SPANDIDOS PUBLICATIONS

Analysis of the differences in structural chromosomal aberrations of the gastric mucosa between *H. pylori* positive and negative gastric cancer patients: Involvement of *H. pylori* in the onset of gastric cancer and examination of the mechanism in gastric carcinogenesis following *H. pylori* eradication

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Abstract. Gene mutations are essential to carcinogenesis. If an evident difference is observed in gastric mucosal chromosomal structure aberrations between *H. pylori* (Hp)-negative and Hp-positive gastric cancer patients, it may be interpreted as suggesting the involvement of Hp in gene mutations. This study was undertaken to compare chromosomal structural aberrations between Hp-negative and Hp-positive gastric cancer patients and to evaluate the effects of Hp eradication on chromosomal structures in clinical cases. The subjects of this study were 40 patients with gastric cancer divided into four groups: Group A was composed of 12 patients with Hp-negative gastric cancer (well-differentiated gastric cancer in 5 cases and poorly-differentiated in 7 cases), Group B of 8 patients with Hp-negative gastric cancer following Hp eradication (well-differentiated in 4 case and poorly-differentiated in 4 cases), Group C of 13 patients with Hp-positive gastric cancer (well-differentiated in 7 cases and poorly-differentiated in 6 cases) and Group D of 7 patients with gastric cancer (well-differentiated in 5 cases and poorly-differentiated in 2 cases) undergoing Hp eradication at subtotal gastrectomy. In each of the groups A, B and C, the structural chromosomal aberration such as loss of heterozygosity (LOH) and microsatellite instability (MSI) was analyzed. In Group D, changes in structural chromosomal aberrations after Hp eradication as compared to the pre-eradication structures were also analyzed. LOH and MSI were examined by PCR, using DNA extracted from the cancer-affected and intact gastric mucosal tissue specimens from each patient. In A, B and C groups, structural chromosomal aberrations were noted, and these aberrations tended to be more marked in cases of poorly-differentiated gastric cancer in each group. In terms of structural chromosomal aberrations, there was no marked difference between Group A and either Group B or C. Hp eradication resulted in no change in chromosomal structure as compared to the pre-eradication structure in Group D. These results suggest the possibility that Hp eradication does not affect chromosomal structures and Hp is involved in gastric carcinogenesis as an additive environmental factor rather than as a factor acting at the gene level.

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Introduction

One important factor regarding the genes responsible for carcinogenesis is structural chromosomal aberrations. All cancer tissues have some structural chromosomal aberrations (1-7). We had examined *H. pylori* (Hp)-positive gastric cancer patients for structural chromosomal aberrations such as loss of heterozygosity (LOH) and microsatellite instability (MSI) and showed changes in the chromosomal structure to be more marked in cases with poorly-differentiated than in those with well-differentiated gastric cancer (8). Various studies have been

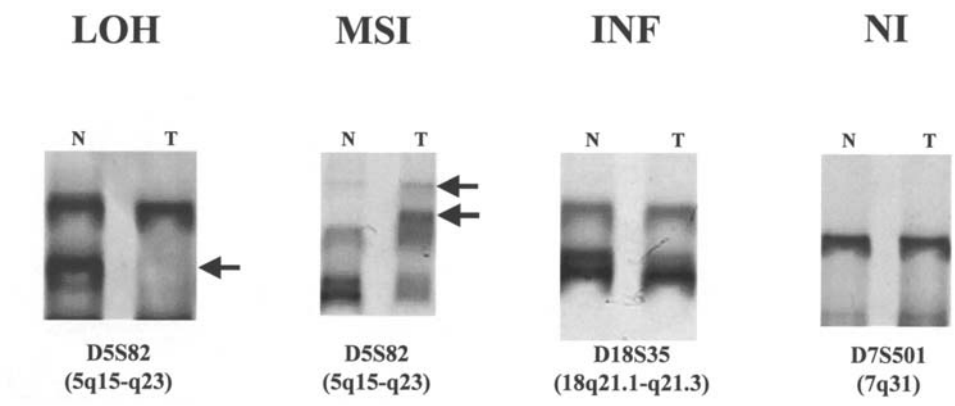


Figure 1. PCR gel electrophoresis. LOH, MSI, INF and NI indicates loss of heterozygosity, microsatellite instability, informative and non-informative, respectively. N and T indicates normal DNA and tumor DNA, respectively. Arrowhead indicates structural chromosomal aberrations as LOH or MSI.

