maintenance form of DNMT that copies methylation patterns after DNA replication. However, some workers have proposed that DNMT1 possesses both maintenance and *de novo* DNA methylation activity *in vivo*, regardless of its *in vitro* substrate preference.[13]

Overexpression of DNMT1 has been detected in several human cancers.[14–16] With regard to gastric cancer, we have reported that DNMT1 mRNA expression levels were significantly higher in cancer tissues than in normal gastric mucosae.[16] Moreover, during this previous study, we found that increased DNMT1 mRNA expression correlated significantly with the CpG island methylator phenotype (defined as frequent DNA hypermethylation of C-type CpG islands that are methylated in a cancer-specific but not an age-dependent manner[17]) in gastric and colorectal cancers.[16] However, most previous analyses concerning DNMT1 expression in human cancers have been performed at the mRNA level. To our knowledge, DNMT1 protein expression in gastric cancers has never been reported. The aim of this study was therefore to evaluate the significance of aberrant DNMT1 protein expression during gastric carcinogenesis. Firstly, we searched for correlations between DNMT1 protein expression and the clinicopathological features of gastric cancers. Secondly, to determine the targets of aberrantly expressed DNMT1 during gastric carcinogenesis, we examined the correlations between DNMT1 protein expression on the one hand and the DNA methylation status of multiple C-type CpG islands and E-cadherin expression on the other. Thirdly, to clarify the background behind aberrant DNMT1 protein expression, we investigated correlations with the proliferative activity of cancer cells (as indicated by the PCNA-labeling index) and with etiological factors that are believed to be involved in gastric carcinogenesis, such as *Helicobacter pylori*[18] and Epstein-Barr virus (EBV) infection.[19]

Materials and Methods

Patients and Tissue Samples

Cancerous tissues and corresponding noncancerous mucosae were obtained from 134 patients with primary gastric cancer. These patients underwent surgery at the National Cancer Center Hospital, Tokyo, Japan, between 1998 and 2002. They included 92 men and 42 women with a mean (± SD) age of 61 ± 7 years (range, 45 to 83 years). None of the patients received any preoperative treatment, such as radiation or chemotherapy. Based on histological examinations, the 134 tumors were classified as 23 well differentiated, 31 moderately differentiated, and 80 poorly differentiated (including signet ring cell and mucinous carcinomas) adenocarcinomas.

Immunohistochemistry

Five-μm-thick sections of formalin-fixed, paraffin-embedded tissue specimens from all 134 patients were deparaffinized and dehydrated. For antigen retrieval, the sections were heated for 10 minutes at 120°C in an autoclave. Nonspecific reactions were blocked with 2% normal swine serum. All sections were incubated with specific primary antibodies that recognized DNMT1 (goat polyclonal antibody, sc-10219, dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), Muc2 (mouse monoclonal antibody, Ccp58, dilution 1:100; Novocastra, Newcastle-on-Tyne, UK), human gastric mucin (mouse monoclonal antibody, 45M1, dilution 1:50; Novocastra), E-cadherin (mouse monoclonal antibody, HECD-1;[20] dilution 1:500), PCNA (mouse monoclonal antibody, p56720, dilution 1:200; Transduction Laboratories, Lexington, KY) and *H. pylori* (rabbit polyclonal antibody, B0471, dilution 1:20; DAKO, Glostrup, Denmark), respectively. We previously confirmed the specificity of the goat anti-human DNMT1 polyclonal antibody by Western blotting analysis: an immunoreactive band of ~193.5 kd, corresponding to the molecular mass of DNMT1, was detected in human cancer cells, but no nonspecific bands were detected with this antibody.[21] All primary antibody incubations were conducted at 4°C overnight and were followed by incubation with biotinylated secondary antibodies (anti-goat IgG, anti-mouse IgG, or anti-rabbit IgG, dilution 1:200; Vector Laboratories, Burlingame, CA) at room temperature for 30 minutes. The sections were then treated with Vectastain Elite ABC reagent (Vector Laboratories). 3.3′-Diaminobenzidine tetrahydrochloride was used as the chromogen. All sections were counterstained with hematoxylin.

The gastric cancers were classified into three phenotypes according to previously described criteria:[22] the gastric type (positive for human gastric mucin), the intestinal type (positive for Muc2), and the mixed type (positive for both human gastric mucin and Muc2). For the evaluations of DNMT1 and PCNA expression, nuclear immunoreactivity in the proliferative zones of noncancerous foveolar epithelia was used as a positive internal control for all sections. Similarly, immunoreactivity in the cell membranes of noncancerous foveolar epithelia was used as a positive internal control for all sections during the evaluation of E-cadherin expression. As a negative control, the primary antibodies were omitted from the reaction sequence.

Methylation-Specific Polymerase Chain Reaction (MSP) and Combined Bisulfite Restriction Enzyme Analysis (COBRA)

High-molecular-weight DNA was extracted from 105 fresh paired samples of cancerous tissues and their corresponding noncancerous mucosae by phenol-chloroform extraction and dialysis. Bisulfite conversion was performed using 1 μg of genomic DNA and the reagents provided in the CpGenome DNA modification kit (Intergen, Purchase, NY). This process converts unmethylated cytosine residues to uracil, whereas methylated cytosine residues remain unchanged. The DNA methylation status of the CpG islands of the *p16*,

MutL homologue 1 (hMLH1), and *E-cadherin* genes was determined by MSP. This technique is based on the principle that the DNA sequences of methylated and unmethylated genomic regions differ after bisulfite conversion and can thus be distinguished by sequence-specific polymerase chain reaction (PCR) primers.[23] The bisulfite-modified DNA of the *p16* gene was amplified using the primer sets provided in the CpG WIZ amplification kit (Intergen) and that of the *hMLH1*[24]and *E-cadherin*[23] genes was amplified using the previously described primers. The DNA methylation status of the *thrombospondin-1 (THBS-1)* gene and the methylated in tumor (MINT)-1, -2, -12, and -31 clones was determined by COBRA.[25] Bisulfite-modified DNA was amplified by PCR using previously described primers that were designed to amplify methylated and unmethylated genomic regions equally.[17] The amplified fragments were digested with restriction enzymes that digest DNA only if the CpG sites in their recognition sequences are methylated: *Taq*I for the *THBS-1* gene and the MINT-1 and -2 clones, *Mae*II for the MINT-12 clone and *Bst*UI for the MINT-31 clone, respectively. The reaction products were separated electrophoretically on a 3% agarose gel and stained with ethidium bromide. Signal intensities were measured using an image analyzer (model FMBIO-2; Takara, Ohtsu, Japan).

In Situ *Hybridization*

Five-μm-thick sections of formalin-fixed, paraffin-embedded tissue specimens from all 134 patients were deparaffinized, dehydrated, and predigested with proteinase K. The sections were then hybridized with a digoxigenin-labeled EBV encoding RNA (EBER) 1 oligonucleotide probe (EBV detection kit; Nichirei, Tokyo, Japan) for 2 hours at 37°C. Anti-digoxigenin antibody-alkaline phosphatase was used with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate to detect the EBER1 signal. Positive control specimens were provided by the manufacture. As a negative control, the EBER1 probe was omitted from the reaction sequence.

Statistics

Correlations between the incidence of DNMT1 immunoreactivity and variables including clinicopathological parameters, the DNA methylation status of CpG islands, E-cadherin expression, and *H. pylori* and EBV infection were analyzed using the chi-square test. Correlations between the PCNA-labeling index and clinicopathological parameters or DNMT1 immunoreactivity were analyzed using the Mann-Whitney *U*-test or the Kruskal-Wallis test. Differences with P values <0.05 were considered significant.

Results

Clinicopathological Significance of DNMT1 Protein Overexpression in Gastric Cancers

Immunoreactivity for DNMT1 was detected in the nuclei, but not in the cytoplasm or cell membranes, of cells in the proliferating zones of foveolar epithelia, lymphocytes, and cancer cells (Figure 1). To discriminate definitely positive cases from cases with leaky background level signal, if more than 30% of the cells in a tissue sample exhibited nuclear staining the sample was considered to show positive immunoreactivity. None (0%) of the 134 noncancerous epithelia exhibited DNMT1 immunoreactivity (except in the proliferative zones, which acted as the positive internal control for the analysis), whereas 97 (72%) of the 134 gastric cancers were DNMT1-positive.

Correlations between the incidence of nuclear immunoreactivity for DNMT1 and the clinicopathological features of the gastric cancers are shown in Table 1. DNMT1 protein overexpression was significantly associated with the degree of histological differentiation ($P < 0.001$).

Next, we evaluated cellular phenotypes based on immunohistochemistry for Muc2 and human gastric mucin, as shown in Figure 2. Fifty (37%) of the cancers showed a gastric phenotype, 34 (26%) showed an intestinal phenotype, and a further 50 (37%) showed a mixed cellular phenotype. There was no significant correlation between DNMT1 protein overexpression and the cellular phenotypes.

We then focused on the histological features of the noncancerous mucosae, as intestinal metaplasia is considered to be a precancerous lesion for adenocarcinomas with an intestinal phenotype. There was no significant correlation between DNMT1 protein overexpression in the gastric cancers and the presence or absence (or, if present, the degree) of intestinal metaplasia in the corresponding noncancerous mucosae (data not shown).

DNMT1 protein overexpression was not significantly associated with other parameters relating to cancer aggressiveness, such as the depth of invasion, vascular involvement, or lymph node metastasis.

