Prognostic Significance of Immunohistochemical Expression of p53 and Retinoblastoma Gene Protein (pRB) in Curatively Resected Gastric Cancer

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Background : The aim of this study was to determine the prognostic significance of the expression of p53 and retinoblastoma (Rb) gene products in cases of curatively resected gastric adenocarcinoma, by immunohistochemical analysis.

Methods : Between January 1996 and December 2001, 736 curatively resected gastric cancer patients underwent immunohistochemical staining for p53 or Rb proteins (pRb), and we retrospectively analyzed the correlation of our results with the clinical outcomes of these cases.

Results : High levels of expression of p53 (>25% p53-positive cells) and Rb (>50% Rb-positive cells) proteins were detected in 40.1% and 43.7% of cases, respectively. Tubular type was found to frequently exhibit higher levels of p53 expression (high expression in 44.2%) than signet ring cell type (high expression in 26.0%) (p=0.042). The incidence of vascular invasion was lower in the high pRb expressors (43.2%) than in the pRb low expressors (56.8%), but this was not a statistically significant discrepancy (p=0.063). Preoperative CEA levels were found to be low in high pRb expressors: initial CEA level in the high pRb expressors was 2.31±3.30 ng/mL, and was 5.18±24.80 ng/mL in the low pRb expressors (p=0.033). Tumor depth and node metastasis were both independent of the levels of expression of p53 and Rb proteins. The seven-year overall survival rate and relapse-free survival rates of patients were 87.2% and 75.7%, respectively. Multivariate Cox regression analysis indicated that tumor stage, tumor size, patient age and pRb expression were the significant prognostic factors with regard to overall survival, and tumor stage and age were both significant factors with regard to relapse-free survival.

Conclusion : Immunohistochemical staining of retinoblastoma gene products was an independent prognostic factor for the prediction of overall survival in curatively resected gastric cancer patients.

Key Words : Gastric cancer, Prognosis, p53, Rb gene, Immunohistochemistry

INTRODUCTION

Gastric cancer is one of the most common cancers, representing the second leading cause of cancer deaths in Korea. A population-based cancer registry was established on January 1, 1997 to estimate the incidence of cancer in Daegu. The age-standardized incidence rates (ASR) of gastric cancer were 73.5 per 100,000 males and 28.9 per 100,000 females, as reported by the Daegu Cancer Registry in 2002. Recently, the proportion of gastric cancer cases among all malignancies is declining: annual reported cases of gastric cancer constituted 24.1% of all cancers in 1990, and 20.8% of all cancers in 2000,

as reported by the Korea Cancer Registry Program. This decline appears to be due principally to changes in diet and food preparation, as well as an increased incidence of early diagnosis of gastric cancer.

Greater insight has recently been gained into the biological properties of tumor cells. Tumor suppressor gene products are of specific interest, as they play important roles in cell cycle regulation. The *p*53 tumor suppressor gene normally regulates cell proliferation[1, 2] and programmed cell death[3, 4]. Abnormalities of the *p*53 tumor suppressor gene have been implicated in both tumorigenesis and tumor progression. The retinoblastoma gene is a prototype of the tumor suppressor gene which controls the cell cycle at the G1 phase[5, 6], and the Rb gene product (pRb) functions as a signal transducer, connecting the cell cycle with the transcriptional machinery. The Rb gene product, pp110Rb, is a nuclear phosphoprotein which exhibits DNA binding properties[6], and it is cyclically phosphorylated and dephosphorylated during the cell cycle, playing a significant role in regulation[5-7]. Loss of pRB function deprives the cell of an important mechanism for halting proliferation[8]. The importance of *p*53 expression has been extensively analyzed with regard to a plethora of human malignancies, including gastric cancer, by immunohistochemical methods, while the role of retinoblastoma gene protein expression has been studied, but certainly not to so significant a degree[9]. The prognostic roles of the above gene expressions with regard to gastric cancer remain controversial. The purpose of this study was to determine the prognostic significance of *p*53 and Rb protein expression in curatively resected gastric adenocarcinoma.

MATERIALS AND METHODS

Patients and setting

From January 1996 to December 2001, 2,104 pathologically confirmed gastric cancer patients were registered in our hospital. Of these, 1,158 were curatively resected patients. In 736 of these patients, immunohistochemical analyses of p53 or Rb proteins were performed. We reviewed the clinicopathological parameters of TNM stage, the World Health Organization classifications, histological grades, Lauren classifications, Ming classifications, vascular invasion rates, and nerve invasion rates in all 736 patients. Staging evaluation was done according to the 5th edition guidelines published by the American Joint Committee of Cancer.