conducted concerning the relationship between Hp infection and gastric cancer, demonstrating the high probability that Hp infection is involved in the onset of gastric cancer (9-13), however, the involving site of Hp infection in gastric carcinogenesis remains unknown. A number of cases of gastric cancer occurring after Hp eradication have been reported (14-16), and the evaluation of Hp eradication therapy for a prevention against gastric cancer onset is unknown. Factors responsible for carcinogenesis can be roughly divided into genetic and environmental (17-19). If a difference in structural chromosomal aberration is found between Hp-negative and Hp-positive gastric cancer patients, it may be interpreted as suggesting the involvement of Hp as a factor inducing gene mutations in carcinogenesis. If no evident difference in structural chromosomal aberrations is found between Hp-negative and Hp-positive gastric cancer cases, it would suggest that Hp is involved in carcinogenesis as an additive environmental factor which works with gene mutations. In other words, we can say that the analysis of differences in structural chromosomal aberrations between Hp-positive and Hp-negative gastric cancer cases is clinically significant since it may allow us to evaluate the role played by Hp in gastric carcinogenesis (either as a genetic or an additive environmental factor). Although Hp eradication has been attempted in many cases as a means of preventing the onset of gastric cancer, there are no studies dealing with the effects of eradication on chromosomal structures. If eradication affects the chromosomal structures, the validity of eradication therapy itself may be questioned.

The present study was undertaken to examine structural chromosomal aberrations in Hp-negative gastric cancers and Hp-negative gastric cancers occurring following Hp eradication in comparison to those in Hp-positive gastric cancers in clinical cases, and to examine the influence of Hp eradication for chromosomal structures. Therefore, we investigated the site involved in Hp infection in gastric carcinogenesis and to what extent Hp eradication can suppress gastric cancer onset.

Patients and methods

The subjects of this study were 40 patients with gastric cancer divided into four groups: Group A was composed of 12 patients with Hp-negative gastric cancer (7 males and 5 females with a mean age of 61.7 years; well-differentiated gastric cancer in 5 cases and poorly-differentiated in 7 cases), Group B of 8 patients with Hp-negative gastric cancer developing after Hp eradication (5 males and 3 females with a mean age of 60.3 years; well-differentiated in 4 cases and poorly-differentiated in 4 cases), Group C of 13 patients with Hp-positive gastric cancer (7 males and 6 females with a mean age of 63.6 years; well-differentiated in 7 cases and poorly-differentiated in 6 cases) and Group D of 7 patients with gastric cancer (5 males and 4 females with a mean age of 62.4 years; well-differentiated in 5 cases and poorly-differentiated in 2 cases) undergoing Hp eradication at surgical treatment as subtotal gastrectomy. Group D is an examination group whether or not the Hp eradication affects to structural chromosomal aberrations. In each of three the groups A, B and C, structural chromosomal aberrations such as LOH and MSI were analyzed and changes in each group were compared. In Group D, changes of structural chromosomal aberrations after eradication as compared to the pre-eradication structures were analyzed. LOH and MSI were assessed by PCR, using DNA extracted from cancer-affected and intact gastric mucosal tissue specimens endoscopically collected from each patient. Hp infection was diagnosed based on the results of cultures and the urea breath test (UBT), with subjects testing negative to both judged to be negative for Hp infection. Informed consent was obtained from all the patients prior to the study.

Tissue samples. After obtaining informed consent from individual subjects, sample tissues were taken from cancer-affected and cancer-free areas in patients with gastric cancer and from atrophic mucosa and atrophy-free mucosa in patients with chronic gastritis. All samples were divided into two segments; one was frozen immediately after collection and stored at -80˚C until extraction of DNA, and the other was fixed in 10% formalin for pathologic diagnosis. Histopathological diagnosis was performed according to the International Classification of Tumors (20) for gastric cancer patients.

DNA extraction. All patients with gastric cancer had histologically confirmed adenocarcinoma of the stomach. Tumor samples for DNA extraction were checked to ensure that they consisted of >80% tumor. DNA extraction was as follows:

PCR primers.

Markers	Locations	Size of PCR products (bp)	Sequence of primers	Oncogenes/tumor sup. genes
D1S116	1p31-p21	89-101	5/-TACAAGGCAACCACATAATT-3/ 5/-CTTTTCCTAATTGTGTGTGT-3/	N-ras
D1S159	1p32	147	5/-TCCTTTACATAAATCATTGTCGTG-3/ 5/-CGACTCTGCATTACCTTGATAGC-3/	
D1S162	1p32	134	5/-GGGGGAAGAAGTCCGAGTAG-3/ 5/-ATAAGGGGAACAGGTCTGGG-3/	L-myc
D1S188	1p32	149-173	5/-AACCAATCAAGGTGCCTGCA-3/ 5/-TCCCCTAGTGTCCTGGCAG-3/	p18
MYCL1	1p32	140-209	5/-TGGCGAGACTCCATCAAAG-3/ 5/-CCTTTTAAGCTGCAACAATTTC-3/	
D5S346	5q21-q22	96-122	5/-ACTCACTCTAGTGATAAATCGGG-3/ 5/-AGCAGATAAGACAGTATTACTAGTT-3/	
D5S82	5q15-q23	169-179	5/-ATCAGAGTATCAGAATTTCT-3/ 5/-CCCAATTGTATAGATTTAGAAGTC-3/	APC
D5S318	5q15-q23	176-188	5/-TCTAGAGGATCTTCCCTCTT-3/ 5/-CCATCGOTAGAACTAATTTC-3/	
D5S299	5q15-q23	156-182	5/-GCTATTCTCTCAGGATCTTG-3/ 5/-GTAAGCCAGGACAAGATGACAG-3/	
MCC	5q21-q22	168-176	5/-CCAATGAAACTTCGCTTTAATCAG-3/ 5/-GTGGAATTTGTATCATGCTCTG-3/	
D7S501	7q31	163-179	5/-CACCGTTGTGATGGCAGAG-3/ 5/-ATTTCTTACCAGGCAGACTGCT-3/	
D7S523	7q31	224-240	5/-CTGATTCATAGCAGCACTTG-3/ 5/-AAAACATTTCCATACCACTG-3/	Met
D7S486	7q31	114-146	5/-AAAGGCCAATGGTATATCCC-3/ 5/-GCCCAGGTGATTGATAGTGC-3/	PPP1R3
D7S677	7q31.2-q31.3	278	5/-ATCATTCACTATGGGATAGC-3/ 5/-GAATTACAAGTCACTCTATACAAAA-3/	
D7S522	7q31	217-229	5/-GCCAAACTGCCACTTCTC-3/ 5/-ACGTGTTATGCCACTCCC-3/	