Correlation between DNMT1 Protein Overexpression and the DNA Methylation Status of Multiple CpG Islands

Figure 3 shows examples of the PCR products from MSP and COBRA. The incidence of DNA methylation of the C-type CpG islands of each of the genes and MINT clones tested in the noncancerous mucosae and gastric cancers is summarized in Table 2. DNA methylation of at least one C-type CpG island was seen in 50 (48%) of the 105 noncancerous mucosae and 83 (80%) of the 105 gastric cancers examined. For all patients showing DNA methylation of a certain CpG island in both their

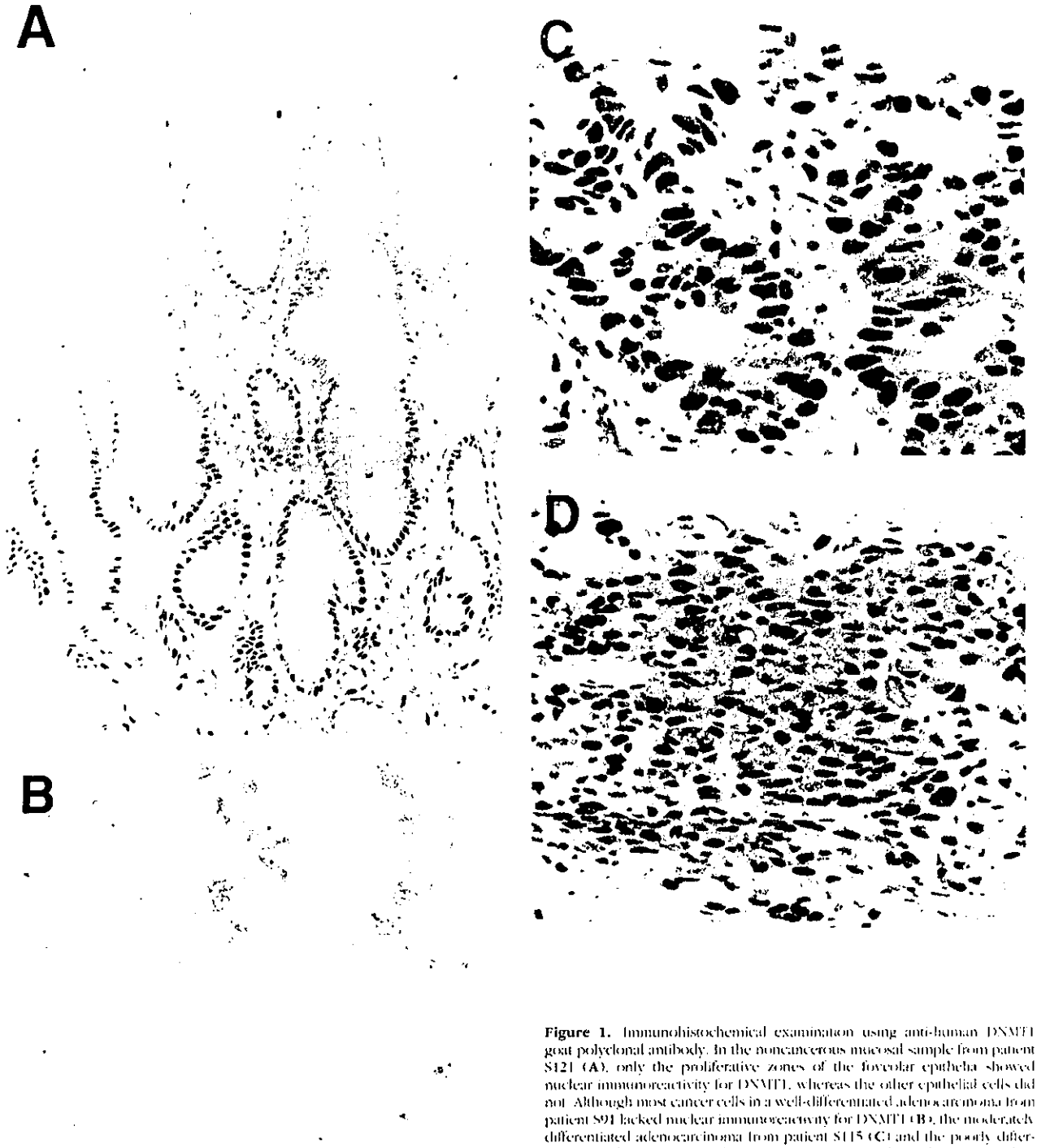

Figure 1. Immunohistochemical examination using anti-human DNMT1 goat polyclonal antibody. In the noncancerous mucosal sample from patient S121 (**A**), only the proliferative zones of the foveolar epithelia showed nuclear immunoreactivity for DNMT1, whereas the other epithelial cells did not. Although most cancer cells in a well-differentiated adenocarcinoma from patient S91 lacked nuclear immunoreactivity for DNMT1 (**B**), the moderately differentiated adenocarcinoma from patient S115 (**C**) and the poorly differentiated adenocarcinoma from patient S130 (**D**) showed strong nuclear immunoreactivity. Original magnifications: ×180 (**A**); ×360 (**B–D**).

noncancerous mucosa and cancer, the signal intensity of the reaction products reflecting the presence of methylated DNA was increased in the cancer compared with the corresponding noncancerous mucosa (Figure 3A). The DNA methylation status of each C-type CpG island in the gastric cancers is shown in Figure 4. When DNA hypermethylation was seen on three or more C-type CpG islands, we regarded the patient as being CpG island methylator phenotype (CIMP)-positive, based on previously described criteria [17] Twenty-five (24%) of the 105 gastric cancers were considered CIMP-positive. Furthermore, there were significant correlations between DNMT1 protein overexpression and DNA hypermethylation of each CpG island of the *hMLH1* (P = 0.024) and *THBS-1* (P = 0.043) genes in the gastric cancers (Table 3). There was also a significant correlation between DNMT1 protein overexpression and CIMP for the gastric cancer tissues (P = 0.007, Table 3).

Table 1. DNMT1 Protein Expression and the PCNA-Labeling Index in the Gastric Cancers

Variables	Analyzed	DNMT-1 positive [number of cases (%)]	$P^{\dagger}$	PCNA-labeling index [mean ± SD (%)]	P
Tumor differentiation					
Well differentiated	23	7 (30%)	<.001	62 ± 25	0.165‡
Moderately differentiated	31	18 (58%)		64 ± 17	
Poorly differentiated	80	72 (90%)		58 ± 27	
Phenotype*					
Gastric type	50	38 (76%)	0.137	60 ± 29	0.793‡
Intestinal type	34	21 (62%)		57 ± 29	
Mixed type	50	34 (68%)		62 ± 23	
Depth of invasion					
Mucosa/submucosa	38	28 (74%)	0.423	56 ± 32	0.125‡
Muscularis propria/subserosa	11	6 (55%)		70 ± 18	
Serosa	85	63 (74%)		60 ± 24	
Vascular involvement					
Negative	55	41 (75%)	0.494	56 ± 30	0.533§
Positive	79	56 (71%)		63 ± 23	
Lymphnode metastasis					
Negative	81	60 (74%)	0.931	59 ± 27	0.465§
Positive	53	37 (70%)		61 ± 26	

*Cellular phenotypes are defined as described in the Materials and Methods section.
†Chi-square test, ‡Kruskal-Wallis test, §Mann-Whitney *U*-test.

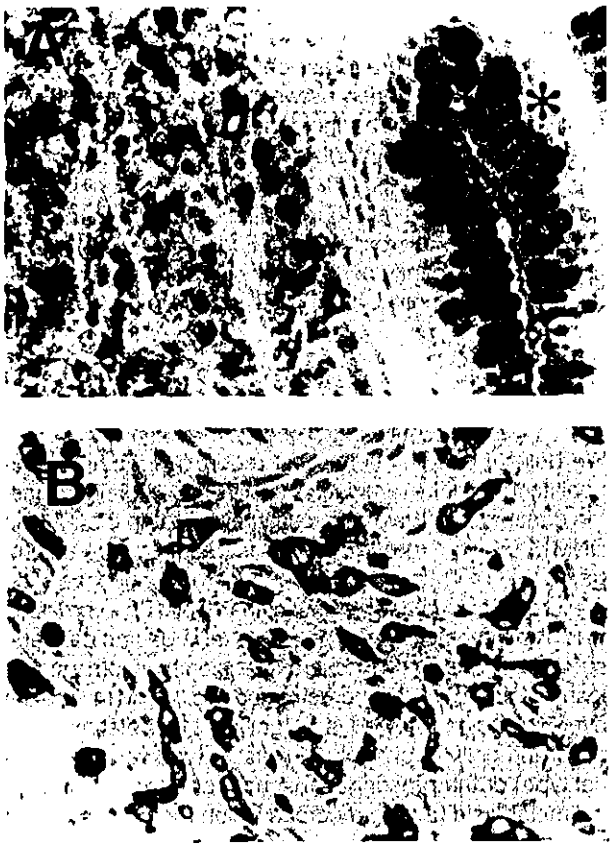

Figure 2. Immunohistochemical examination using anti-human gastric mucin (**A**) and Muc2 (**B**) mouse monoclonal antibodies. **A:** An adenocarcinoma with a gastric phenotype and noncancerous foveolar epithelia (*) from patient S19. Both showed strong cytoplasmic immunoreactivity for human gastric mucin. **B:** An adenocarcinoma with an intestinal phenotype from patient S78, showing strong cytoplasmic immunoreactivity for Muc2. Original magnifications, ×360.

