Presence of human mycoplasma DNA in gastric tissue samples from Korean chronic gastritis patients

Hyuk-Joon Kwon,[1] Jeong-Ok Kang,[1] Sun-Hee Cho,[1] Hee-Bum Kang,[1] Kyung-Ah Kang,[2] Jeong-Ki Kim,[2] Yoon-Suk Kang,[2] Byung-Cheol Song,[3] Hyun-Wook Kang,[4] Mi-Ja Shim,[5] Hee-Sun Kim,[6] Young-Bae Kim,[7] Ki-Baeg Hahm,[8] Bum-Joon Kim,[9] Myeong-Cherl Kook,[10] Myung-Hee Chung[11] and Jin-Won Hyun[2, 12]

[1]Institute of iNtRON Biotechnology, Seongnam; Departments of [2]Biochemistry, [3]Internal Medicine and [4]Forensic Medicine, College of Medicine, Cheju National University, Ara 1-Dong, Teju 690-756; [5]Department of Life Science, Seoul University, Jeonnong-Dong, Seoul 130-743; [6]Department of Neuroscience, College of Medicine, Ewha Women's University, Jongno 6-Ga, Seoul 110-783; Departments of [7]Pathology and [8]Internal Medicine, College of Medicine, Ajou University, Wonchun-Dong, Suwon 443-749; and Departments of [9]Microbiology, [10]Pathology and [11]Pharmacology, Seoul National University College of Medicine, 28 Yongon-Dong, Seoul 110-799, Korea

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We aimed to determine whether mycoplasmas are present in Korean chronic gastritis, and to understand their roles in gastric cancer tumorigenesis, because mycoplasmas resemble *Helicobacter pylori* in terms of ammonia production and induction of inflammatory cytokines in immune and non-immune cells. The presence and identity of mycoplasmas were assessed by semi-nested PCR and sequencing, and the results were compared with pathologic data. Fifty-six samples collected from Korean chronic gastritis patients were used for this study. Twenty-three (41.1%) were positive for mycoplasmas. Eighteen sequenced samples contained a single human mycoplasma or two mycoplasmas, which were identified as *Mycoplasma faucium* (13/18), *M. fermentans* (3/18), *M. orale* (1/18), *M. salivarium* (2/18), and *M. spermatophilum* (1/18). Mycoplasma-infected chronic gastritis samples showed significantly more severe neutrophil infiltration than non-infected samples (P=0.0135). Mycoplasma profiles in the oral cavity (*M. salivarium* is major) and stomach were different, and the presence of significant proinflammatory responses in mycoplasma-positive patients suggests that the mycoplasmas are not simply contaminants. Further studies are required to understand whether mycoplasmas play a role in gastric tumorigenesis. (Cancer Sci 2004; 95: 311–315)

Gastric cancer can be caused by chronic *Helicobacter pylori* infection, which induces inflammatory cytokines and oxidative stress, and results in the accumulation of genetic alterations leading to transformation. However, only a proportion of *H. pylori*-positive patients develop a tumor, and it has been shown that *vac A* and *cag A* of *H. pylori* and host factors, such as single nucleotide polymorphisms (SNPs) in the promoter region of interleukin-1β (IL-1β) and IL-1β receptor antagonist (IL-1β Ra), are not related to gastric tumorigenesis[1–4]; it therefore remains worthwhile to investigate other risk factors.

Mycoplasmas are the smallest self-replicating bacteria. Although mycoplasmas are generally commensal parasites in humans, some species are real pathogens and are capable of causing a wide variety of diseases.[5] Mycoplasmas present in the human oropharynx include *Mycoplasma orale*, *M. salivarium*, *M. faucium*, which produce ammonia and cause tissue damage,[6, 7] and *M. fermentans*, which induces inflammatory cytokines from macrophages[8] and epithelial cells.[9] Among them, *M. salivarium* and *M. orale* are major mycoplasmas found in throat specimens of adults, but *M. faucium* is relatively rare.[6] The presence of mycoplasmas in gastritis and gastrointestinal tumors has been reported.[10–12] These reports provoked concern about the epidemiological role of mycoplasmas in gastric cancer, though the fact that the identified species was of porcine origin, *M. hyorhinis*, was unexpected.[10–12] Pathobiological similarities between mycoplasmas and *H. pylori*, and questions on additional risk factors in the tumorigenesis of Korean gastric cancer encouraged us to further investigate the detection and identification of mycoplasmas in chronic gastritis.[2]

