Microsatellite Alterations and Protein Expression of 5 Major Tumor Suppressor Genes in Gastric Adenocarcinomas ⊙CrossMark

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Abstract

PURPOSE: In gastric adenocarcinoma (GC), the major tumor suppressor genes (TSGs) such as p16, PTEN, Rb, E-cadherin, and p53, may play important roles in various regulatory pathways and in tumor suppression. This study evaluated the loss of heterozygosity (LOH) of microsatellite and protein expression of 5 TSGs and the results were examined for their correlation with clinicopathological factors. *METHODS:* LOH analysis was carried out using polymerase chain reactions with 15 polymorphic microsatellite markers of 5 chromosomes containing TSGs in 100 surgically resected tumors. Protein expression was evaluated by immunohistochemistry (IHC). *RESULTS:* LOH was detected in 83% of GCs. LOH of 9p21, 10q23, 13q14, 16q22, and 17p13 were detected in 26%, 31%, 24%, 22%, and 35% of cases, respectively. Protein expression of p16, PTEN, Rb, E-cadherin, and p53 were found to be 31%, 39%, 28%, 32%, and 46% of cases. Advanced GCs showed significantly higher rates of 17p13 LOH and p53 expression. 9p21 LOH and E-cadherin IHC were correlated with higher tumor grade. Lymph node metastasis was correlated with the LOH of 9p21, 16q22, and 17p13 and IHC of the Rb and p53. A higher stage was correlated with 10q23 and 17p13 in LOH and p53 for IHC. *CONCLUSION:* These results suggest that LOH and protein expression of various TSGs are important in carcinogenesis and tumor invasion. Additionally, LOH and IHC may be useful clinical indicators for determining the prognosis of patients with GCs. In particular, the 17p13 LOH and p53 for IHC can be applied as simple evaluations in the clinic.

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Introduction

Gastric cancer is one of the most common cancers in Korea [1]. Although it has been extensively studied in the fields of preclinical and clinical medicine and clinical treatment methods have been developed, the incidence and mortality rates of gastric cancer remain very high [2]. Accordingly, the importance of clinical and molecular biological approaches for investigating the early diagnosis, progression, and metastasis mechanism of gastric cancer has been emphasized [3]. Recent studies have mostly focused on specific tumor biological factors, and studies on the complex process of carcinogenesis and central regulators of cell growth and differentiation aside from the P53 and PTEN genes are rare [4–6].

In addition, patients who have undergone similar surgical procedures and share the same pathological diagnosis and TNM stage can still show clinical variations in their prognoses, as well as different characteristics in the oncological approach [7]. Therefore, studies of comprehensive methods for detecting various oncological factors that can easily be applied after surgical treatment of gastric cancer are needed [8].

P53, PTEN, p16, Rb, and E-cadherin are the most well-known and widely researched tumor suppressor genes [9]. Of these, p53 is the most extensively studied. The mechanism of carcinogenesis involving this gene has been reported in most types of cancer, including gastric cancer, and p53 mutations are known the most common in malignancies. Numerous mechanisms of this gene have recently been reported [5,10,11].

Under normal physiological conditions, the expression of tumor suppressor genes is precisely regulated. These genes play a role in cell maintenance by regulating the cell cycle, activating other genes, and being involved in cellular reactions against DNA damage, while also inhibiting carcinogenesis. Mutation or deletion of these tumor suppressor genes can lead to loss of tumor suppression and subsequently to malignancies [12]. Mutations in tumor suppressor genes have been reported to be associated with highly malignant tumors, poorly differentiated type tumors, and high recurrence rates of tumor in numerous studies [13,14].

Advances in molecular biology and cytogenetics have enabled the localization of genes associated with tumor development across all chromosomes, and, accordingly, studies of the association between tumor development and chromosomal mutations have been conducted [15,16]. After the correlation between microsatellite locations and tumor suppressor genes was identified, it was subsequently found that allelic loss or the loss of heterozygosity (LOH) of polymorphic markers contributes to tumor suppressor gene deletions [17,18]. Inactivation of a tumor suppressor gene generally occurs because of a deletion or mutation in both alleles of the tumor suppressor gene [3,12,19]. This process can be examined through LOH analysis using microsatellite markers [20]. LOH on various chromosomes in patients with gastric cancer has been reported both within and outside Korea [21–26].

In a series of studies, Tamura et al. detected LOH on chromosomes 2q, 4p, 5q, 6p, 7q, 11q, 14q, 17p, 18q, and 21q and suggested that LOH at multiple chromosomes may be involved in gastric cancer development [12,19]. In Korea, Choi et al. reported LOH detection rates of 11.5% to 48% for chromosomes 9p, 10q, 13q, 16q, and 17p in patients with gastric cancer [21]. Similarly, Kim et al. found LOH rates of 15% to 60% for chromosomes 3p, 4p, 5q, 8p, 9p, 13q, 17p, and 18q [24]. Sugai et al. and Grundai et al. also reported LOH on multiple chromosomes of patients with gastric cancer [23,25]. However, few studies have investigated the correlation between multiple chromosomes or microsatellite markers and clinicopathologic characteristics. In our study, we used three microsatellite markers associated with major suppressor genes including *p16*, *PTEN*, *Rb*, *E-cadherin*, and *p53*, which are present in chromosomes 9p21, 10q23, 13q14, 16q22, and 17p13, respectively, for LOH analysis and to investigate the correlation between these markers and clinicopathologic findings.

In this study, we used two approaches to analyze the inactivation mechanism of tumor suppressor genes. First, we analyzed microsatellite alterations in five tumor suppressor genes in 100 cases of early and advanced gastric cancer. Second, we performed immunohistochemical analysis on the protein expression of each tumor suppressor gene. In addition, we compared microsatellite alterations and protein expression of major suppressor genes to those of clinicopathologic factors and compared survival rates to investigate their correlations and clinical usefulness.

Material and Methods

Subjects

Among patients who underwent surgical resection after being diagnosed with gastric adenocarcinoma at Kangdong Sacred Heart Hospital, Seoul, Korea between 2000 and 2006, 100 patients whose follow-up records and paraffin blocks were in good condition were included in the study. Tumor paraffin blocks without necrosis and a tumor cell distribution greater than 60% were selected for each patient.

Pathologic Examination

All samples were fixed in 10% neutral formalin and subjected to a treatment process using paraffin-embedded tissues for pathological diagnosis. Paraffin blocks that included cancerous and non-cancerous areas were selected and subjected to hematoxylin and eosin (H&E) staining.

H&E slides of all 100 patients were reexamined under an optical microscope and their clinical and pathological characteristics were investigated. All tissues were reexamined by two specialists in pathology. Clinical information was recorded according to the histologic grade and TNM stage defined by the UICC 6th edition. All clinical records were retrospectively analyzed.

Detection of Microsatellite Alteration on Major Tumor Suppressor Genes

Microdissection and DNA Extraction.   All DNA was obtained from paraffin blocks. Three paraffin-embedded tissues, including cancerous, non-cancerous, and normal (control), were prepared as 6-μm-thick sections and paraffin was removed. H&E staining of the tissue samples were weakened, and a resin solution was smeared on a slide for observation of the samples under a microscope at low power. Cancerous and non-cancerous tissues were scraped off the slide. Next, each sample was placed in a 1.5-mL tube containing DNA extraction buffer solution (100 mM Tris–HCl, pH 8.0; 1% Tween 20, 0.1 ㎎/mL proteinase K). To prevent tissue contamination during each slide replacement, a knife was cleaned with an alcohol swab and a new knife was used for each sample. The tubes were stored in a water bath at 52°C for 2 days. Reacted samples were placed in boiling water for 10 min to inactivate proteinase K, and 500 μl of these samples was moved to a new tube. Next, 500 ml of phenol/chloroform (1:1) was added to the tube and DNA was extracted. This process was repeated until a clear DNA solution was obtained. Subsequently, 50 μl of 3.5 M sodium acetate and 1 mL of 100% ethanol were added to the DNA solution, which was stored at –20°C for 30 min and then centrifuged at 14,000 rpm and 4°C for 30 min for DNA precipitation. The DNA sample was rinsed with 1 mL of 70% ethanol and dried. The concentration of the DNA sample was measured and the sample was used as a DNA template.

Analysis of Microsatellite Alterations on Tumor Suppressor Gene.   In our analysis of microsatellite alterations, microsatellite markers of five chromosomal loci (9p21, 10q23, 13q14, 16q22, 17p13) containing major tumor suppressor genes were used. The markers of each chromosomal locus were as follows, with a total of 15 markers used: D9S104, D9S162, and D9S165 associated with the p16 gene located on the chromosome 9p21 locus; D10S1765, D10S1692, and D10S1483 associated with the *PTEN* gene on the chromosome 10q23 locus; D13S118, D13S153, and D13S273 associated with the *Rb* gene on the chromosome 13q14 locus; D16S419, D16S3106, and D16S498 associated with the *E-cadherin* gene on the chromosome 16q22 locus; and D17S796, TP53, and D17S513 associated with the *p53* gene on the chromosome 17p13 locus. Primers were manufactured by GIBCO Co. (Grand Island, NY, USA). Primer sequences are shown in Table 1.