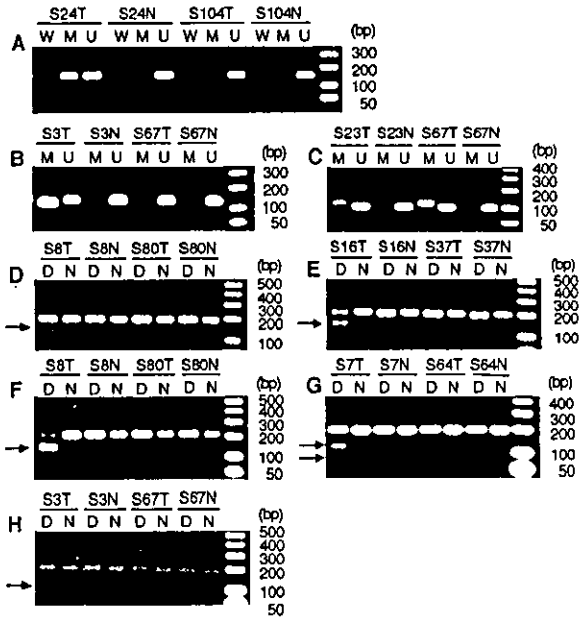

Figure 3. Examples of PCR products from DNA methylation analyses of multiple CpG islands in patients with gastric cancer (S). N, noncancerous mucosae; T, cancer tissue. The DNA methylation status of the CpG islands of the *p16* (**A**), *hMLH1* (**B**), and *E-cadherin* (**C**) genes was evaluated by MSP. In this analysis, the PCR products generated by primer sets M and U reflect the presence of methylated and unmethylated genes, respectively. Primer set W was used to confirm the completeness of the bisulfite modification. **A:** Methylated gene was detected in S24N, but the signal intensity of the M fragment was markedly increased in S24T compared with S24N. This indicates that a greater number of cells had undergone DNA hypermethylation in T than in N, although N can contain precursor cells for cancers and/or precancerous lesions. DNA methylation of the CpG islands of the *THBS-1* (**D**) gene and the MINT-1, -2, -12, and -31 clones (**E**, **F**, **G**, and **H**, respectively) was evaluated by COBRA. In this analysis, only methylated genes (**arrows**) were digested by the restriction enzymes.

Table 2. The DNA Methylation Status of the C-Type CpG Islands of Various Genes and Clones in Noncancerous Mucosae and Gastric Cancers

	Number of tissue samples (%)			
	Noncancerous mucosae		Gastric cancers	
CpG islands	Analyzed	DNA hypermethylation detected	Analyzed	DNA hypermethylation detected
p16	104	18 (17)	103	23 (22)
hMLH1	105	15 (14)	105	18 (17)
THBS-1	105	2 (2)	104	25 (24)
MINT1	105	23 (22)	105	39 (37)
MINT2	105	1 (1)	105	26 (25)
MINT12	104	6 (6)	104	18 (17)
MINT31	105	0 (0)	105	10 (10)

Correlation between DNMT1 Protein Overexpression and Reduced E-Cadherin Expression

E-cadherin was detected in the cell membranes of epithelia from all (100%) of the 134 noncancerous mucosae. However, E-cadherin protein expression was considered to be reduced when more than 50% of the gastric cancer cells in a particular patient's sample lacked or showed only slight membranous E-cadherin immunoreactivity. Reduced E-cadherin expression was observed in 59 (44%) of the 134 gastric cancers (Figure 5). The incidence of reduced E-cadherin expression was 22% in well, 23% in moderately, and 59% in poorly differentiated adenocarcinomas, respectively, and reduced E-cadherin expression was significantly associated with poorer tumor differentiation ($P = 0.002$). DNA methylation of CpG island of the *E-cadherin* gene was seen in 20 (19%) of the 105 gastric cancers examined (Figure 3C) and there was a significant correlation between DNA hypermethylation of CpG island of the *E-cadherin* gene and reduced E-cadherin expression in the gastric cancers ($P < 0.001$). Furthermore, there was a significant correlation between DNMT1 protein overexpression and reduced E-cadherin expression in gastric cancers ($P = 0.014$, Table 3). In fact, coincidence of nuclear immunoreactivity of DNMT1 and lack of membrane immunoreactivity of E-cadherin was frequently observed in individual cancer cells.

DNMT1 Protein Expression and the PCNA-Labeling Index

Examples of the results of immunohistochemistry for PCNA are shown in Figure 6. To evaluate the PCNA-labeling index, ~300 cells per specimen were examined at a magnification of ×400 under a microscope and the cells that did and did not show nuclear immunoreactivity for PCNA were counted. The PCNA-labeling index was expressed as the percentage of the total cells that showed nuclear immunoreactivity. The PCNA-labeling index was increased even in well-differentiated adenocarcinomas, in which the incidence of overexpression of DNMT1 protein was still low (Table 1), and coincidence of nuclear immunoreactivity of PCNA and lack of nuclear immunoreactivity of DNMT1 was frequently observed in individual well-differentiated cancer cells. Thus, DNMT1 protein overexpression was not significantly associated with the PCNA-labeling index in the gastric cancers ($P = 0.309$, Figure 7).

Correlation between DNMT1 Protein Overexpression and Etiological Factors

To understand the background behind DNMT1 protein overexpression, etiological factors considered to be involved in gastric carcinogenesis were examined. Although 44 (46%) of the 96 patients examined showed *H. pylori* infection in their noncancerous mucosae, there was no significant correlation between *H. pylori* infection and DNMT1 protein overexpression ($P = 0.113$). Similarly, there was no correlation between *H. pylori* infection and CIMP ($P = 0.146$). The incidence of EBV infection was examined by *in situ* hybridization in the same cohort (Figure 8). Four patients (4%) had EBV infection in their cancer cells, and all four of these cancers showed DNMT1 protein overexpression. Furthermore, EBV infection was significantly associated with DNA hypermethylation of five or more C-type CpG islands ($P < 0.001$).

Discussion

We believe that this is the first report describing immunohistochemical examination for DNMT1 protein in gastric cancers. We have previously reported an increase in DNMT1 mRNA expression levels in gastric cancers compared with corresponding noncancerous mucosae.[16] In the present study, we showed that DNMT1 expression was also increased at the protein level in gastric cancers, suggesting that DNMT1 overexpression has some significance during gastric carcinogenesis. DNMT1 protein overexpression showed no significant correlations with either the cellular phenotype (gastric type *versus* intestinal type) or the presence, absence, or degree of intestinal metaplasia (a precancerous lesion for adenocarcinomas with an intestinal phenotype) in corresponding noncancerous mucosae, suggesting that DNMT1 protein overexpression is associated with gastric carcinogenesis regardless of the cellular origin or phenotype. Our results also suggest that DNMT1 may particularly affect the stage of development at which cancers begin to show

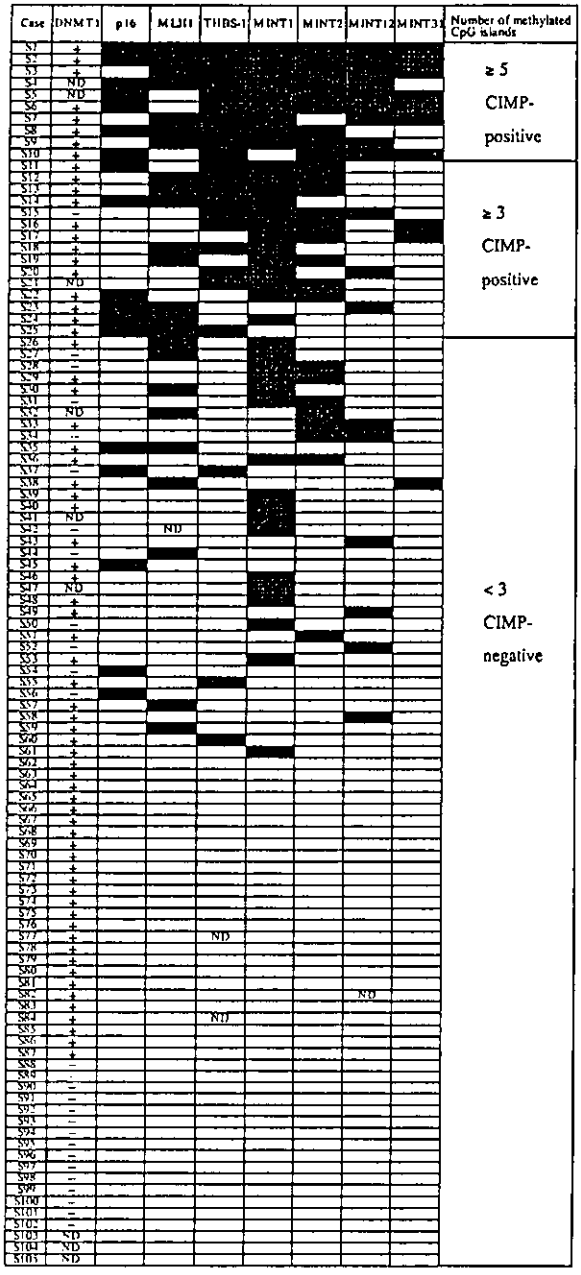

Figure 4. Protein expression of DNMT1 and DNA methylation profiles for seven C-type CpG islands in 105 gastric cancers. The DNA methylation status was examined using MSP or COBRA (see Figure 3). Patient numbers are indicated on the **vertical columns** and the seven CpG islands are indicated on the **top row**. +, DNMT1 protein overexpression-positive; −, DNMT1 protein overexpression-negative; **solid box**, methylated; **open box**, unmethylated; ND, not done. When DNA hypermethylation was seen in three or more CpG islands, the patient was regarded as being CIMP-positive.

poorer differentiation. However, in a previous study, overexpression was detected even in precancerous conditions when we used quantitative reverse transcriptase-PCR analysis to examine DNMT1 mRNA expression levels in a cohort with hepatocellular carcinomas (HCCs).[15] Although there appears to be a discrepancy between the previous and present findings, when the same cohort was examined by immunohistochemistry protein overexpression was not detected in precancerous conditions but only in moderately or poorly differentiated HCCs.[21] This may be attributable to methodological differences: quantitative reverse transcriptase-PCR is so sensitive that it can detect small elevations in DNMT1 mRNA levels in precancerous conditions, whereas immunohistochemistry cannot detect such elevations until protein expression reaches a certain level in more malignant HCCs.[21] By analogy with hepatocarcinogenesis, we cannot rule out the possibility that a small elevation in DNMT1 expression had already occurred in the earlier stages of gastric carcinogenesis, before the DNMT1 expression level reached the threshold of detection for the immunohistochemical methods used.

