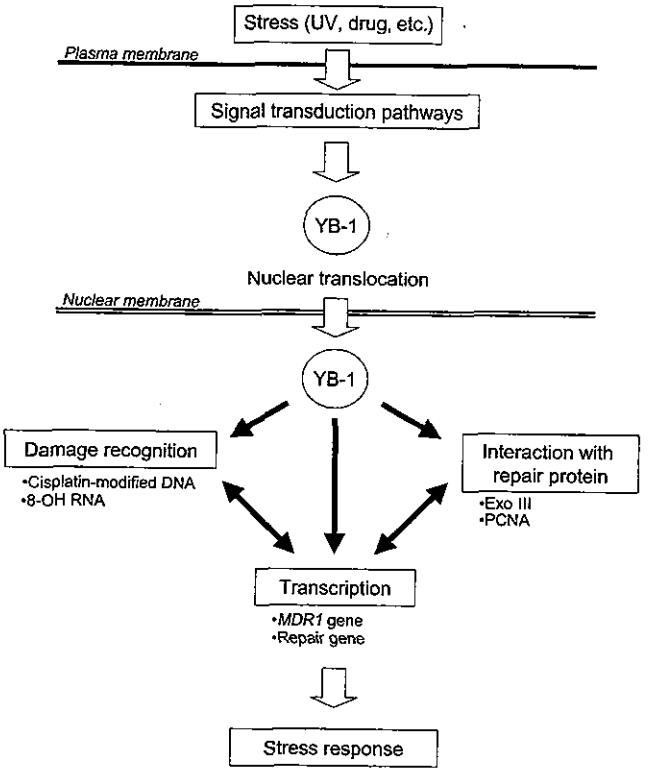

Figure 3. Schematic summary of MDR mediated by PGP or non-PGP. YB-1 is normally present in the cytoplasm but is translocated to the nucleus by treatment with anticancer agents, hyperthermia, or UV light irradiation. YB-1 in the nucleus functions as a transcription factor, which can bind to the Y-box and transactivate promoters, such as the *MDR1* gene or repair genes. By contrast, YB-1 can bind directly to cisplatin-modified DNA and interact with repair proteins including NTH1 (*Exo III*) and proliferating cell nuclear antigen (*PCNA*). These functions might be advantageous for the acquisition of drug resistance.

that are exposed to anticancer and other cytotoxic DNA-damaging agents (Fig. 3). In one response pathway to environmental stimuli, YB-1 is translocated to the nucleus and up-regulates *MDR1* gene expression through binding to the Y-box on the promoter. Alternatively, YB-1 might operate its DNA repair pathway through interactions with p53 (71), proliferating cell nuclear antigen (72), and other molecules (77) when DNA is damaged (Fig. 3). Further research is needed to fully understand the role of YB-1 in cancer and drug resistance.

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Microsatellite instability and hMLH1 and hMSH2 expression analysis in soft tissue sarcomas

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Abstract. Alterations of the size of microsatellite DNA sequences, namely microsatellite instability (MSI), have been demonstrated in some types of malignancies. We analyzed the MSI of five microsatellite markers in 40 cases of soft tissue sarcoma (STS) using high resolution fluorescent microsatellite analysis. In addition, we examined the expression of hMLH1 and hMSH2 proteins of DNA mismatch repair (MMR) genes by immunohistochemistry, and promoter methylation of the *hMLH1* gene by methylation-specific PCR (MSP). MSI was recognized in 10 of 40 STS cases (25%), which consisted of 2 MSH-high (MSI-H) tumors and 8 MSI-low (MSI-L) tumors. A loss of hMLH1 expression was recognized in 7 of 40 STS cases (18%), and loss of hMSH2 expression was recognized in 3 of 40 STS cases (8%). One case showed a loss of both hMLH1 and hMSH2 expression. Promoter hypermethylation of the *hMLH1* gene was detected in only 3 of 40 STS cases (8%). Of 10 cases with MSI, 5 (50%) showed a loss of hMLH1 and/or hMSH2 expression. There was a statistically significant correlation between MSI-positive tumors and the loss of hMLH1 and/or hMSH2 expression (p=0.0286). Although the frequency of MSI (25%) or a loss of hMLH1 and/or hMSH2 expression (23%) was relatively low in STS cases, a loss of hMLH1 and/or hMSH2 was recognized in 5 out of 10 MSI-positive cases (50%). These findings suggest that the inactivation of *MMR* gene expression might be the cause of MSI in STS cases.

Introduction

Microsatellite instability (MSI) comprises length mutations in tandem oligonucleotide repeats that occur in a large subset of human tumors (1-3). This type of mutation is believed to be caused by altered DNA mismatch repair (MMR) (1-6). Although several human MMR genes have been implicated in the predisposition to cancer, the *hMSH2* and *hMLH1* genes are the ones most often defective in MSI cancer cells (4-8). Defective DNA mismatch repair is most commonly associated with the functional loss of *hMLH1* and *hMSH2* genes and results in the mutator phenotype characterized by MSI. Some investigators have shown a close association between the presence of MSI and reduced expression of hMLH1 and/or hMSH2 protein by immunohistochemistry in some types of malignancies (4,9,10). Furthermore, in MSI-positive sporadic colorectal and endometrial tumors, hypermethylation of the *hMLH1* gene promoter is extremely frequent and often accompanied by down-regulation of the expression of these genes (4-6). Although some investigators have examined MSI in soft tissue sarcomas (11-16), the frequencies of MSI and the correlation between MSI and altered MMR expression or epigenetic alteration of *hMLH1* gene remain unknown in soft tissue sarcomas (STSs).

In this study, we examined MSI of 5 microsatellite markers in 40 STS cases using high resolution fluorescent microsatellite analysis. In addition, we performed an immunohistochemical analysis for the 2 MMR genes, *hMLH1* and *hMSH2* and examined the correlation between the presence of MSI and MMR expression or promoter methylation of *hMLH1* by MSP in the same series.

Materials and methods

Snap-frozen tumor samples and corresponding normal tissues from 40 cases of STS were obtained from the collection of soft-tissue tumors registered in the Department of Anatomic Pathology, Pathological Sciences, Graduate School of Medical Sciences, Kyushu University, Japan. Diagnosis in each of the 40 cases was based on light microscopic examinations with hematoxylin and eosin staining of paraffin blocks. Immunohistochemical analysis, using conventional markers of differentiation routinely used in the diagnosis of soft-tissue neoplasms, was performed in almost all of the cases. Histologic diagnosis of the 40 cases of STS in this study revealed 12 cases of liposarcoma (5 cases of well-differentiated lipoma-like type, 6 of myxoid type, and 1 of pleomorphic

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type), 11 cases of malignant fibrous histiocytoma (MFH) (9 cases of storiform-pleomorphic type and 2 of myxoid type), 4 cases each of leiomyosarcoma (LMS) and malignant peripheral nerve sheath tumor (MPNST), 2 cases each of fibrosarcoma and low-grade fibromyxoid sarcoma, and 1 case each of alveolar soft part sarcoma (ASPS), rhabdomyosarcoma, solitary fibrous tumor, extraskeletal mesenchymal chondrosarcoma, and extraskeletal myxoid chondrosarcoma (Table I).

Immunohistochemical analyses were performed for the 40 STS cases for which formalin-fixed, paraffin-embedded materials were available. In addition, genomic DNA was extracted using standard proteinase K digestion and phenol/chloroform extraction methods.

Immunohistochemistry. Immunohistochemical analysis was performed using mouse IgG monoclonal antibodies against hMLH1 (G168-728, 1:100; BD Biosciences, USA) and hMSH2 (1:100, Oncogene Science, New York, NY, USA). Histologic sections (n=4) of 4-μm thickness were cut, mounted on glass slides coated with 3-aminopropyltriethoxysilane, and air-dried overnight at room temperature. The sections were deparaffinized in xylene and dehydrated in ethanol. After dehydration, the endogenous peroxidase was blocked by methanol containing 3% H_2O_2 for 30 min. For staining with the above antibody, specimens were pretreated with citrate buffer (0.01 mol/l citric acid: pH 6.0) 4 times, with each pretreatment consisting of 5 min at 100°C in a microwave oven. Sections were incubated with the primary antibody at 4°C overnight, followed by staining with a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan). Finally, the sections were reacted in a 3,3' diaminobenzidine, peroxytrichloride substrate solution, counterstained with hematoxylin, and mounted. Tumors were considered to demonstrate inactivation of hMLH1 or hMSH2 when there was complete absence of detectable nuclear staining in neoplastic cells (14,17). Intact nuclear staining of adjacent normal tissue or lymphocytes served as an internal positive control.