In this study, we investigated whether human mycoplasmas are associated with chronic gastritis by means of semi-nested PCR and direct sequencing. The results were then compared with pathologic data. Here, we report that human mycoplasmas are present in a high proportion of chronic gastritis (41.1%) cases, and the mycoplasma profiles are different from those of the oropharynx. In addition, mycoplasma-infected chronic gastritis recruits significantly more neutrophils than uninfected chronic gastritis (P<0.05), with no significant differences in the load of *H. pylori* (P>0.05). Therefore, we suggest that mycoplasmas are not simple contaminants, and further studies are needed to understand their possible roles in gastric tumorigenesis.

Materials and Methods

Chronic gastritis samples and pathologic examinations. Our experiments were carried out in accordance with the principles embodies in the Declaration of Helsinki and all volunteers gave written informed consent. Fifty-six gastric mucosal biopsy samples were collected during gastrofibroendoscopy from the same number of patients suffering from gastric symptoms in Korea. Each sample consisted of one to six pieces of biopsied tissues from representative areas. Fifty-one cases were biopsied from the antrum and five from the body. Tissues for pathologic examination were fixed in 10% neutral buffered formalin and processed routinely for embedding in paraffin and staining with hematoxylin and eosin. The severity of the pathologic findings was assessed according to the updated Sydney system.[13]

DNA preparation and semi-nested PCR. All samples were tested more than once for the presence of mycoplasmas using primers directed to conserved regions of the 16S ribosomal mycoplasma DNAs: P1F (1st forward primer, 5′-GGGAGCAAACAGGATTAGATACCCT-3′), MF (2nd forward primer, 5′-CGC CTG AGT AGT ATG CTC GC-3′) and P1R (common reverse primer, 5′-TGC ACC ATC TGT CAY TCT GTT AAC CTC-3′). Total DNA of the gastric tissue was extracted using a G-spin genomic DNA extraction kit (iNtRON Biotechnology Co., Seoul, Korea) according to the manufacturer's protocol. The PCR solution was composed of 10× buffer 2 μl, dNTPs (2.5 mM) 0.4 μl, P1F/P1R (10 pmol/μl) for 1st PCR and MF/P1R (10 pmol/μl) for 2nd PCR 0.5 μl each, *Taq* DNA polymerase (5 U/μl; iNtRON Biotechnology Co.) 0.2 μl, distilled water 15.4 μl and template DNA (50 ng/μl) or the 1st PCR product 1 μl. The 1st and 2nd PCR procedures were performed as follows: 94°C for 5 min; followed by 35 cycles of 94°C for

[12]To whom correspondence should be addressed. E-mail: jinwonh@cheju.ac.kr

20 s, 55°C for 10 s, and 72°C for 20 s; and a final extension step at 72°C for 5 min. Amplicons were analyzed by electrophoresis on 1.5% agarose gels, and a 100-bp ladder (iNtRON Biotechnology Co.) was used as the molecular weight marker.

Sequencing and analysis of sequence data. The PCR products were sequenced using an ABI 377 automatic DNA sequencer and dye terminator kit (Perkin Elmer Co., Foster City, CA) as previously reported.[14)]

The identities of the PCR products were confirmed by comparing sequences with the database of the National Center for Biotechnology Information BLAST network server.[15)] Nucleotide sequences were aligned and pair-wise distances were calculated using the Clustal method in the MegAlign package (Windows version 3.12e; DNASTAR, Madison, WI).