Regional DNA hypermethylation of CpG islands was detected even in noncancerous mucosae, which can contain precursor cells for cancers and/or precancerous lesions, such as intestinal metaplasia. However, the incidence and degree of DNA hypermethylation was increased in the gastric cancers compared with the noncancerous mucosae. These data are consistent with previous findings in precancerous conditions and cancers of various organs.[3–8] Twenty-four percent of the gastric cancers were CIMP-positive, confirming that aberrant DNA methylation is associated with the multistage development of certain subgroups of gastric cancers.

Table 3. Correlations between DNMT1 Protein Overexpression and the DNA Methylation Status of Each C-Type CpG Island, CIMP and E-Cadherin Protein Expression in Gastric Cancers

	DNMT1 expression (number of tissue samples)		
	Positive	Negative	*P**
CpG islands			
p16			
Methylated	13	3	0.361
Unmethylated	56	24	
hMLH1			
Methylated	20	2	0.024
Unmethylated	49	25	
THBS-1			
Methylated	18	2	0.043
Unmethylated	51	25	
MINT1			
Methylated	28	6	0.091
Unmethylated	41	21	
MINT2			
Methylated	18	4	0.237
Unmethylated	51	23	
MINT12			
Methylated	13	3	0.361
Unmethylated	56	24	
MINT31			
Methylated	10	0	0.503
Unmethylated	59	27	
CIMP			
Positive	10	1	0.007
Negative	59	26	
E-cadherin expression			
Maintained	48	27	0.014
Reduced	49	10	

*Chi-square test. Reduced: over 50% of the cancer cells in a particular patient's sample lacked or showed only slight E-cadherin immunoreactivity.

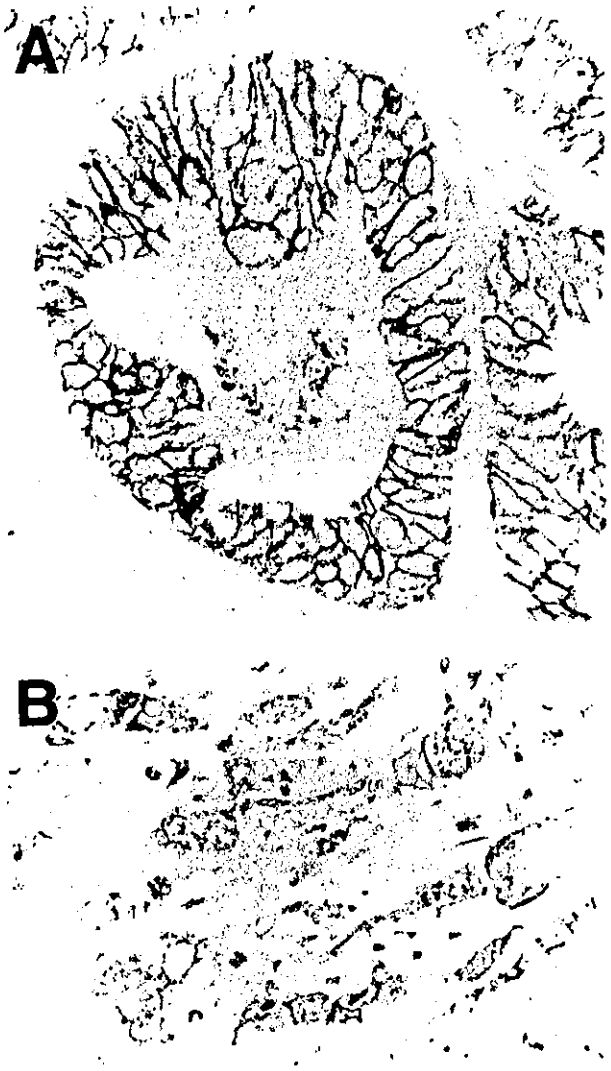

Figure 5. Immunohistochemical examination using the anti-human E-cadherin mouse monoclonal antibody HECD-1. Although the well-differentiated adenocarcinoma from patient S31 maintained strong E-cadherin immunoreactivity at the cell-cell borders (**A**), E-cadherin expression was reduced in the poorly differentiated adenocarcinoma from patient S64 (**B**). Original magnifications, ×360.

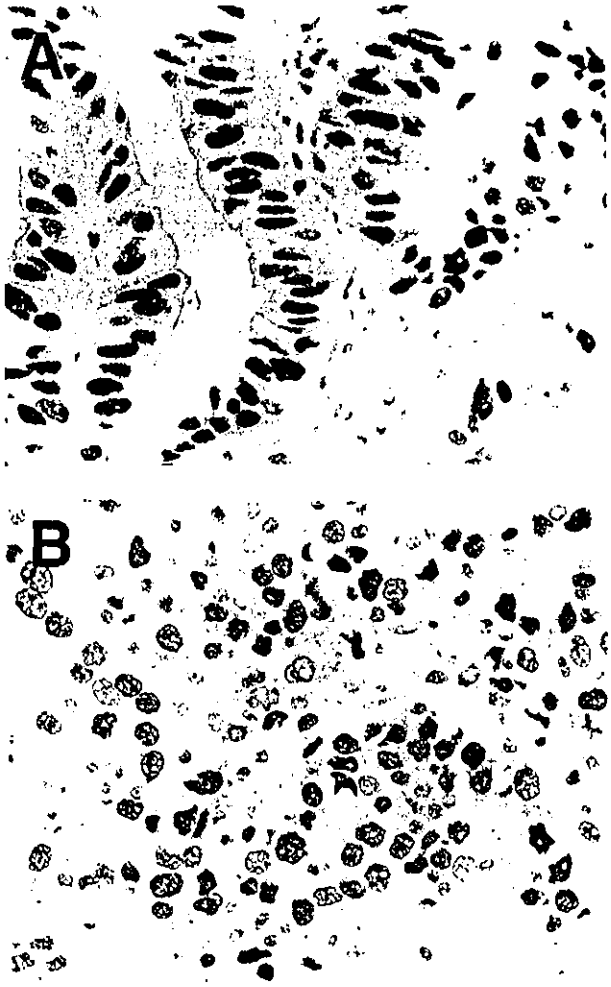

Figure 6. Immunohistochemical examination using anti-human PCNA mouse monoclonal antibody. **A:** A well-differentiated adenocarcinoma from patient S125. **B:** A poorly differentiated adenocarcinoma from patient S30. Original magnifications, ×360.

Targeting of the substrate DNA by DNMT1 may be disrupted by mechanisms such as dysfunction of p21WAF1,[26] which competes with DNMT1 for binding to PCNA, in cancer cells.[12] Moreover, it has recently been suggested that DNMT1 is capable of *de novo* methylating activity as well as having a maintenance function.[13] Therefore, it is feasible that, in cancers, DNMT1 participates in the DNA hypermethylation of CpG islands that are not methylated in normal cells. We have previously reported that DNMT1 mRNA overexpression correlates significantly with CIMP in gastric and colorectal cancers.[16] In the present study, we demonstrated a significant correlation between DNMT1 expression and CIMP in gastric cancers, even at the protein level. Moreover, among the C-type CpG islands examined, those of the *hMLH1* and *THBS-1* genes may be particularly targeted by overexpressed DNMT1. This is compatible with previ-

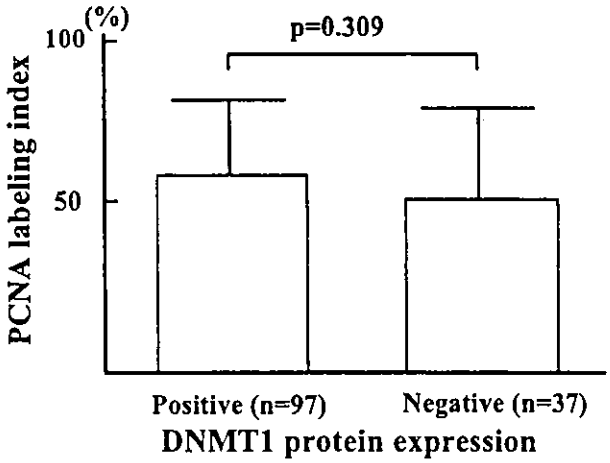

Figure 7. Average PCNA-labeling indices in DNMT1 protein overexpression-positive (n = 97) and -negative (n = 37) gastric cancers. Error bar, SD. DNMT1 protein overexpression was not significantly associated with the PCNA-labeling index in the gastric cancers (P = 0.309, Mann-Whitney U-test).