First, DNA extracted from cancerous and normal areas was used as a template for PCR. Each 12-μl PCR solution contained 1 μl template DNA, 0.2 mM dNTPs, 10× buffer, 10 pmol/μl primer, 1 U Taq polymerase, and distilled water. For each sample, 35–40 cycles of PCR were performed. All samples were reacted in a thermal cycler (Perkin Elmer 9700, Waltham, MA, USA). For each cycle, denaturation was conducted for 30 s at 94°C, annealing for 30 s at 55°C to 60°C, and extension for 40 s at 72°C. The final extension step

Table 1. Primer sequences of 15 microsatellite markers in LOH analysis

D9S162	(f) 5′-GCAATGACCAGTTAAGGTTC-3′
	(r) 5′-AATTCCCACAACAAATCTCC-3′
D9S104	(f) 5′-GATCTGGGTATGTCTTTCTG-3′
	(r) 5′-ACTGGGACTCTAACTAATGT-3′
D9S165	(f) 5′-GACTTTGGCTGCT AGATGTG-3′
	(r) 5′-CAGAGGAGTTACAAATATAGACAGG-3′
D10S1765	(f) 5′–TTACATAGTGCTTTCTGCGGTC-3′
	(r) 5′–TGATCTCGAACTCCTGACCTC-3′
D10S1692	(f) 5′-AGCCTGTGTGAAAGAGCAAGAG-3′
	(r) 5′-CAGTTAGGAAGGAGGCTGTTGT-3′
D10S1483	(f) 5′-TTCCAATGCTATCCCGGCTATG-3′
	(r) 5′-GAGTGGAGGTGGTTAAGAGGT-3′
D13S118	(f) 5′-GCTCCCGGCTGGTTTT-3′
	(r) 5′-GCAGGAAATCGCAGGAACTT-3′
D13S153	(f) 5′-ATTAGCCCAGGTATGGTGAC-3′
	(r) 5′-GCTGTGGTATGAGTTACTTAAACAC-3′
D13S273	(f) 5′-CTGNGGCAAAAACAACTCTT-3′
	(r) 5′-ATCTGTATGTCCTCCTTTCAATG-3′
D16S421	(f) 5′-ACATGAACCGATTGGACTGA-3′
	(r) 5′-CCGTTCCCTATATTTCCTGG-3′
D16S512	(f) 5′-TGAGAGCCAAATAAATAAATGG-3′
	(r) 5′-TCACGTTGTGAATGCAAGT-3′
D16S3106	(f) 5′-GAGACCTACAGTCTTTTGCATTTAC-3′
	(r) 5′-TTTTGAAGCTGAGCAGAAGG-3′
TP53	(f) 5′-TGGATCCTCTTGCAGCAGCC-3′
	(r) 5′-TP53.A2 AACCCTTGTCCTTACCAGAA-3′
D17S796	(f) 5′-CAATGGAACCAAATGTGGTC-3′
	(r) 5′-AGTCCGATAATGCCAGGATG-3′
D17S513	(f) 5′-TTCACTTGTGGGCTGCTGTC-3′
	(r) 5′-TAAGAAAGGCTCCCACAAGCA-3′

was conducted for 10 min at 72°C. Next, 3 μl of amplified PCR products were subjected to 2% agarose gel electrophoresis.

Next, 3 μl of the PCR products and 3 μl formamide loading dye (95% formamide, 20 mM EDTA, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanole) were mixed and boiled at 98°C for denaturation, and then rapidly cooled on ice. Next, 3 μl of this solution was separated by electrophoresis at 1400 V for 90 min followed by silver staining. During silver staining, the glass side attached to the gel by the binding solution was fixed in 10% acetic acid for 30 min. After two washes with distilled water (3 min each), the sample was reacted in silver nitrate solution for 30 min. Next, the

Table 2. Clinicopathologic features of 100 gastric adenocarcinomas

Clinicopathologic features	No. of patients
Gender	
Male	74 (74%)
Female	26 (26%)
Lauren's classification	
Intestinal type	63 (63%)
Diffuse type	37 (37%)
EGC/AGC	
EGC	38 (38%)
AGC	62 (62%)
Depth of invasion	
Mucosa	19 (19%)
Submucosa	19 (19%)
Proper muscle	34 (34%)
Subserosa, serosa	28 (28%)
Lymph node metastasis	
Positive	31 (31%)
Negative	69 (69%)
Stage	
I	32 (32%)
II	35 (35%)
III	27 (27%)
IV	6 (6%)

EGC, early gastric cancer; AGC, advanced gastric cancer.

Table 3. LOH of 9p21 and p16 immunohistochemical expression according to clinicopathological factors

	Total	9p21 LOH	P	p16 IHC	P
Tumor vs normal					
Tumor	100	26 (26%)	<.001	31 (31%)	<.001
Normal	100	0 (0%)		0 (0%)	
Lauren's classification					
Intestinal type	63	11 (17.5%)	.001	21 (33.3%)	.755
Diffuse type	37	15 (40.5%)		10 (27%)	
EGC vs AGC					
EGC	38	9 (23.7%)	.858	9 (23.7%)	.342
AGC	62	17 (27.4%)		22 (35.5%)	
Tumor depth					
Mucosa	19	4 (21.1%)	.650	5 (26.3%)	.443
Submucosa	19	5 (26.3%)		4 (21.1%)	
Proper muscle	34	9 (26.5%)		13 (38.2%)	
Subserosa, serosa	28	8 (28.6%)		9 (32.1%)	
Lymph node metastasis					
Positive	31	12 (38.7%)	.035	9 (29.0%)	.351
Negative	69	14 (20.3%)		22 (31.9%)	

Values are presented as number (%).
LOH, loss of heterozygosity; IHC, immunohistochemistry; EGC, early gastric cancer; AGC, advanced gastric cancer.

sample was rapidly washed in distilled water and the color was developed in sodium carbonate solution until clear bands appeared. The reaction was stopped with 10% acetic acid. The gel was examined for any loss of bands.

Immunohistochemical Staining

Tissues fixed in 10% neutral formalin and subjected to paraffin embedding were cut into continuous 5-μm-thick sections. The sections were deparaffinized with 100% xylene for 3–5 min and rinsed with distilled water. Immunohistochemical staining of the proteins expressed by the five tumor suppressor genes was performed. During immunohistochemical staining, the samples were mixed with phosphate-buffered saline (PBS) and boiled in a microwave or pressure cooker for 5 min to maintain the antigenicity of the proteins. Next, the samples were treated with PBS at 4°C for 5 min, with 3% hydrogen peroxide added to inhibit intrinsic peroxidase activities.

Table 4. LOH of 10q23 and PTEN immunohistochemical expression according to clinicopathological factors

	Total	10q23 LOH	P	PTEN IHC	P
Tumor vs normal					
Tumor	100	31 (31%)	<.001	39 (39%)	<.001
Normal	100	0 (0%)		0 (0%)	
Lauren's classification					
Intestinal type	63	20 (31.7%)	.771	23 (36.5%)	.355
Diffuse type	37	11 (29.7%)		16 (43.2%)	
EGC vs AGC					
EGC	38	10 (26.3%)	.454	13 (43.2%)	.854
AGC	62	21 (33.9%)		26 (41.9%)	
Tumor depth					
Mucosa	19	5 (26.3%)	.659	6 (31.6%)	.763
Submucosa	19	5 (26.3%)		7 (36.8%)	
Proper muscle	34	10 (29.4%)		13 (38.2%)	
Subserosa, serosa	28	11 (39.3%)		13 (46.4%)	
Lymph node metastasis					
Positive	31	14 (45.2%)	.431	13 (41.9%)	.656
Negative	69	17 (34.8%)		26 (37.7%)	

Values are presented as number (%).
LOH, loss of heterozygosity; IHC, immunohistochemistry; EGC, early gastric cancer; AGC, advanced gastric cancer.

Table 5. LOH of 13q14 and Rb immunohistochemical expression according to clinicopathological factors

	Total	13q14 LOH	P	Rb IHC	P
Tumor vs normal					
Tumor	100	24 (24%)	<.001	28 (28%)	<.001
Normal	100	0 (0%)		0 (0%)	
Lauren's classification					
Intestinal type	63	15 (23.8%)	.501	19 (30.2%)	.501
Diffuse type	37	9 (24.3%)		9 (24.3%)	
EGC vs AGC					
EGC	38	10 (26.3%)	.658	11 (28.9%)	.842
AGC	62	14 (22.6%)		17 (27.4%)	
Tumor depth					
Mucosa	19	4 (21.1%)	.850	6 (31.6%)	.803
Submucosa	19	6 (31.6%)		5 (26.3%)	
Proper muscle	34	8 (23.5%)		10 (29.4%)	
Subserosa, serosa	28	6 (21.4%)		7 (25.0%)	
Lymph node metastasis					
Positive	31	9 (29.0%)	.033	6 (19.4%)	.044
Negative	69	15 (21.7%)		22 (31.9%)	

Values are presented as number (%).
LOH, loss of heterozygosity; IHC, immunohistochemistry; EGC, early gastric cancer; AGC, advanced gastric cancer.