Sequence accession numbers. The nucleotide sequences of amplicons were registered at GenBank. The accession numbers of nucleotide sequences used for identifying mycoplasmas in samples were as follows: *M. buccale* (AF125586), *M. faucium* (AF125590), *M. fermentans* (M24289), *M. hominis* (M96660), *M. pneumoniae* (U95297), *M. salivarium* (AF125583), *M. spermatophilum* (AF221119), *M. hyorhinis* (AF412982), *M. lipophilum* (M24581), *M. orale* (M24659), *M. penetrans* (L10839), *M. pirum* (M23940), *M. genetalium* (X77334), Chronic gastritis (CG)5 (AY371310), CG13 (AY371300), CG16 (AY371301), CG17 (AY371302), CG20 (AY371303), CG28 (AY371304), CG30 (AY371305), CGS31 (AY371294), CGSP31 (AY371295), CG34 (AY371306), CG36 (AY371307), CG41 (AY371308), CG43 (AY371309), CG97 (AY371311), CGF104 (AY371312), CGFA104 (AY371313), CG105 (AY371296), CG106 (AY371297), CG111 (AY371298), CG113 (AY371299).

Statistical analysis. Differences of inflammation (degree of lymphoplasmacytic infiltration), activity (degree of neutrophilic infiltration), atrophy, metaplasia and *H. pylori* load between the mycoplasma-positive and negative groups were analyzed using the Mann-Whitney *U* test (95% confidence intervals).

Results

Detection and identification of mycoplasmas in chronic gastritis. Specific and expected bands were amplified in 23 (41.1%) of the 56 chronic gastritis (CG) samples (Table 1). Of the 23 positive cases, 22 were tissues biopsied from the antrum and one was from the body. The nucleotide sequences of 18 samples were determined and compared to those of other mycoplasmas. Sixteen of these 18 were single nucleotide sequences and the others were a mixture of two different sequences. Twelve (CG5, CG13, CG16, CG17, CG20, CG30, CG36, CG41, CG43, CG97, CG105, and CG111) were 100% homologous to *M. faucium*, and CG28 was 100% homologous to *M. salivarium*. CG106 and CG113 most resembled *M. fermentans* (98.7%), and CG34 resembled *M. orale* (88.0%). CG31 contained a mixture of *M. spermatophilum* (100%) and *M. salivarium* (100%) and CG104 a mixture of *M. fermentans* (98.7%) and *M. faucium* (100%) (Fig. 1, Table 2). All nucleotide sequences de-

Table 1. Pathologic characteristics of mycoplasma-negative chronic gastritis samples

Sample	Inflammation (0–3)	Activity (0–3)	Atrophy (0–3)	Metaplasia (0–3)	HP[2)] (0–3)	Biopsy site
CG[1)]1	2	1	2	0	1	Body
CG 2	1	0	1	0	1	Antrum
CG 3	2	2	0	0	3	Antrum
CG 4	3	3	0	0	3	Body
CG 6	1	0	0	0	1	Antrum
CG 7	1	0	3	0	1	Antrum
CG 8	3	1	0	0	2	Antrum
CG 10	0	0	0	0	1	Antrum
CG 11	1	0	2	0	2	Antrum
CG 12	1	0	3	0	1	Antrum
CG 18	1	0	2	0	0	Antrum
CG 19	1	0	1	0	0	Antrum
CG 21	3	2	0	0	3	Antrum
CG 25	1	0	1	3	1	Antrum
CG 26	1	0	1	3	2	Antrum
CG 29	1	0	2	2	2	Antrum
CG 32	1	0	1	0	1	Antrum
CG 33	1	0	3	0	1	Antrum
CG 35	1	0	0	0	1	Body
CG 38	1	0	1	0	1	Antrum
CG 39	3	1	0	0	1	Antrum
CG 42	3	1	1	1	1	Antrum
CG 44	3	2	0	0	1	Antrum
CG 45	1	0	1	0	0	Antrum
CG 46	2	1	2	0	3	Antrum
CG 48	1	1	0	0	1	Antrum
CG 50	2	1	1	0	1	Antrum
CG 51	1	0	1	3	0	Antrum
CG 52	2	1	0	0	1	Antrum
CG 56	2	2	0	0	3	Antrum
CG 107	1	1	0	0	1	Antrum
CG 109	2	2	0	0	1	Body
CG 115	3	2	0	0	3	Antrum