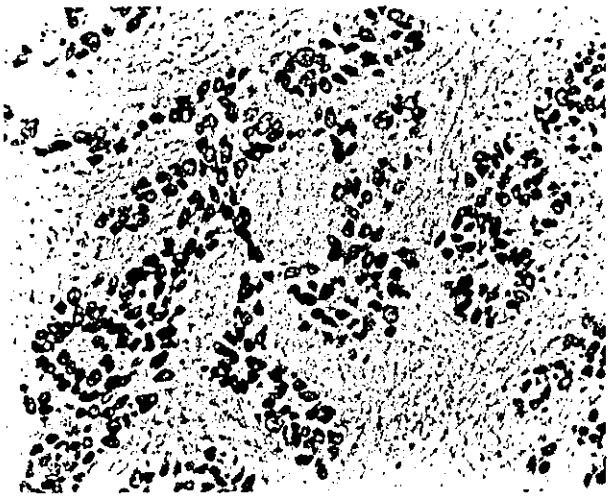

Figure 8. *In situ* hybridization investigation for EBV infection using the EBER1 oligonucleotide probe. A poorly differentiated adenocarcinoma from patient S45, showing diffuse positivity in the nuclei. Original magnification, ×180.

ous reports that silencing of the *hMLH1* gene results in frequent microsatellite instability in gastric cancers.[27,28]

E-cadherin is one of the most important molecules involved in intracellular adhesion and cancer morphogenesis.[29] In addition to the development of mutations,[30–32] it has become apparent that the *E-cadherin* gene can be silenced by DNA hypermethylation around its promoter regions in gastric cancers.[33,34] Indeed, reduced E-cadherin protein expression associated with DNA hypermethylation around the promoter region of the *E-cadherin* gene was significantly correlated with poorer glandular differentiation of the cancers examined in this study. We showed a significant correlation between DNMT1 protein overexpression and reduced E-cadherin protein expression in gastric cancers, indicating that the *E-cadherin* gene may also be a target of overexpressed DNMT1.

Although DNMT1 is a major DNMT in humans, so far two other enzymes, DNMT3a and DNMT3b, have also been shown to possess DNMT activity.[1] Genomic methylation patterns may be established through cooperation among these three DNMTs, even in cancer cells.[13] Moreover, it may be that unknown co-factors potently target these DNMTs to unmethylated substrates in a sequence-specific manner in cancers. Thus, DNMT1 overexpression alone may not explain regional DNA hypermethylation in cancers. Further studies on the cooperation between DNMT1 and other components of the DNA methylation machinery in tissue specimens may further our understanding of the basis of regional DNA hypermethylation during gastric carcinogenesis. Although the degree of DNA hypermethylation in noncancerous mucosae seemed slight, DNA methylation of CpG islands actually occurred in a considerable number of noncancerous mucosae that can contain precursor cells for cancer. However, DNMT1 overexpression was not detected immunohistochemically in noncancerous mucosae except for proliferative zones, in which DNMT1 performs maintenance methylation at the replication foci. The protein expression levels of DNMT1 in noncancerous mucosae may not reach the threshold that can be detected by the present immunohistochemical method, even though it promotes slight DNA hypermethylation. Otherwise, slight DNA hypermethylation in noncancerous mucosae may be attributable to alterations in components of the DNA methylation machinery other than DNMT1. Although frequent regional DNA hypermethylation has been reported in gastric type tumors,[35] DNMT1 protein expression was not different between gastric and intestinal phenotypes in the present study. We assume again that unknown components of the DNA methylation machinery may potently target DNMT1 to substrate DNA, or that DNMTs other than DNMT1 may also participate in regional DNA hypermethylation in cancers showing gastric phenotype.

On the other hand, the overall 5-methylcytosine level is lower in cancer tissues than in normal tissues.[1] In common with the previously reported immunohistochemical findings for DNMT1 in colorectal cancers[36] and HCCs,[21] some cancer cells exhibited very weak or no DNMT1 immunoreactivity, even in a gastric cancer that was considered DNMT1-positive according to the criteria described in the Materials and Methods section. Cancer cells with low DNMT1 levels may be prone to overall DNA hypomethylation.

An initial increase in DNMT1 mRNA expression in colon cancers became far more modest when the expression level was normalized according to that of a cell proliferation marker.[37] DNMT1 mRNA is expressed mainly during the S-phase and, because tumor tissue is presumed to contain a greater proportion of dividing cells than normal tissue, some debate has arisen as to whether increased DNMT1 expression is because of an increase in the proportion of dividing cells or to an acute increase in DNMT1 expression per individual cell. This continuing discussion prompted us to compare DMNT1 immunoreactivity and the PCNA-labeling index in gastric cancers. In the present study, DNMT1 protein overexpression was not significantly associated with increased proliferative activity of gastric cancer cells; we have previously observed a similar discrepancy between DNMT1 expression and cell proliferative activity in precancerous conditions for urinary bladder carcinomas[38] and in certain subgroups of HCCs.[21] These findings suggest that DNMT1 protein overexpression does not result entirely from increased numbers of dividing cells during carcinogenesis.

Finally, we focused on etiological factors believed to be involved in gastric carcinogenesis to clarify the background behind DNMT1 protein overexpression. Although DNMT1 protein overexpression was not significantly associated with the incidence of *H. pylori* infection in corresponding noncancerous mucosae, all four of the EBV-positive cancers showed DNMT1 protein overexpression. EBV infection was significantly associated with the DNA hypermethylation of five or more C-type CpG islands, in accordance with a previous report that the average number of methylated CpG islands was higher in EBV-positive gastric cancers than in EBV-negative ones.[39] These observations indicate that DNMT1 may play an important

role in EBV-related gastric carcinogenesis via aberrant DNA methylation. Although it has previously been reported that transfection of latent membrane protein-1 of EBV induces the expression and activity of DNMT1,[40] latent membrane protein-1 is not typically expressed in EBV-associated gastric cancers.[41] Although the molecular mechanism governing how EBV infection results in overexpression of DNMT1 protein requires further elucidation, and our present findings must be interpreted with caution because of the small number of EBV-positive gastric cancers studied, EBV infection and other etiological factors may be associated with DNMT1 overexpression and contribute toward gastric carcinogenesis by inducing frequent DNA hypermethylation of multiple CpG islands.

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Focus on gastric cancer

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Epidemiology and incidence statistics

Gastric cancer is the second most common cancer in the world (Ferlay et al., 2001). It is unique in that its time trend and geographical distribution are very informative in estimating its risk factors. In the US, the crude mortality rate in Caucasian males was 33/100,000 in the early 20th century, and this declined to 5/100,000 in the late 20th century. The declining trend is worldwide, and the decline began earlier in developed countries. However, even among them, mortality is still high in Korea (43/100,000), Russia (35/100,000), Japan (31/100,000), and Portugal (22/100,000). The age-adjusted incidence reaches as high as 70/100,000 in Korean and Japanese males. The male to female ratio is consistently two to one in many geographical regions.

The decline took place following the popularization of refrigerators, which resulted in a decreased intake of salt and an increased intake of fruit and vegetables (Palli, 2000; Potter et al., 1997). The preventive effects of fruit and vegetables are consistently confirmed by many epidemiological studies. Most epidemiological studies have shown the promoting effect of salt and the preventive effect of vitamin C. The effects of salt were also shown by animal experiments. Some epidemiological studies suggest that consumption of grilled meat/fish increases the risk, and that the consumption of carotenoids and green tea reduce the risk. Epidemiological data linking *N*-nitrosamines to gastric cancers have so far been inconclusive, although their carcinogenicity at high doses is proven.

Infection by *Helicobacter pylori* is prevalent in areas with high incidences of gastric cancers, and increases the risk of gastric cancer. However, in some Asian countries, such as India and Thailand, incidences of gastric cancers are not high in spite of the high *H. pylori* infection rates, a phenomenon known as the "Asian Enigma" (Miwa et al., 2002). Possible explanations for this include host genetic factors, different virulence among strains of *H. pylori*, and dietary factors. Polymorphisms of proinflammatory cytokine genes have been shown to associate with risk of *H. pylori*-related gastric cancers (El-Omar et al., 2000).

Animal models

A rat model for gastric cancers induced by a chemical carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, has been widely used for a variety of purposes, such as evaluation of various promoting and preventing factors and clarification of genes involved in genetic susceptibility (Yamashita et al., 2002). A model in which *H. pylori* could infect an animal was established using Mongolian gerbils, which contributed to clarification of the strong promoting effect of *H. pylori* (Shimizu et al., 1999).

In addition, there have been more than 10 lines of genetically modified mice that show hyperplasia of the gastric epithelium and/or intestinal metaplasia (Gut et al., 2002). These mouse models were created by targeting genes involved in ion transport, signal transduction, transcriptional regulation, and cell adhesion. Development of gastric cancers was observed in mice lacking the pS2 trefoil protein, those lacking *Smad4/Dpc4*, those lacking the SHP2 binding site on the Il-6 family corepressor gp130, and those lacking *RUNX3* (Judd et al., 2004; Lefebvre et al., 1996; Xu et al., 2000; Li et al., 2002).