Samples were powdered with liquid nitrogen. After they had been spun down to be deposited once, genomic DNAs were added to 1000 μl of TNES buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 0.1% SDS) and 30 μl Proteinase K (100 μg/ml), stirred, digested, and incubated in a water-bath overnight at 50˚C. After DNAs had been extracted by phenol-chloroform extraction and refined, they were washed and precipitated with ethanol (21). The concentration of extracted DNA was estimated by a spectrophotometric method, and the samples were kept frozen at -80˚C. From each DNA sample, 50 ng/μl was used for the template of the polymerase chain reaction (PCR) amplification procedure.

PCR and microsatellite analysis. We used 31 primers corresponding to chromosomal regions known to contain major oncogenes and tumor suppressor genes (N-ras, L-myc, p18, APC, Met, PPP1R3, p53, NF1, BRCA1, Smad2, Smad4/DPC4, DCC, ANA, AML1), namely, 1q, 5q, 7q, 17p, 17q, 18q and 21q. The following 31 primers used were: D1S116, D1S159, D1S162, D1S188, MYCL1, D5S346, D5D82, D5S318, D5S299, MCC, D7S501, D7S523, D7S486, D7S677, D7S522, D17S261, D17S1176, TP53, D17S250, D17S1320, D17S1329, D18S46, DCC, D18S35, D18S38, D18S64, D21S369, D21S236, D21S11, D21S1436 and D21S1254 (Table IA and B). The primers were obtained from

Table IB. PCR primers.

Markers	Locations	Size of PCR products (bp)	Sequence of primers	Oncogenes/tumor sup. genes
D17S261	17p12-p11.1	157-171	5/-CAGGTTCTGTCATAGGACTA-3/ 5/-TTCTGGAAACCTACTCCTGA-3/	Unknown
D17S1176	17p13.1	95-109	5/-ACTTCATATACATATCACGTGC-3/ 5/-TCAATGGAGAATTACGATAGTG-3/	p53
TP53	17p13.1	103	5/-TTGCCTCTTTCCTAGCACTG-3/ 5/-CCAAGACTTAGTACCTGAAG-3/	
D17S250	17q11.2-12	151-169	5/-GGAAGAATCAAATAGACAAT-3/ 5/-GCTGGCCATATATATATTTAAACC-3/	NF1
D17S1320	17q21	180	5/-ACTTTCCAGAAAATCTCTGCTC-3/ 5/-CCACGTCTTTTCTGTGTTCC-3/	BRCA1
D17S1329	17q21	170	5/-GACTCTGAAGGTAAAGAGCAA-3/ 5/-CTCCCCTGCCTTGGGAGTAG-3/	
D18S46	18q21.1	129-153	5/-GAATAGCAGGACCTATCAAAGAGC-3/ 5/-CAGATTAAGTGAAAACAGCATATGTG-3/	Smad2 Smad4/DPC4
DCC	18q21.1-q21.3	90	5/-CATCCAAGCACTAACAGG-3/ 5/-CAACGATGATTATAGGCAAT-3/	DCC
D18S35	18q21.1-q21.3	104-124	5/-AGCTAGATTTTTACTTCTCTG-3/ 5/-CTGGTTGTACATGCCTGAC-3/	
D18S38	18q21.3	162-172	5/-TACACGCTCACACTTCAACC-3/ 5/-AGGATACAAGTAGTGAGAGC-3/	
D18S64	18q21.3	188-208	5/-ATACTGGTGGTGGTTATACAACAT-3/ 5/-AAATCAGGAAATCGGCA-3/	
D21S369	21q11.1	173-201	5/-ATGGCCTTGGCTAAATGCTG-3/ 5/-CTAAGCTGATATGGTAAGTACA-3/	ANA
D21S236	21q11.1	104-128	5/-CCCAAATAAAAAAGAGAACAG-3/ 5/-CTAAAGAGGACTTCAGAGTAAGG-3/	
D21S11	21q21	172-264	5/-GTGAGTCAATTCCCCAAG-3/ 5/-GTTGTATTAGTCAATGTTCTCC-3/	
D21S1436	21q21	178	5/-AGGAAAGAGAAAGAAAGGAAGG-3/ 5/-TATATGATGAAAGTATATTGGGGG-3/	
D21S1254	21q22.1	148	5/-AAGAGCCAATTACAGAAAGTCA-3/ 5/-GTTGTTTTAAGGATGGTCATTC-3/	AML1