Curative resection was defined as the removal of all gross tumors, and the demonstration of tumor-negativity by microscopic examination, in both proximal and distal surgical margins. Total gastrectomy was performed in 114 patients (15.5%), subtotal gastrectomy in 622 patients (84.5%), D1 and D1+α resection in 232 patients (31.5%), and D2 and D2+α resection in 504 patients (68.5%). 494 (67.1%) of these patients were male, and 242 (32.9%) were female. The mean age of the subjects was 57.1 years (range: 19-80 years). Staging was as follows: IA in 338 (45.9%) patients, IB in 104 patients (14.1%), II in 115 patients (15.6%), IIIA in 106 patients (14.4%), and IIIB in 73 patients (9.9%). The median follow-up duration was 37.8 months (range: 0.8 to 97.3 months) (Table 1).

Table 1. Clinical characteristics of curatively resected gastric cancer patients

Number of patients	Male	736
Sex	Male	494 (67.1%)
	Female	242 (32.9%)
Age, years	Mean	57.1
	Range	19-80
Operation method	Total gastrectomy	114 (15.5%)
	Subtotal gastrectomy	622 (84.5%)
	D1, D1 + α	232 (31.5%)
	D2, D2 + α	504 (68.5%)
Stage	T1/T2/T3/T4	378/138/217/3
	N0/N1/N2	467/173/96
	IA	338 (45.9%)
	IB	104 (14.1%)
	II	115 (15.6%)
	IIIA	106 (14.4%)
	IIIB	73 (9.9%)
Follow-up period, months	Median	37.8
	Range	0.8-97.3

Immunohistochemical staining

Immunohistochemical staining was performed using the avidin-biotin-peroxidase complex with monoclonal antibodies against *p*53 (NCL-p53-D07, Novocastra Laboratories, Newcastle, United Kingdom), and Rb (14001A, Pharmingen, USA). Representative paraffin blocks containing tumors isolated from each case subject were sectioned into 5 μm slices and affixed to slides, then dried for 1 hour at 60℃. The sections were deparaffinized in xylene, and rehydrated with a descending series of alcohol concentrations. Endogenous peroxidase activity was blocked by 3% hydrogen peroxidase for 15 minutes, followed by washing with phosphate buffered saline (PBS), at a pH of 7.2. The sections were then subjected to a heat antigen retrieval process, by autoclaving with 1% zinc sulfate solution for 5 minutes. After 20 minutes of cooling at room temperature, the sections were incubated with 10% normal horse serum (Vectastain Elite kit) for 30 minutes. After decanting away the excess serum, sections were incubated with primary antibody for 2 hours at 37℃. In the p53 study, DO7 monoclonal antibody was used at a 1:100 dilution (Novocastra, Newcastle, UK), and in the Rb study, the monoclonal antibody NCL-Rb1 was used, at a 1:500 dilution (Pharmingen, USA). The sections were subsequently incubated with prediluted biotinylated anti-mouse immunoglobulin (Vectastain Elite kit) for 30 minutes at 37℃. After washing with PBS, the sections were allowed to react with peroxidase-conjugated streptoavidin (Dako, USA) at a dilution of 1:500 for 30 minutes at 37℃. After washing with PBS, peroxidase activity was evaluated with 3,3'-diaminobenzidine tetrahydrochloride (DAB), and the sections were counterstained with Mayer's hematoxylin.

All sections were examined by 2 pathologists, who were blind to the clinical outcomes and features of the patients. The sections were scored according to the percentage of positive cells using the following categories: 0=negative, 1=1~10%, 2=11~25%, 3=26~50%, 4=51~75% and 5=76~100%. A breast cancer biopsy specimen showing intense uniform positivity for pRb was used as a positive control, as was a squamous cell lung cancer biopsy specimen exhibiting uniform positivity for *p*53.