Disease mechanism and molecular targets

Histological classification and gastric/intestinal phenotypes

Histological classification of gastric cancers is different between Japan and Western countries. Generally, "differentiated" and "undifferentiated" types in Japanese classification correspond to "intestinal" and "diffuse" types, respectively, in the Western classification established by Lauren. It has been considered that intestinal-type gastric cancers are associated with intestinal metaplasia, whereas diffuse-type gastric cancers are originated from gastric mucosa proper. Recent analysis of gastric and intestinal phenotypes in early gastric cancers has shown that cancer cells with gastric phenotypes were present in both intestinal and diffuse types of gastric cancer. Furthermore, phenotypic expression in gastric cancer cells was shown to be independent of phenotypic changes in the surrounding gastric mucosa (Tatematsu et al., 2003).

Gastric cancer predisposition

Germline mutations of *E-cadherin* were first found in a large family from New Zealand in which diffuse-type gastric cancers took place at an early age (Guilford et al., 1998). Although *E-cadherin* germline mutations are very rare, the finding provided to be useful information for clinicians to manage high-risk patients. Gastric cancers, mainly of the intestinal type, can be associated with hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, most cases of which are caused by germline mutations of mismatch repair genes *hMLH1* or *hMSH2*, and are more prominently manifested in older generations of HNPCC patients. Patients with familial adenomatous polyposis, which is caused by germline mutations of *APC*, and Peutz-Jeghers syndrome also have increased risk for gastric cancer (Oberhuber and Stolte, 2000).

Molecular alterations in gastric cancer

Many genes have been analyzed in attempts to understand the molecular bases for human gastric cancers, but only a few with frequent alterations have been identified (Table 1). Oncogenic activations of β-*catenin* (17%–27% in intestinal type) and K-*ras* (0%–18% in both histological types) have been found in human gastric cancers (Lee et al., 2002; Park et al., 1999). In addition, amplifications of the c-*erb*B2 and the c-*met* genes have each been found in approximately 10% of both histological types.

As for tumor-suppressor genes, *p53* mutations are repeatedly reported in gastric cancers of the diffuse type (0%–21%) and intestinal type (36%–43%) (Maesawa et al., 1995).

Table 1. Histology and genetic alterations of gastric cancers

	Diffuse type (%)	Intestinal type (%)
Oncogene activation		
β-*catenin*	0	17–27
K-*ras*	0–6	0–18
c-*erb*B2	12–13	12–13
Inactivation of tumor suppressor genes		
p53	0–21	36–43
APC	0–5	0
E-cadherin		
Mutation	33–50	0
Methylation	79	55
p16		
Mutation	0	0
Methylation	11*	50*
Microsatellite instability	5–32	23–41

*Incidences are overestimated due to analysis of CpG islands in exons.

Mutations of the *APC* tumor suppressor gene are found frequently in gastric adenomas, but only rarely in gastric cancers; this is clearly different from the similar frequencies of *APC* mutations in colorectal adenomas and carcinomas (Lee et al., 2002; Maesawa et al., 1995). Somatic mutations of *E-cadherin* are observed specifically in sporadic diffuse-type gastric cancers (33%–50%) (Becker et al., 1994). *RUNX3* was recently shown to be a tumor-suppressor gene of gastric cancers, although its mutations were rare (Li et al., 2002).

Microsatellite instability (MSI) is observed in 5%–10% of diffuse-type gastric cancers and in 15%–40% of intestinal-type gastric cancers. The major mechanism for the MSI in gastric cancer is inactivation of the mismatch repair gene *hMLH1* resulting from hypermethylation of its promoter (Fang et al., 2003). Similarly, mutation of the *p16* gene is infrequent, but hypermethylation of *p16* is common (25%–42% overall) in gastric cancer, with the intestinal type having higher incidence (Ding et al., 2003; Oue et al., 2002).

Factors that induce molecular alterations

Although *hMLH1* and *p16* can be inactivated in gastric cancers by mutations or by promoter hypermethylation, inactivation by methylation is much more frequent than mutation in sporadic gastric cancers. The second hit in *E-cadherin* germline mutation carriers is also generally due to methylation (Machado et al., 2001). A genome-wide scan for aberrant methylations revealed silencing of nine genes in gastric cancers (Kaneda et al., 2002). Even in noncancerous gastric mucosae (Waki et al., 2002), aberrant methylation can be present. These findings suggest that aberrant methylation is deeply involved in gastric carcinogenesis, and aberrant methylation seems to be useful as a new target for diagnostics and prevention of gastric cancers.

The presence of Epstein-Barr virus (EBV) is observed in 7%–20% of gastric cancers, being slightly more frequent in diffuse-type gastric cancers. EBV is clonal in cancer tissue, and is maintained as a plasmid. EBV has been shown to extend cell generations of gastric epithelial cells in in vitro cell culture, but it cannot immortalize them (Takada, 2000). Recently, EBV-associated gastric cancers were shown to be more frequently associated with promoter methylation of *p16* (Kang et al., 2002).

There has been discussion about whether intestinal metaplasia (IM) is a precancerous lesion for gastric cancers. Although gastric cancers are frequently accompanied by IM, no molecular alterations that cause both IM and gastric cancers have been identified. It is thus more likely that factors that induce molecular alterations for IM, such as *H. pylori* infection (Uemura et al., 2001), also induce molecular alterations for gastric cancers.

Diagnosis of gastric cancers

Most patients with gastric cancer are diagnosed when they undergo endoscopy and biopsy after exhibiting symptoms. In Japan, about 25% of patients are diagnosed by mass screening or a personal health check (Japanese National Gastric Cancer Registry). In high-risk areas of this disease, the most important issue is the education of general practitioners and the public to make them aware of the risk of this cancer. Early diagnosis used to be made by a barium meal study, especially in mass screening in Japan (Oshima, 1997). Endoscopy is being used more and more for secondary prevention in combination with a serum test of pepsinogen subtypes. However, there is a consensus that the efficacy of mass screening itself should be reevaluated (Tsubono and Hisamichi, 2000).

At an early phase of development, a well-differentiated carcinoma (WDC) replaces the mucosa of atrophic gastritis or IM without showing any invasion. As tumors progress, they start to invade the lamina propria mucosae or the muscularis mucosae, then the submucosal layer. As these invasive parts are often missed by biopsy, the lesions are often diagnosed as dysplasia. Thus, many lesions initially diagnosed as severe dysplasia turned out to be an invasive cancer, sometimes invading even the muscularis propria, after histological evaluation of resected materials (Fertitta et al., 1993).

Diagnostic criteria for early gastric cancers and endoscopic mucosal resection

Diagnostic criteria of WDC differs to some extent between the West and the East (Schlemper et al., 1997). In Western countries, the diagnosis of adenocarcinoma is made only when pathologists can recognize the evidence of invasion, while the term cancer is used in the East when cellular or structural atypia is evident, even without evidence of invasion. WHO classification now clearly states that the lesions called severe dysplasia/adenoma in the West are the same as noninvasive mucosal carcinoma in the East, and this is a result of pathologists' mutual communication and cooperation (Fenoglio-Preiser et al., 1997). The Western policy runs the risk of overlooking true cancers, but the Eastern policy may induce overtreatment. However, as the result of the development of the technique of endoscopic mucosal resection (EMR), the majority of such lesions are now treated endoscopically in Japan (Ono et al., 2001). Thus, paradoxically, "severe dysplasia" is often treated by surgery in the West, and "noninvasive mucosal carcinoma" is treated by EMR. This treatment can be applied exclusively to mucosal cancer, for which endoscopic ultrasound (EUS) is sometimes helpful. Because the histology of the entire specimen resected using EMR can be examined in detail, additional surgery can be applied without much delay if a patient's tumor is found to have submucosal invasion. Because of these potential advantages, distribution of the EMR technique to the West is urgently needed.

Metastases and their diagnosis

Gastric cancer remains a localized disease for a long time and metastasizes slowly. Table 2 shows the incidence of metastasis to lymph nodes, the liver, and the peritoneum according to tumor depth. Metastasis to sites other than these three sites is rare. Systemic metastasis seldom occurs until the late phase of

Table 2. Incidence of metastasis and five-year survival rates by tumor size and depth

Depth		Number of cases	Incidence (%)			Five-year survival rate (%)
			LN metastasis	Liver metastasis	Peritoneal metastasis	
pT1	M	1063	3.3	0.0	0.0	93.3
	SM	881	17.4	0.1	0.0	88.9
pT2	MP	436	46.4	1.1	0.5	81.3
	SS	325	63.7	3.4	2.2	65.8
pT3	SE	1232	78.9	6.3	17.8	35.5
pT4	SI	724	89.8	15.5	41.6	10.1
Overall		4683	47.8	4.5	11.5	60.3

Patients operated on between 1972–1991 at National Cancer Center Hospital, including exploratory laparotomy. pT1: pathologically confirmed tumor invasion of mucosa and/or muscularis mucosa (M) or submucosa (SM). pT2: pathologically confirmed tumor invasion of muscularis propria (MP) or subserosa (SS). pT3: pathologically confirmed tumor penetration of serosa (SE). pT4: pathologically confirmed tumor invasion of adjacent structures (SI).

local invasion (T3/4). By deeper invasion, nodal metastasis occurs more massively and to more distant areas. Nearly 20% of T2 tumors have metastasis at the second tier nodes. Systemic and local recurrences of T1/T2 lesions are rare when treated by proper lymph node dissection, while local recurrence is frequent after limited surgery (Sasako, 2003).

Conventional CT scanning is useful in detecting enlarged nodes, which are often irresectable. However, 25% of metastatic nodes are 5 mm or less and undetectable by any imaging diagnostic tool, including MRI, PET scan, or EUS (Noda et al., 1998).