Bisulfite modification and methylation-specific PCR for hMLH1. Bisulfite modification was performed using a DNA modification kit (Intergen) according to the manufacturer's protocol. The modified DNA was used for the methylation-specific PCR. The sequences of primers and PCR conditions are given in a previous report (18). DNA of normal skeletal muscle was used as a negative control. Each PCR product (8 μl) was directly loaded onto 2% agarose gels, stained with ethidium bromide, and directly visualized under UV illumination.

MSI assay. For microsatellite analysis, five dinucleotide microsatellite markers (D2S123, D5S107, D10S197, D11S904, and D13S175) were selected according to previous reports on the detection of MSI (16,19,20). The primer sequences were as described previously (16). The 5' PCR primers were labeled with 6-carboxyfluorescein (6-FAM) or 4, 7, 2', 4', 5', 7'-hexachloro-6-carboxyfluorescein (HEX). Size markers were labeled with 6-carboxy-X-rhodamine (ROX). DNA derived from tumor was amplified with a 6-FAM labeled 5' primer and a cold 3' primer, while DNA from normal tissue was amplified with a HEX labeled 5' primer and 3' primer using a thermocycler (Tgradient, Biometra, Germany). A 50 μl reaction mixture contained 100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 2.5 mM dNTP, 2.5 U Taq DNA polymerase (Perking-Elmer, Norwalk, CT), 2 μM of each primer, and 25 ng of genomic DNA. The thermal conditions were as follows: one cycle at 95°C for 5 min; 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec; and a final cycle at 72°C for 10 min. One microliter of 6-FAM-labeled product, 1 μl of HEX-labeled product, and 0.5 μl of

Table I. Clinicopathologic parameters in 40 cases of STSs.

Age (years)		
≥60		14
<60		26
Sex		
Male		16
Female		24
Location		
Thigh		23
Buttock		6
Lower leg		3
Shoulder		2
Knee		2
Chest wall		2
Upper arm		1
Finger		1
Histologic subtype		
Liposarcoma		12
Well-differentiated lipoma like type	5	
Myxoid/round cell type	6	
Pleomorphic type	1	
Malignant fibrous histiocytoma		11
Storiform pleomorphic type	9	
Myxoid type	2	
Leiomyosarcoma		4
Malignant peripheral nerve sheath tumor		4
Fibrosarcoma		2
Low-grade fibromyxoid sarcoma		2
Alveolar soft part sarcoma		1
Rhabdomyosarcoma		1
Solitary fibrous tumor		1
Extraskeletal mesenchymal chondrosarcoma		1
Extraskeletal myxoid chondrosarcoma		1
Size (cm)		
≥10		15
<10		25
Tumor type		
Primary		28
Recurrent		12

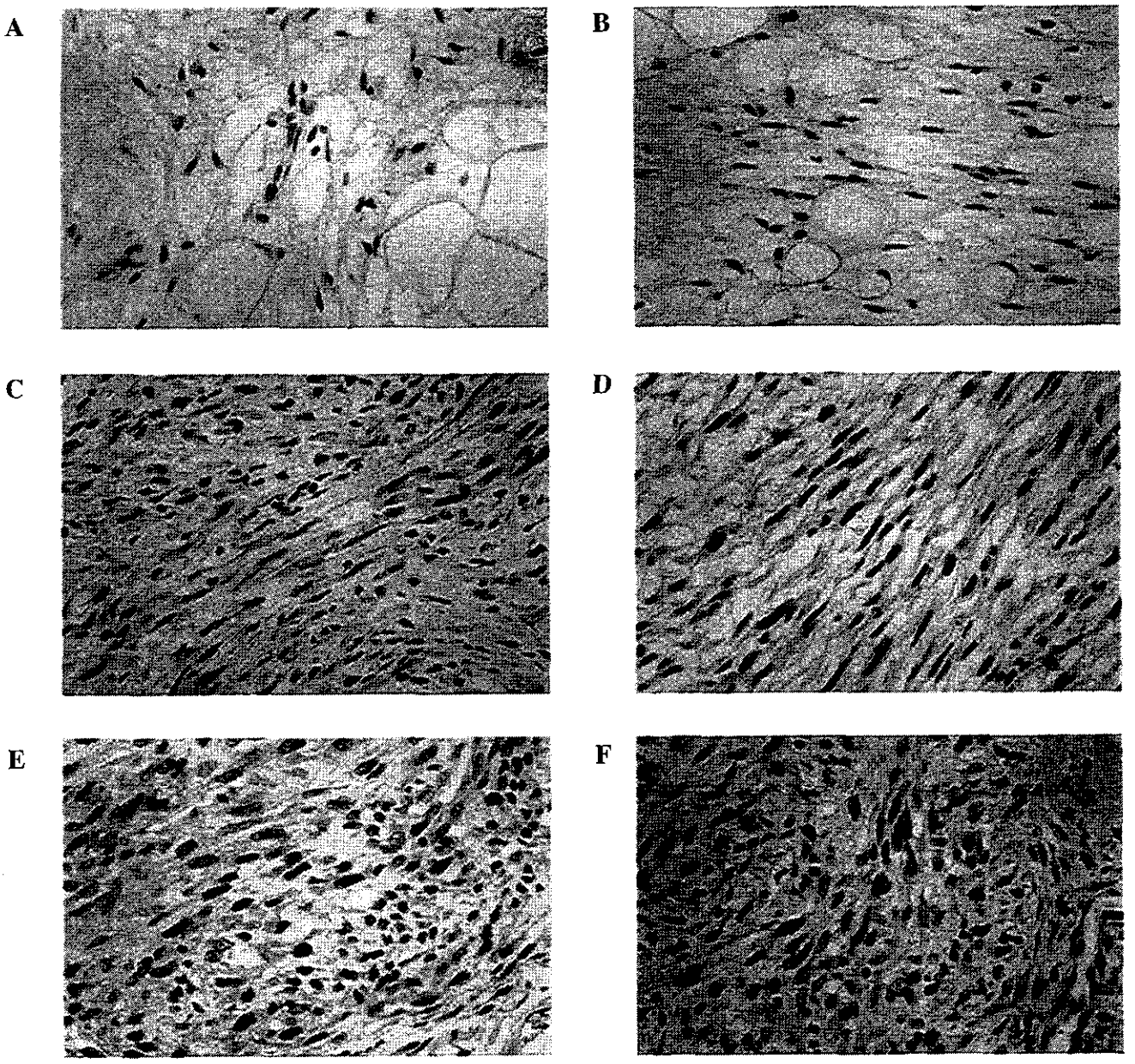

Figure 1. Immunohistochemical staining for hMLH1 or hMSH2 protein. (A) There was a loss of *hMLH1* expression in the majority of tumor cells (x400), and (B) hMSH2 expression also was not observed in the majority of tumor cells (x400), (case 7, myxoid/round cell liposarcoma). This case showed MSI-H. (C) A loss of hMLH1 expression was observed (x400), however, (D) hMSH2 expression was diffusely recognized in tumor cells (x400) (case 38, LMS). This case showed MSI-L. (E) Both hMLH1 and (F) hMSH2 expression were observed in the majority of tumor cells (x400) (case 33, MFH). This case showed MSS.

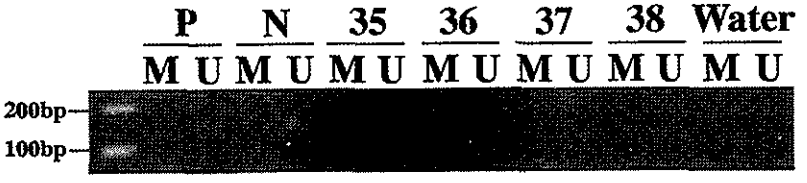

Figure 2. Methylation status of the promoter region of the *hMLH1* gene by MSP. PCR products were amplified by unmethylated (U) and methylated (M) specific primers. Case 28 shows hypermethylation of the *hMLH1* gene promoter.

ROX-labeled size marker were mixed with 22.5 µl tumor suppressor reagent (Applied Biosystems, Foster City, CA). After denaturization, the mixture was electrophoresed in the same lane using an ABI Prism 310 sequencer. The data were processed using ABI software, GeneScan. With regard to the evaluation of microsatellite status, cases showing an additional peak in the tumor DNA compared with their respective normal sample were regarded as 'microsatellite

Table II. Microsatellite instability status in STSs.

