Altered microsatellites in incomplete-type intestinal metaplasia adjacent to primary gastric cancers

T Hamamoto, H Yokozaki, S Semba, W Yasui, S Yunotani, K Miyazaki, E Tahara

Abstract

Aim—To investigate the presence of genetic instability in precancerous lesions of the stomach.

Methods—Fifteen cases of sporadic gastric cancers with a background of intestinal metaplasia were studied by microsatellite assay at nine loci. Altered metaplastic mucosa was microdissected, reconstructed topographically, and examined immunohistochemically with an anti-p53 antibody, comparing its positive area with foci of microsatellite instability in each individual.

Results—Alterations at one or more loci were observed in seven of 15 cancers (46.7%) and four of 15 intestinal metaplasias (26.7%). Two cases of replication error positive phenotype had no microsatellite alterations in their metaplastic mucosa. All the microsatellite alterations in the metaplastic mucosa were restricted to incomplete-type intestinal metaplasia around the respective cancers. Moreover, in one case, an identical pattern of microsatellite alteration was detected in the cancer tissue and in the adjacent metaplastic mucosa, suggesting the sequential development of gastric cancer from intestinal metaplasia. Frequent alteration was found at the locus D1S191 (1q), indicating that this locus might be altered early in the development of intestinal-type gastric cancer. No significant association between microsatellite instability and p53 immunoreactivity was observed in the cases examined.

Conclusion—These results indicate that microsatellite instability may be an early event in stomach carcinogenesis, especially in intestinal-type cancers.

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Keywords: microsatellite instability; replication error; intestinal metaplasia; gastric cancer

First Department of Pathology, Hiroshima University School of Medicine, Hiroshima, Japan
H Yokozaki
S Semba
W Yasui
E Tahara

Department of Surgery, Saga Medical School, Saga, Japan
T Hamamoto
S Yunotani
K Miyazaki

Correspondence to:
Professor Tahara, First Department of Pathology, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan.

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Gastric adenocarcinoma is classified histologically into two subtypes: well differentiated or intestinal-type, and poorly differentiated or diffuse-type.[1][2] Frequently, the former is accompanied by widespread intestinal metaplasia in the vicinity of the tumour, and is generally believed to arise via a multistage process, including chronic gastritis and intestinal metaplasia.[3] These morphological changes from normal gastric epithelium may occur sequentially as a result of exogenous and endogenous factors that cause genetic alterations. Several molecular genetic studies have revealed that some well differentiated types of gastric cancer may develop by a cumulative series of gene changes similar to those that occur in colorectal cancer.[4] In fact, in addition to cancer tissue, some gastric intestinal metaplasias show inactivation of the p53[5] and the APC genes,[6] and reactivation of telomerase,[7] indicating that metaplastic glands might be one of the precancerous lesions of the stomach. Moreover, we have detected microsatellite instability in one in three intestinal metaplasias of the stomach.[8]

Mutations in mismatch repair genes, such as hMSH2, hMLH1, hPMS1, hPMS2, and GTBP, responsible for maintaining the fidelity of DNA replication increase spontaneous mutation rates greatly.[9-12] Microsatellites are simple oligonucleotide repeat units, distributed randomly and widely throughout the genome. The appearances of additions or deletions within the microsatellites are known as replication errors or microsatellite instability, and are believed to reflect derangement of the mismatch repair system.[13][14] Such genomic instability at microsatellite loci has been observed not only in hereditary non-polyposis colorectal cancer (HNPCC) associated tumours but also in certain sporadic cancers.[15-17] As for gastric cancer, microsatellite instability has been reported with frequencies ranging from 18% to 38%.[16][18-21]

However, little is known about genomic instability in precancerous lesions of the stomach. To elucidate the role of genetic instability in the development of intestinal-type gastric cancer, we conducted a microsatellite assay on both resected intestinal-type sporadic gastric cancer tissues and the surrounding intestinal metaplastic mucosa. In addition, as microsatellite markers are useful as clonal markers, clonality of the somatic mutation within individual metaplastic mucosas and cancer tissues was also analysed by microdissection and topographical mapping.