Treatment of gastric cancers and its recent advances

Tumors without distant metastasis are potentially curable, and treatment comprises resection of the primary tumor and control of lymph node metastasis. For differentiated-type T1 mucosal cancers, EMR is often successful, as metastasis does not generally occur (Gotoda et al., 2000). The Japanese Gastric Cancer Treatment Guideline indicates the criteria for EMR as follows: mucosal cancer of intestinal type, no ulcer nor ulcer scar in the lesion, and size smaller than 21 mm (Nakajima, 2002). For more advanced lesions, gastrectomy of over 2/3 of the stomach with proper lymph node dissection is regarded as standard treatment even in the West (Sasako, 2003; Allum et al., 2002; NCCN Guideline [http://www.nccn.com/physician_gls/f_guidelines.html]), in spite of the negative results of two large randomized trials (Bonenkamp et al., 1999; Cuschieri et al., 1999).

Tumors with distant metastasis are mostly incurable at present, with the rare exceptions of those with solitary liver metastasis or peritoneal nodules. For these advanced or recurrent tumors, chemotherapy shows a modest effect, and cure by medical treatment is rare, even in combination with radiotherapy. Combination chemotherapy using 5-fluorouracil with other agents remains the most popular regimen.

Chemoradiotherapy and D2 surgery

Recently, chemoradiotherapy (CRT) after a potentially curative operation was shown to improve the results of surgery without lymph node dissection (MacDonald et al., 2001). As adjuvant chemotherapy has not proven its efficacy over surgery alone, these results strongly suggest the efficacy of radiotherapy to achieve good local control. However, the results achieved by limited surgery followed by CRT are still worse than those of extended surgery, so-called D2 nodal dissection. Currently, questions regarding whether CRT in combination with limited surgery can replace D2 surgery and whether CRT can improve the results of D2 surgery alone remain to be answered. The former should be evaluated in the Western specialized centers, where D2 surgery can be carried out safely with sufficient quality. If this proves the efficacy of CRT, both questions should be investigated in Japan. Meta-analysis evaluating the effect of adjuvant chemotherapy without irradiation after curative surgery for gastric cancer suggested strongly the effect of the treatment. As none of the large sized trials has proven the effect of adjuvant chemotherapy, it is urgent to establish standard adjuvant treatment. At the moment, a large randomized trial is going on using TS-1, which showed the highest response rate as a single agent in the past. In Western countries, neoadjuvant chemotherapy for advanced gastric cancer is now being tested in a few large phase III trials. Neoadjuvant CRT is just now under investigation as a phase II trial in some American institutions.

New chemotherapeutic agent

Some new chemotherapeutic agents, such as Irrinotecan, TS-1, and Docetaxel, show promise as being more effective than conventional drugs. A combination chemotherapy including TS-1 has shown a response rate of over 70% (Koizumi et al., 2003). Further studies may change the chemotherapy for gastric cancer.

Future challenges

Severe dysplasia/noninvasive mucosal carcinoma could contain different entities that have different abilities to invade the lamina propria mucosae. However, key molecular alterations that determine this progression are unknown. The presence of lymph node or distant metastasis is a very important factor in deciding a treatment strategy, but accurate diagnosis is still difficult. Clarification of molecular alterations that are closely linked with these characteristics will be beneficial to decide on a treatment strategy for individual cases. Popular use of EMR raises a new question, whether or not a secondary cancer will arise from the remnant stomach, and prediction of risk for developing gastric cancer is becoming more important. Recent genomic approaches demonstrate great potential for addressing these issues (Hasegawa et al., 2002). The more important and appropriate questions we ask, the more useful these new approaches will be.

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Original article

Feasibility study of adjuvant chemotherapy with S-1 (TS-1; tegafur, gimeracil, oteracil potassium) for gastric cancer

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Abstract

Background. **We conducted a feasibility study using S-1, a novel oral derivative of 5-fluorouracil, as postoperative adjuvant chemotherapy for curatively resected gastric cancer patients.**

Methods. **Adjuvant chemotherapy consisted of eight courses (4-week administration and 2-week withdrawal) of S-1, at 80–120 mg/body per day. Forty-one patients from 11 institutions were enrolled in this pilot study, from November 1999 to October 2000.**

Results. **Thirty-five patients were eligible. In 7 patients, S-1 administration was discontinued due to recurrence. Among the 28 patients without recurrence, the planned eight courses of S-1 were administered to 17 patients (60.7%). In 4 patients, S-1 administration was discontinued due to subjective symptoms, such as anorexia, in the first course. Adverse reactions such as neutropenia, leukopenia, elevated total bilirubin, anorexia, general fatigue, diarrhea, nausea, and stomatitis were seen in more than half of the patients. Although grade 3 neutropenia (29.3%), leukopenia (9.8%), and diarrhea (9.8%) were observed, no grade 4 adverse effects appeared. Compared with the treatment of unresectable or recurrent gastric cancer with S-1, the incidence of adverse reactions in the adjuvant setting was slightly higher, probably due to the influence of gastrectomy.**

Conclusion. **Except for the early development of anorexia, most likely due to adverse effects of surgery, postoperative administration of S-1 for 1 year seems feasible as adjuvant chemotherapy for gastric cancer.**

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Key words Gastric cancer · S-1 · Adjuvant chemotherapy · Feasibility study

Introduction

The results of surgical treatment for gastric cancer have been improved by early detection and meticulous surgical procedures in Japan. However, we still face recurrence in patients with advanced gastric cancer, even with extended surgical treatment [1].

Many clinical trials of adjuvant chemotherapy could not prove survival benefits. Only a few metaanalyses of adjuvant trials reported the possibility of survival benefits [2,3]. According to the guidelines from the Japanese Gastric Cancer Association [4], the efficacy of adjuvant chemotherapy after curative resection for gastric cancer has not been established. A well-designed large-scale phase III trial is needed, with a surgery-alone arm, to prove the benefit of adjuvant chemotherapy.

S-1 is a dihydropyrimidine dehydrogenase (DPD)-inhibitory fluoropyrimidine (DIF), which showed the highest response rate among many oral anticancer agents against unresectable advanced gastric cancer in early and late phase II studies [5–7]. In these phase II trials, S-1 showed 40% and higher response rates with acceptable low toxicity. Based on these results, a phase III trial to compare S-1 with two other regimens is underway as a JCOG (Japan Clinical Oncology Group) trial for unresectable and recurrent gastric cancer.

At the same time, the high response rate and low toxicity of S-1 has led to its adjuvant use. Oral anticancer drugs are also attractive for outpatient use. A feasibility study to confirm the safety of S-1 for adjuvant chemotherapy after curative surgery was necessary before starting the phase III trial.

Patients and methods

Design of the trial

The trial was non-blinded and open-label. The primary endpoint was the rate of accomplishment of the scheduled adjuvant chemotherapy. Secondary endpoints were the incidence and grade of adverse reactions.

In this trial, the sample size was 50, without any calculations based on statistical assumptions.

Patient eligibility

Patient eligibility required compliance with the following criteria: gastric adenocarcinoma with histological proof; operative findings revealing advanced gastric cancer (T2 or more); curability B resection, defined in the *Japanese classification of gastric carcinoma* [8]; sufficient oral intake; no prior treatment except for surgery; and more than 19 and less than 76 years of age. Patients also had to have adequate organ function ($4000 \leq$ leukocytes $< 12000/mm^3$; thrombocytes, $\geq 100000/mm^3$; total bilirubin, ≤ 1.5 mg/dl; GOT and GPT, less than twice the normal limits at each institution; creatinine, ≤ 1.5 mg/dl). Patients expected to receive medication and to be followed-up regularly for more than 48 weeks. Patients with a history of drug hypersensitivity, serious surgical and non-surgical complications, or active secondary cancer were excluded. Pregnant or lactating women were excluded. This study was approved by the institutional review board at each site, and written informed consent was obtained from all patients.

Treatment schedule

Chemotherapy consisted of eight courses (4-week administration and 2-week withdrawal) of S-1 (tegafur, gineracil, oteracil potassium; Taiho Pharmocentical, Tokyo, Japan) at 80–120 mg/body per day according to the body surface area (BSA): BSA $< 1.25\,m^2$, 80 mg/day; $1.25 \leq$ BSA $< 1.5\,m^2$, 100 mg/day; $1.5\,m^2 \leq$ BSA, 120 mg/day. S-1 was administered orally, twice daily after meals for 4 weeks after surgery. Doses were modified in accordance with the following guidelines. When adverse reactions appeared, the dose was reduced from 120 to 100 mg/day or from 100 to 80 mg/day, or administration was temporarily discontinued. The withdrawal period due to adverse reactions was less than 16 days in the same course, with a maximum of 28 days' administration of the drugs in total. Treatment was discontinued when the patient showed recurrence of disease or adverse reactions that were uncontrollable by dose modification and temporary withdrawal of drug administration. A 48-week period of temporary drug withdrawal was the limit set for a patient not being able to enter a new course.

Evaluation of toxicity

The National Cancer Institute Common Toxicity Criteria (NCI-CTC; 1998) were adopted to determine the toxicity of the chemotherapy.

Results

In November 2000, an interim analysis was performed in order to determine whether to continue further recruitment of patients. Because information from the periodic safety report of use-results surveillance (September 24, 2000) from Taiho Pharmaceutical to the Ministry of Health, Labor, and Welfare revealed there were no specific adverse reactions in up to two courses in 110 patients who received S-1 within 30 days after surgical resection of gastric cancer, and because a feasible result was obtained by monitoring the patients in the present study, we decided to complete recruitment at 41 patients.