The samples were reacted with primary antibody diluted 1:100 and rinsed with PBS. The following five primary antibodies were used:

> p16 antibody (monoclonal mouse, #sc-56,330; Santa Cruz Biotechnology, Santa Cruz, CA, USA)
> PTEN antibody (monoclonal rabbit, Y184; Epitomics, Burlingame, CA, USA)
> Rb antibody (polyclonal mouse; Neomarker, Fremont, CA, USA)
> E-cadherin antibody (monoclonal mouse anti-E-cadherin; Zymed, Carlsbad, CA, USA)
> p53 antibody (monoclonal mouse, DO-7; Novocastra, Buffalo Grove, IL, USA)

The samples were then reacted with biotinylated secondary antibodies (LSAB kit, DAKO, Glostrup, Denmark) for 20 min and then rinsed with PBS. A solution containing streptavidin (Zymed) bound to peroxidase was then reacted with the samples. Color was developed using 3,3′-diaminobenzidine tetrachloride. Contrast

Table 6. LOH of 16q22 and E-cadherin immunohistochemical expression according to clinicopathological factors

	Total	16q22 LOH	P	E-cadherin IHC	P
Tumor vs normal					
Tumor	100	22 (22%)	<.001	32 (32%)	<.001
Normal	100	0 (0%)		0 (0%)	
Lauren's classification					
Intestinal type	63	12 (19.0%)	.155	6 (9.5%)	.001
Diffuse type	37	10 (27.0%)		26 (70.3%)	
EGC vs AGC					
EGC	38	10 (26.3%)	.573	11 (28.9%)	.513
AGC	62	21 (33.9%)		21 (33.9%)	
Tumor depth					
Mucosa	19	2 (10.5%)	.043	6 (31.6%)	.655
Submucosa	19	4 (21.1%)		5 (26.3%)	
Proper muscle	34	9 (26.5%)		12 (35.3%)	
Subserosa, serosa	28	7 (25.0%)		9 (32.1%)	
Lymph node metastasis					
Positive	31	14 (45.2%)	.010	11 (35.5%)	.540
Negative	69	8 (11.6%)		21 (30.4%)	

Values are presented as number (%).
LOH, loss of heterozygosity; IHC, immunohistochemistry; EGC, early gastric cancer; AGC, advanced gastric cancer.

staining was performed by using Mayer's hematoxylin. The samples were washed in running water, dried at room temperature, and sealed.

Determination of Results and Statistical Analysis

Determination of LOH of Microsatellites on Major Tumor Suppressor Genes. LOH was determined by densitometry analysis of bands on the gel. Loss greater than 50% on cancerous tissue relative to normal tissue indicated LOH. Samples showing homozygosity or samples in which PCR was incomplete for tumor cells or normal cells and genetic variations could not be examined were excluded.

Determination of Immunohistochemical Staining Results. Areas showing the highest density of stained cells in 10 samples were examined under an optical microscope at 400× magnification. To evaluate the protein expression of p53, p16, and Rb, the number and percentage of tumor cells with dark reddish-brown nuclei were calculated. If more than 10% of all cells were stained, the result was considered positive. For PTEN protein expression, dark brown cells in the cytoplasm and nuclear membrane indicated positive results.

For E-cadherin protein expression, the results were considered positive if reddish-brown granules appeared along the cell membrane of tumor cells, which is when proteins were expressed at the same intensity as those in the epithelium of normal gastric tissue of the control group, and were stained in more than 70% of all tumor cells. Results were considered negative if the expression intensity was weak or less than 70% of tumor cells were stained.

Statistical Analysis. For each sample, LOH for each microsatellite marker and immunohistochemical protein expression results were expressed as percentages. Correlations between patients' clinical stage and clinicopathologic features as well as prognostic factors were compared and tested by the chi-square test. The correlation between LOH and protein expression was tested by Fisher's exact test. A survival curve was drawn using the Kaplan Meier method and tested by a log-tank test. Statistical significance was set at a $P < .05$.

Results

Clinical and Pathological Findings

A total of 100 cases of gastric adenocarcinoma (74 male, 25 female) were analyzed. There were 38 cases of early gastric cancer and 62 cases of advanced gastric cancer (Table 2).

Regarding depth of tumor invasion, there were 19 cases (19%) of mucosa-confined cancer, 19 (19%) cases of submucosa invasion, 34 cases (34%) of proper muscle layer invasion, and 28 cases (28%) involving more than subserosal layer.

Based on Lauren's classification of gastric cancer, there were 63 cases (63%) of intestinal type and 37 cases (37%) of diffuse type. Thirty-one patients (31%) had lymph node metastasis and 69 patients (69%) did not.

LOH Patterns of Microsatellite Markers on Five Chromosomes

LOH was detected on all 15 markers (3 markers per chromosome) across the five chromosomes (9p21, 10q23, 13q14, 16q22, and 17p13) studied. At least one LOH was detected in 83 of 100 cases of gastric adenocarcinoma. LOH was not found in 17 cases (17%). LOH was not observed in 10 of 38 cases (26.3%) of early gastric cancer (EGC) and 7 of 62 cases (11.3%) of advanced gastric cancer (AGC). LOH was not detected at a significantly higher rate in EGC than in AGC ($P = .033$).

In 40 of 83 cases (40%), LOH was found on only one chromosome. And LOH was found on two chromosomes in 33 of 83 cases (33%), three chromosomes in 8 of 83 cases (8%), and four

Table 7. LOH of 17p13 and p53 immunohistochemical expression according to clinicopathological factors

	Total	17p13 LOH	P	p53 IHC	P
Tumor vs normal					
Tumor	100	35 (35%)	<.001	46 (46%)	<.001
Normal	100	0 (0%)		0 (0%)	
Lauren's classification					
Intestinal type	63	23 (36.5%)	.601	30 (47.6%)	.722
Diffuse type	37	12 (32.4%)		16 (43.2%)	
EGC vs AGC					
EGC	38	10 (26.3%)	.041	13 (34.2%)	.029
AGC	62	25 (40.3%)		33 (53.2%)	
Tumor depth					
Mucosa	19	4 (21.1%)	.039	6 (31.6%)	.031
Submucosa	19	6 (31.6%)		7 (36.8%)	
Proper muscle	34	13 (38.2%)		18 (52.9%)	
Subserosa, serosa	28	12 (42.9%)		15 (53.6%)	
Lymph node metastasis					
Positive	31	15 (48.4%)	.036	19 (61.3%)	.020
Negative	69	20 (29.0%)		27 (39.1%)	

Values are presented as number (%).
LOH, loss of heterozygosity; IHC, immunohistochemistry; EGC, early gastric cancer; AGC, advanced gastric cancer.

chromosomes in two of 83 cases (2%). However, there were no cases in which LOH was detected in all 5 chromosomes. Thus, LOH was found on at least two chromosomes in a total of 43 cases (43%).

The LOH detection rate for each chromosome was 26% (n = 26) for 9p21, 31% (n = 31) for 10q23, 24% (n = 24) for 13q14, 22% (n = 22) for 16q22, and 35% (n = 35) for 17p13. The rate of LOH detection was highest on the chromosome 17p13 locus, where the p53 gene is located, and lowest on the chromosome 16q22 locus, where the E-cadherin gene is located.

Table 8. Correlation of loss of heterozygosity and immunohistochemical method

A. LOH of 9p21 vs p16 immunohistochemical expression

p16 IHC	9p21 LOH (+)	(–)	Total
(+)	13 (41.9%)	18 (58.1%)	31
(–)	13 (18.8%)	56 (81.2%)	69
Total	26	74	P = .174

B. LOH of 10q23 vs PTEN immunohistochemical expression

PTEN IHC	10q23 LOH (+)	(–)	Total
(+)	21 (53.8%)	18 (46.2%)	39
(–)	10 (16.4%)	51 (83.6%)	61
Total	31	69	P = .043

C. LOH of 13q14 vs Rb immunohistochemical expression

Rb IHC	13q141 LOH (+)	(–)	Total
(+)	10 (35.7%)	18 (64.3%)	28
(–)	14 (19.4%)	58 (80.6%)	72
Total	24	76	P = .211

D. LOH of 16q22 vs E-cadherin immunohistochemical expression

E-cadherin IHC	16q22 LOH (+)	(–)	Total
(+)	13 (40.6%)	19 (59.4%)	32
(–)	9 (13.2%)	59 (86.8%)	68
Total	22	78	P = .171

E. LOH of 17p13 vs p53 immunohistochemical expression

p53 IHC	17p13 LOH (+)	(–)	Total
(+)	20 (43.5%)	26 (56.5%)	46
(–)	15 (27.8%)	39 (72.2%)	54
Total	35	65	P = .342

LOH, loss of heterozygosity; IHC, immunohistochemistry.

Table 9. Summary of statistically significant parameters of loss of heterozygosity and immunohistochemical expression of 5 tumor suppressor genes

	p16		PTEN		Rb		E-cadherin		p53	
	LOH	IHC	LOH	IHC	LOH	IHC	LOH	IHC	LOH	IHC
Tumor vs normal	p	p	p	p	p	p	p	p	p	p
Lauren's classification	p							p		
EGC vs AGC									p	p
Tumor depth						p			p	p
LN metastasis	p					p	p		p	p
Survival rate						p			p	

Statistically significant parameters are listed as p.
LOH, loss of heterozygosity; IHC, immunohistochemistry; EGC, early gastric cancer; AGC, advanced gastric cancer; LN, lymph node.