Case	Sex	Age	Diagnosis	Location	Size (cm)	MSI status	hMLH expression	hMSH expression	*hMLH1* methylation
7	F	53	Myxoid/round cell liposarcoma	Thigh	12	MSI-H	D	D	-
14	F	64	Leiomyosarcoma	Thigh	6	MSI-L	P	P	-
20	F	74	MFH	Thigh	8	MSI-L	P	P	-
28	F	27	MPNST	Lower leg	15	MSI-H	P	P	Methylation
32	F	52	MFH	Thigh	8	MSI-L	D	P	-
34	M	38	MFH	Knee	7	MSI-L	P	P	-
35	M	84	Leiomyosarcoma	Thigh	16	MSI-L	D	P	Methylation
36	M	42	MFH	Thigh	10	MSI-L	P	P	-
38	F	84	Leiomyosarcoma	Thigh	5	MSI-L	D	P	-
39	F	58	MFH	Buttock	2	MSI-L	D	P	-

MFH, malignant fibrous histiocytoma; MPNST, malignant peripheral nerve sheath tumor; P, preserved; D, decreased.

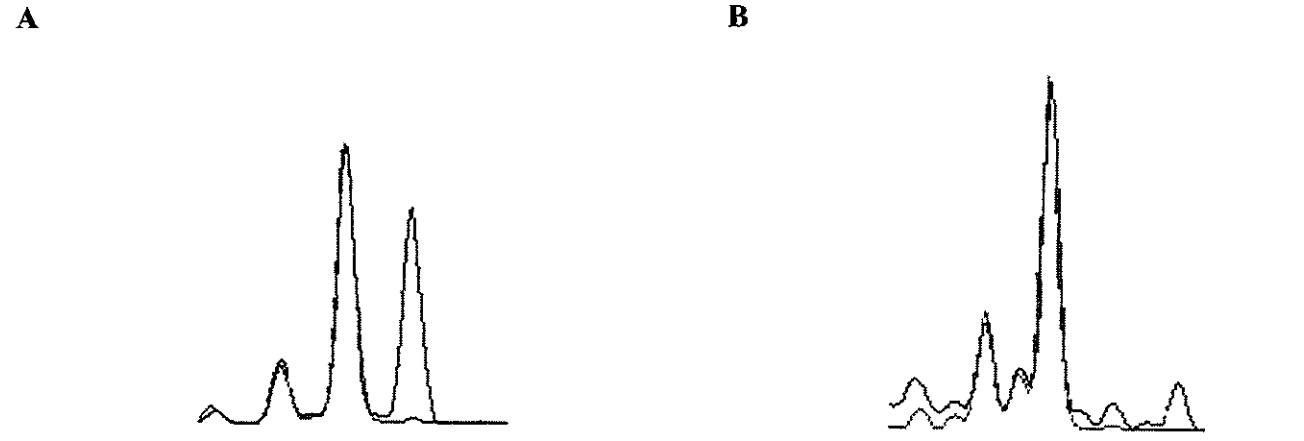

Figure 3. (A) MSI-positivity for the D13S175 microsatellite marker in case 7 (green, DNA derived from normal tissue; red, DNA derived from tumor tissue). (B) MSI-positivity for the D2S123 microsatellite marker in case 32 (green, DNA derived from normal tissue; red, DNA derived from tumor tissue).

unstable' for a given marker. When the percentage of markers having an additional peak among markers that could be analyzed (%MSI) was >40%, the cases were regarded as 'MSI-high' (MSI-H). The cases whose microsatellite was stable at all of the successfully examined markers were regarded as 'microsatellite stable' (MSS). The remaining cases were regarded as 'MSI-low' (MSI-L).

Statistical analysis. Fisher's exact test was used to evaluate the association between two dichotomous variables. A p-value of <0.05 was considered to indicate statistical significance.

Results

Clinical and histological findings. Clinicopathologic characteristics of the 40 cases are summarized in Table I. The age of the patients ranged from 13 to 84 years (average, 52.4 years) and consisted of 16 male and 24 female patients. Among them, 23 patients were affected in the thigh, 6 in the buttock, 3 in the lower leg, 2 each in the shoulder, knee, and chest wall, and 1 each in the upper arm and finger. Tumor size was available for 40 cases, and ranged from 2 to 22 cm (average, 9.3 cm). The tumors were primary in 28 cases and recurrent in 12 cases.

Immunohistochemical analysis of hMLH1 and hMSH2. A loss of hMLH1 expression was recognized in 7 of 40 STS cases (18%) (Fig. 1). These 7 cases consisted of 3 cases of MFH, and 2 cases of leiomyosarcoma, and 1 case each of myxoid/round cell liposarcoma and low-grade fibromyxoid sarcoma. In contrast, a loss of hMSH2 expression was detected in 3 of 40 cases (8%), or 1 case each of MFH, MPNST, and myxoid/round cell liposarcoma. One case, that of myxoid/round cell liposarcoma, showed a loss of both hMLH1 and hMSH2 expression. In the current study, a loss of hMLH1 and/or hMSH2 expression was identified in 9 out of 40 STS cases (23%). The loss of hMLH1 and/or hMSH2 expression had no statistically significant correlation with the clinicopathological findings (data not shown).

Promoter hypermethylation of the hMLH1 gene. MSP revealed promoter hypermethylation of the *hMLH1* gene in 3 out of 40 STS cases (8%) (Fig. 2). Both unmethylated and methylated signals were seen in all 3 of these cases, which

Table III. Correlation between MSI and MMR expression (n=40).

	hMLH1 and/or hMSH2 IHC		
	Decreased	Preserved	p-value
MSI-positive	5	5	0.0286[a]
MSS	4	26	

IHC, immunohistochemistry, [a]Statistically significant.

were histologically shown to consist of 2 LMS cases and 1 MPNST case. Of the 3 cases with promoter hypermethylation, 1 showed a loss of hMLH1 expression.

Microsatellite instability analysis. MSI was observed in 10 of the 40 STS cases (25%) (Table II and Fig. 3). Among them, 8 were MSI-L and 2 were MSI-H. The 2 cases with MSI-H were MPNST and myxoid/round cell liposarcoma. A loss of hMLH1 and/or hMSH2 expression was recognized in 5 of 10 MSI-positive cases (50%). There was a statistically significant correlation between the presence of MSI and a loss of hMLH1 and/or hMSH2 expression (p=0.0286) (Table III). Two cases with MSI showed promoter hypermethylation of the *hMLH1* gene. There was no statistically significant correlation between the presence of MSI and clinicopathologic parameters (data not shown).

Discussion

Microsatellites are short oligonucleotide repeats that disperse throughout the human genome and exhibit length polymorphism. MSI is characterized by small deletions or expansions within short tandem repeats in tumor DNA as compared with matching normal DNA. MSI has been shown to be a signal of defective DNA mismatch repair in several types of tumors. Several studies have described the presence of MSI in bone and soft tissue sarcomas (11-16). In the present study, we analyzed 5 microsatellite loci and detected MSI in STSs using a dual fluorescence co-electrophoresis system (20) in which the samples derived from both tumor and normal tissues were electrophoresed concurrently in order to minimize migration errors and perform a precise and objective assessment. Our results showed that MSI was present in 10 of 40 STS cases (25%), which consisted of 2 MSI-H tumors and 8 MSI-L tumors, and these findings were in agreement with previous studies that reported a low frequency of MSI in STS (11-15). The patterns of microsatellite changes observed in various human cancers can be classified into 2 subtypes: i) Those showing relatively small changes within 6 base pairs (type A) and ii) those exhibiting drastic changes over 8 base pairs (type B) (21-23). In the current study, 9 cases were type A, and the remaining one was type B.

The human DNA repair system plays an important role in reducing mutation and maintaining genomic stability. Defective DNA mismatch repair is most commonly associated with the functional loss of *hMLH1* and *hMSH2* genes and results in the mutator phenotype characterized by MSI. Previous studies on certain malignancies have shown a high correlation between the presence of MSI and the loss of hMLH1 and/or hMSH2 expression, as determined by immunohistochemistry (4,9,10). In sarcomas, some previous studies have demonstrated a loss of hMLH1 or hMSH2 expression, with the frequency of hMLH1 loss ranging from 12-25% and that for hMSH2 loss ranging from 0-16% (24,25). In the current study, although the frequencies of the loss of hMLH1 (18%) or hMSH2 (8%) expression were relatively low, a loss of hMLH1 and/or hMSH2 expression was observed in 5 of 10 MSI-positive cases (50%); on the other hand, such a loss was observed in only 5 of 30 MSS cases (17%). This result indicates that the inactivation of MMR genes, particularly hMLH1 and hMSH2, may play an important role in the origin of MSI in STSs. In other MSI-positive cases without a loss of hMLH1 and/or hMSH2, it is still necessary to assess whether there is altered expression in other MMR genes, such as *hMSH3*, *hMSH6*, or *hPMS2*.