Of the 41 patients, 6 patients were ineligible. Four patients had received curable A resection, 1 patient had received intraoperative chemotherapy with cisplatin (CDDP), and the other patient had distant metastasis at the time of the operation. Thirty-five patients were eligible (full analysis set [FAS]).

Table 1 shows the characteristics of the FAS. Thirty of the 35 patients (85.7%) had rather advanced stage disease (TNM stage IIIA or more; Table 1).

Table 2 shows drug compliance in each course and the reasons for discontinuation of drug administration. In 7 of the 35 patients (FAS), administration of S-1 was discontinued due to recurrence. The planned eight courses of S-1 were administered to 17 patients (60.7%). In 4 patients, drug administration was discontinued in the first course at the patient's request, due to anorexia. The main reason for discontinuation was recurrence of disease.

Table 3 shows the drug compliance of the FAS (days and total amount of the drug). In every course, drug compliance was maintained at more than 85% (86.0%–90.4%). In the total of 35 patients (FAS), the percentage of actual administration days against the total number of planned administration days (28 days × 8;

Table 1. Patient characteristics

		Number of patients	Percentage
Sex	Male	20	57.1
	Female	15	42.9
Age (years)	20–29	1	2.9
	30–39	2	5.7
	40–49	5	14.3
	50–59	4	11.4
	60–69	12	34.3
	70–79	11	31.4
	Mean, 60.3; median, 65.0		
BSA (m^2)	1.20–1.39	10	28.6
	1.40–1.59	20	57.1
	1.60–1.79	5	14.3
	Mean, 1.47; median 1.47		
Lymph node dissection	D2	24	68.6
	D3	11	31.4
Type of resection	Distal gastrectomy	15	42.9
	Total gastrectomy	19	54.3
	Proximal gastrectomy	1	2.9
Combined resection	No	11	31.4
	Yes	24	68.6
Reconstruction	Billroth I	5	14.3
	Billroth II	8	22.9
	Roux-Y	20	57.1
	Interposition	1	2.9
	Other	1	2.9
Japanese Stage	IB	1	2.9
	II	3	8.6
	IIIA	12	34.3
	IIIB	9	25.7
	IV	10	28.6
TNM Stage	IB	2	5.7
	II	3	8.6
	IIIA	9	25.7
	IIIB	8	22.9
	IV	13	37.1

BSA, body surface area

224) was 79.0% (median) and 69.7% (mean). Concerning the amount of the drug, compliance was 75.2% (median) and 67.3% (mean).

Table 4 shows summaries of the adverse reactions that developed in more than 10% of the 41 patients in total, grouped as laboratory findings-based and clinical findings-based. Of the laboratory findings-based adverse reactions, neutropenia was the most frequent, in 35 of the 41 patients (85.4%) followed by leukopenia (75.6%), increase in serum total bilirubin (53.7%), GOT (41.5%), anemia (hemoglobin [Hb]; 41.5%), anemia (RBC; 34.1%), alkaline phosphatase (ALP; 26.8%), GPT (26.8%), lactate dehydrog-enase (LDH; 24.4%), thrombocytopenia (24.4%), proteinuria (24.4%), lymphopenia (19.5%), anemia (hematocrit [Hct]; 14.6%), hyperkalemia (12.2%), blood urea nitrogen (BUN; 12.2%), and hypoalbuminemia (12.2%). Among the clinical findings-based adverse reactions, anorexia was the most frequent (68.3%), followed by fatigue (61.0%), diarrhea (58.5%), nausea (51.2%), stomatitis (51.2%), pigmentation changes (46.3%), weight loss (39.0%), rash (31.7%), and vomiting (19.5%). Concerning the incidence and grade of laboratory findings-based adverse reactions, grade 3 adverse reactions were seen with neutropenia, leucopenia, lymphopenia, anemia (Hb), GOT, and GPT. However, there were no grade 4 adverse reactions. In the clinical findings-based adverse reactions, grade 3 adverse reactions were observed with anorexia, fatigue, diarrhea, and weight loss. There were also no grade 4 adverse reactions.

Figure 1 shows comparisons of the incidences of the main adverse reactions in a late-phase II study [9] and those in the present study. When compared with the

Table 2. Drug compliance (each course)

Course no.	FAS full analysis set; $n = 35$		Excluding patients with recurrence ($n = 28$)		Reasons for discontinuation of drug administration
	Number of patients entering the course	Percentage	Number of patients entering the course	Percentage	
1	35	—	28	—	Patient refusal (anorexia; $n = 4$) Complication, (varicose; $n = 1$)
2	30	85.7	23	82.1	
3	30	85.7	23	82.1	Recurrence ($n = 1$)
4	29	82.9	23	82.1	Patient refusal (anorexia; $n = 1$) Dr's judgment (poor general condition; $n = 1$)
5	27	77.1	21	75.0	Recurrence ($n = 3$)
6	24	68.6	21	75.0	Recurrence ($n = 2$) Adverse reaction (arrhythmia; $n = 1$)
7	21	60.0	20	71.4	Recurrence ($n = 1$) Unable to enter the eight course ($n = 3$)[a]
8	17	48.6	17	60.7	Patient refusal (adverse reaction; $n = 1$)

[a]Because of prolongation of the period during which the drug was temporarily withdrawn

Table 3. Drug compliance (days and total amount of the drug)

Course no.	No. of patients entering the course	Percent administration days (mean)[a]	Percent administration amount (mean)[b]
1	35	85.9	87.9
2	30	91.4	90.4
3	30	91.7	88.1
4	29	92.3	87.4
5	27	96.1	90.4
6	24	92.1	89.0
7	21	93.7	86.0
8	17	91.8	87.1
Overall mean ($n = 35$)		69.7	67.3
Overall median ($n = 35$)		79.0	75.2

[a]Days actually administered as a percentage of planned number of days
[b]Amount of drug as actually administered a percentage of planned amount

data from the late-phase II study [9], a higher incidence of adverse reactions was observed in the present study.

Discussion

As mentioned in the "Introduction", S-1 is an attractive oral anticancer agent for advanced gastric cancer, with a high response rate and low toxicity. The possibility of outpatient use of S-1 has increased the convenience for both doctors and patients, and it has led to the idea of using S-1 as an adjuvant chemotherapeutic agent. Up to 1999, there were no trials of S-1 use in the adjuvant setting. Therefore, as a prerequisite to conducting a large-scale clinical trial of adjuvant S-1, the present study was carried out to confirm the feasibility of adjuvant S-1 given after curative gastrectomy.

Survival benefits of adjuvant chemotherapy after curative resection of gastric cancer have not yet been proved by a large-scale prospective randomized trial, as stated in the guidelines of the Japanese Gastric Cancer Association (Japanese guidelines). Even though

Table 4. Adverse reactions (n = 41)

	Grade				
	4	3	2	1	Total (incidence; percentage)
Laboratory findings					
Neutropenia		12	16	7	85.4
Leukopenia		4	15	12	75.6
Lymphopenia		2	5	1	19.5
Thrombocytopenia			2	8	24.4
Anemia (Hb)		3	8	6	41.5
Anemia (RBC)			5	9	34.1
Anemia (Hct)			3	3	14.6
GOT		2	1	14	41.5
GPT		1	2	8	26.8
LDH				10	24.4
ALP			1	10	26.8
Total bilirubin			8	14	53.7
Hypoalbuminemia			1	4	12.2
Hyperkalemia				5	12.2
BUN				5	12.2
Proteinuria			1	9	24.4
Clinical findings					
Anorexia		4	5	19	68.3
Nausea			3	18	51.2
Vomiting				8	19.5
Diarrhea		4	5	15	58.5
Stomatitis			2	19	51.2
Fatigue		1	5	19	61.0
Pigmentation changes			3	16	46.3
Rash			5	8	31.7
Weight loss		1	9	6	39.0

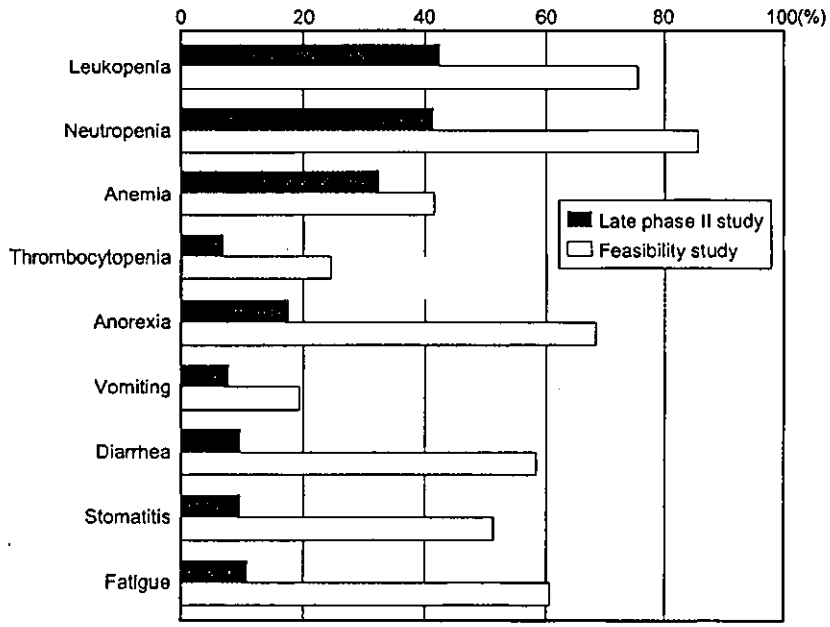

Fig. 1. Comparison of incidence of adverse reactions with that in a late phase II study [9]