Because LOH was not detected on any of the markers on the five chromosomes above in the control group, all five types of LOH were detected specifically in cases of gastric cancer (*P* < .001).

Correlation Between LOH on Each Chromosome and Clinicopathologic Features

LOH on Chromosome 9p21 Locus. Microsatellite alterations in the p16 gene associated with the chromosome 9p21 locus were detected in 26 cases, using three markers, D9S104, D9S162, and D9S165.

LOH was found in 11 of 63 cases (17.5%) of intestinal type and 15 of 37 cases (40.5%) of diffuse type per Lauren's classification; the LOH detection rate was significantly lower for intestinal type (*P* = .001). LOH was found in 9 of 38 cases (23.7%) of EGC and 17 of 62 cases (27.4%) of AGC; no significant difference in the LOH detection rate was found between the two groups.

Regarding LOH detection rates in terms of the depth of invasion, LOH was found in 4 of 19 (21.1%) of mucosa-confined cancer, 5 of 19 (26.3%) of submucosa invasion cases, 9 of 34 (26.5%) of proper muscle invasion cases, and 8 of 28 (28.6%) of more than subserosal layer invasion cases; no significant difference in the LOH detection rate was found with respect to the depth of invasion.

Regarding LOH detection rates in terms of the presence of lymph node metastasis, LOH was found in 12 of 31 lymph node-positive cases (38.7%) and 14 of 69 lymph node-negative cases (20.3%); the LOH detection rate was significantly higher for lymph node-positive cases (*P* = .035).

The five-year survival rate of patients with LOH on the chromosome 9p21 locus was 73.1%. No significant difference in the survival rate was found with respect to the presence or absence of LOH (*P* = .565, Figure 2A).

Detection of Microsatellite Alterations on the Chromosome 10q23 Locus. Microsatellite alterations of the PTEN gene associated with the chromosome 10q23 locus were detected in 31 cases, using three markers, D10S1765, D10S1692, and D10S1483.

Regarding LOH detection rates in terms of the type of gastric cancer determined by Lauren's classification, LOH was found in 20 of 63 cases (31.7%) of intestinal type and 11 of 37 cases (29.7%) of diffuse type; no significant difference in LOH detection rates was found between the two groups.

LOH was found in 10 of 38 cases (26.3%) of EGC and 21 of 62 cases (33.9%) of AGC; the LOH detection rate was higher for AGC, but not significantly.

Regarding LOH detection rates in terms of the depth of invasion, LOH was found in 5 of 19 cases (26.3%) of mucosa confined gastric cancer, 10 of 34 cases (29.4%) of proper muscle invasion, and 11 of
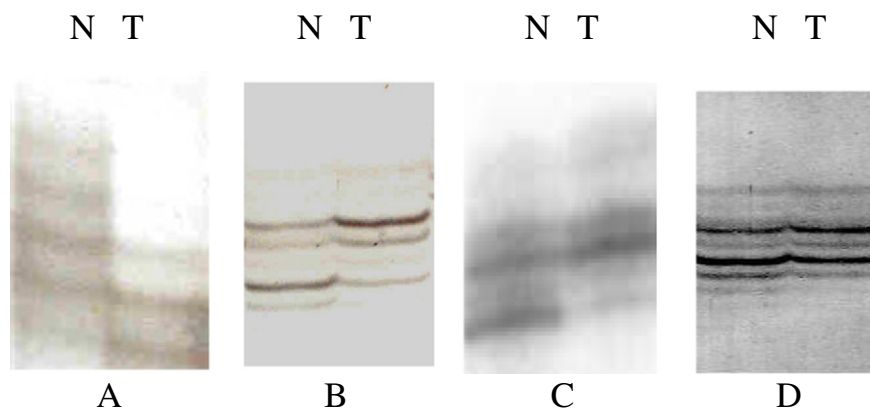
N T   N T   N T   N T

A   B   C   D

Figure 1. Representative loss of heterozygosity band pattern in gastric adenocarcinomas; A. D9STP53 B. D10S1692 C. D13S273 D. D16S512 markers. Each T lane shows lower or upper band loss. N (normal), T (tumor).

28 cases (39.3%) of more than subserosal layer invasion; no significant difference in the LOH detection rate was found with respect to the depth of invasion.

Regarding LOH detection rates in terms of the presence of lymph node metastasis, LOH was found in 14 of 31 lymph node-positive cases (45.2%) and 17 of 69 lymph node-negative cases (34.8%); no significant difference in the LOH detection rate was found with respect to the presence or absence of lymph node metastasis (Table 4, Figure 1B).

The five-year survival rate of patients with LOH on the chromosome 10q23 locus was 68.8%. This was lower than that of LOH-negative patients (68.8%), but not significantly (P = .169, Figure 3A).

Detection of Microsatellite Alterations on the Chromosome 13q14 Locus. Microsatellite alterations of the Rb gene associated with the chromosome 13q14 locus were detected in 24 cases, using three markers, D13S118, D13S153, and D13S273.

Regarding LOH detection rates in terms of the type of gastric cancer determined by Lauren's classification, LOH was found in 15 of 63 cases (23.8%) of intestinal type and 9 of 37 cases (24.3%) of diffuse type gastric cancer; no significant difference in the LOH detection rate was found between the two groups.

LOH was detected in 10 of 38 cases (26.3%) of EGC and 14 of 62 cases (22.6%) of AGC; the LOH detection rate was lower for AGC, but not significantly.

Regarding LOH detection rates in terms of the depth of invasion, LOH was found in 4 of 19 cases (21.1%) of mucosa-confined gastric cancer, 6 of 19 cases (31.6%) of submucosa invasion, 8 of 34 cases (23.5%) of proper muscle invasion, and 6 of 28 cases (21.4%) of more than subserosal layer; no significant difference in the LOH detection rate was found with respect to the depth of invasion.

Regarding LOH detection rates in terms of the presence of lymph node metastasis, LOH was found in 9 of 31 lymph node-positive cases (29.0%) and 15 of 69 lymph node-negative cases (21.7%); no significant difference in the LOH detection rate was found with respect to the presence or absence of lymph node metastasis (Table 5, Figure 1C).

No significant difference in the five-year survival rate was found with respect to the presence or absence of LOH on the chromosome 13q14 locus (P = .567, Figure 4A).

Detection of Microsatellite Alterations on the Chromosome 16q22 Locus. Microsatellite alterations of the E-cadherin gene associated with the chromosome 16q22 locus were detected in 22 cases, using three markers, D16S419, D16S3106, and D16S498.

Regarding LOH detection rates in terms of the type of gastric cancer determined by Lauren's classification, LOH was detected in 12 of 63 cases (19.0%) of intestinal type and 10 of 37 cases (27.0%) of diffuse type gastric cancer; the LOH detection rate was higher for diffuse type gastric cancer, but not significantly.

LOH was found in 10 of 38 cases (26.3%) of EGC and 21 of 62 cases (33.9%) of AGC; the LOH detection rate was higher for AGC, but not significantly.

Regarding LOH detection rates in terms of the depth of invasion, LOH was detected in 2 of 19 cases (10.5%) of mucosa-confined gastric cancer, 4 of 19 cases (21.1%) of submucosa invasion, 9 of 34 cases (26.5%) of proper muscle invasion, and 7 of 28 cases (25.0%) of more than subserosal layer invasion; no significant difference in the LOH detection rate was found with respect to the depth of invasion. However, the LOH detection rate was significantly low for mucosa confined EGC (P = .043).

Regarding LOH detection rates in terms of the presence of lymph node metastasis, LOH was detected in 14 of 31 lymph node metastasis-positive cases (45.2%) and 8 of 69 lymph node metastasis-negative cases (11.6%); the LOH detection rate was significantly high for lymph node metastasis-positive cases (P = .01) (Table 6, Figure 1D).

The 5-year survival rate of patients without LOH on the chromosome 16q22 locus was 83.3%, while that of patients with LOH on the same chromosome was 54.5%; a significant difference existed between the two survival rates (P = .008, Figure 5A).

Detection of Microsatellite Alterations on the Chromosome 17p13 Locus. Microsatellite alterations of the p53 gene associated with the chromosome 17p13 locus were detected in 35 cases, using three markers, D17S796, TP53, and D17S513.

Regarding LOH detection rates in terms of the type determined by Lauren's classification, LOH was found in 23 of 63 cases (36.5%) of intestinal type and 12 of 37 cases (32.4%) of diffuse type gastric cancer; no significant difference in the LOH detection rate was found between the two groups.

LOH was found in 10 of 38 cases (26.3%) of EGC and 25 of 62 cases (40.3%) of AGC; the LOH detection rate was significantly higher for AGC (P = .041).

Regarding LOH detection rates in terms of the depth of invasion, LOH was detected in 4 of 19 cases (21.1%) of mucosa-confined gastric cancer, 6 of 19 cases (31.6%) of submucosa invasion, 13 of 34 cases (38.2%) of proper muscle invasion, and 12 of 28 cases (42.9%)

of more than subserosal layer invasion; LOH detection significantly increased as the depth of invasion increased ($P = .039$).

