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ARTICLE

Prognostic Significance of Loss of c-Fos Protein in Gastric Carcinoma

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c-fos was first identified as a viral oncoprotein, and has been studied in terms of its oncogenic function in tumorigenesis. Many experimental and clinical data indicated that c-fos expression plays a role in the progression of several types of carcinomas. However, some recent studies challenge this view as they indicate that c-fos has tumor suppressor activity. In the present study, we assessed c-fos protein expression in 625 consecutive gastric cancers immunohistochemically, and analyzed its relationship with clinicopathologic factors and survival. We found that a loss of c-fos expression is correlated with a more advanced stage, lymph node metastasis, lymphatic invasion and shorter survival, indicating that c-fos expression in gastric cancer cells is lost during progression and that this loss is associated with a poor prognosis. The above findings suggest that loss of c-fos expression has tumor suppressor activity in gastric cancer and we suspect that this suppressor activity might be related to the pro-apoptotic function of c-fos. (Pathology Oncology Research Vol 13, No 4, 284–289)

Key words: stomach neoplasms, immunohistochemistry, survival analysis, proto-oncogene protein c-fos, tumor suppressor protein, tissue array analysis

Introduction

The c-fos protein encoded by FOS (v-fos FBJ murine osteosarcoma viral oncogene homolog) is a regulatory protein that has a basic leucine-zipper region for binding with many proteins. c-fos can dimerize with c-jun family members to form various trans-activating or trans-repressing complexes of the AP-1 proteins. The proteins then bind to specific DNA segments that control target gene expression.

AP-1 subunits are formed by either jun-fos heterodimerization or jun-jun homo-dimerization and in vitro studies have shown that jun-fos heterodimers are more stable and have stronger DNA-binding activity than jun-jun homodimers.[1] Moreover, in F9 teratocarcinoma cells, c-fos enhanced the trans-activating and transforming properties of c-jun family members such as JUN and JUNB.[2] Thus, expression of c-fos might be crucial for the activity of AP-1-regulated genes.

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Since c-fos was first identified as a viral oncoprotein, many studies focused on its oncogenic functions and showed that c-fos-regulated genes include important regulators of tumorigenesis, i.e. proliferation, invasion and metastasis. c-fos can change DNA methylation patterns by regulating the DNA cytosine-5-methyltransferase gene (DNMT1), and thereby cause the down-regulation of tumor suppressor genes.[3] When overexpressed in mice, c-fos induced osteosarcoma formation by transforming chondroblasts and osteoblasts.[4] In a c-fos-deficient cell line derived from primary embryonic fibroblasts of *fos*-knockout mice, c-fos was found to be required for the expression of matrix metalloproteinases, which are required for the invasive growth of cancer cells.[5] In addition, c-fos can induce a loss of cell polarity and epithelial-mesenchymal transition, a known hallmark of metastatic and invasive growth in mammary epithelial cells.[6] In addition to these experimental data, several reports dealt with the oncogenicity of c-fos protein in human tumor tissues. In human osteosarcoma, c-fos overexpression was associated with high-grade lesions and with frequent relapse,[7-9] whereas in endometrial carcinoma, c-fos overexpression on Western blots correlated with a high histological grade and with a negative estrogen receptor and progesterone

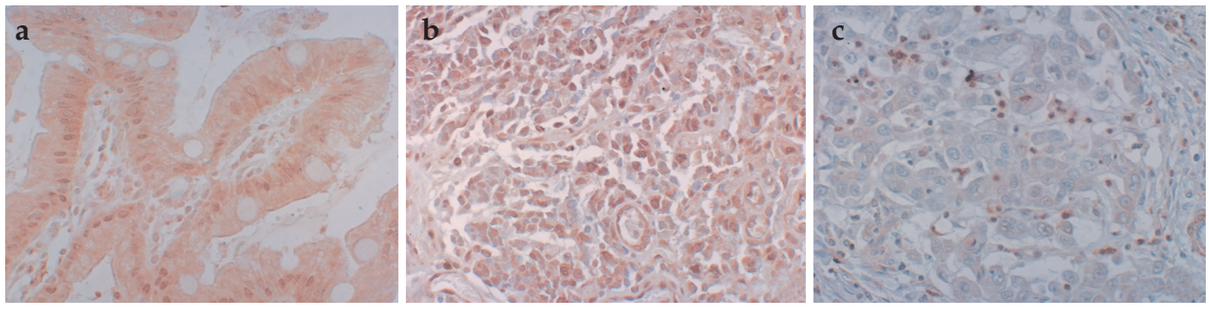

Figure 1. *Microscopic features of the immunohistochemical staining of c-fos in consecutive gastric cancers (×200).* ***(a)*** *c-fos in metaplastic gastric tissue, expressed in the nuclei of epithelial cells.* ***(b)*** *c-fos-positive cancer; c-fos expression is intense in the nuclei.* ***(c)*** *c-fos-negative cancer. The cancer cells lost nuclear c-fos expression.*

receptor status.[10] In cervical precancerous lesions and cancer, c-fos expression was relatively low in precancerous lesions such as cervical intraepithelial neoplasia (CIN), but high in invasive cervical cancer,[11] and in an immunohistochemical study on hepatocellular carcinoma, c-fos expression was found to be significantly higher in tumor tissues than in non-tumor tissues.[12] In pancreatic tumors, FOS mRNA and protein overexpression were found in the majority of carcinomas.[13] In addition, c-fos has been identified as an independent predictor of survival by multivariate analyses in breast cancer (relative risk 4.214).[14] All of the above results suggest that c-fos participates in tumor progression.