In the statistical analysis of *p*53 and Rb expression, the tmors were further categorized, into three and two groups respectively. Tumors expressing *p*53 protein in over 25% of cells with strong staining were designated as high *p*53 expressors, and tumors expressing *p*53 protein in 1~25% of cells or weak staining were designated as low *p*53 expressors. Tumors exhibiting no *p*53 expression were designated negative *p*53 expressors. With regard to pRb expression, tumors expressing pRb in over 50% of cells with strong staining were designated high pRb expressors, and tumors expressing pRb in less than 50% of cells or weak staining were designated as low
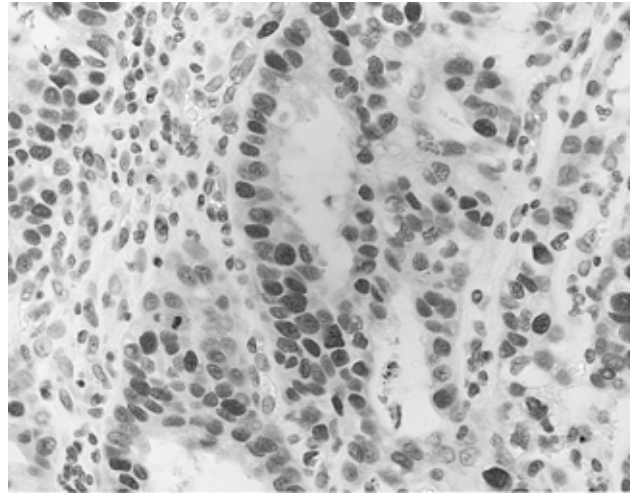

Figure 1. Immunohistochemical staining for Rb evidences high expression in the nuclei of tumor cells (×400).

pRb expressors (Figure 1). These classifications were predicated on the observations of previous studies[10].

Statistics

Statistical analysis was performed using $\chi-2$ tests, in order to compare percentages in cross tabulations, and independent sample *t*-tests were used to compare the means. Survival curves were generated by the Kaplan-Meier method, and compared using the log-rank test. To determine the significant prognostic factors in terms of survival and relapse-free survival, multivariate analysis was performed with the Cox proportional hazards regression model. All significance levels listed refer to two-sided tests. *p* values of <0.05 were considered to be significant.

Statistical analyses were performed using SPSS for Windows 11.0 (Chicago, II, USA).

RESULTS

Tumor samples with high, low and no *p*53 protein expression were found in 287 (40.1%), 120 (16.8%) and 309 (43.2%) of the 716 samples. High and low pRb expressors were found in 317 (43.7%) and 409 (56.3%) of the 726 samples examined, respectively (Table 2).

Correlation with clinicopathologic parameters

According to the classifications provided by the World Health Organization, tubular-type adenocarcinoma (low and high *p*53 expressors in 17.9% and 44.2%) was frequently more *p*53 positive than signet ring cell type (low and high *p*53 expressors in 15.0% and 26.0%) (*p*=0.042). The incidence of vascular

Table 2. Immunohistochemical expression rates according to histologic characteristics of curatively resected gastric cancer patients

		p53			Rb	
		Negative	low	high	low	high
Total		309 (43.2%)	120 (16.8%)	287 (40.1%)	409 (56.3%)	317 (43.7%)
Vascular	positive	98 (41.2%)	38 (16.0%)	102 (42.9%)	150 (60.5%)	98 (39.5%)
invasion	negative	115 (43.6%)	40 (15.2%)	109 (41.3%)	141 (52.2%)	129 (47.8%)
WHO	tubular	146 (37.9%)	69 (17.9%)	170 (44.2%)	222 (55.8%)	176 (44.2%)
classification	signet−ring	59 (59.0%)	15 (15.0%)	26 (26.0%)*	61 (59.8%)	41 (40.2%)
Histologic	well differentiated	27 (35.5%)	15 (19.7%)	34 (44.7%)	47 (58.8%)	33 (41.3%)
grade	moderate differentiated	105 (40.1%)	44 (16.8%)	113 (43.1%)	158 (59.8%)	106 (40.2%)
	poorly differentiated	132 (44.0%)	52 (17.3%)	116 (38.7%)	162 (52.9%)	144 (47.1%)
Lauren	intestinal	152 (40.0%)	63 (16.6%)	165 (43.4%)	215 (55.7%)	171 (44.3%)
classification	diffuse	135 (48.6%)	39 (14.0%)	104 (37.4%)	154 (55.0%)	126 (45.0%)
Nerve	positive	95 (40.1%)	33 (13.9%)	109 (46.0%)	131 (54.4%)	110 (45.6%)
invasion	negative	144 (43.0%)	56 (16.7%)	135 (40.3%)	197 (56.8%)	150 (43.2%)

*$p<0.05$: WHO classification according to p53 expression

invasion was lower in the high pRb expressors (43.2%) than in the low pRb expressors (56.8%), but this was not a statistically significant difference (p=0.063). Preoperative CEA levels were low in the high pRb expressors: the initial CEA level in the high pRb expressors was 2.31±3.30 ng/mL, and 5.18±24.80 ng/mL in the low pRb expressors (p=0.033).

