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c-myc Expression is Related with Cell Proliferation and Associated with Poor Clinical Outcome in Human Gastric Cancer

We underwent protein assay for Myc expression in 76 human gastric cancer tissues using immunohistochemistry. Expression of Myc protein was analyzed according to proliferative indices measured by flow cytometry. Levels of Myc protein expression was evaluated by correlating with biologic and clinical parameters. In 36 (47.4%) of 76 primary gastric cancers, overexpression of Myc was observed. We could observe expression of Myc protein in a significant portion of early gastric cancer (42.9%). Expression of Myc protein was demonstrated to be more frequent in poorly differentiated cancer cells (p=0.043). However, expression of Myc protein had little influence over progress or extent of the disease. Expression of Myc protein was significantly correlated with increased proliferative activity (p=0.032) and patients with high levels of Myc expression had poor disease-free survival. In a certain proportion of human gastric cancer, Myc protein may function as a regulator of cancer cell growth and expression of Myc may represent an aggressive phenotype of gastric cancer.

Key Words : *Cell division; Stomach neoplasms; Immunohistochemistry; Proto-oncogene proteins, myc; Prognosis; Survival*

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INTRODUCTION

Myc, one of the most extensively investigated transcriptional regulators in human cancers, rarely undergoes DNA amplification in stomach cancer, but accumulation of the *c-myc* gene product, c-myc protein, has been detected in 20-50% of patients with stomach cancer (1-4). This conflicting result can be explained by the fact that amplification of a gene is not necessarily associated with or required for its overexpression. Overexpression of c-myc is also possible in the absence of detectable gene amplification. Furthermore, the final functional unit of molecular events is the protein product of specific gene.

Various genetic alterations and chromosomal aberrations are involved in carcinogenesis and cancer progression. It would be more practical to elucidate the clinical markers that provide cancer cells with an aggressive biologic nature rather than to simply identify the numerous oncogenes in clinical settings. The ultimate aim to find molecular markers, which represent an aggressive phenotype or modest one in human cancers, is to identify the patients who need more intensive adjuvant treatments because of high risk of recurrence.

Proliferative activity of cancer cells has been investigated as a promising prognostic marker in many human cancers. Flow cytometry is the most widely available method to estimate the proliferative activity of cells by calculating specific stage of the cell cycle. If c-myc is the key transcriptional regulator of stomach cancer, expression of c-myc in stomach cancer could be closely associated with proliferative activity of the cancer cells. So we did a protein assay for c-myc and evaluated the correlation between c-myc expression and proliferative activity of cancer cells in stomach cancer. We also estimated the biologic implication of c-myc expression in stomach cancer by correlating it with other clinical and histologic parameters such as extent of disease, degree of differentiation, and histologic types of stomach cancer.

MATERIALS AND METHODS

Medical records and archival pathology tissues from 76 stomach cancer patients who underwent gastric resection at Inje University Sanggye Paik Hospital between January 1994 and December 1996 were evaluated. Important criteria for selection of study patients were feasible freshness of cancer tissues for flow cytometry and immunohistochemical assay of c-myc protein. All patients underwent radical subtotal or total gastrectomy with more than D2 lymph node dissection. Mean age of the patients was 61 years ranging from 23 to 75 years, and male pa-

tients were 48 (63.2%) and female patients were 28 (36.8%). The stage of each patient was determined according to TNM classification of the International Union against Cancer; 30 with stage I (39.5%), 12 with stage II (15.8%), 19 with stage III (25.0%), and 16 with stage IV (19.7%). Postoperative adjuvant chemotherapy, which was applied to all patients except those who had early gastric cancer (EGC), included 5-FU (600 mg/m^2), mitomycin (20 mg/m^2), and leucovorin (15 mg/m^2) every three weeks for six cycles.

Flow cytometry analysis was performed on cell suspensions from stomach cancers obtained by mechanical disaggregation of tumor materials. After centrifugation, supernatants were discarded, and the cell pellets were resuspended in 250 μL of Buffer solution (10 mM Citrate, pH 7.5, 20 mM NaCl, 20 mM $MgCl_2$). After adding 10 μL/mL of trypsin, trypsin inhibitor and DNase-free RNase, nuclei were incubated at room temperature for 30 min. DNA staining was obtained with 500 μL of propidium iodide solution (PI; Molecular Probes, Eugine, OR, U.S.A.) in PBS (100 μL/mLPI, 0.1% Triton X-100, 1% FCS) for 1 hr at 4℃ in the dark, followed by flow analysis. DNA fluorescence was analyzed using a FACScan (Becton-Dickinson, Bedford, MA, U.S.A.). Data acquisition was performed using the Cell Fit software (Becton-Dickinson) and data analysis done using the Phoenix Flow System Multicycle AV software.

