the cDNA corresponding to 16S rRNA of *M. hyorhinis*, but PCR amplification analysis showed that the sequence was identical not to that of *M. hyorhinis*, but to that of *M. orle* (data not shown).

A 1.7 kb DNA fragment corresponding to a major band in the Southern blot of DNA from case 3261 was cloned and sequenced. The sequence of the 1.7 kb DNA fragment was identical to that of 16S rDNA of *S. anginosus*, and had 69% homology to that of 16S rRNA of *M. hyorhinis*. The nucleotide sequence from 88 to 191 of the *S. anginosus* 16S rRNA gene had 90% homology to 16S rRNA of *M. hyorhinis*. This explains why *Streptococcus* DNA could be detected by Southern blot analysis using *Mycoplasma* DNA as a probe under a highly stringent condition. Southern blot analysis with the 1.7 kb *S. anginosus* 16S rDNA probe indicated presence of *Streptococcus* or its related DNA sequences in 9 (20%) of 43 gastric cancer samples. PCR analysis showed that all these positive samples had *Streptococcus* DNA or its related DNA sequences (unpublished data). It should be noted that some specimens positive for *Streptococcus* DNA could be positive for *Mycoplasma* DNA.

The nature of the DNA fragments other than the 1.7 kb DNA that hybridized with *M. hyorhinis* 16S rDNA probe remains unknown. It is possible that there are multicopies of the rRNA gene in *S. anginosus* and/or superinfection of different strains of *Streptococcus* showing various sizes of DNA bands, depending on the samples. Although no report indicating the integration of bacterial DNA into mammalian cells *in vivo* has been published, it is also possible that DNAs of *S. anginosus* are integrated into the host genome. These points remain to be clarified.

Streptococcus DNA or its related DNA sequences were detected predominantly in cancer tissues, which are different from the colonization of *H. pylori* in normal tissues, and it should also be noted that the *Streptococcus* or its related bacterial infection was not always observed only in necrotic lesions. The *S. milleri* group, including *S. anginosus, S. constellatus* and *S. intermedius*, is known to be present in various sites of the body, including the buccal cavity, intestine and vagina, and to cause suppurative diseases,[17] including dental periapical abscesses[18,19] and liver abscesses in elderly patients.[20] However, there has been no report on presence of *Streptococcus* or its related bacteria in cancer tissue.

Although epidemiological studies and identification of the strain of *Streptococcus* cultured from surgical specimens of gastric cancers are needed, our present data suggest that *Streptococcus* or its related bacteria could be associated with gastric cancer.

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