Some investigators have reported an association between reduced expression of the MMR proteins and either advanced tumor stage or tumor invasiveness (26,27). In STS patients, Taubert *et al* described that reduced expression of the hMSH2 protein by Western blot analysis was closely associated with poor prognosis (24). In our study, the loss of hMLH1 and/or hMSH2 expression was not associated with clinicopathologic parameters of tumor aggressiveness in STS cases, such as tumor type (primary versus recurrent) or tumor size. Further detailed analysis is necessary to determine the potential role of MMR genes in STS.

hMLH1 promoter methylation, with associated reduced protein expression, has been described in some malignancies displaying a high level of MSI (4-6). On the other hand, this association has not been observed in MSS tumors (28,29). Some studies have reported that up to 90% of sporadic MSI-positive colorectal carcinomas are due to inactivation of the *hMLH1* gene, principally via transcriptional silencing, and the remainder are consistent with inactivation of *hMLH1* and *hMSH2* by somatic mutation (8,30,31). In bone and soft tissue sarcomas, no *hMLH1* promoter methylation has been detected by MSP (14,32). In our study, promoter methylation of *hMLH1* was only detected in 3 of 40 STS cases (8%), and in only 1 of 7 cases with a loss of hMLH1 expression. As for mechanisms of the inactivation of *hMLH1*, other molecular alterations, such as somatic mutations in *hMLH1*, should be examined in a future study. In addition, methylation of CpG in other specific regions of the *hMLH1* promoter, reported in recent studies (33), may be associated with the silencing of its expression. In 2 cases with promoter methylation showing preserved hMLH1 expression, *hMLH1* methylation could be monoallelic or less intense. Although further detailed analyses are necessary, the results of this study support the hypothesis that promoter methylation of the *hMLH1* gene might play a minor role in MSI in STSs.

In conclusion, we analyzed 40 STS cases and detected MSI in 10 of them (25%). Although the frequency of the loss of hMLH1 and/or hMSH2 expression was relatively low, a loss of hMLH1 and/or hMSH2 expression was recognized in 5 of 10 MSI-positive cases (50%), indicating that inactivation of the *MMR* gene might be the origin of MSI in STS cases. Promoter methylation of *hMLH1* was a rare event in STSs (8%), and thus other possible mechanisms of inactivation of the *hMLH1* gene should also be considered.

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Multiple gastric carcinoids and endocrine cell micronests in type A gastritis: Nuclear morphometric and immunohistochemical analysis

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Abstract. While the hyperplasia-neoplasia sequence of enterochromaffin-like (ECL) cells has been proposed in the pathogenesis of type I gastric carcinoids, the criteria for distinction between hyperplastic endocrine cell micronest (ECM) and neoplastic ECM have not been established. The aims of this study were to clarify differences between the hyperplasia and neoplasia of ECL cells and determine the optimal classification system for gastric ECL cell proliferations in type A gastritis. Endocrine cell lesions (n=531) from 8 surgically-resected stomachs with type A gastritis were reclassified as either atrophic ECM (n=333), hyperplasic ECM (n=168), neoplastic ECM (all ECM ≥0.1 mm in size, n=15), or typical carcinoid (n=15). Hematoxylin and eosin-stained sections were semiautomatically analyzed by nuclear morphometry. Immunohistochemical expression of bcl-2, p53 and Ki-67 was also investigated. As the histologic grade of histology advanced, the morphometric values of area, circumference and largest diameter of the nuclei significantly increased ($p<0.0005$), while the frequency of diffuse expression of bcl-2 significantly decreased ($p<0.0001$). Significant differences were also observed in all morphometric parameters and in bcl-2 positivity between the hyperplasic ECM and neoplastic ECM group. There was no expression of p53 in any of the lesions. The Ki-67 index did not differ between the neoplastic ECM and typical carcinoid groups. These results suggest that our system of classification for gastric endocrine cell proliferations in type A gastritis is appropriate. Nuclear morphometry and bcl-2 immunoexpression are useful parameters for the distinction of neoplastic ECMs from hyperplastic ECMs.

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Key words: gastric carcinoid, type A gastritis, endocrine cell micronest, nuclear morphometry, bcl-2, Ki-67, p53

Introduction

Gastric carcinoid is a rare neoplasm, which mainly develops from enterochromaffin-like (ECL) cells in the corpus mucosa (1). The current clinicopathologic classification proposed by Rindi *et al* (2-4) identified three subtypes of ECL cell-derived carcinoid tumors of the stomach. Among them, type I gastric carcinoid is the most common type, which develops in atrophic gastritis type A and is accompanied by multiple endocrine cell micronests (ECMs). Type A gastritis is characterized by mucosal atrophy of the corpus associated with some autoimmune processes (5,6). The fundic mucosal atrophy causes achlorhydria or hypochlorhydria, and brings about hyperplasia of antral gastrin-producing cells (G-cell hyperplasia) and subsequent hypergastrinemia. Hypergastrinemia induces the proliferation of ECL cells by a trophic effect, which ultimately results in the development of carcinoid tumors (7-12). Thus, the hyperplasia-neoplasia sequence of ECL cells has been used as a provisional pathogenesis of type I gastric carcinoids (7,8,13). To date, however, few authors have reported detailed histopathologic analysis of a large number of ECL cell lesions of type A gastritis on the basis of the hyperplasia-neoplasia sequence (7,8,13).

While Solcia *et al* (8) proposed a standard classification of gastric ECL cell proliferations (simple or diffuse hyperplasia, linear hyperplasia, micronodular hyperplasia, adenomatoid hyperplasia, dysplasia, and neoplasia), these lesions are usually classified as either atrophic ECM, hyperplastic ECM, or neoplasia (carcinoid) in Japan (7). However, the criteria for a clear distinction between hyperplastic ECM and neoplastic ECM (microcarcinoid) have not yet been fully established.

The aims of our current study were to determine the optimal classification system for gastric ECL cell proliferation, and clarify the differences between the hyperplasia and neoplasia of ECL cells in type A gastritis. In this study, we performed extensive histopathologic analyses on gastrectomy specimens from type A gastritis patients with multiple carcinoids. All detectable ECMs and carcinoids were reclassified, and then evaluated by means of nuclear morphometry with a semiautomatic image analyzer. In addition, these lesions were assessed immunohistochemically with

Table I. Clinicopathologic findings in the 8 cases of type A gastritis.

Case	Age/ Gender	Anemia	Serum gastrin (ng/ml)	Achlorhydria	No. of endocrine cell lesions				Size of largest lesion (cm)	Depth of largest lesion
					A	B	C	D		
1	66/M	-	2600	+	26	12	1	1	0.3	MM
2	61/F	IDA	373	NT	14	13	0	2	1.5	SM
3	59/M	-	785	NT	112	28	2	1	0.4	SM
4	51/F	-	2000	NT	28	6	3	3	0.8	SM
5	42/F	IDA	NT	NT	77	12	2	2	0.7	SM
6	39/M	PA	22955	+	28	10	1	1	0.4	M
7	38/F	PA	1048	+	36	58	3	3	1.0	SM
8	33/F	-	NT	NT	12	29	3	2	0.8	SM

PA, pernicious anemia; IDA, iron-deficiency anemia; NT, not tested. Normal level of serum gastrin, 40-200 (ng/ml). A, atrophic ECM; B, hyperplastic ECM; C, neoplastic ECM; D, typical carcinoid; M, mucosa; MM, muscularis mucosae; SM, submucosa.