The results were expressed as the frequency distribution of DNA cell content; normal DNA histograms were characterized by a peak corresponding to the DNA content of G0/G1 diploid cells. Clonal DNA abnormality (aneuploidy) was identified by the presence of an accessory peak generally shifted to the right of the G0/G1 diploid peak. The percentage of aneuploid cells was defined as the percentage of cells in the G0/G1 aneuploid peak with respect to those in the G0/G1 diploid peak. Diploid tumors were considered as those with 0% aneuploid cells.

Fresh frozen tissues of gastric cancer patients were retrieved and neoplastic tissues of these gastric cancers were examined for expression of c-myc protein, using the avidin-biotin complex (ABC) immunoperoxidase method. We used commercially available mouse monoclonal antibody to human c-myc protein, NCL-cMYC, for c-myc protein assay (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). Immune staining was performed as described previously (5). Counterstaining with hematoxylin was done after ABC immune staining and two pathologists evaluated immunohistochemical staining separately without information of patients' outcome data. Two pathologists reviewed the slides if interpretation of the immunohistochemical analysis was different. Three separate blocks, containing malignant cells, were stained and scored by calculating the percentage of stained cancer cells. Sections of stomach cancer, observed to express homogenous and/or intense immunohistochemical staining for Myc protein in more than 5% of the observed field, were considered to be positive for overexpression (Fig. 1A). The stained cancer cells was calculated since staining of normal growing cells was not infrequent (Fig. 1B).

Data for the immunohistochemical assay were com-

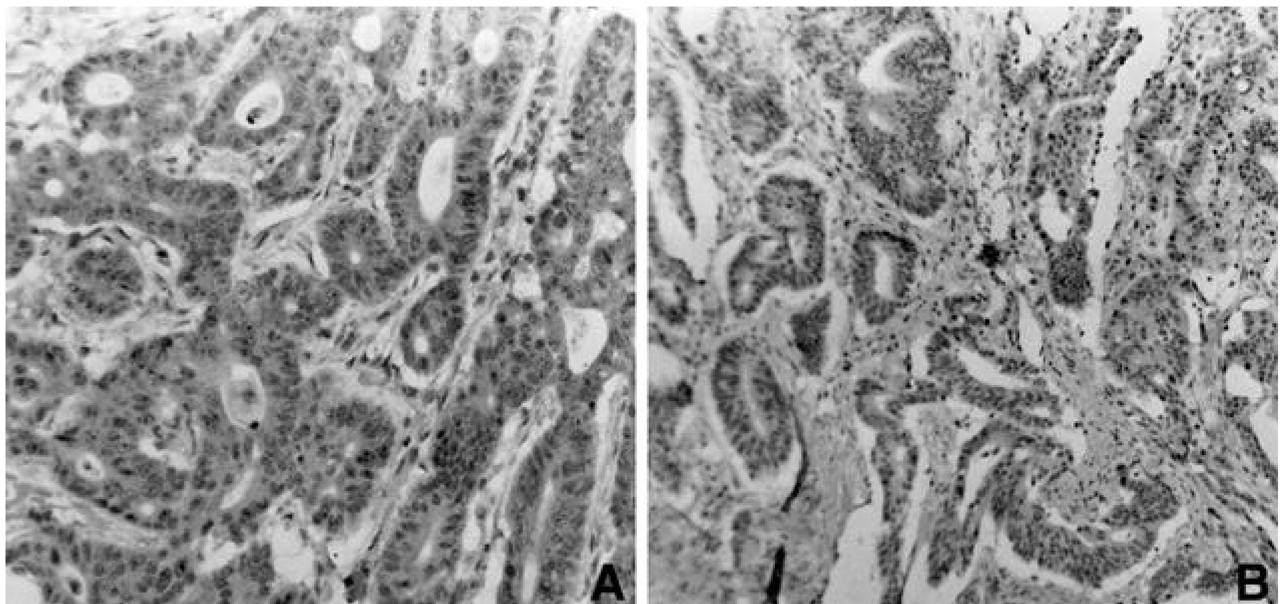

Fig. 1. A: Intense cytoplasmic staining of Myc in human gastric cancer cells (×400). B: expression of Myc protein is apparent in growing cells of normal gastric tissues (×100). In contrast, terminally differentiated epithelial cells do not show Myc expression.

bined with clinicopathologic characteristics of patients to determine the frequency of Myc protein overexpression in human gastric cancer, and to evaluate the Myc for their implication as cell cycle regulator in human stomach cancer by correlating with proliferative activity. Statistical analyses were performed, using SPSS personal computer statistics software. Correlation between clinical parameters and c-myc protein overexpression was estimated by Chi-square test. Survival curve of studied patients was plotted by Kaplan-Meier method and statistical anaysis was performed by log-rank test.