1) Chronic gastritis.
2) *Helicobacter pylori.*

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
100.0	94.7	86.7	69.3	96.0	89.3	74.7	81.3	77.3	74.7	74.7	96.0	85.3	94.7	85.3	96.0	90.7	**1**	*M. buccale*
	100.0	92.0	70.7	96.0	94.7	81.3	86.7	80.0	76.0	73.3	98.7	88.0	100.0	90.7	98.7	96.0	**2**	*M. faucium*
		100.0	70.7	86.7	97.3	84.0	82.7	80.0	68.0	68.0	90.7	96.0	92.0	98.7	90.7	92.0	**3**	*M. fermentans*
			100.0	74.7	66.7	56.0	61.3	81.3	93.3	80.0	70.7	65.3	70.7	69.3	70.7	68.0	**4**	*M. genetalium*
				100.0	89.3	74.7	82.7	82.7	80.0	80.0	97.3	82.7	96.0	85.3	97.3	92.0	**5**	*M. hominis*
					100.0	86.7	85.3	82.7	72.0	72.0	93.3	93.3	94.7	96.0	93.3	94.7	**6**	*M. hyorhinis*
						100.0	84.0	70.7	62.7	64.0	80.0	81.3	81.3	82.7	80.0	81.3	**7**	*M. lipophilum*
							100.0	72.0	68.0	70.7	85.3	80.0	86.7	81.3	85.3	88.0	**8**	*M. orale*
								100.0	89.3	86.7	78.7	76.0	80.0	78.7	78.7	78.7	**9**	*M. penetrans*
									100.0	88.0	76.0	64.0	76.0	68.0	76.0	72.0	**10**	*M. pirum*
										100.0	76.0	65.3	73.3	68.0	76.0	73.3	**11**	*M. pneumoniae*
											100.0	86.7	98.7	89.3	100.0	94.7	**12**	*M. salivarium*
												100.0	88.0	94.7	86.7	86.7	**13**	*M. spermatophilum*
													100.0	90.7	98.7	96.0	**14**	CG13
														100.0	89.3	90.7	**15**	CG113
															100.0	94.7	**16**	CG28
																100.0	**17**	CG34

Fig. 1. Pairwise distances of nucleotide sequences (16S ribosomal DNA, 92 nucleotides) of mycoplasmas. The accession numbers of nucleotide sequences are as follows: *M. buccale* (AF125586), *M. faucium* (AF125590), *M. fermentans* (M24289), *M. hominis* (M96660), *M. pneumoniae* (U95297), *M. salivarium* (AF125583), *M. spermatophilum* (AF221119), *M. hyorhinis* (AF412982), *M. lipophilum* (M24581), *M. orale* (M24659), *M. penetrans* (L10839), *M. pirum* (M23940), *M. genetalium* (X77334), chronic gastritis (CG) 5 (AY371310), CG13 (AY371300), CG16 (AY371301), CG17 (AY371302), CG20 (AY371303), CG28 (AY371304), CG30 (AY371305), CGS31 (AY371294), CGSP31 (AY371295), CG34 (AY371306), CG36 (AY371307), CG41 (AY371308), CG43 (AY371309), CG97 (AY371311), CGF104 (AY371312), CGFA104 (AY371313), CG105 (AY371296), CG106 (AY371297), CG111 (AY371298), CG113 (AY371299).

Table 2. Pathologic characteristics of mycoplasma-positive chronic gastritis samples and identified *Mycoplasma* species

Sample	Mycoplasma species	Inflammation (0–3)	Activity (0–3)	Atrophy (0–3)	Metaplasia (0–3)	HP (0–3)	Biopsy site
CG 5	*M. faucium*	3	1	0	0	1	Antrum
CG 13	*M. faucium*	2	1	0	0	2	Antrum
CG 14	NS[1)]	1	0	1	0	1	Antrum
CG 16	*M. faucium*	2	2	0	0	2	Antrum
CG 17	*M. faucium*	1	1	0	0	2	Antrum
CG 20	*M. faucium*	2	1	0	0	1	Antrum
CG 28	*M. salivarium*	1	0	1	0	2	Antrum
CG 30	*M. faucium*	1	1	3	2	2	Antrum
CG 31	*M. spermatophilum*/*M. salivarium*	1	1	3	0	1	Antrum
CG 34	*M. orale*	1	0	1	0	1	Antrum
CG 36	*M. faucium*	3	3	0	0	1	Antrum
CG 37	NS	2	1	2	0	3	Antrum
CG 41	*M. faucium*	1	2	0	0	1	Body
CG 43	*M. faucium*	2	3	0	0	1	Antrum
CG 47	NS	1	1	0	0	0	Antrum
CG 53	NS	1	0	1	2	1	Antrum
CG 97	*M. faucium*	3	1	2	0	2	Antrum
CG 104	*M. fermentans*/*M. faucium*	2	2	3	0	0	Antrum
CG 105	*M. faucium*	2	1	0	0	1	Antrum
CG 106	*M. fermentans*	3	3	0	1	2	Antrum
CG 108	NS	2	1	0	0	1	Antrum
CG 111	*M. faucium*	3	3	0	0	2	Antrum
CG 113	*M. fermentans*	3	3	0	0	2	Antrum