Regarding LOH detection rates in terms of the presence of lymph node metastasis, LOH was found in 15 of 31 lymph node metastasis-positive cases (48.4%) and 20 of 69 lymph node metastasis-negative cases (29.0%); the LOH detection rate was significantly high for lymph node metastasis-positive cases ($P = .036$) (Table 7).

A comparison of five-year survival rates in terms of the presence of LOH showed lower survival rates for patients with LOH compared to those without LOH ($P = .02$, Figure 6A).

Correlation Between Immunohistochemical Protein Expression of Each Gene and Clinicopathological Features

p16 Protein Expression.   p16 protein expression was observed in a total of 31 cases (31%). Expression was observed in 21 of 63 cases (33.3%) of intestinal type and 10 of 37 cases (27.0%) of diffuse type gastric cancer determined per Lauren's classification; no significant difference in the level of protein expression was found with respect to the Lauren's classification type.

Nine of 38 cases (23.7%) of EGC and 22 of 62 cases (35.5%) of AGC were p16 protein expression positive; the level of protein expression was higher for AGC, but not significantly.

Regarding the levels of p16 protein expression in terms of the depth of invasion, p15 proteins were expressed in 5 of 19 cases (26.3%) of mucosa-confined gastric cancer, 4 of 19 cases (21.1%) of submucosa invasion, 13 of 34 cases (38.2%) of proper muscle invasion, and 9 of 28 cases (32.1%) of more than subserosal layer; no significant difference in the level of protein expression was found with respect to the depth of invasion.

Regarding the levels of p16 protein expression in terms of the presence of lymph node metastasis, p16 proteins were expressed in 9 of 31 lymph node metastasis-positive cases (29.0%) and 22 of 69 lymph node metastasis-negative cases (31.9%); no significant difference in the level of protein expression was found with respect to the presence or absence of lymph node metastasis (Table 3).

No significant difference in the five-year survival rate was found with respect to the level of p16 protein expression (Figure 2B).

PTEN Protein Expression.   PTEN protein expression was observed in a total of 39 cases (39%). PTEN proteins were expressed in 23 of 63 cases (36.5%) of intestinal type and 16 of 37 cases (43.2%) of diffuse type gastric cancer determined by Lauren's classification; no significant difference in the level of protein expression was found with respect to Lauren's classification type.

Thirteen of 38 cases (34.2%) of EGC and 26 cases of AGC (41.9%) were PTEN protein positive; no significant difference in the level of protein expression was found between the two groups.

Regarding the levels of PTEN protein expression in terms of the depth of invasion, PTEN proteins were expressed in 6 of 19 cases (31.6%) of mucosa-confined gastric cancer, 7 of 19 cases (36.8%) of submucosa invasion, 13 of 34 cases (38.2%) of proper muscle invasion, and 13 of 28 cases (46.4%) of more than subserosal layer invasion; no significant difference in the level of protein expression was found with respect to the depth of invasion.

Regarding the levels of PTEN protein expression in terms of the presence of lymph node metastasis, PTEN proteins were expressed in 13 of 31 lymph node metastasis-positive cases (41.9%) and 26 of 69 lymph node metastasis-negative cases (37.7%); no significant difference in the level of protein expression was found with respect to the presence or absence of lymph node metastasis (Table 4).
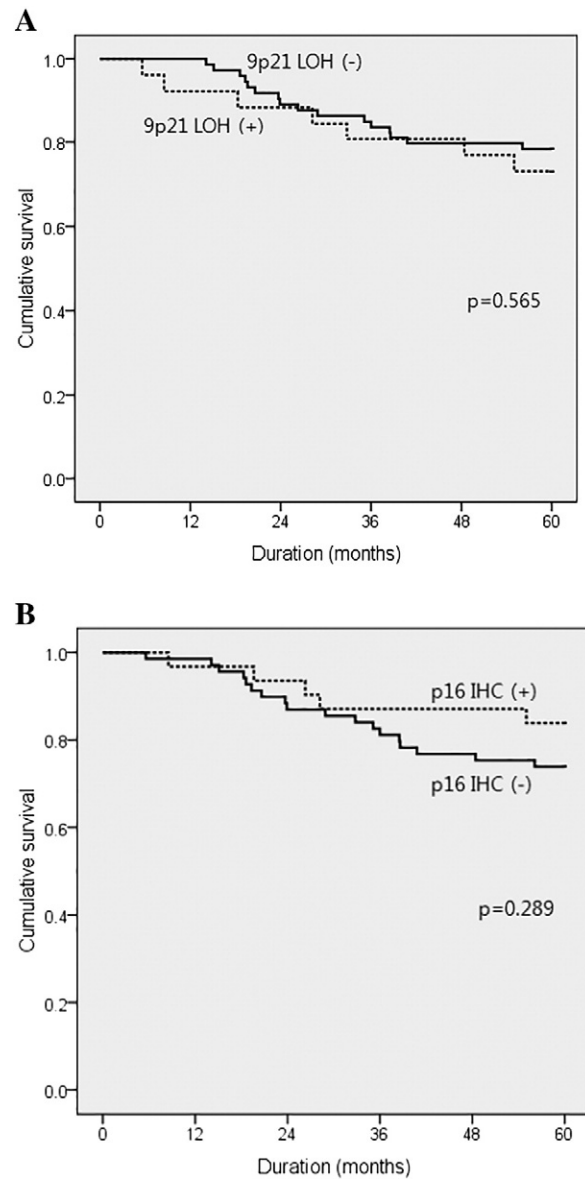
Figure 2. Cumulative overall survival of patients according to LOH status of 9p21 (A) and p16 immunohistochemical expression (B). LOH, loss of heterozygosity; IHC, immunohistochemistry.

No significant difference in the survival rate was found with respect to the level of PTEN protein expression (Figure 3B).

Rb Protein Expression.   Rb protein expression was observed in a total of 28 cases (28%). Rb proteins were expressed in 19 of 63 cases (30.2%) of intestinal type, and 9 of 37 cases (24.3%) of diffuse type gastric cancer determined by Lauren's classification; no significant difference in the level of protein expression was found between the Lauren's classification types.

Eleven of 38 cases (28.9%) of EGC, and 17 of 62 cases (27.4%) of AGC were Rb protein-positive; no significant difference in the level of protein expression was found between the two groups.

Regarding the levels of Rb protein expression in terms of the depth of invasion, Rb proteins were expressed in 6 of 19 cases (31.6%) of mucosa-confined gastric cancer, 5 of 19 cases (26.3%) of submucosa invasion, 10 of 34 cases (29.4%) of proper muscle invasion, and 7 of 28 cases (25.0%) of more than subserosal layer invasion; no significant
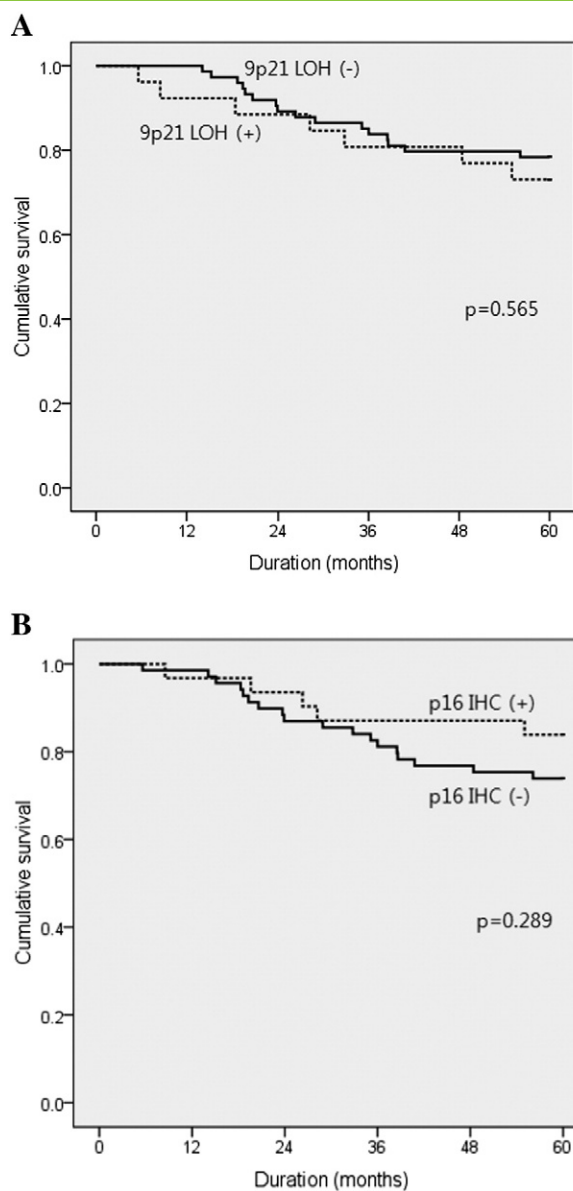
Figure 3. Cumulative overall survival of patients according to LOH status of 10q23 (A) and PTEN immunohistochemical expression (B). LOH, loss of heterozygosity; IHC, immunohistochemistry.
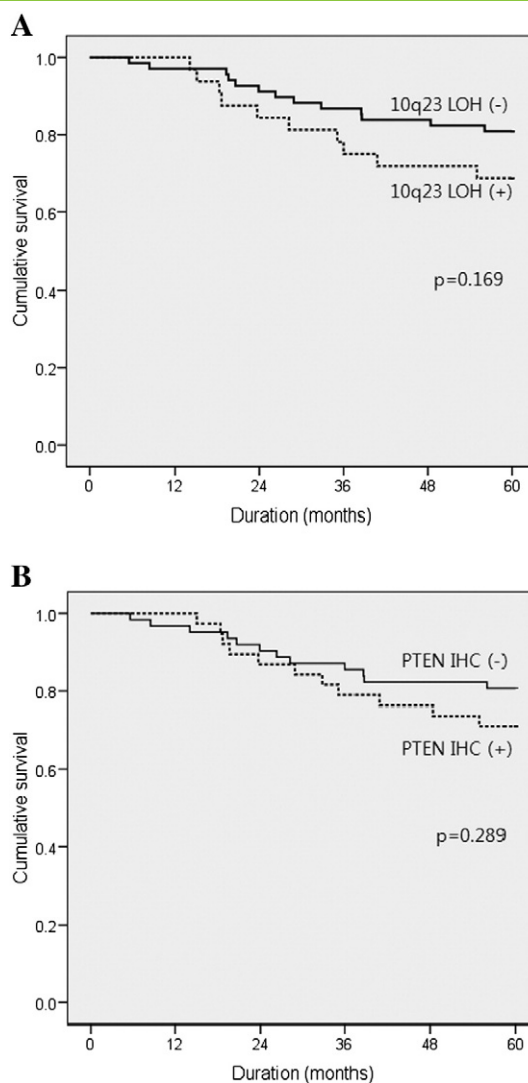
Figure 4. Cumulative overall survival of patients according to LOH status of 13q14 (A) and Rb immunohistochemical expression (B). LOH, loss of heterozygosity; IHC, immunohistochemistry.