No significant correlations were observed with regard to the overexpression of p53 and Rb proteins, and Lauren classifi− cations, Ming classifications, tumor size, depth of invasion (pT category), lymph node involvement (pN category), or pathologic stages.

Survival analysis

The 7−year overall survival rate was 87.2% among the total patient population (Table 3) and the relapse−free survival rate (Table 4) was 75.7% among the total patient population. According to stage, the 7−year overall survival rate of stage IA was 97.5%, stage IB 92.7%, stage II 86.2%, IIIA 66.5% and

Table 3. Overall survival rate of curatively resected gastric cancer patients

		1 year	3 year	5 year	7 year	Mean	(months)
Total		96.1%	89.6%	88.0%	87.2%	87.8	
Stage	IA	98.2%	97.5%	97.5%	97.5%	90.6	
	IB	96.1%	92.7%	92.7%	92.7%	91.2	
	II	96.4%	90.3%	86.2%	86.2%	81.2	p=.000
	IIIA	94.0%	76.5%	73.9%	66.5%	67.9	
	IIIB	88.4%	63.7%	56.6%	56.6%	57.3	
p53	high	95.0%	90.0%	90.0%	90.0%	79.5	
	low	96.6%	90.7%	85.3%	85.3%	87.1	p=.914
	negative	96.7%	88.7%	87.7%	86.2%	83.5	
Rb	high	96.8%	92.7%	92.7%	92.7%	82.4	
	low	95.2%	87.2%	84.8%	83.3%	81.5	p=.013

Table 4. Relapse free survival rate of curatively resected gastric cancer patients

		1 year	3 year	5 year	7 year	Mean	(months)
Total		97.5%	86.9%	79.2%	75.7%	81.9	
p53	high	98.1%	88.2%	78.1%	78.1%	74.5	
	low	98.1%	91.1%	82.2%	72.6%	82.0	p=.719
	negative	96.6%	84.3%	78.9%	75.3%	78.2	
Rb	high	98.7%	87.9%	78.1%	72.9%	74.7	
	low	96.5%	86.0%	79.4%	78.1%	79.0	p=.741

Table 5. Multivariate analysis of the prognostic factors for total survival and relapse free survival in curatively resected gastric cancer patients

	Overall	survival		Relapse	free	survival
	RR	95%	CI	RR	95%	CI
Age	1.030	1.007	1.053	1.019	1.000	1.037
Stage I vs. II	2.144	0.945	4.867	7.032	3.311	14.931
I vs. IIIA	5.561	2.737	11.295	14.676	7.178	30.003
I vs. IIIB	8.305	3.932	17.542	25.379	12.110	53.187
Tumor size	1.107	1.001	1.224	1.044	0.958	1.139
Rb	0.557	0.333	0.933	0.925	0.617	1.385

stage IIIB 56.6% (p=0.000).

There were no significant differences detected in terms of total survival (p=0.914) and relapse-free survival rates (p=0.719) between patients with no, low, and high p53 expression characteristics. The 7-year overall survival rate of high pRb expressors (92.7%) was better than in the low pRb expressors (83.3%) (p=0.013) but the 7-year relapse-free survival rate was similar between the two.

According to multivariate Cox regression analysis, tumor stage, tumor size, patient age, and pRb expression rates (RR 0.557, 95% CI 0.333~0.933) were the important prognostic factors in overall survival rates (Table 5).

DISCUSSION

Among the various malignancies, gastric cancer represents the second most common cause of cancer death throughout the world, and the prognosis for advanced gastric cancer remains quite poor. The expression of the p53 and Rb genes can be easily detected in a variety of human malignancies, including gastric cancer, by immunohistochemical methods, but its prognostic roles in gastric cancer remain controversial. The p53 tumor suppressor gene, which is localized in the short arm of human chromosome 17 (17p13.1), plays an important role in the control of tumors, by regulating the expression of vascular endothelial growth factor (VEGF)[11]. The levels of wild-type p53 protein are normally extremely low and, due to its short half-life, it is undetectable by standard immunohistochemical staining in normal cells and tissues. Conversely, the mutated p53 protein accumulates in the nucleus, either by binding with other oncogenic proteins, or by prolonging its half-life[12]. The nuclear accumulation of p53 was consistent with p53 mutation rates, and exhibited the same subtype specificity[13]. About 40% of our gastric cancer cases (287/716) evidenced high reactivity to p53, which is consistent with previous studies, in which p53 overexpression was found to be common in gastric cancers[14-18].