Research Genetics (Huntsville, AL). PCR amplification was performed in a total reaction volume of 20 μl, as described previously (22). Each PCR reaction mixture contained 250 ng of sample DNA, 20 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 2 mM dNTP, and 0.5 units of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Thirty-five cycles of denaturation at 94˚C for 1 min, and extension at 72˚C for 1 min were performed with a DNA thermal cycler (Perkin-Elmer Cetus). After dilution with an adequate volume of formamide-dye mixture (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), the PCR products were heat-denatured (98˚C, 5 min), chilled on ice, and electrophoresed on 6% urea-formamide-polyacrylamide gel at 3 W for 2-3 h, depending on fragment size. Silver staining of the gels was performed using a DNA silver staining kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden). To ensure reproducibility in each case with LOH or MSI, all tests were performed under the same conditions.

Assessment of LOH and MSI. LOH in tumor DNA samples was assessed by scanning densitometry and analyzed with

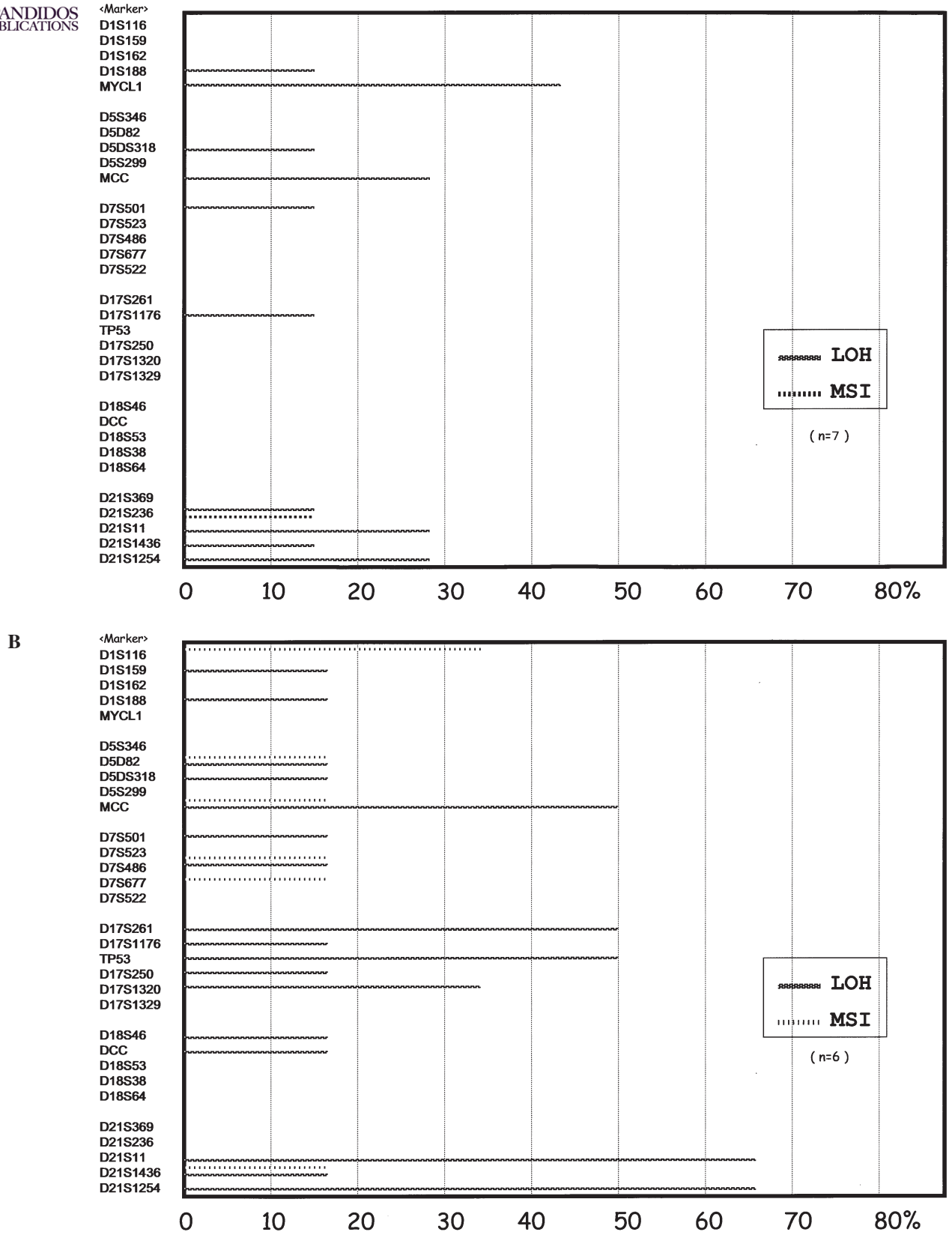