Methods

PATIENTS AND TISSUE SAMPLES
Fifteen gastric cancers and corresponding samples of intestinal metaplasia were studied. All samples were obtained from surgery at Hiroshima University Hospital and Hiroshima Memorial Hospital. None of the patients met the Amsterdam criteria for HNPCC[22] according to their family history collected from a review of medical records. Either non-

Table 1   Results of microsatellite assay in gastric cancers and intestinal metaplasia

Case	Intestinal metaplasia	Microsatellite loci								
		D1S191	D7S486	D11S29	TP53	D17S855	BAT-RII	BAT-25	BAT-26	BAT-40
538	Complete	+[T]	+[T]	+[T]	—	—	+[T]	+[T]	—	+[T]
805	Complete	+[T]	NI	—	NI	ND	—	+[T]	+[T]	—
804	Complete	+[T]	—	—	ND	—	—	—	—	—
662	Complete	ND	—	—	ND	ND	—	—	—	—
753	Complete	—	—	—	NI	—	—	—	—	—
336	Complete	ND	LOH[M]	ND	—	NI	—	—	—	—
834	Incomplete	+[TM]	ND	—	LOH[TM]	—	—	—	—	—
545	Incomplete	+[TM]	—	—	LOH[T]	—	—	—	—	—
742	Incomplete	+[TM]	—	—	LOH[T]	—	—	—	—	—
969	Incomplete	—	—	—	+[TM]	ND	—	—	—	—
784	Incomplete	—	ND	—	ND	—	—	—	—	—
337	Incomplete	—	ND	—	NI	—	—	—	—	—
368	Incomplete	—	ND	—	—	—	—	—	—	—
665	Incomplete	—	—	—	—	—	—	—	—	—
312	Incomplete	—	NI	—	—	—	—	—	—	—

+, microsatellite altered; —, microsatellite not altered; LOH, loss of heterozygosity; NI, not informative; ND, not determined; T, detected in tumour; M, detected in intestinal metaplasia; TM, detected in both tumour and intestinal metaplasia.

metaplastic normal mucosa or non-metastatic regional lymph node of the stomach was taken as a source of constitutive normal DNA. Tissues were fixed in 10% buffered formalin and embedded in paraffin. All tumours were intestinal-type gastric cancers comprising eight well differentiated and seven moderately differentiated tubular adenocarcinomas. Among these 15 subjects, 13 had single early cancers, one had single advanced cancer (extending into the muscularis propria), and one had double early cancers. Of the non-neoplastic mucosa, six revealed complete-type intestinal metaplasia and nine had incomplete-type intestinal metaplasia. The definitions of histological classification, stage grouping, and depth of tumour invasion were according to those of the Japanese Research Society for Gastric Cancer.[23] Intestinal metaplasia was classified by the morphological definition of Ming.[24] Complete-type intestinal metaplasia is characterised by the presence of goblet cells, absorptive-type enterocytes, and Paneth cells, and incomplete-type intestinal metaplasia is characterised by the loss of Paneth cells, with mucous columnar cells in place of enterocytes.

DNA EXTRACTION

Haematoxylin and eosin stained serial 10 μm sections of cancer tissues and non-neoplastic mucosa were dissected finely under the microscope. After deparaffinisation, DNA was extracted as described previously.[8]

MICROSATELLITE ANALYSIS

Nine microsatellite loci containing $(CA)_n$ dinucleotide or poly $(A)_n$ repeat sequences were analysed in this study. Markers for $(CA)_n$ repeat were D1S191, D7S486, D11S29, TP53 and D17S855 (Research Genetics, Huntsville,

Alabama, USA) on chromosomes 1, 7, 11, and 17; markers for poly $(A)_n$ tract were BAT-RII, BAT-25, BAT-26, and BAT-40[25] on chromosomes 1, 2, and 4. The extracted DNA was amplified by polymerase chain reaction (PCR) using the same conditions as described previously.[26] After 35–40 cycles of PCR with $[r^{32}P]$ ATP labelled primer, the aliquots were fractionated on 6% polyacrylamide/8 M urea/32% formamide gels. Following electrophoresis, the gels were fixed briefly in 10% methanol/10% acetic acid, dried, and exposed at −80°C to *x* ray film. Tumours and metaplasias were judged as microsatellite instability positive if they demonstrated alterations in the size of the microsatellite sequences, compared with the product from the matched normal tissue. All markers for unstable cases were repeated for PCR and gel electrophoresis to confirm the results.