difference in the level of protein expression was found with respect to the depth of invasion.

Regarding the levels of Rb protein expression in terms of the presence of lymph node metastasis, Rb proteins were expressed in 6 of 31 lymph node metastasis-positive cases (19.4%) and 22 of 69 lymph node-metastasis negative cases (31.9%); the level of Rb protein expression was significantly lower in the lymph node-positive cases (*P* = .044) (Table 5).

No significant difference in the survival rate was found with respect to the level of Rb protein expression (*P* = .427, Figure 4*B*).

E-Cadherin Protein Expression. Samples were considered E-cadherin protein-positive if there was any protein loss. Protein loss was observed in a total of 32 cases (32%), including in 6 of 63 cases (9.5%) of intestinal type and 26 of 37 cases (70.3%) of diffuse type gastric cancer determined by Lauren's classification; the rate of protein loss was significantly higher for diffuse type gastric cancer (*P* = .001).

Eleven of 38 cases (28.9%) of EGC and 21 of 62 cases (33.9%) of AGC showed protein loss; no significant difference in the rate of protein loss was found between the two groups.

Regarding the rates of protein loss in terms of the depth of invasion, protein loss was observed in 6 of 19 cases (31.6%) of mucosa-confined gastric cancer, 5 of 19 cases (26.3%) of submucosa invasion, 12 of 34 cases (35.3%) of proper muscle invasion, and 9 of 28 cases (32.1%) of more than subserosal layer invasion; no significant difference in the level of protein expression was found with respect to the depth of invasion.

Regarding the rates of protein loss in terms of the presence of lymph node metastasis, protein loss was observed in 11 of 31 lymph node metastasis-positive cases (35.5%) and 21 of 69 lymph node metastasis-negative cases (30.4%); no significant difference in the level of protein expression was found with respect to the presence of lymph node metastasis (Table 6).

No significant difference in the survival rate was found with respect to the level of E-cadherin protein expression (Figure 5*B*).

p53 Protein Expression. p53 protein expression was observed in a total of 46 cases (46%). p53 proteins were expressed in 30 of 63 cases
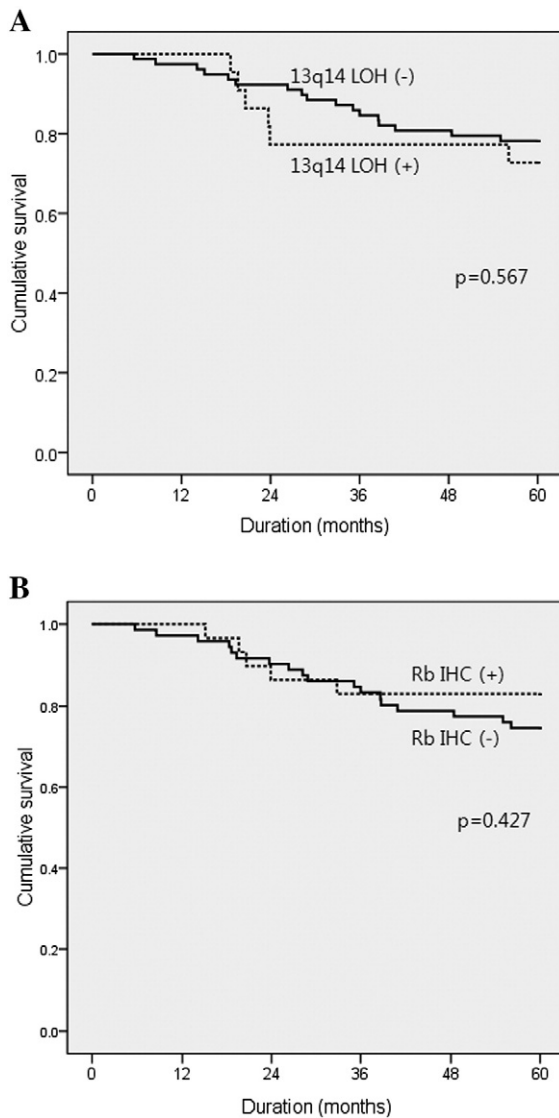
Figure 5. Cumulative overall survival of patients according to LOH status of 16q22 (A) and E-cadherin immunohistochemical expression (B). LOH, loss of heterozygosity; IHC, immunohistochemistry.

Figure 6. Cumulative overall survival of patients according to LOH status of 17p13 (A) and p53 immunohistochemical expression (B). LOH, loss of heterozygosity; IHC, immunohistochemistry.

(47.6%) of intestinal type and 16 of 30 cases (47.6%) of diffuse type gastric cancer; no significant difference in the level of p53 protein expression was found between the Lauren's classification types.

Thirteen of 38 cases (34.2%) of EGC and 33 of 62 cases (53.2%) of AGC were p53-positive; the level of p53 protein expression was significantly higher for AGC (P = .029).

Regarding the levels of p53 protein expression in terms of the depth of invasion, 6 of 19 cases (31.6%) of mucosa-confined gastric cancer, 7 of 19 cases (36.8%) of submucosa invasion, 18 of 34 cases (52.9%) of proper muscle invasion, and 15 of 28 cases (53.6%) of more than subserosal layer invasion exhibited p53 protein expression; the level of p53 protein expression was significantly higher for the cases of invasion at and above proper muscle layer (P = .031).

Regarding the levels of p53 protein expression in terms of the presence of lymph node metastasis, 19 of 31 lymph node metastasis-positive cases (61.3%) and 27 of 69 lymph node metastasis-negative cases (39.1%) showed p53 protein expression;
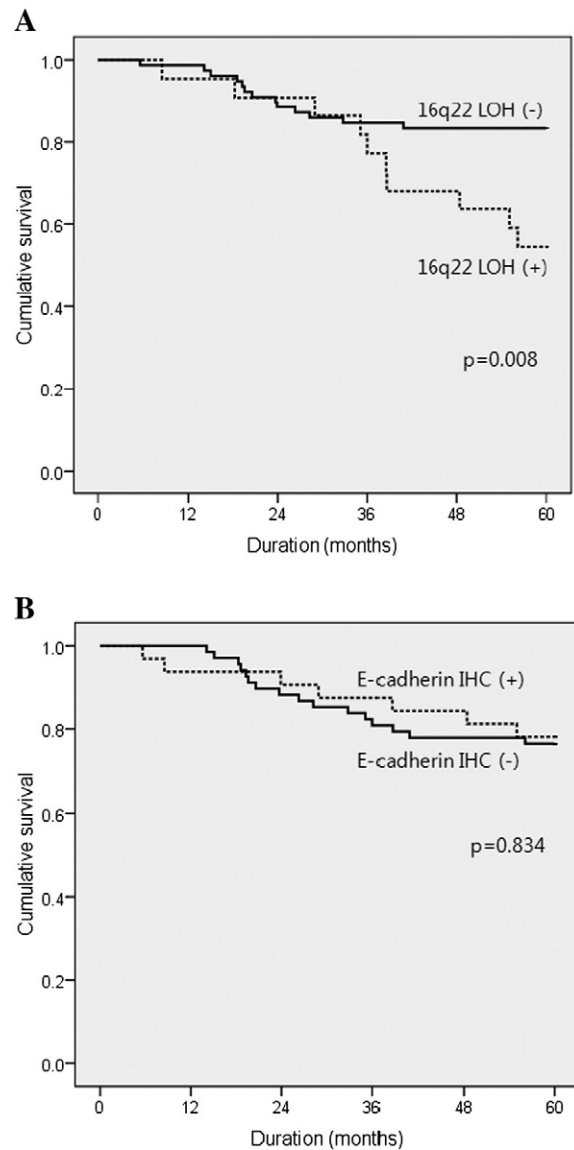
the p53 protein expression was significantly higher in lymph node metastasis cases (P = .020) (Table 7).