Figure 2. (A) Frequency of the deletion of chromosome 1p, 5q, 7q, 17p, 17q, 18q and 21q in Hp-positive well-differentiated adenocarcinoma with LOH or MSI. (B) Frequency of the deletion of chromosome 1p, 5q, 7q, 17p, 17q, 18q and 21q in *H. pylori* positive poorly-differentiated adenocarcinoma with LOH or MSI.

National Institute of Health (NIH) software (Image version 1.62, Dr W. Rasband, NIH, Bethesda, MD, USA). Signal intensities in tumor DNA were compared with those of the corresponding normal DNA. A reduction in signal intensity of >50% was required for LOH. Commonly, deleted regions were defined by considering the loci most frequently showing LOH, together with multiple interstitial deletions. MSI in DNA samples was also assessed as positive in cases with

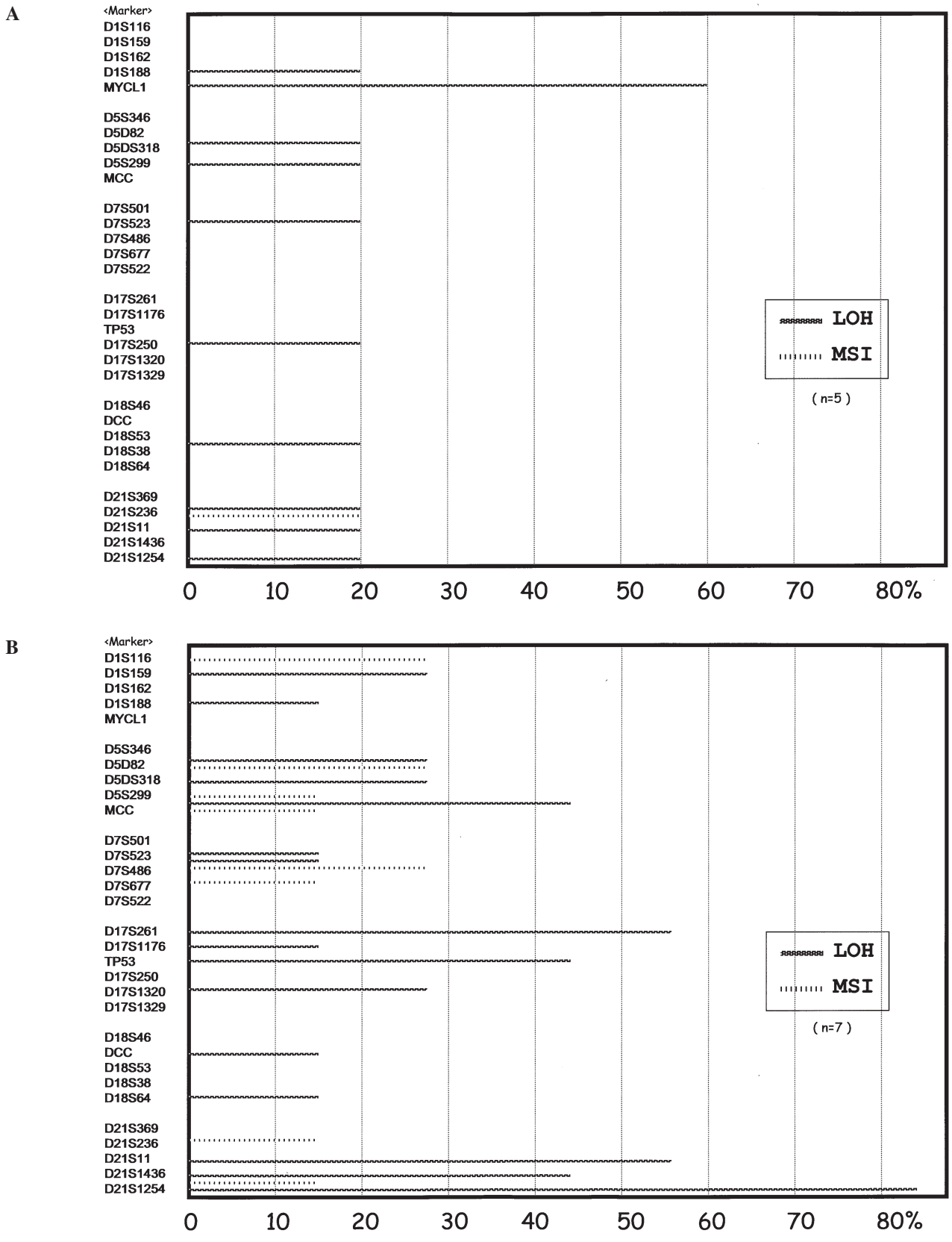


Figure 3. (A) Frequency of the deletion of chromosome 1p, 5q, 7q, 17p, 17q, 18q and 21q in Hp-negative well-differentiated adenocarcinoma with LOH or MSI. (B) Frequency of the deletion of chromosome 1p, 5q, 7q, 17p, 17q, 18q and 21q in Hp-negative poorly-differentiated adenocarcinoma with LOH or MSI.

additional bands in the tumor sample that were not observed in the corresponding normal sample or in cases with a band shift in the tumor sample that contrasted with those of the corresponding normal bands (Fig. 1).