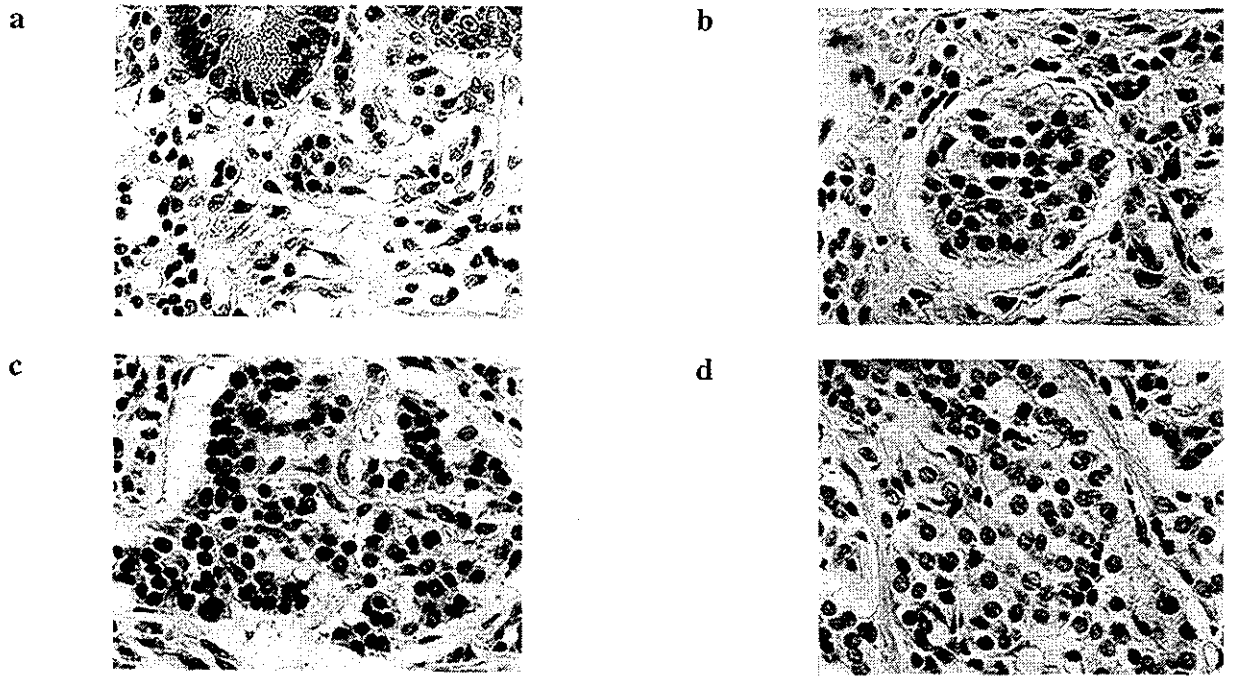

Figure 1. Light microscopic features of the lesions associated with ECL cells. (a) Atrophic ECM, which is composed of ≤10 endocrine cells; (b) hyper-plastic ECM, which is composed of >10 endocrine cells and <0.1 mm in diameter; (c) neoplastic ECM (microcarcinoid), which ranged from 0.1 to 0.5 mm in diameter; and (d) typical carcinoid, which measured >0.5 mm in diameter. (a-d) H&E stain, magnification x680).

proliferation marker Ki-67 and apoptosis-related oncoproteins bcl-2 and p53.

Materials and methods

Subjects. Surgically-resected stomachs (n=8) diagnosed as having carcinoid tumors with type A gastritis (type I) at the authors' institution from 1985 to 2001 were retrospectively examined. Cases of sporadic gastric carcinoid (type III) and endocrine cell carcinoma were excluded. There were no cases of type II carcinoid associated with multiple endocrine neoplasia type 1 or Zollinger-Ellison syndrome. The clinical features of the patients are summarized in Table I. There were 3 males and 5 females ranging in age from 33 to 66 years (mean 49 years). Of them, 2 patients (cases 6 and 7) were complicated by pernicious anemia with parietal cell antibody, and 2 other cases (cases 2 and 5) exhibited iron-deficiency anemia. Hypergastrinemia was detected in all 6 patients (cases 1-4, 6 and 7) in whom serum gastrin levels were examined preoperatively, and 3 of 6 patients had achlorhydria. There were 7 patients that underwent total gastrectomy (cases 1-4, 6 and 7), and the remaining one underwent partial fundic resection (case 5). Survival data were available for all patients except for case 2. All patients were alive without recurrence of carcinoid during the follow-up period ranging from 12 to 197 months (mean 104 months).

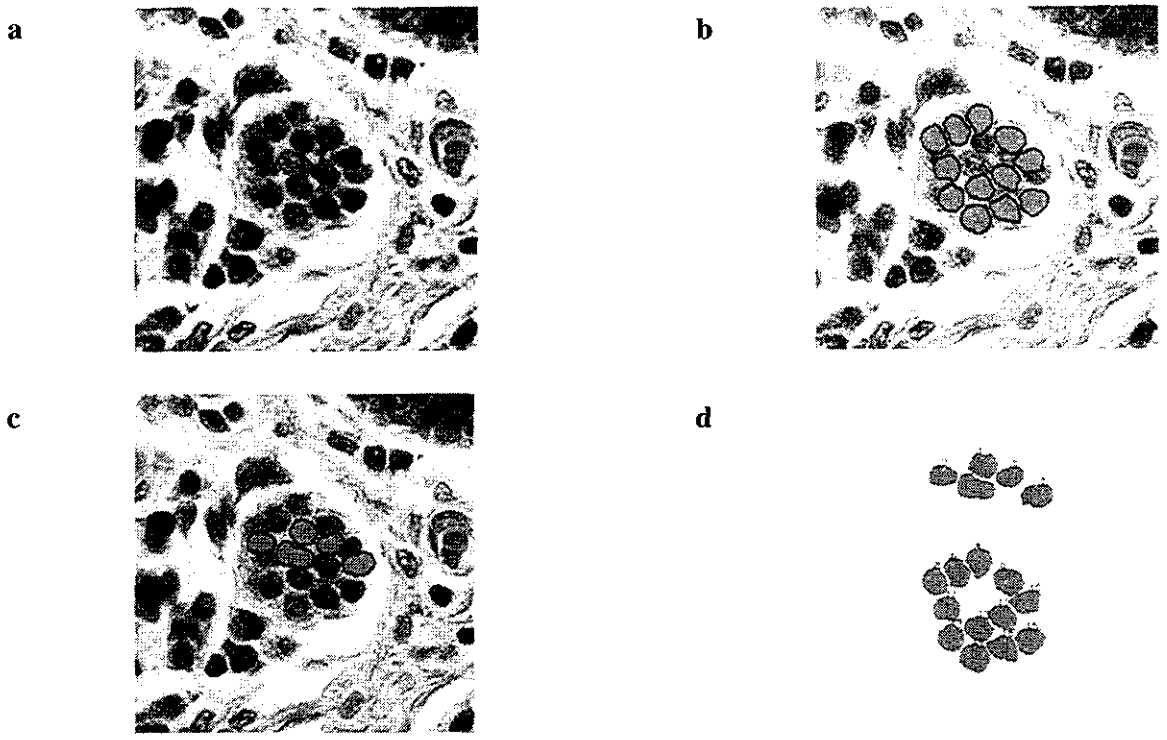

Figure 2. (a) Case 1, the H&E-stained section visualized by light microscopy at an original magnification of x400. A nest comprising 17 endocrine cells can be observed. (b) The complete outlines of the 12 nuclei are traced over the Photoshop file. (c) The remaining 5 nuclei are also traced. (d) Traced molds were exported to the image analysis processor, Mac Scope. Morphometric parameters studied were nuclear area, circumference and largest diameter.