RESULTS

Expression of Myc protein was observed in 36 patients (47.4%) out of a total 76 patients. In non-cancerous portion of studied tissues, Myc expression was frequently observed in regenerating or growing cells. However, we could not observed Myc expression in terminally differentiated cells. Forty-seven patients (61.8%) had dipoid tumor while 29 patients (38.2%) had aneuploid tumor. Mean value of S-phase fraction measured by flow cytometry was 17.4%. When we classified histologic type of the tumor according to Lauren's classification, 45 patients (59.2%) had intestinal type tumor and 29 (40.8%) had diffuse type tumor. Frequency of Myc protein expression was 42.9% (12 of 28) in EGC and there was no significant correlation between Myc protein expression and depth of tumor infiltration. However, Myc protein expression tended to increase in tumors which infiltrate beyond the serosal layer. When we analyzed expression of Myc protein according to the extent of the disease, frequency of Myc protein expression increased only in the far advanced stage of the disease. The expression of Myc protein tended to increase in node positive disease, but statistical analysis exhibited only marginal significance ($p=0.054$).

Frequency of Myc protein expression increased significantly as tumor cells differentiated poorly ($p=0.043$) whereas Myc is expressed only in two cases (28.6%) of well-differentiated cancers. However, analysis of Myc protein expression showed no significant correlation with Lauren's classification (Table 1). Increased expression of Myc protein was observed more frequently in tumors with high levels of S-phase fraction and the difference was statistically significant ($p=0.032$). When we analyzed the pattern of Myc protein expression according to the G0/G1 fraction of the tumor cells, expression of Myc protein inversely correlated with levels of G0/G1 fraction

Table 1. Correlation of c-myc overexpression with clinicopathologic characteristics of stomach cancer

Variables	Myc expression		x^2-test	*p*-value
	Negative	Positive (%)		
Sex			1.432	0.231
Male	23/48 (47.9)	25/48 (52.1)		
Female	17/28 (62.9)	11/28 (37.1)		
Age			0.043	0.834
<60 yr	26/37 (70.2)	11/37 (29.8)		
≥60 yr	29/39 (74.3)	10/39 (25.7)		
Depth of invasion			2.025	0.392
T1	16/28 (57.1)	12/28 (42.9)		
T2	9/15 (60.0)	6/15 (40.0)		
T3	9/16 (56.2)	7/16 (43.8)		
T4	6/17 (35.2)	11/17 (64.8)		
L/N metastasis			7.643	0.054
Negative	17/29 (58.6)	12/29 (41.4)		
Positive	23/47 (48.9)	24/47 (51.1)		
Stage			3.747	0.911
I	16/29 (55.1)	13/29 (44.9)		
II	7/12 (58.3)	5/12 (41.7)		
III	11/19 (57.8)	8/19 (42.2)		
IV	6/16 (37.5)	10/16 (62.5)		
Lauren's classification			0.375	0.540
Intestinal	25/45 (55.6)	20/45 (44.4)		
Diffuse	14/29 (48.3)	15/29 (51.7)		
Differentiation				
Well	5/7 (71.4)	2/7 (28.6)	5.466	0.043
Moderate	19/31 (61.2)	12/31 (38.8)		
Poor	16/38 (42.1)	22/38 (57.9)		

Table 2. Correlation between expression of c-myc and proliferative activity of gastric cancer

Variables	c-myc expression		x^2-test	*p*-value
	Negative (%)	Positive (%)		
Ploidy			0.121	0.727
Diploid	24/47 (51.1)	23/47 (48.9)		
Aneuploid	16/29 (55.1)	13/29 (44.9)		
S-phase			4.832	0.032
<15%	30/43 (69.7)	13/43 (30.2)		
≥15%	10/33 (30.3)	23/33 (69.7)		
G0/G1			5.362	0.021
≥80%	27/36 (75.0)	9/36 (25.0)		
<80%	13/40 (32.5)	27/40 (67.5)		

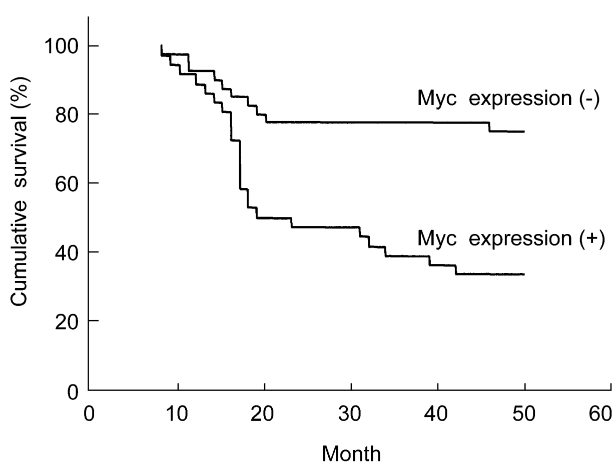

Fig. 2. Kaplan-Meier curve of patient survival, according to expression of Myc protein. The patients with high expression of Myc protein have a poorer survival than other patients ($p<0.001$).