Results

In the groups A, B and C, at least one structural chromosomal aberration such as LOH and MSI was identified. These

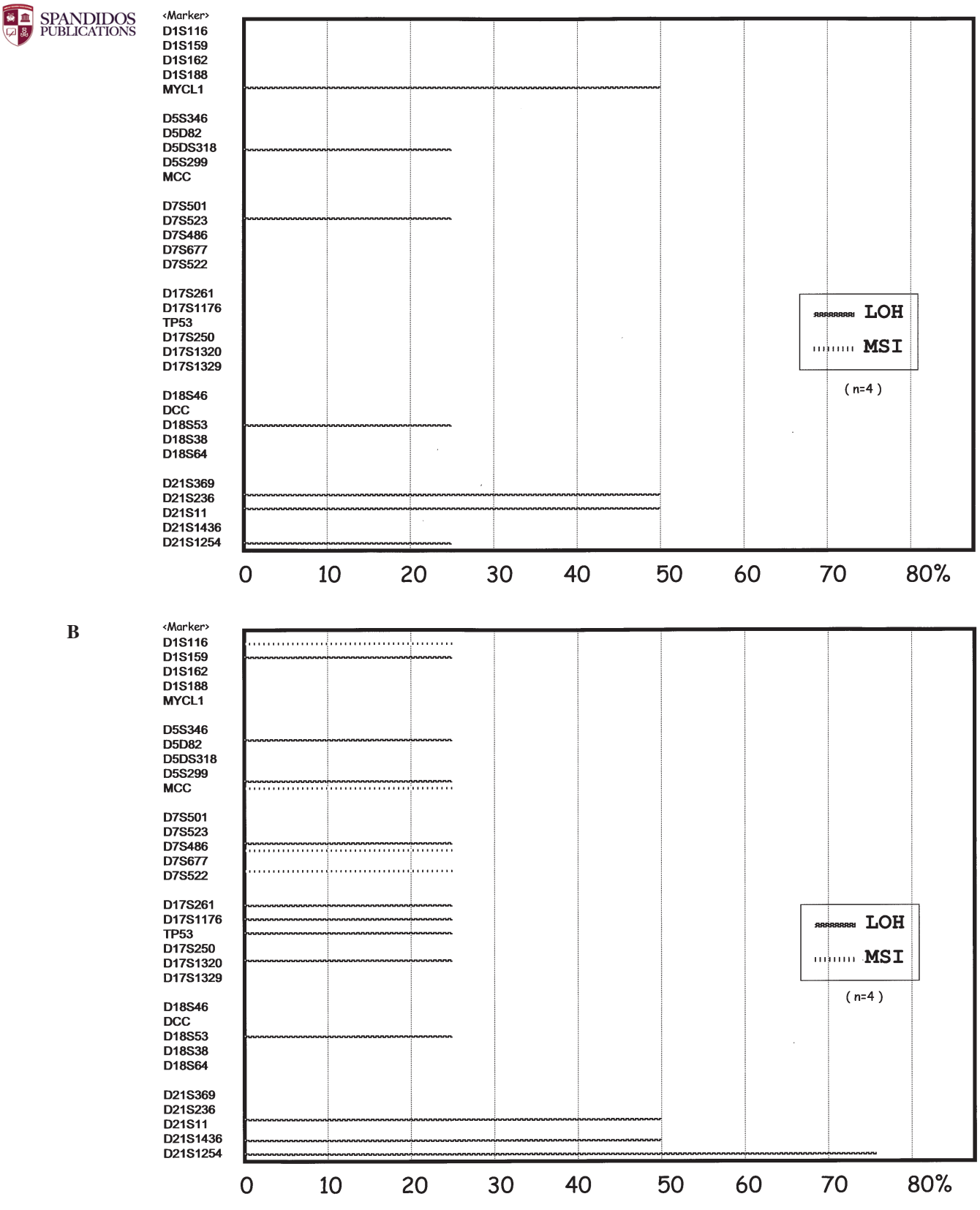
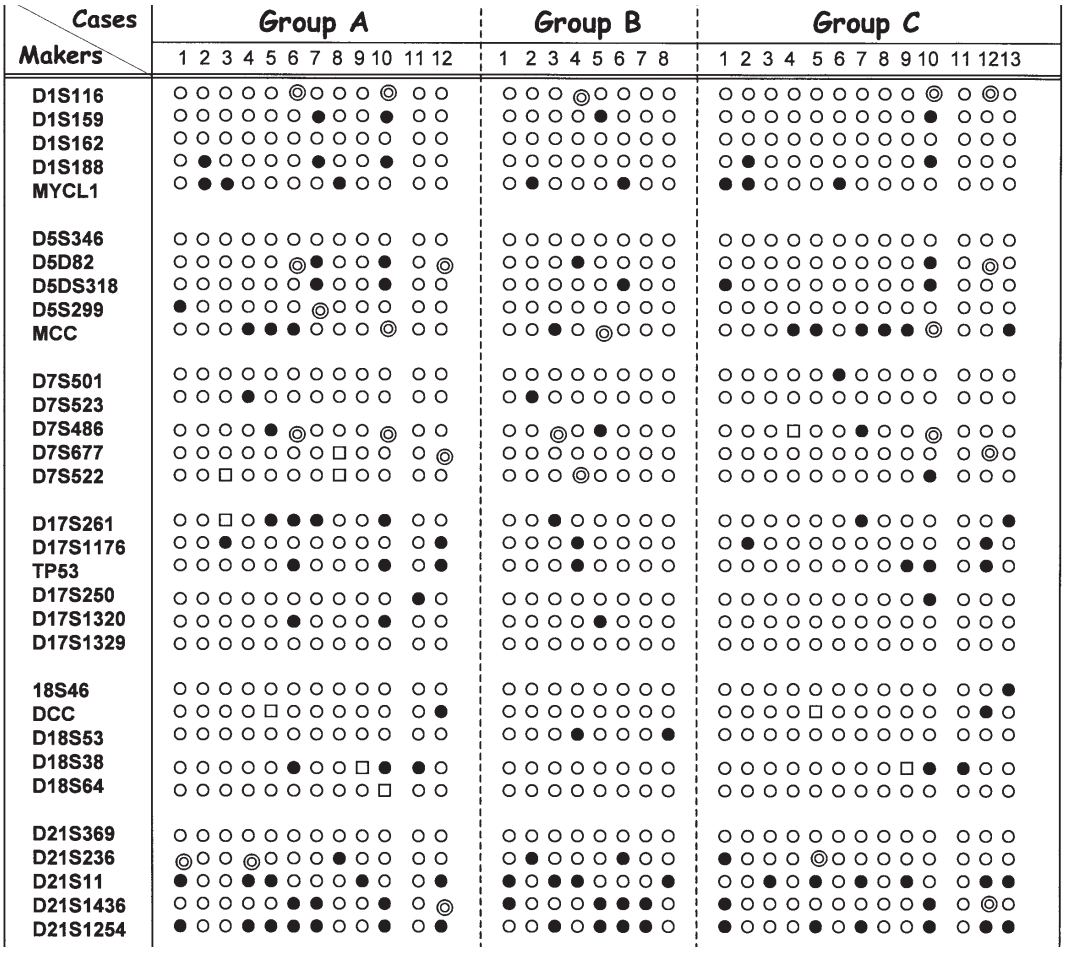