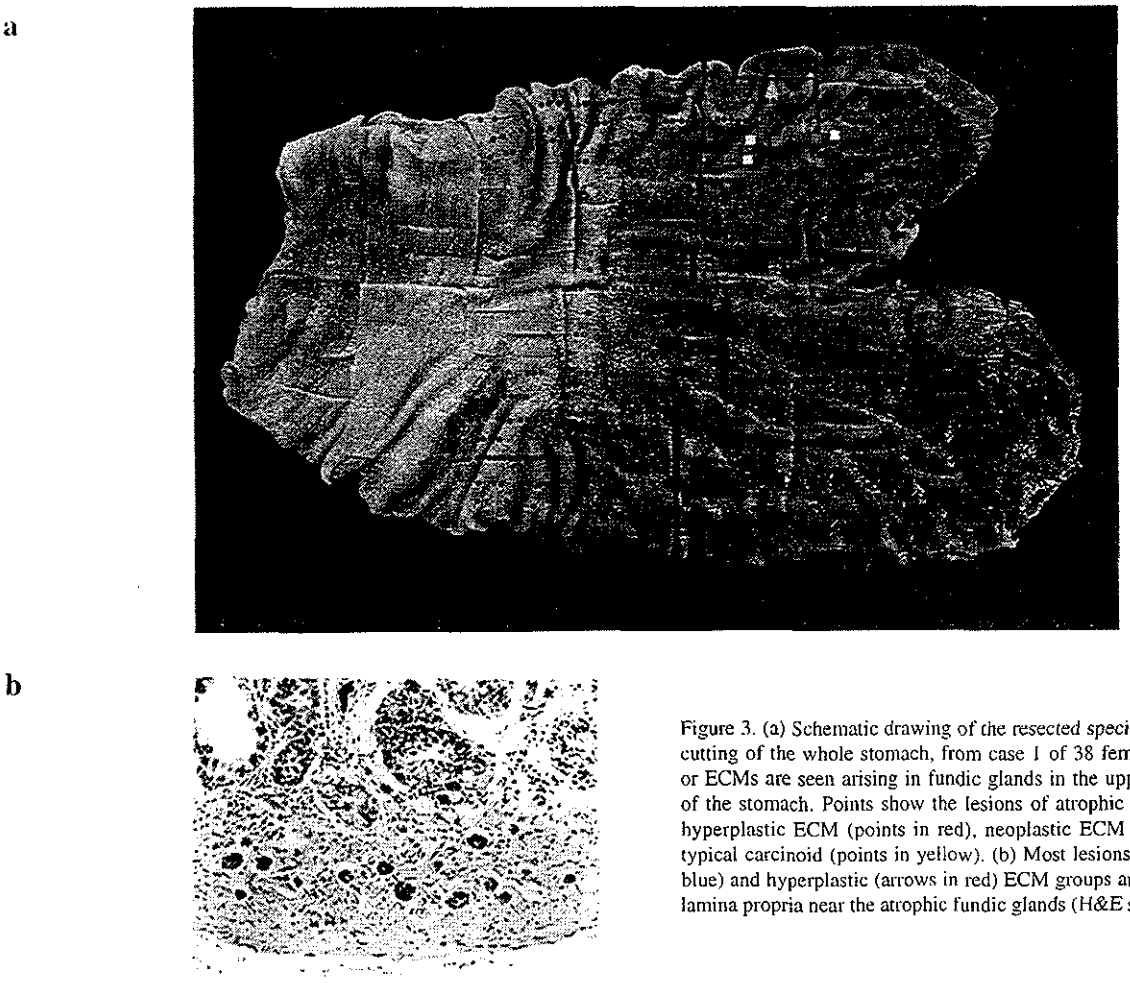

Figure 3. (a) Schematic drawing of the resected specimen, obtained by step-cutting of the whole stomach, from case 1 of 38 females; carcinoid tumors or ECMs are seen arising in fundic glands in the upper and middle portion of the stomach. Points show the lesions of atrophic ECM (points in blue), hyperplastic ECM (points in red), neoplastic ECM (points in green), and typical carcinoid (points in yellow). (b) Most lesions in atrophic (arrows in blue) and hyperplastic (arrows in red) ECM groups are observed in the deep lamina propria near the atrophic fundic glands (H&E stain, x71).

Histologic assessment. All 8 resected stomachs were opened along the greater curvature and fixed in 10% formalin, and the whole stomachs were then cut in 4-5 mm-wide serial sections. All of the cut specimens were embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin (H&E), followed by immunohistochemical staining. We examined all sections under a light microscope and selected 6 sections per stomach to evaluate in detail.

Based on a modification of the previous definition by Itsuno *et al* (7) and Solcia *et al* (8), all detectable ECMs and carcinoids were classified into the following 4 groups: 1) atrophic ECM, which was composed of ≤10 endocrine cells, and regarded to be consistent with 'pseudohypreplasia' by Solcia *et al* (8) (Fig. 1a); 2) hyperplastic ECM, which was composed of ≥10 endocrine cells and <0.1 mm in diameter, and regarded to be 'micronodular and adenomatoid hyperplasia' (8) (Fig. 1b); 3) neoplastic ECM (microcarcinoid), which ranged from 0.1 to 0.5 mm in diameter, and was consistent with the Solcia 'dysplasia' (8) (Fig. 1c); 4) typical carcinoid, which measured >0.5 mm in diameter, as defined by Solcia *et al* (8) (Fig. 1d). In the present study, intraglandular hyperplasia of ECL cells ('simple or diffuse hyperplasia' and 'linear hyperplasia' by the Solcia classification) was not evaluated.

Immunohistochemical analysis. Immunohistochemical staining was carried out on formalin-fixed paraffin-embedded sections by the streptavidin-biotin-peroxidase complex method using a Histofine SAB-PO Kit (Nichirei, Tokyo, Japan). The primary antibodies applied included monoclonal antibodies against Ki-67 (MIB-1, Immunotech, Marseille, France; diluted 1:100), bcl-2 (clone 124, Dako, Glostrup, Denmark; diluted 1:1000), p53 (Pab1801, Oncogene Science, Uniondale, NY; diluted 1:100) and a polyclonal antibody raised against chromogranin A (Dako; diluted 1:1500). After deparaffinization, the sections were dehydrated and blocked for endogenous peroxidase using 0.3% hydrogen peroxide. For antigen retrieval, the sections for Ki-67, bcl-2 and p53 were pretreated in a microwave oven at 99°C for 20 min in citrate buffer (pH 6.0) for Ki-67, 99°C for 15 min in citrate buffer (pH 6.0) for bcl-2, and 100°C for 10 min in phosphate-buffered saline for p53. Sections for chromogranin A were heated in sodium citrate buffer (pH 6.0) for 30 min. After incubation with 10% normal rabbit (for Ki-67, bcl-2 and p53) or goat (for chromogranin A) serum for 10 min, the sections were incubated with a primary antibody overnight at 4°C, followed by secondary biotinylated antibody for 20 min, and then a streptavidin-biotin-peroxidase complex reagent for 20 min at room temperature. Diaminobenzidine tetrahydrochloride was used as the chromogen and Mayer's hematoxylin was used for counterstaining. The Ki-67 labeling index in ECM and carcinoid was determined as the percentage of positively-labeled nuclei among the chromogranin A positive cells. Based on the proportion of cytoplasmic positive cells for bcl-2, the expression of bcl-2 was recorded as either negative (0-5%), sporadic (5-10%), focal (10-50%), or diffuse (≥50%).

Nuclear morphometry. Morphometric analyses was performed semiautomatically on H&E sections using a Macintosh G3 computer (Apple Computer Japan, Tokyo) with imaging

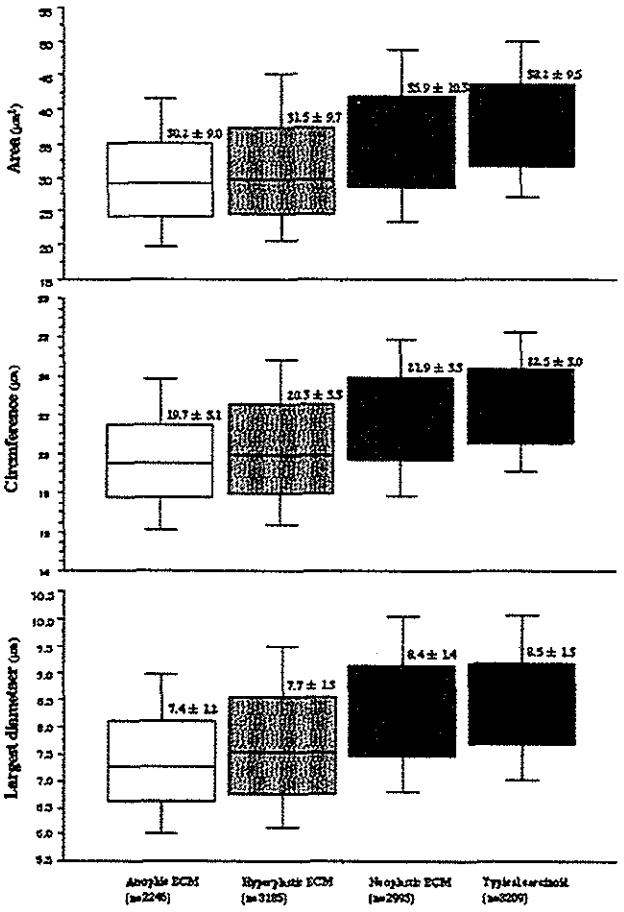

Figure 4. The comparison of nuclear morphometric data (area, circumference and largest diameter) between each group. Data are shown for all 8 cases.

software Photoshop 4.0 (Adobe Systems Co., Tokyo, Japan) and a Mac Scope image analysis processor (Apple Computer). The histologic images were photographed using a DMRXA light microscope (Leica, Tokyo, Japan) with an original magnification of x400, connected to a digital camera system Binary Planner 4490 (Kontron, GmbH, Germany), and saved as Photoshop files (Fig. 2a). The outlines of nuclei of the chromogranin A immunoreactive cells were traced over the Photoshop image, using a pen-type mouse FAVO (Wacom, Tokyo, Japan) attached to the computer. The nuclei that could be completely outlined were chosen (Fig. 2b and c). The area, circumference and length of major axes of all nuclei were measured using the scaler of the computer (Fig. 2d). In the atrophic ECM and hyperplastic ECM groups, all the appropriate endocrine cells were measured, while in the neoplastic ECM and typical carcinoid groups, the measurements were done in two randomly-selected fields in each lesion. The mean number of the measured nuclei was 207 (range, 171-230) per lesion in neoplastic ECM and typical carcinoid groups.