IMMUNOHISTOCHEMISTRY

A monoclonal antibody to p53 (DO7) was purchased from Novocastra (Newcastle upon Tyne, UK). A modification of the immunoglobulin enzyme bridge technique was used, as described previously.[27] Deparaffinised and rehydrated sections were heated by microwave oven for 10 minutes in citrate buffer to retrieve antigen and were treated with anti-p53 antibody (diluted 1/1000) at 4°C for eight hours. The immunoreactivity in the tissues was graded from − to +++, according to the number of cells stained and the intensity of the reaction in individual cells. Grades were defined as follows: −, almost no positive cells; +, 10–30% of tumour/metaplastic cells showing weak to moderate immunoreactivity; ++, 30–60% of tumour/metaplastic cells showing moderate immunoreactivity and/or 10–30% of tumour/metaplastic cells showing intense immunoreactivity; +++, > 60% of tumour/metaplastic cells showing intense immunoreactivity.

Results

MICROSATELLITE ALTERATIONS IN INTESTINAL-TYPE GASTRIC CANCERS

The results of the microsatellite assay in gastric cancers and intestinal metaplasia are

Table 2   Frequency of microsatellite instability in gastric cancer and intestinal metaplasia

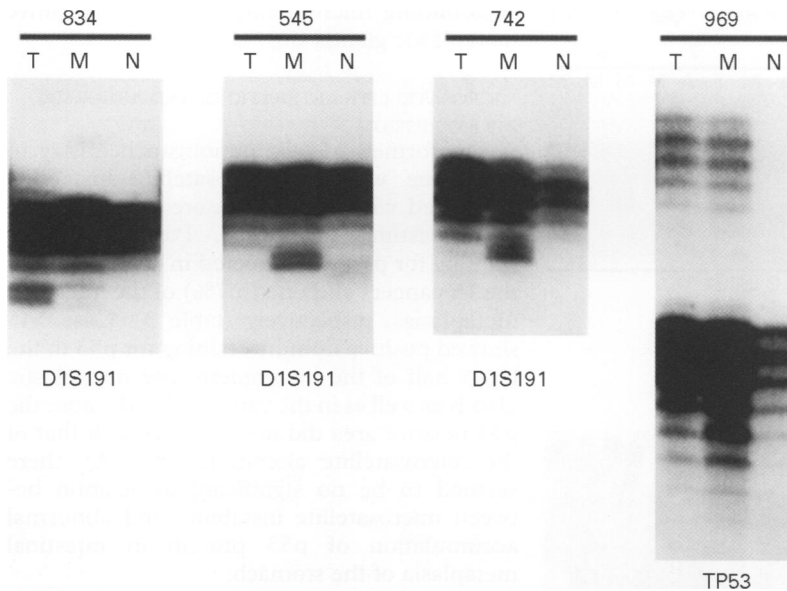|	Cases (n)	MI at single locus	MI at multiple loci	MI at least one locus
Cancer	15	5 (33.3%)	2 (13.3%)	7 (46.7%)
Intestinal metaplasia	15	4 (26.7%)	0	4 (26.7%)
Complete	9	0	0	0
Incomplete	6	4	0	4

Figure 1   Microsatellite alterations detected in intestinal metaplasia of the stomach at loci D1S191 and TP53. Cases are numbered according to table 1. T, tumour; M, intestinal metaplasia; N, normal mucosa. Both the tumours and intestinal metaplasia from these four cases (834, 545, 742, and 969) displayed altered alleles at respective loci.
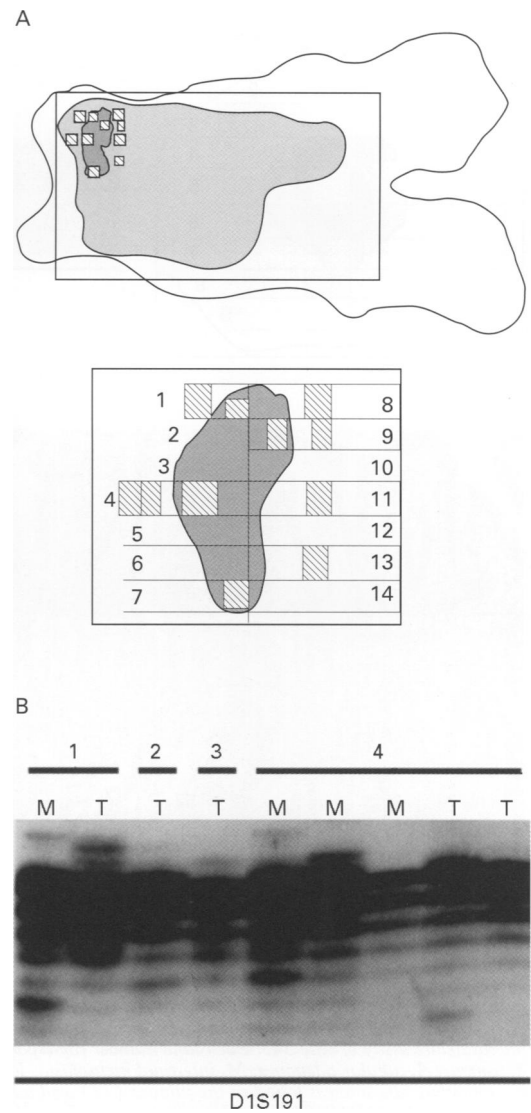