The Rb gene was the initial tumor suppressor gene discovered in childhood retinoblastomas[19]. The Rb gene normally codes for a nuclear phosphoprotein, but both copies of the gene are inactivated in retinoblastomas[19]. It is now believed that the Rb protein (pRb), the product of the Rb gene, functions as a signal transducer, connecting the cell cycle with the transcriptional machinery. The Rb gene can be inactivated by DNA mutations, deletions, undetectable or reduced mRNA expression, or undetectable or aberrant protein production. Loss of pRB function deprives the cell of an important mechanism for halting proliferation[8]. pRb phosphorylation induces the inactivation of its growth inhibitory functions, via the release of transcription factor E2F, which is required for progression into the S phase[8, 20]. Ogawa et al.[9] reported finding overexpression of the Rb gene in 51.1% of gastric cancer cases, and in our study, 43.7% (317/726) of cases exhibited a high degree of reactivity to Rb.

The majority of tumors exhibited Rb and p53 located in the nucleus. This is consistent with previous studies[21] and appears to occur via the regulation of transcriptional factors.

Regarding the WHO histopathologic classifications, positive immunohistochemical staining of c-erbB-2 protein was significantly higher in the tubular type of gastric cancer than in the signet ring cell type[22]. However, positive nuclear p53 expression was found to be significantly higher in the tubular type of gastric cancer in our study; positive p53 expression was detected in 62.1% of tubular type gastric cancer cases, but only in 41.0% of the signet ring cell type (p=0.042). This is similar to the results reported by Uchino et al.[12], in which nearly 40% of papillary adenocarcinomas were associated with the nuclear accumulation of the p53 protein. The incidence of c-erbB-2 expression was higher in intestinal-type carcinomas than in diffuse carcinomas[16], but in our study, we detected no differences between the Lauren classifications and p53 and Rb expression.

In the previous studies, p53 expression was associated with vascular invasion[14, 17, 23] and in our study, vascular invasion was less frequent in high pRb expressors than in low pRb expressors. However, this was not a statistically significant difference (p=0.063). No significant correlations were detected with regard to age, sex, tumor size, depth of tumor invasion, lymph node metastasis, or the overexpression of p53 and Rb proteins in our study of curatively resected gastric cancer

patients.

In our study, the 7-year survival rate was 87.2% and the relapse-free survival rate was 75.7%. The 7-year survival rates, according to stage, were as follows: stage IA 97.5%, IB 92.7%, stage II 86.2%, stage IIIA 66.5% and stage IIIB 56.6%.

According to multivariate Cox regression analyses, tumor stage, tumor size, age, and Rb expression were the relevant prognostic factors in terms of total survival, and tumor stage and age were the most important prognostic factors with respect to relapse-free survival. Currently, the most accurate prognostic indicator for gastric cancer is tumor stage[24]. Age is also an important prognostic factor in gastric cancer; however, it was associated with increased treatment-related mortality in our study[25]. In our study, p53 overexpression was not found to be an independent prognostic marker. In previous studies, p53 expression was a useful marker in intestinal-type only[26, 27]. Our study also determined no significant differences to exist with regard to Lauren histological types.

We did, however, demonstrate a significant difference in survival curves between high- and the low- expressors of the Rb gene. The Rb gene was found to be an independent prognostic factor in the prediction of total survival. It has been determined that reduced Rb expression was associated with poor prognoses in stage I and II non-small cell lung carcinoma[28], and that Rb-positive tumors were associated with significantly better survival rates inboth ovarian cancer[10] and gastric cancer[9].

In conclusion, immunohistochemical staining for the Rb protein proved to be a clinically useful prognostic indicator in cases of curatively resected gastric cancer. Conversely, high p53 expression is not an independent indicator for survival. Further prospective studies are warranted, in order to clarify the relationship between tumor suppressor proteins and gastric cancer prognoses, and cell cycle regulation associated with the action of these proteins.

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