1) Not sequenced.

termined in this study shared only 93.3–96.0% similarity with *M. hyorhinis*.

Correlations between mycoplasma infection and pathologic factors. Inflammation (degree of lymphoplasmacytic infiltration), activity (degree of neutrophilic infiltration), atrophy, metaplasia and *H. pylori* loading were compared in the mycoplasma-positive and negative groups. Inflammation (P=0.2245), atrophy (P=0.4142), metaplasia (P=0.7425) and *H. pylori* loading (P=0.6089) showed no significant difference; however, activity (P=0.0135) was significantly different in the two groups (Table 3).

Discussion

In the present study, we examined the presence of mycoplasmas in human chronic gastritis and their effect on chronic inflammation. Twenty-three (41%) out of 56 chronic gastritis samples were positive for several human mycoplasmas. The presence of microorganisms other than *H. pylori* in gastric cancer and in the tissues of patients with other gastric disorders has been reported.[16–18)] *Streptococcus anginosus* and *M. hyorhinis* were identified in Japanese gastric cancer tissues by means of an in-

Table 3. Differences of inflammation (degree of lymphoplasmacytic infiltration), activity (degree of neutrophilic infiltration), atrophy, metaplasia, and *H. pylori* load between the mycoplasma-positive and negative groups (Mann-Whitney *U* test, 95% confidence intervals)

	Probability	Significance
Inflammation	0.2245 ($P>0.05$)	Not significant
Activity	0.0135 ($P<0.05$)	Significant
Atrophy	0.4142 ($P>0.05$)	Not significant
Metaplasia	0.7425 ($P>0.05$)	Not significant
H. pylori load	0.6089 ($P>0.05$)	Not significant

gel competitive DNA re-association method.[16] *M. hyorhinis* was identified in more than 50% of gastric cancer tissues in a Chinese population by immunohistochemistry and PCR, and the positive rate in gastric cancer was found to be significantly higher than in other gastric diseases, i.e., chronic superficial gastritis (28%), gastric ulcer (30%), and intestinal metaplasia (37%).[11, 12] *M. hyorhinis* is a swine mycoplasma and is very commonly found in the nasal and tracheobronchial secretions of young swine.[15] Human mycoplasmas are frequently detected on various mucous membranes, but to date *M. hyorhinis* has never been detected in other human tissue.[3] Normally mycoplasmas in the oral cavity can enter the stomach with food and saliva, and thus it is more likely that human mycoplasmas will be detected in gastric tissues than swine mycoplasmas. Therefore, the unexpected presence of *M. hyorhinis* in human gastric cancer raises a question on identification methods, i.e., immunohistochemistry and PCR, and should be further confirmed by nucleotide sequencing. We determined the nucleotide sequences of amplicons by direct sequencing and their nucleotide sequences were compared with those of known human mycoplasmas. Sixteen were found to be single nucleotide sequence, but two of the sequences were mixed. CG5, CG13, CG16, CG17, CG20, CG30, CG36, CG41, CG43, CG97, CG105, CG111, and CG28 were found to be 100% homologous to *M. faucium* and *M. salivarium*, respectively, but CG106, CG113, and CG34 showed the greatest homology to *M. fermentans* and *M. orale* (98.7% and 88.0%, respectively) (Fig. 1). CG106 and CG113 showed only one nucleotide difference from *M. fermentans*, and were identified as *M. fermentans*. The relatively low homology between CG34 and *M. orale* (M24659) was due to several ambiguous nucleotides in M24659, and except for these, CG34 was 100% homologous to *M. orale*. CG31 and CG104 were mixtures of *M. spermatophilum* (100% homology) and *M. salivarium* (100% homology), and *M. fermentans* (98.7% homology) and *M. faucium* (100% homology), respectively. Relatively low nucleotide similarities (93.3–96.0%) between amplicons and *M. hyorhinis* supported the idea that only human mycoplasmas were present in these Korean chronic gastritis tissues (Fig. 1). *M. salivarium* is usually found in 60–80% of throat specimens from adults and *M. orale* in 30–60% but *M. faucium* is isolated infrequently from the human oropharynx.[4, 6] The mycoplasmas in gastric tissues could be regarded as simple contaminants of the oropharynx, but the fact that *M. faucium* is more frequent in chronic gastritis tissues than *M. salivarium* and *M. orale* does not support this possibility. To elucidate the role of *M. faucium* in gastric cancer, further pathobiologic study is required.