No significant difference in the five-year survival rate was found with respect to the level of p53 protein expression (Figure 6B).

Correlation Between LOH on Each Chromosome and Immunohistochemical Protein Expression

The correlation between LOH on each chromosome and level of corresponding protein expression was analyzed by Fisher's exact test. Of the 26 cases in which LOH was detected on the chromosome 9p21 locus, 13 cases (50%) showed p16 protein expression. Of the 31 cases showing LOH on the chromosome 10q23 locus, 21 cases (71%) showed PTEN protein expression. Of the 24 cases showing LOH on the chromosome 13q14 locus, 10 cases (41.7%) showed Rb protein expression. Of the 22 cases showing LOH on the chromosome 16q22 locus, 13 cases (59.1%) showed E-cadherin protein loss. Of the 35 cases showing LOH on the chromosome 17p13 locus, 20 cases

(57.1%) showed p53 protein expression. A significant correlation was found between LOH on the chromosome 10q23 locus and PTEN protein expression (P = .042), while no such correlation was found for LOH on the other four chromosomes (Table 8).

Discussion

Gastric cancer is one of the most common types of cancer worldwide. Notable advancements in the clinical examination and treatment of gastric cancer have been made because of extensive research of risk groups, carcinogenic factors, epidemiological and molecular oncological characteristics, surgical techniques, and chemotherapy. However, the incidence and mortality rates of gastric cancer remain high. The incidence of gastric cancer is particularly high among Koreans and Japanese people and relatively low in the Western world [2,26,27].

There has been much interest in gastric cancer by national health authorities and civilians, and efforts to prevent gastric cancer through early diagnosis have been made. However, many patients are diagnosed with far-advanced stage cancer at their initial diagnosis. Thus, studies of the molecular biological characteristics of gastric cancer and mechanisms of cancer development, tumor progression, and metastasis are very important [7,8]. The oncological pathogenesis of gastric cancer at the molecular level is similar to that of cancer in other organs in that multiple genetic variations and abnormalities are involved at multiple stages of pathogenesis [9,12,13,19].

Recent molecular oncological studies of gastric cancer have mostly focused on investigating the mechanisms and clinical aspects by primarily analyzing mutations in individual oncogenes and suppressant genes or protein expression, and studies that investigating multiple genes or mechanisms at the same time are relatively rare. Therefore, we evaluated aberrations in various tumor suppressor genes simultaneously at the molecular level.

Expression of tumor suppressor genes is precisely regulated under normal physiological conditions. Tumor suppressor genes control enzymatic activities and the cell cycle to maintain important cell activities while suppressing cancer growth. Inactivation of these tumor suppressor genes by numerous factors can lead to the loss of normal tumor suppression [5,6,10]. Inactivation of tumor suppressor genes results not only from somatic mutations, but also from other important mechanisms including microsatellite alterations [3,4,9,12].

Nearly all genes have been mapped onto chromosomes in the last two decades, and extensive research on the correlation between deletions of tumor suppressor genes and chromosomal abnormalities has been conducted. A major mechanism of tumor suppressor gene inactivation is allelic loss. Research of this mechanism has led to the discovery of numerous microsatellites, and it was subsequently revealed that microsatellite alterations are extensively involved in the process of tumor suppressor gene inactivation. Therefore, it has become possible to locate defective areas on chromosomes by detecting allelic loss or LOH [13–20].

To summarize the characteristics of the major tumor suppressor genes analyzed in this study, *p16INK4A* is a tumor suppressor gene located on the chromosome 9p21 locus and p16 acts as a cyclin dependent kinase (CDK) suppressor, which regulates the cell cycle. During a normal cell cycle, CDK binds cyclinD to form a cyclinD/CDK4 protein complex, and this complex phosphorylates pRb protein, the product of the retinoblastoma (*Rb*) tumor suppressor gene. This results in progression of the cell cycle from the late G1 phase to S phase. p16 protein inhibits the binding of CDK to cyclinD by binding to CDK4 protein to ultimately prevent pRb phosphorylation, halting the

cell cycle in the late G1 phase. If mutation, abnormal methylation and LOH occur on *p16INK4A*, the G1 checkpoint is no longer controlled by the cyclinD/CDK4 complex and the cell cycle continuously progresses to S phase, leading to tumor development [28].

PTEN gene is one of the most recently investigated tumor suppressor genes. It has been reported that LOH of the chromosome 10q23.3 locus occurs in nearly all malignant tumors. The PTEN gene shares a similar base sequence as tensin, a cytoskeletal protein. Its products contain tyrosine phosphatase domains and thus act as phosphatases, which control the activation of other genes. The main functions of PTEN proteins are apoptosis induction, cell cycle arrest in G1 phase, inhibition of cell proliferation and growth through the Pl3-kinase/Akt signaling pathways, and inhibition of cell motility and movement through the effects of mitogen-activated protein kinase. Inactivation of the PTEN gene can result from genetic mutations, loss of protein expression, and LOH [6,12].

The products of the E-cadherin gene are 120-kDa molecules involved in intercellular adhesion. They are homophilic and bind other cadherins. They form a complex with catenin and bind actin, an intracellular cytoskeletal protein, to maintain the structure of certain cells. Variations and loss of cadherin and catenin have been extensively researched. It is well known that the expression levels of cadherin and catenin vary depending on the extent of tumor progression across various organs and that their genetic loss or variation contributes to the invasive potential of cancer cells [29].

p53 is known as the most important tumor suppressor gene and is located on the chromosome 17p13.1 locus. Its main roles include regulating the expression of other genes, regulating cell growth and cycle, apoptosis induction, angiogenesis suppression, and damaged DNA repair. However, variations in this gene can lead to inactivation of the normal function of *p53*, ultimately leading to tumor growth. Moreover, *p53* has been the most extensively researched in numerous tumors. This gene is clinically useful, as variations or overexpression of *p53* suggest poor prognosis and the presence of a high-grade malignancy [5,10,11].

LOH, which is a major mechanism of inactivation of these major tumor suppressor genes, is a variation at the introns of DNA and differs from somatic mutations. When large damage to microsatellites occurs in a tumor suppressor gene, the tumor suppressor gene becomes inactivated. Tumor suppressor genes inhibit tumor growth by regulating nucleus transcription, the cell cycle, cell proliferation, and signaling pathways. Studies have reported LOH, a type of allelic loss, is related to tumor suppressor genes on chromosomes within tumors at different rates and that an increased rate of LOH is associated with the invasive potential of a tumor [13,14,21–25].

In this study, we analyzed the LOH of major tumor suppressor genes, *p16*, *PTEN*, *Rb*, *E-cadherin*, and *p53*, located on the 9p21, 10q23, 13q14, 16q22, and 17p13 loci, by using three microsatellite markers per gene and investigated the correlation between LOH and clinicopathological characteristics on the surgically resected gastric cancer. We found at least one LOH in 83 cases (83%), and not even a single LOH was detected using 15 microsatellite markers in 17 cases (17%). The LOH detection rate was highest at the 17p13 locus at 35%, followed by 31% at the 10q23 locus. These rates are lower than those previously reported by Choi et al. [21] and Sugai et al. [23], but are, on average, consistent with previous reports.

In our analysis of the LOH of 9p21 and immunohistochemical staining of p16 proteins, LOH was detected in 26 cases (26%) and protein expression was observed in 31 cases (31%). Comparing our

results with those of other studies, Choi et al. [21] reported a detection rate of 18.8% using single marker (D16S398), Chung et al. [22] reported 32.4% using three markers, Sugai et al. [23] reported 25% using two markers in early gastric cancer, and Kim et al. [24] reported 37% using five markers. In this study, LOH detection rates ranged from 15.5–25.4%, and were similar to the detection rates reported in other studies. However, Zhang et al. [30] reported a significantly high LOH detection rate of 65% using two markers, D9S171 and D9S1604, in Chinese subjects. In our analysis of the correlation between LOH and clinicopathological characteristics, high LOH rates were found for diffuse type cancer determined by Lauren's classification and lymph node metastasis-positive cases. Kim et al. [24] and Zhang et al. [30] reported no significant correlation, while Sugai et al. [23] reported significant differences in the detection rate between mucosa-confined cancer and submucosa invasion cancer in cases of EGC. In our study, p16 protein expression was not significantly correlated with clinicopathological factors. Tsujie et al. [31] reported a low p16 protein expression level of 11.3%, and significant differences in the level of p16 protein expression among undifferentiated type cancer and Stage III and IV cancer.