Figure 4. (A) Frequency of the deletion of chromosome 1p, 5q, 7q, 17p, 17q, 18q and 21q in Hp-negative well-differentiated adenocarcinoma with LOH or MSI following eradication therapy. (B) Frequency of the deletion of chromosome 1p, 5q, 7q, 17p, 17q, 18q and 21q in Hp-negative poorly-differentiated adenocarcinoma with LOH or MSI following eradication therapy.

abnormalities were not associated with the histological type of gastric cancer or any background variable such as Hp infection (Tables II and III). In each of the above groups, structural chromosomal aberrations tended to be more marked in poorly differentiated gastric cancer cases than in those with well-differentiated gastric cancer (Figs. 2A and B, 3A and B, and 4A and B). In terms of structural chromosomal aberrations, there was no marked difference between Group A and either

Table II. The changes of chromosome 1p, 5q, 7q, 17p, 17q, 18q and 21q in the regionof Hp-positive and Hp-negative gastric cancers with LOH or MSI.



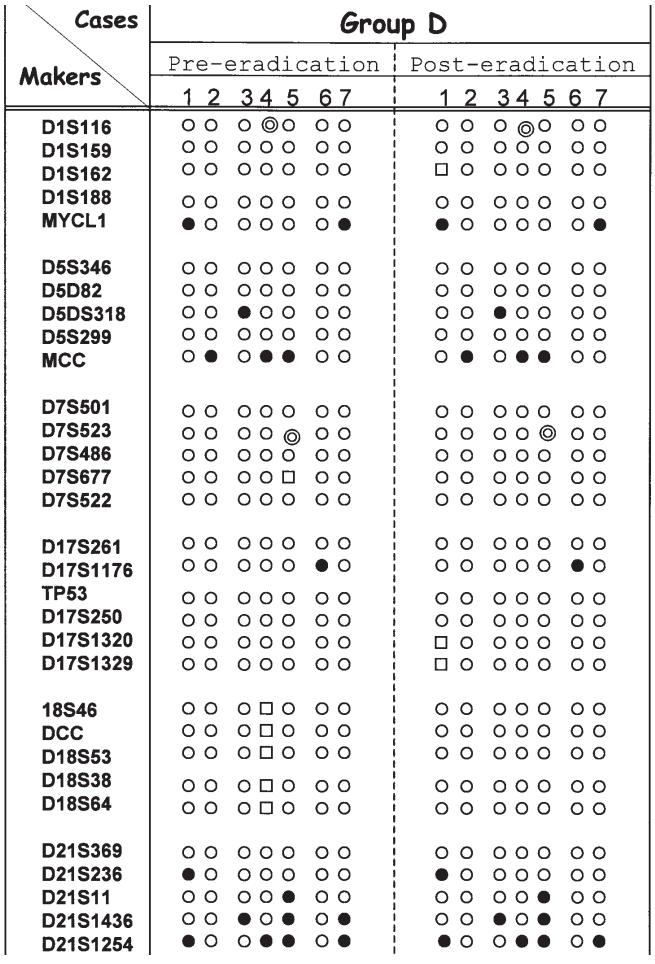
Groups A, B and C indicate Hp-negative cases, Hp-negative incidence cases following eradication therapy and Hp-positive cases, respectively. Cases 1 to 3, 8 and 11 are well-differentiated adenocarcinomas, and the cases 4 to 7, 9 to 10 and 12 are poorly-differentiated adenocarcinomas in group A. Cases 1 to 2, 6 and 8 are well-differentiated adenocarcinomas, and the cases 3 to 5 and 7 are poorly-differentiated adenocarcinomas in group B. Cases 1 to 6 and 8 are well-differentiated adenocarcinomas, and the cases of 7 and 9 to 13 are poorly-differentiated adenocarcinomas in group C. ○, ●, a circle within a circle, and □ indicate retention of heterozygosity (ROH), LOH, MSI and not informative (NI), respectively.

Group B or C (Table III). In Group D, no change in chromosomal structure was observed following Hp eradication as compared to the pre-eradication structure (Table III).

Discussion

Gene mutation is essential for carcinogenesis (1-7). The onset of gastric and other cancers is thought to take place in steps in the presence of gene mutations combined with various environmental factors such as dietary style. Hp infection has been regarded as an important factor associated with the onset of gastric cancer (9-13), and Hp eradication has been conducted to prevent the onset of gastric cancer (23,24). However, many cases of gastric cancer occurring after Hp eradication have been reported (14-16,24), and the extent to which Hp eradication can suppress the onset of gastric cancer and the site involved in Hp infection in gastric carcinogenesis are unknown. The onset mechanism of Hp-negative gastric cancer is also unknown. There are no reports as to whether or not Hp-negative gastric cancers occur in unique features of structural chromosomal aberrations in comparison with Hp-positive gastric cancers. When discussing the mechanism of carcinogenesis, it is important to determine whether or not Hp infection induces gene mutations. In the present study, structural chromosomal aberrations such as LOH and MSI were also identified in Hp-negative gastric cancer patients, as in Hp-positive gastric cancer patients, and there was no evident difference in these abnormalities between the Hp-negative and Hp-positive groups. Eradication of Hp was also shown to be unlikely to affect structural chromosomal aberrations. These two findings from the present study are clinically significant. Factors involved in carcinogenesis can be roughly divided into genetic and various additive/synergistic factors (e.g., environmental factors). In view of the present results, it seems probable that Hp infection is involved in the mechanism of gastric carcinogenesis as an additive/synergistic factor rather than as a factor affecting the genes. Various additive/synergistic factors involved in carcinogenesis are known, in addition to Hp. We consider the results of our present study to also allow us to explain why gastric

The changes of chromosome 1p, 5q, 7q, 17p, 17q, 1q in the region of pre- and post-eradication therapy of gastric cancers with LOH or MSI.



This group indicates pre- and post-eradicated therapy cases of gastric cancers infected with Hp. Cases 1 to 3 and 6 to 7 are well-differentiated adenocarcinomas, and the cases 4 to 5 are poorly-differentiated adenocarcinomas. ○, ●, double circle, and □ indicate retention of heterozygosity (ROH), LOH, MSI and not informative (NI), respectively.

cancer occurs even after Hp eradication. As demonstrated in previous epidemiological studies of patients with gastric cancer, it is highly probable that Hp serves as an important factor involved in the onset of gastric cancer. According to our view, it is possible to reduce the incidence of gastric cancer by eradicating Hp but it is difficult to completely prevent its onset by means of Hp eradication. It cannot be said with certainty that Hp infection had not occurred in all of Hp-negative gastric cancer patients. We cannot rule out the possibility that some Hp cases were negative at the time of gastric cancer onset but had suffered Hp infection previously. As far as Hp eradication therapy is concerned, a clinically significant issue remains; identification of the stage of Hp infection at which Hp eradication is expected to allow greater suppression of gastric cancer onset.

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