Figure 2   (A) Topographical distribution of a microsatellite instability positive lesions in case 834. The top panel is an illustration of a resected stomach and the bottom panel is a magnification of the lesions around the tumour. The square frame in the top panel indicates the area examined by the microsatellite assay. A total of 80 samples was analysed. Specimen numbers are indicated. Light grey, intestinal metaplasia; dark grey, tumour; cross-hatch, microsatellite instability altered lesion. (B) A representative autoradiograph of a microsatellite assay of case 834. Each lane number corresponds to the specimen number. T, tumour; M, intestinal metaplasia. A different pattern of microsatellite alteration is seen in the field of cancer (1T and 4T) compared with that seen in the adjacent metaplastic mucosa (1M and 4M) at the locus D1S191.

summarised in tables 1 and 2. Seven of 15 (46.7%) tumour specimens demonstrated altered mobility of alleles at one or more loci. Out of the seven microsatellite instability positive tumours, two tumour specimens (cases 805 and 538) exhibited microsatellite instability at more than three loci. Among them, case 538 displayed one base pair insertion at the BAT-RII locus. They were interpreted as replication error positive phenotypes, although obtained from non-HNPCC individuals. Their samples were microdissected and re-examined by microsatellite assay at altered loci. All the altered alleles in cancer tissue demonstrated the same pattern of alteration in each individual (data not shown). No significant difference in the incidence of microsatellite instability was found between $(CA)_n$ repeat markers and poly $(A)_n$ tract markers. Interestingly, frequent alteration (six of 15) was detected at the locus D1S191 on the long arm of chromosome 1. Loss of heterozygosity was observed infrequently at several loci, but was not regarded as microsatellite instability.

MICROSATELLITE ALTERATIONS IN INTESTINAL METAPLASIA OF THE STOMACH
Four of 15 intestinal metaplasias (26.7%) revealed microsatellite instability at a single locus, as well as in corresponding cancer tissue (fig 1). All four were incomplete-type intestinal metaplasia lacking Paneth cells (table 2). On the other hand, intestinal metaplasias of two cases with sporadic replication error positive phenotypes in the cancer tissues did not harbour alteration at any microsatellite loci, which was confirmed using microdissecting samples of case 538 (data not shown).