H. pylori infection induces active inflammation with neutrophilic infiltration and chronic inflammation with infiltration of lymphocytes and macrophage/monocytes into the lamina propria of the mucosa of the human gastric antrum.[20, 21] These neutrophils and macrophages/monocytes produce reactive oxygen species or reactive nitrogen species,[22, 23] which have the potential to cause DNA damage to adjacent gastric cells, such as gastric glandular epithelial cells and deep foveolar cells.[24] Moreover, the life-long effect of *H. pylori*-induced inflammation might cause accumulation of DNA damage. This damage could induce gastric cancer. Also, the neutralization of gastric acid by ammonia secreted in *H. pylori* directly and indirectly stimulates immune and non-immune cells to secrete inflammatory cytokines, IL-1β, TNF-α, IL-6 and IL-8, and induces adhesion molecules, which recruit inflammatory cells, i.e., ICAM-1 and VCAM-1.[6, 19, 25] The transcription factor NF-κB plays a pivotal role in inflammatory responses in *H. pylori*-associated gastritis by up-regulating the mRNA expressions of chemokines and adhesion molecules.[26, 27] Mycoplasmas present in tissue samples from Korean chronic gastritis patients resemble *H. pylori* with respect to the role that it plays in the tumorigenesis of gastric cancer. The non-fermentative mycoplasmas, *M. salivarium, M. orale, M. faucium* and *M. spermatophilum*, are known to produce ammonia from arginine, to cause tissue damage and to neutralize gastric acid.[11] *M. salivarium* and *M. fermentans* induce the inflammatory cytokines IL-1β, TNF-α, and IL-6 in monocytes/macrophages,[28] and IL-6 and IL-8 in human gingival fibroblasts.[29] In addition *M. salivarium* and *M. fermentans* can activate the transcription of ICAM-1 mRNA and induce cell surface expression of ICAM-1 in normal human gingival fibroblasts.[30] In view of the lack of an association between the molecular pathotypes of *H. pylori* (*cag A* and *vac A*) and SNPs in the promoter region of interleukin-1β (IL-1β) or with IL-1β receptor antagonist (IL-1β Ra) in the tumorigenesis of Korean gastric cancer, the significantly higher activity ($P=0.0135$) observed in the mycoplasma-positive group compared with the negative group, without differences in severity of inflammation, metaplasia and load of *H. pylori*, may be explained by the pro-inflammatory properties of human mycoplasmas (Table 1 and Table 2).

Moreover, unlike *H. pylori*, mycoplasmal chronic infection can transform normal cells.[31–33] The malignant transformation of embryonic (C3H) cells by mycoplasmas was shown to be a multistage process, involving overexpression of the H-*ras* and *c-myc* oncogenes.[31, 32] In addition, *M. fermentans* and *M. orale* induced the malignant transformation of IL-3-dependent 32D myeloma cells by activating NF-κB.[33]

In conclusion, human mycoplasmas are present in the chronic gastritis tissues of Korean patients, and may exacerbate chronic inflammation status by recruiting neutrophils. In view of the transforming activities of mycoplasmas, we suggest that further study of the putative role of mycoplasmas in gastric tumorigenesis is warranted.

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