Statistical analysis. All statistical analyses were performed using Stat View 4.11 (Abacus Concepts, Inc., Berkeley, CA). Numeric data are expressed as mean ± SD unless stated otherwise. The Kruskal-Wallis or Mann-Whitney U test

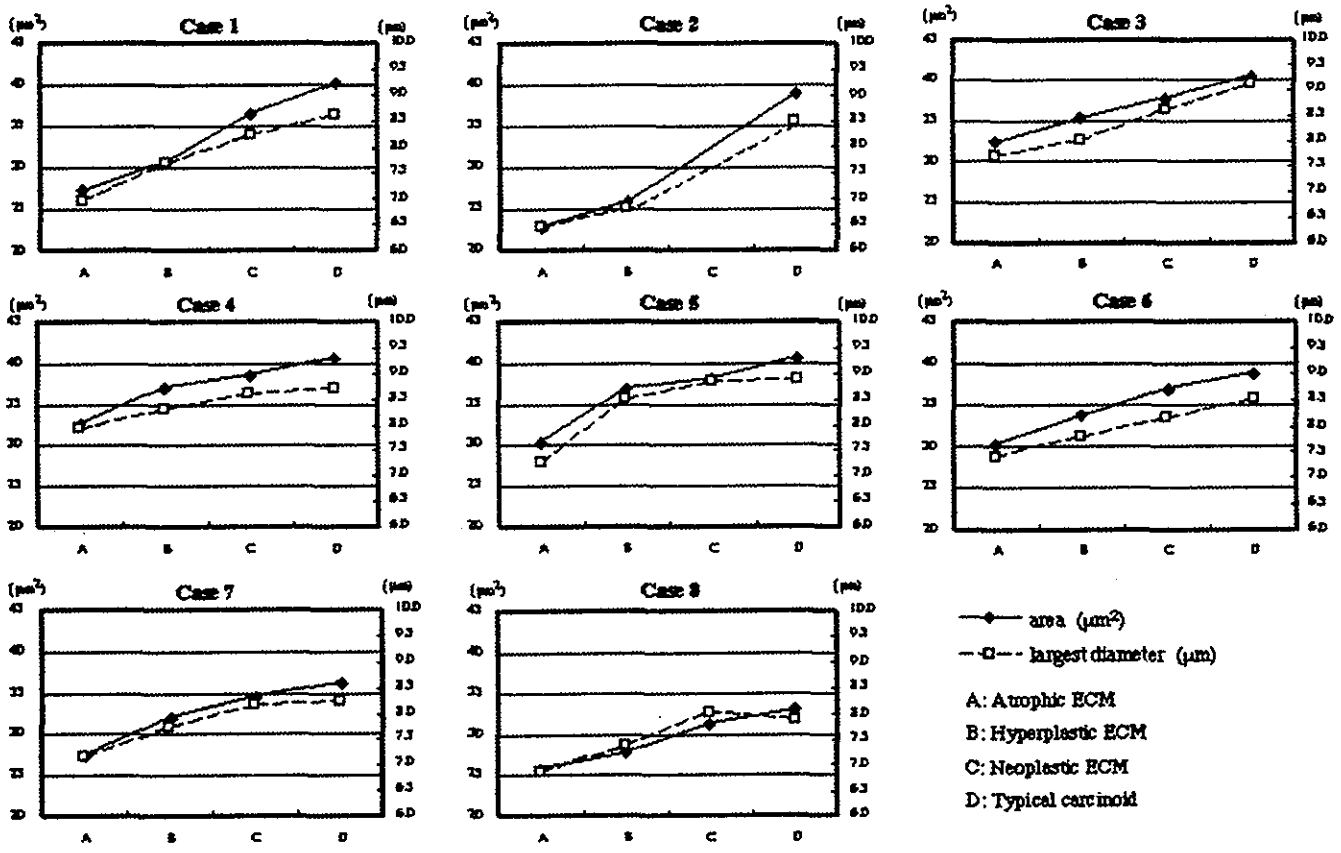

Figure 5. Nuclear morphometry studies in 8 individual cases. The average nuclear size (area and largest diameter) associated with each group increased in proportion to size of lesion.

Table II. Immunohistochemical expression of bcl-2 according to endocrine cell lesion type.

Group	No. of lesions	bcl-2 expression (%)				
		Negative	Sporadic	Focal	Diffuse	
Atrophic ECM	333	19 (6)	0 (0)	3 (1)	311 (93)	a
Hyperplastic ECM	168	28 (17)	10 (6)	12 (7)	118 (70)	b
Neoplastic ECM	15	7 (47)	3 (20)	3 (20)	2 (13)	c
Typical carcinoid	15	9 (60)	5 (33)	1 (7)	0 (0)	

[a]p<0.0001 vs. hyperplastic ECM; [b]p<0.0001 vs. neoplastic ECM. ECM, endocrine cell micronest; [c]NS, not significant.

was used to evaluate statistical differences among the histologic groups. Differences in immunoreactivity for bcl-2 among the groups were assessed by the χ^2 or Fisher's exact probability test. A statistically significant result was defined as a p-value <0.05.

Results

Histopathologic findings. In all of the specimens we examined from the 8 stomachs, a total of 531 lesions that consisted of endocrine cells (ECMs or carcinoids) were identified (Table I). The number of total lesions in each case ranged from 29 to 143 (mean, 66.4), and that of carcinoid tumors (neoplastic ECM plus typical carcinoid groups) ranged from 2 to 6 (mean, 3.8). The average numbers of lesions in the atrophic ECM and hyperplastic ECM groups were 37.5 (range, 12-112) and 25 (range, 6-55) per case, respectively. A representative image (case 7) showing distribution of ECMs and carcinoids is shown in Fig. 3a. In all cases, most lesions in the atrophic, hyperplastic and neoplastic ECM groups were observed in the deep lamina propria near the atrophic fundic glands at the upper and middle portions of the stomach (Fig. 3b). The size of the largest carcinoid in each case ranged from 0.3 to 1.5 cm (mean, 0.7 cm) in diameter.

Table III. Ki-67 index according to endocrine cell lesion type.

Group	No. of lesions	Ki-67 index per lesion	No. of cells	Ki-67 index total cells
Atrophic ECM	333	NT	2246	0.13
Hyperplastic ECM	168	NT	3185	0.47
Neoplastic ECM	15	0.9±1.4	2995	0.90[a]
Typical carcinoid	15	1.2±1.3	3209	1.25[a]

ECM, endocrine cell micronest; NT, not tested. [a]The Ki-67 index was not significantly different between the neoplastic ECM and typical carcinoid groups.

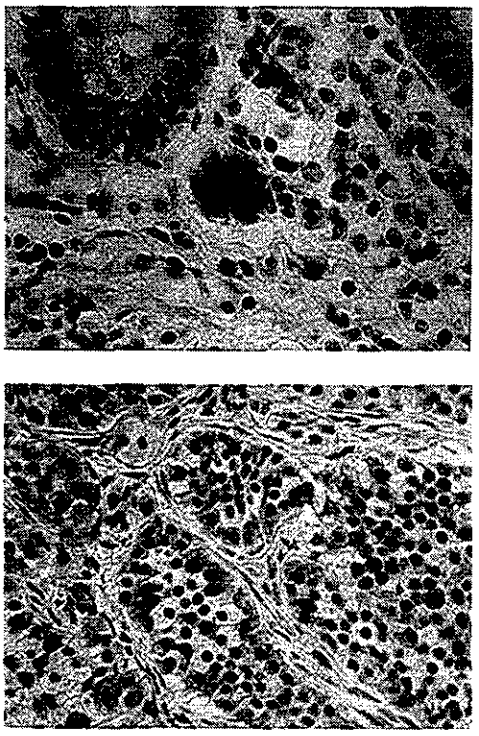

Figure 6. Investigation of bcl-2 immunoactive endocrine cell frequency. Diffusely-positive immunoactivity for bcl-2 protein (50%) was found in (a) lesions in the atrophic ECM group, and (b) lesions in the neoplastic ECM group.

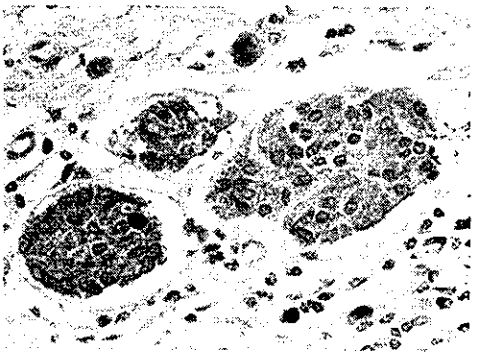

Figure 7. In the atrophic ECM and hyperplastic ECM groups, Ki-67 positive cells were observed in only 3 of 2246 (0.13%) endocrine cells and in 15 of 3185 (0.47%) cells, respectively.