TOPOGRAPHICAL DISTRIBUTION OF MICROSATELLITE ALTERATIONS IN INTESTINAL METAPLASIA OF THE STOMACH
The topographical distributions of microsatellite alteration in microsatellite instability positive metaplasia was investigated. Distributions of microsatellite instability were determined using microdissected samples. Representative cases of microsatellite instability positive metaplasia are shown in figs 2 and 3. A mapping study demonstrated that all the microsatellite alterations in metaplastic mucosa were restricted to small areas, all of which were adjacent to respective cancers (fig 2A). Analysing the pattern of altered alleles, three cases had different alleles with altered microsatellites
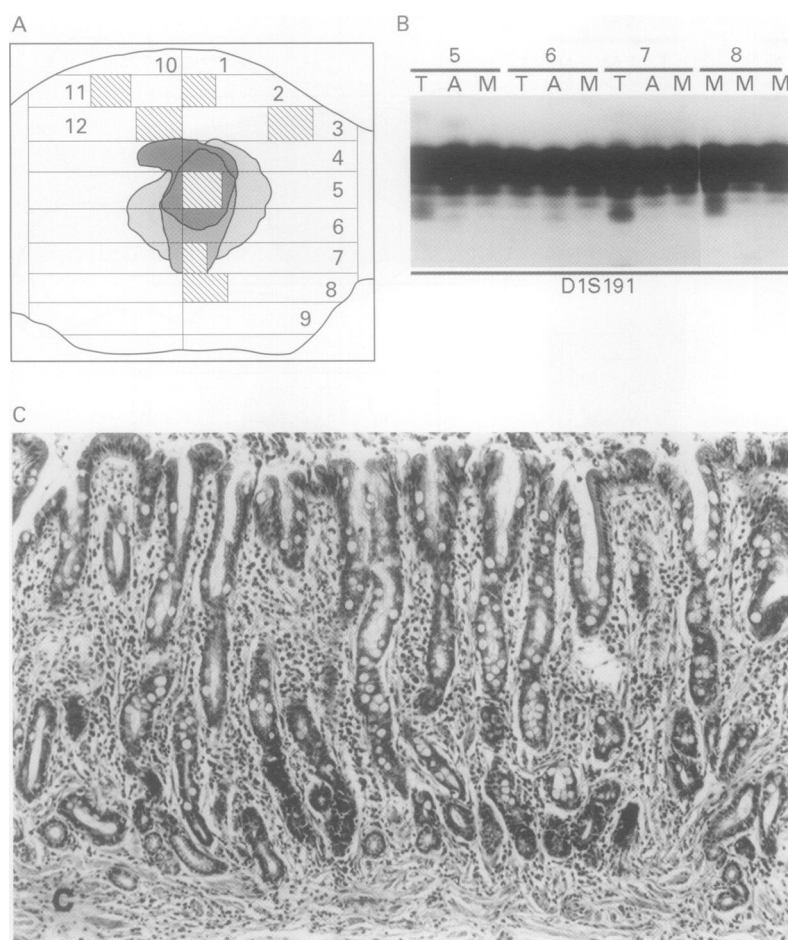
Figure 3   (A) Topographical distribution of microsatellite instability and p53 positive lesions in case 545. A total of 47 samples was analysed. Specimen numbers are indicated. Medium grey, tumour; light grey, tubular adenoma; cross-hatch, microsatellite instability altered lesion; dark grey, p53 positive lesion. (B) A representative autoradiograph of a microsatellite assay of case 545. Each lane number corresponds to the specimen number. T, tumour; A, tubular adenoma; M, intestinal metaplasia. Identical and sequential microsatellite alteration is found in the tumour (7T) and adjacent intestinal metaplasia (8M) at the locus D1S191. (C) Microphotograph of microsatellite instability positive metaplasia, specimen 8 from case 545.

Table 3   Microsatellite instability and p53 status on the cases of gastric cancers with intestinal metaplasia

| | Microsatellite instability | | p53 immunoreactivity | |
Case	Tumour	Intestinal metaplasia	Tumour	Intestinal metaplasia
538	+	—	+	—
805	+	—	—	—
804	+	—	++	—
662	—	—	—	—
763	—	—	—	—
336	—	—	—	—
834	+	+	—	—
545	+	+	++	+
742	+	+	+++	—
969	+	+	—	—
784	—	—	—	—
337	—	—	++	—
368	—	—	—	—
665	—	—	++	—
312	—	—	—	—

in the field of cancer tissue and adjacent metaplastic mucosa (fig 2B). Interestingly, in case 545, identical and sequential microsatellite alteration was observed in both the cancer tissue and adjacent intestinal metaplasia at the locus D1S191 (figs 3A and B). Histologically, most of the altered epithelium demonstrated intestinal metaplasia with chronic inflammation and mild atypia compared with the surrounding microsatellite instability negative metaplastic glands (fig 3C).

MICROSATELLITE ALTERATIONS AND ABNORMAL P53 EXPRESSION

We performed p53 immunohistochemistry to determine whether microsatellite instability correlated with p53 immunoreactivity in gastric intestinal metaplasia. Positive nuclear staining for p53 was detected in six (40.0%) of the 15 cancers and one (6.7%) of the intestinal metaplasias, respectively (table 3). Case 545 showed positive immunostaining for p53 in the lower half of the incomplete-type metaplastic glands as well as in the cancer cells. Because the p53 positive area did not coincide with that of the microsatellite alterations (fig 3A), there seemed to be no significant association between microsatellite instability and abnormal accumulation of p53 protein in intestinal metaplasia of the stomach.