In our analysis of LOH of 10q23 and immunohistochemical staining of PTEN protein, at least one LOH was detected in 31 cases (31%) and protein expression was observed in 39 cases (39%). However, the LOH and protein expression were not significantly correlated with clinicopathological factors. The LOH detection rate was 18.2–26.4% for each LOH marker. Choi et al. [21] reported a LOH detection rate of 11.5% using a single microsatellite marker (D10S469), which was significantly lower than the rate found in this study. Oki et al. [32], in their study of 113 patients, used a single microsatellite marker (D10S796) and found an LOH detection rate of 17.1%, which was similar to that found in this study. They reported no significant correlation between LOH and clinicopathological factors and a low survival rate of the LOH group. Li et al. [33], in a study of Chinese subjects, used three markers and reported LOH detection rates of 33.3% for advanced gastric cancer and 20% for early gastric cancer. These rates were similar to our findings. Byun et al. [34] detected LOH in 126 cases by using polymorphisms at a high rate of 47%, and reported high detection rates for advanced cancer and undifferentiated type cancer.

In our analysis of LOH of 13q14 and immunohistochemical staining of Rb protein, at least one LOH was detected in 24 cases (24%) and protein expression was observed in 28 cases (28%). The level of Rb protein expression was significantly high for lymph node metastasis-positive cases. The LOH detection rate was 15.6–23.0% for each marker. Sugai et al. [23] reported an LOH detection rate of 15.8% using a single marker (D13S162), Chung et al. [22] reported 22.8% using three markers, Choi et al. [21] reported 38.1% using three markers, and Kim et al. [24] reported 30% using five markers. These rates are similar to those found in this study. Kim et al. reported a significantly higher detection rate for advanced cancer relative to early cancer.

In our analysis of the LOH of 16q22 and immunohistochemical staining of E-cadherin protein, at least one LOH was detected in 22 cases (22%) and protein expression loss was observed in 32 cases (32%). LOH detection rates were significantly low for mucosa-confined EGC and lymph node metastasis-negative cancer. The level of protein expression was significantly high for diffuse type cancer. The LOH detection rate was 15.5–25.4% for each LOH marker. Choi et al. [21] reported a similar LOH detection rate of 18.8% using a single marker

(D16S398). Grundei et al. [25] reported a detection rate of 8% using a single marker (D16S301), which was significantly lower than the rate found in our study. Huiping et al. [35] reported significantly high rates of LOH detection and protein expression loss (75% and 42%, respectively). Because of the large variations in the LOH detection rate across studies, it was difficult to accurately assess our results.

In our analysis of the LOH of 17p13 and immunohistochemical staining of P53 protein, at least one LOH was found in 35 cases (35%) and protein expression was observed in 46 cases (46%). LOH was detected at significantly high rates in advanced gastric cancer, lymph node metastasis-positive cases, and deeper invasion cases. The LOH detection rate was 18.5–26.6% for each LOH marker. Sugai et al. [23] reported a high LOH detection rate of 45.5% using TP53 marker in early cancer. Chung et al. and Choi et al. [21,22] reported LOH detection rates of 41.9% and 48%, respectively, using three of the markers used in our study. These rates were somewhat higher than those found in our study. The LOH detection rate of the TP53 marker was particularly high at 55.3% in Choi et al.'s study. Ohmura et al. [36] found a detection rate of 33.3% using the TP53 marker. Regarding the correlation between LOH and clinicopathological factors, Sugai et al. reported significant differences in the LOH detection rate between mucosa-confined EGC and submucosa layer-involved EGC. In their study, the level of p53 protein expression was 45.2% for EGC, which was higher compared to our finding (34.2%). The expression level found by Ohmura et al. was 48%, which is closer to that found in our study.

We investigated the correlation between five-year survival rates with LOH and protein expression, and found that the LOH of 16q22 and 17p13 were significantly correlated with prognosis. Although there are few studies available to verify our results, Oki and Kakeji et al. [37] previously reported similar results regarding the correlation between LOH of 10q23 containing the PTEN gene and five-year survival rates. By confirming survival rates for a larger number of cases, more objective results regarding survival rates can be obtained.

The LOH detection rates found in our study were consistent with those of previous studies for some, but not all, major tumor suppressor genes. However, we found that high LOH rates were associated with the high invasive potential of cancer and poor prognosis, and various genetic variations play a role in gastric tumor growth and malignant transformation. Furthermore, while much research has been conducted on genetic variations and LOH in gastric, comprehensive studies of the correlation between LOH of suppressor genes and clinicopathological factors are limited. However, previous studies in which LOH detection rates significantly differed from those of our study only used one or two markers; therefore, the reliability of their results is questionable. When only a single marker is used, non-informative cases are excluded from the denominator when calculating the LOH detection rate, which may produce values that are higher than the actual rates. To prevent this phenomenon, we used three markers for each chromosome, which is an appropriate quantity for rapid and inexpensive clinical application.

Regarding the correlation between LOH and protein expression results for each gene, p16, PTEN, Rb, E-cadherin, and p53 were expressed in 50%, 71%, 42%, 59%, and 57% of all cases with LOH, respectively; only PTEN was significantly correlated with LOH. These findings suggest that LOH and protein expression are independent factors in the context of a tumor suppressor gene.

In our results, the LOH of 17p13 and p53 protein expression were correlated with the depth of tumor invasion and lymph node metastasis, and the former was also correlated with five-year survival

rates. LOH of 17p13 may be quickly and easily applicable in clinical settings.

This study had several limitations. First, there were relatively few cases of lymph node metastasis and Stage IV cancers. Second, we could have gained more information regarding the stage-by-stage process of tumor growth by detecting LOH on dysplasias and metastatic lymph nodes for each case of gastric cancer. Third, we performed silver staining to detect LOH. Higher LOH detection rates may be expected from detection techniques using radioisotopes or fluorescence. Fourth, more meaningful results may be obtained by using gene mutation and methylation techniques in addition to LOH analysis and immunohistochemical staining used in our study.

Conclusion

In this study, microsatellite alterations of tumor suppressor genes, *p16*, *PTEN*, *Rb*, *E-cadherin*, and *p53*, across five chromosomes (9p21, 10q23, 13q14, 16q22, 17p13, respectively) were analyzed at the molecular level. The protein expression of each gene was studied by immunohistochemistry, and the following results were obtained.

1. LOH was not detected in 17 of 100 cases of gastric adenocarcinoma (17%). The rate of non-detection of LOH was significantly higher for early gastric cancer than for advanced cancer (P = .033). In addition, two or more LOHs were detected in 43 of 83 cases (43%). The LOH detection rate was highest at the chromosome 17p31 locus, where *p53* is located, and lowest at the chromosome 16q22 locus, where *E-cadherin* is located.
2. The rate of detecting LOH associated with the chromosome 9p21 locus was significantly high for diffuse type cancer and lymph node metastasis cases (P = .001, 0.035).
3. The rate of detecting LOH associated with the chromosome 16q22 locus was significantly low for mucosa-confined early gastric cancer (P = .043) and significantly high for lymph node metastasis cancer (P = .01). Patients in whom LOH was detected showed significantly poor prognoses (P = .008).
4. The rate of detecting LOH associated with the 17p13 was significantly higher for advanced gastric cancer than for early gastric cancer and was significantly positively correlated with the depth of tumor invasion and presence of lymph node metastasis (P = .041, 0.039, 0.036). Moreover, the five-year survival rate was significantly low for patients with LOH at the chromosome 17p13 locus (P = .02).
5. No significant correlation was found between LOH associated with the chromosome 10q23 and 13q14 loci and clinicopathological factors.
6. In immunohistochemical analysis, protein expression of the five tumor suppressor genes was not significantly correlated with clinicopathological factors. However, Rb expression was significantly low in lymph node metastasis-positive cases (P = .044). Significant loss of E-cadherin expression was observed in patients with diffuse type gastric cancer determined by Lauren's classification (P = .001). Significantly high levels of p53 protein expression were observed for advanced cancer and lymph node metastasis-positive cases (P = .029, 0.031, 0.020). However, no significant correlations were found between protein expression of all five tumor suppressor genes and patient survival.
7. Comparing microsatellite alterations and the protein expression of each gene, p16, PTEN, Rb, E-cadherin, and p53 proteins were expressed in 50%, 71.0%, 41.7%, 59.0%, and 57.1% of all cases in which LOH was detected. PTEN protein expression was correlated with the mechanism of LOH at the chromosome 10q23 locus.

Based on these results, LOH and protein overexpression of various tumor suppressor genes may be involved in the pathogenesis and malignant transformation of gastric adenocarcinoma. The LOH of 9p21 and E-cadherin protein expression loss affected tumor grade in gastric cancer. The LOH of 17p13 and p53 protein expression may be clinically useful factors for evaluating the progression of early gastric cancer to advanced cancer. The LOH of 16q22 and 17p13 and p53 protein expression may be useful for differentiating the depth of tumor invasion, and the LOH of 9p21, 16q22, and 17p13, and Rb and p53 protein expression for differentiating lymph node metastasis (Table 9). Therefore, the LOH of 17p13 and immunohistochemical staining of p53 may be the most clinically useful factors.

In addition, LOH and protein expression of each tumor suppressor gene may be involved in the process of tumor growth and progression in an independent manner, while the LOH and protein expression of the PTEN gene may be highly correlated with one another.

Conflicts of Interests

There are no potential conflicts of interest to disclose.

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