In 2 cases (cases 1 and 6), all carcinoids were confined to the mucosa and muscularis mucosae, while the other 6 cases had carcinoid tumors invading the submucosa (cases 2-5, 7 and 8). Neither lymphatic or venous invasion, nor lymph node metastasis was observed in any of the cases.

Nuclear morphometric findings. Measurements were performed in 11635 cells from all 531 lesions. The nuclear areas of the cells ranged from 7.0 to 94.8 μm^2 (mean, 34.2 μm^2). The mean nuclear circumference and length of major axis were 21.2 μm (range, 8.9-44.9 μm) and 8.0 μm (range, 3.5-16.2 μm), respectively. All 3 parameters (area, circumference and maximum diameter) significantly increased in proportion to the advance in histologic grade (Kruskal-Wallis test, $p<0.0001$, Fig. 4). Significant differences were also found between hyperplastic and neoplastic ECM, between atrophic and hyperplastic ECM, and between neoplastic ECM and typical carcinoid (Mann-Whitney U test, $p<0.0005$). These trends were consistently observed in each of the 8 cases (Fig. 5).

Expression of bcl-2, p53 and Ki-67 labeling index. Results of immunohistochemistry for bcl-2 and Ki-67 in 531 lesions are shown in Tables II and III. The expression of p53 protein was not detected in any of the lesions.

Diffusely-positive immunoreactivity for bcl-2 protein was found in 311 of 333 (93%) lesions in the atrophic ECM group (Fig. 6a), 118 of 168 (70%) lesions in the hyperplastic ECM group, 2 of 15 (13%) lesions in the neoplastic ECM group (Fig. 6b), and 0 of 15 lesions in the typical carcinoid group (χ^2 test, $p<0.0001$). Significant differences were observed between the atrophic ECM and hyperplastic ECM groups (Fisher's exact probability test, $p<0.0001$), and between the hyperplastic ECM and neoplastic ECM groups (Fisher's exact probability test, $p<0.0001$), but no difference was found between the neoplastic ECM and typical carcinoid groups ($p=0.4642$).

In the atrophic ECM and hyperplastic ECM groups, Ki-67 positive cells were observed in only 3 of 2246 (0.13%) endocrine cells and in 15 of 3185 (0.47%) cells, respectively (Fig. 7). The mean Ki-67 labeling index was did not differ between the neoplastic ECM (0.9±0.6%) and typical carcinoid (1.2±0.5%) groups ($p=0.1300$). The indices in these 2 groups were higher than those in hyperplastic ECM group; however, statistical analyses could not be performed due to the small sample size.

Discussion

In the current study, we performed a detailed examination of 531 endocrine cell lesions, including carcinoids and ECMs from 8 surgically-resected stomachs, and reclassified them as atrophic ECM, hyperplastic ECM, neoplastic ECM (micro-carcinoid) or typical carcinoid according to the size of the lesions and the number of endocrine cells. We demonstrated nuclear morphometric and immunohistochemical differences among the 4 groups.

It has been suggested that the hyperplasia-neoplasia sequence is the main route for the development of type I gastric carcinoids (7,8,13). However, it is sometimes difficult to distinguish non-neoplastic ECM from microcarcinoid by

microscopic findings, since the definitive criteria for such a distinction have not been fully established. Solcia *et al* (8) described that ECM, which show infiltrative or solid growth >0.5 mm in diameter, should be regarded as carcinoid. This definition was consistent with our typical carcinoid group. The Solcia classification (8), however, also includes dysplasia, which was defined as enlarged ECM with a size between 150 μm and 0.5 mm. We believe that this category is ambiguous, and it would be better to include 'dysplasia' in our neoplastic ECM category.

On the other hand, Itsuno *et al* (7) determined the diameter of 0.1 mm to be a criterion that distinguishes neoplastic ECM from hyperplastic ECM. Their definition was based on the finding that most of the ECMs and distinct microcarcinoids >0.1 mm in diameter frequently infiltrated into the muscuaris mucosae or submucosa. The neoplastic ECM group in our study was consistent with the Itsuno criteria. We found significant differences in nuclear morphometric parameters, in bcl-2 immunoreactivity and in Ki-67 index (Tables II and III) between hyperplastic ECM and neoplastic ECM. In addition, both bcl-2 immunoreactivity and Ki-67 index did not differ between neoplastic ECM and typical carcinoid (Table III). These results support the validity of the Itsuno criteria for distinction between hyperplastic ECM and neoplastic ECM. However, we could not confirm as to whether these lesions in our 'neoplastic ECM group' actually show autonomous proliferation.

A morphometric analysis of the nuclear shape and size has been reported to be useful in predicting prognosis in various malignant neoplasms, such as breast cancer, prostate cancer, renal cell carcinoma, urinary bladder carcinoma, pancreatic tumor and rhabdomyosarcoma (14-21). A number of reports have concluded that malignant tumors with a large nuclear area show a significant potential for malignancy and a poor prognosis. As for gastric ECL proliferations, some investigators have conducted cellular morphometric analyses of the number, area and volume of non-neoplastic ECL cells (22-25). To our knowledge, however, there has only been a single study on nuclear morphometric findings with respect to the hyperplasia-neoplasia sequence of ECL cell proliferations (26).

In our nuclear morphometric study, all parameters significantly increased in proportion to the advance in the histologic grade of ECM (Fig. 4). This observation indicates that the nuclear size of endocrine cells closely correlated with the size of the lesion (ECMs or carcinoids). It is noteworthy that each parameter differed between the hyperplastic ECM group and neoplastic ECM group (Fig. 4). Thus, ECL cell lesions with large nuclei should be considered to be neoplastic rather than hyperplastic.

The bcl-2 proto-oncogene encoded protein enhances cell survival by inhibition of apoptosis (programmed cell death). Overexpression of bcl-2 protein has been found in various types of malignant lymphoma (27,28) and epithelial neoplasms, (29,30) as well as in non-neoplastic lymphoid tissue (31). In contrast, only 2 studies have examined the expression of bcl-2 in gastric carcinoids (4,32). Rindi *et al* (4) detected bcl-2 expression in 34 of 94 (36%) gastric endocrine tumors including carcinoids and endocrine cell carcinomas. Moyana *et al* (33) observed bcl-2 expression in 14 of 58 (24%) carcinoid tumors occurring in various sites of the gastrointestinal tract. The frequency of bcl-2 positivity in carcinoids in our study (neoplastic ECM plus typical carcinoid groups; 14 of 30, 47%) seems to be higher than those reported previously; however, diffusely positive expression of bcl-2 was observed in only 2 of 30 microcarcinoids (7%).

It is of interest that the frequency of bcl-2 expression decreased as the histologic grade progressed (Table II). To our knowledge, only one study by Azzoni *et al* (34) has investigated the bcl-2 expression in endocrine cells, in relation to the hyperplasia-neoplasia sequence. They demonstrated that the degree of bcl-2 expression increased in hyperplastic ECL cells in corpus atrophic gastritis, while the degree tended to decrease in ECL cell carcinoids. Overexpression of bcl-2 may contribute to tumor growth by inhibiting apoptosis of hyperplastic ECL cells and, accordingly, to exposure of the cells to oncogenic factors (35). A marked difference in diffusely positivity of bcl-2 between hyperplastic ECM and neoplastic ECM in our study (70% and 13%, respectively) seems to be comparable to the results of Azzoni *et al* (34).

Conversely, expression of p53 protein was never found in ECMs or carcinoids in our study. This result was comparable to that of Rindi *et al* (4) in which p53 expression was only detected in 1 of 52 (2%) type I gastric carcinoids and 0 of 10 type II carcinoids, while 8 of 16 (50%) type III carcinoids were positive for p53.

For the management of ECL cell neoplasia, surgical antrectomy has been a recommended procedure, as it induces the regression of both hyperplastic and neoplastic ECM by removing the bulk of gastrin producing G cells (24,36-39). However, it remains unknown as to whether all cases with type I carcinoid respond to antrectomy. We speculate nuclear morphometry and/or bcl-2 expression may be predictive of the therapeutic efficacy of antrectomy. This speculation awaits validation in future clinical trials.

In conclusion, we demonstrated the validity of our simple classification for gastric endocrine cell proliferations in type A gastritis, which is based mainly on the size of the lesions. It seems reasonable to define ECMs measuring ≥0.1 mm in size as carcinoid tumors. Nuclear morphometry and bcl-2 immunoexpression may be useful procedures for distinguishing between hyperplastic and neoplastic ECMs.

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