Discussion

In this series of intestinal-type gastric cancers, we found microsatellite instability in seven of 15 cancers (46.7%) in at least one locus after examining nine loci; this frequency is slightly higher than that reported previously.[8 21] Two cases with microsatellite instability at more than three loci were regarded as replication error positive cancers because widespread microsatellite instability was comparable to the typical microsatellite instability described in HNPCC associated tumours and represented defects in the mismatch repair system.[28] In addition, one of them had an alteration of 10 base pair poly $(A)_n$ tract of the transforming growth factor β type II receptor, which is a critical target of inactivation in mismatch repair deficient tumours.[25 29]

The main purpose of the present study was to clarify the existence of microsatellite instability in intestinal metaplasia, which is implicated in stomach carcinogenesis. We detected microsatellite alterations in four of 15 foci of metaplasia (26.7%) at a single locus, indicating that irreversible genetic changes exist in metaplastic glands of the stomach.

In addition, we investigated microsatellite instability in intestinal metaplasia of the stomach by analysing its topographical distribution and allelic pattern. First, microsatellite instability positive metaplasias were separated into small foci, but all of them were restricted to adjacent areas of the respective cancers. Also, the majority of altered alleles exhibited heterogeneous patterns, suggesting that they might not have a common clonal origin. Interestingly, in case 545, an identical and sequential alteration pattern was observed in the cancer tissue as well as in the adjacent metaplastic mucosa, indicating single clonal origin and sequential tumour development from intestinal metaplasia. From the histological point of view, microsatellite instability positive metaplasias of four cases were incomplete-type with regenerative atypia and chronic inflammation.

Our observations suggest that microsatellite instability may occur multiclonally within certain fields of the gastric epithelium showing

incomplete-type intestinal metaplasia, and some of the gastric cancers may develop from intestinal metaplasia. Therefore, microsatellite instability may represent an early genetic alteration in the development of intestinal-type gastric cancer. Moreover, p53 mutations have been detected in incomplete-type intestinal metaplasia.[30] Taken together, incomplete-type metaplastic glands of the stomach can be viewed as genetically unstable.

What is more interesting is the altered number of microsatellite loci in intestinal metaplasia. All microsatellite instabilities in intestinal metaplasia were detected at a single locus. On the other hand, we could not detect microsatellite instability in intestinal metaplasia surrounding two replication error positive phenotype gastric cancers. Gleeson *et al* described two types of genomic instability; widespread microsatellite instability and low level microsatellite instability, based on the mutated number of microsatellite markers tested and such low level instability may reflect the inherent instability at microsatellite markers.[28] Microsatellite instability in intestinal metaplasias may reflect the inherent instability at each microsatellite locus. Seruca *et al* found that most replication error positive gastric cancers displayed abundant T lymphocyte infiltration.[31] Gastric epithelium with continuous inflammation might have been exposed to a variety of carcinogens that could facilitate increased mutagenesis, leading to the low level microsatellite instability. In the present study, frequent instability in both intestinal-type cancer and intestinal metaplasia of the stomach was observed at D1S191 on chromosome 1q. It is likely that this locus is a specific fragile site that may be linked with stomach carcinogenesis.

However, it is still unclear whether microsatellite instability plays a pivotal role in stomach carcinogenesis, because most microsatellite instability occurs in non-coding DNA, that is, within introns or intergenic regions of the genome. On the other hand, alterations in the p53 gene are observed in over 60% of gastric cancer cases, regardless of histological type. Moreover, they take place even in the mucosal cancers and in intestinal metaplasia.[32] [33] Among 15 intestinal metaplasias of the stomach, we observed positive nuclear staining for p53 in only one case (case 545), which revealed microsatellite instability at different metaplastic mucosa. However, it was difficult to distinguish morphologically between microsatellite instability positive metaplastic glands and p53 positive metaplastic glands. Therefore, there seems to be no apparent association between microsatellite instability and p53 alteration.

In conclusion, our results demonstrate that microsatellite instability occurs frequently in the incomplete type of intestinal metaplasia, a condition that can be viewed as a precancerous lesion of the stomach. Furthermore, it is suggested that microsatellite alterations could be useful as a biomarker for the clinical evaluation of the malignant potential of gastric non-neoplastic mucosa.

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