($p=0.021$). We could observe that expression of Myc protein increased with prominent proliferative activity of cancer cells (Table 2).

We analyzed the influence of Myc expression on patients' outcome. Of 76 patients, 34 (44.7%) had recurrence of the disease with a median follow-up period of 38 months, ranging from 24 to 43 months. Twenty-four patients (70.6%) out of 34 who had recurrent disease showed high levels of Myc expression in cancer tissues, while only ten patients (25.0%) out of 40 who did not express Myc protein had recurrence of the disease. The difference between two groups was statistically significant ($p<0.001$). Survival curve according to the expression status of c-myc is plotted in Fig. 2.

DISCUSSION

Myc expression is known to be highest in proliferating cells (6) and increased Myc expression is associated with the proliferative phases of development. Myc is continuously expressed in proliferating cells, but not expressed in most quiescent or terminally differentiated cells (7). From the results of the present study, we demonstrated that expression of Myc protein was repressed in differentiated gastric cancers, whereas we could observe a significant increase of Myc protein expression in poorly differentiated gastric cancers. This result is in line with findings from previous in vitro studies which showed that Myc expression is down-regulated with cell differentiation (7, 8).

Constitutive expression of Myc prevents growth arrest of serum-depriven fibroblast and reduction of Myc levels lengthens G1 phase, whereas ectopic expression of Myc has been shown to shorten G1 phase (9). In the present study, S-phase fraction measured by flow cytometry increased significantly in the cancer cells expressing Myc protein. This finding coincided with G0/G1 fraction prolongation in tumor cells which lacked the Myc protein expression. However, it has not been determined whether cell proliferation is totally dependent on Myc expression, since we did not performed genetic assay for Myc at DNA or RNA level.

In breast cancer, Myc amplification is maintained in the transition from carcinoma in situ to invasive cancer, but not necessarily in the transition to metastatic tumors (10). Activation of Myc unlikely initiates cellular transformation. However, it seems likely that genetic changes of Myc may be rather early events in human gastric carcinogenesis, since a considerable number of early stage gastric cancers expressed high levels of Myc protein in the present study. Moreover, we could observe expression of Myc protein in the nests of normal cells adjacent to tumors and in regenerative epithelial cells (Fig. 1B). Although the incidence of cells with *Myc* DNA amplification is low (less than 12%) in carcinogenesis of the stomach, Onoda et al. reported that *Myc* mRNA and Myc protein were overexpressed in the early stage of stomach cancer: mRNA overexpression in 54.8% of advanced cases and in 90.0% of early cases (11). They speculated that the overexpression of Myc mRNA was not caused by *Myc* DNA abnormality or amplification, but the dif-

ference in mRNA and protein levels could be caused by translational inhibition or stabilization of mRNA. Theoretically, this discrepancy can be explained as follows; 1) Southern blotting analysis does not take into consideration variation in cellular composition, 2) DNA analysis may underestimate the frequency of amplification, 3) amplification of a gene is not necessarily associated with or required for its overexpression, 4) myc could be overexpressed in the absence of detectable gene amplification. Consequently, Myc protein levels may be regulated primarily at the transcriptional and post-transcriptional stages. So, we performed protein assay for Myc, which is the final functional unit of *Myc* gene, using immunohistochemical staining and analyzed the results with proliferative indices calculated by flow cytometry.

Myc plays a central role in normal growth and development, as well as in cellular transformation and carcinogenesis in vitro (12). Initial bursts of Myc expression are likely to be a critical event in cell-cycle entry, and this was supported by the results of the present study. Expression of Myc protein correlated with proliferative activity of gastric cancer cells. However, we could not demonstrate the relationship between expression of Myc protein and progression or extent of disease. Impact of Myc on cell proliferation may be through control of cell-cycle regulatory proteins such as the cyclin family and numerous cyclin dependent kinase (cdk) inhibitors in human gastric cancer.

In survival analysis, patients with high expression of Myc showed increased recurrence rate. However, the number of studied patients was not sufficient for multivariate analysis and the result only revealed one possible use of Myc expression in clinical setting. When we consider that 42 patients enrolled in the current study received adjuvant chemotherapy with same regimen, expression of Myc may be an indicator of cell clones which are resistant to conventional adjuvant chemotherapy.

In conclusion, expression of Myc is closely linked to proliferative activity of human gastric cancer. Expression of Myc may represent an aggressive phenotype of gastric cancer as poorly differentiated gastric cancer cells exhibited higher expression of Myc than differentiated cancer cells, and patients with high expression of Myc had poor survival.

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