In contrast, some recent studies have concluded that c-fos has tumor suppressor activity. The overexpression of c-fos was found to inhibit cell cycle progression and stimulated murine hepatocyte cell death and, furthermore, c-fos repressed anchorage-independent growth driven by oncogenic H-ras in vitro and strongly suppressed tumor formation in vivo.[15] However, few reports addressed the tumor suppressor activity of c-fos in human tumor tissues.

In this study, we evaluated the expressional status of c-fos in a large number of human gastric cancers using a tissue array method, and then went on to evaluate the significance of c-fos expression on clinico-pathologic characteristics, including survival data.

Materials and methods

Tissue specimens

A total of 625 consecutive, surgically resected cases of gastric cancer were identified from the files of the Department of Pathology, Seoul National University College of Medicine during 1995. Age, gender, tumor location, lymphatic invasion, vascular invasion and pTNM (Tumor-Node-Metastasis) stage[16] were evaluated by reviewing medical charts and pathologic records. Tissue slides were reviewed for histologic classification (according to the WHO classification and Lauren's classification),[17] and clinical outcome was followed from the date of surgery to the date of death or until the end of 2003. Cases lost during follow-up and those that died from any cause other than gastric cancer were regarded as censored data for the survival analysis. Seven cases that were lost just after the surgery were deleted during the survival analysis.

Tissue array methods

Core tissue samples of 2.0 mm diameter were taken from representative paraffin blocks of each gastric cancer and arranged in a new recipient paraffin block, as previously described.[18,19] A total of 12 blocks were used for this study and each block contained 55 cases of gastric cancer plus three non-neoplastic gastric mucosa samples from body, antrum and intestinal metaplasia. Because there has been an excellent agreement between staining results obtained from different intra-tumoral areas of gastric carcinomas,[18] one core was sampled per case. An adequate case was defined as one with tumor occupying more than 10% of the core area. Four-μm-thick slices were cut from each tissue array block, deparaffinized and then rehydrated.

Immunohistochemistry

Immunohistochemical staining for c-fos was performed using antibody against c-fos (rabbit polyclonal, 1:100, Santa Cruz, CA, USA) using the labeled streptavidin-biotin method after microwave antigen retrieval as described previously.[18] Intensities of immunolabeling were scored as positive (strong staining), equivocal (faint staining), or negative (absence of staining), and patterns of immunolabeling were scored as diffusely positive (≥50% of the tumor cells), focally positive (10~49% of the tumor cells), or negative (<10% of the tumor cells) according to tumor cell nuclear staining. For statistical analysis, we defined loss of expression as positive nuclear staining in less than 10% of tumor cells.

Statistical analyses

The Chi-square test and Fisher's exact test (2-sided) were used to examine the correlation between the expression of c-fos and clinicopathologic factors. Survival curves were plotted using the Kaplan-Meier product-limit method, and differences between survival curves were tested using the log-rank test. Multivariate survival analysis was performed using the Cox proportional hazards model. Results were considered to be statistically significant when p values were less than 0.05. All statistical analyses were conducted using SPSS Version 12.0 (SPSS, Chicago, IL).

Results

In non-neoplastic mucosa, c-fos was expressed in the nuclei of epithelial cells, stromal cells and inflammatory cells. Faint staining was noted in the cytoplasm of most epithelial cells including tumor cells (*Fig. 1*).

Of the 625 consecutive gastric cancers, 388 cases (62.1%) showed loss of nuclear c-fos expression (*Fig. 1*). Correlations between c-fos expression status and clinicopathologic characteristics are summarized in *Table 1*. Loss of c-fos expression was significantly more frequent in the intestinal type than in the diffuse type (p=0.006). The mean age of the c-fos-negative patients (55.8 years) was slightly older than that of the c-fos-positive patients (52.8 years) (p=0.005). Regarding tumor progression, loss of c-fos expression was positively associated with depth of invasion (p<0.001), lymph node metastasis (p<0.001), and an advanced stage (p<0.001). However, no significant correlation was found between loss of c-fos expression and vascular invasion (p>0.05).

Univariate survival analysis using log rank test showed that patients with c-fos-positive tumors achieved significantly better survival than those with c-fos-negative tumors (p<0.001) (*Fig. 2*). The 5-year survival rate (95% CI) was 72.9±5.8% and 57.1±5.2% for patients with positive and negative c-fos expression, respectively. By multi-

Table 1. **Correlation between c-fos expression and clinico-pathologic characteristics in consecutive gastric carcinomas**

Characteristics	*Total*	*c-fos expression*		*p value*
		Negative (N=388)	*Positive (N=237)*	
Gender				0.001
Male	412	275 (66.7%)	137 (33.3%)	
Female	213	113 (53.1%)	100 (46.9%)	
Location				0.086
lower	284	186 (65.5%)	98 (34.5%)	
middle	278	164 (59.0%)	114 (41.0%)	
upper	16	13 (81.2%)	3 (18.8%)	
whole	47	25 (53.2%)	22 (46.8%)	
Lauren classification				0.006
intestinal	235	158 (67.2%)	77 (32.8%)	
diffuse	345	196 (56.8%)	149 (43.2%)	
mixed	45	34 (75.6%)	11 (24.4%)	
Depth of invasion				<0.001
early (EGC)	199	86 (43.2%)	113 (56.8%)	
advanced (AGC)	426	302 (70.9%)	124 (29.1%)	
Lymph node metastasis				<0.001
absent	237	108 (45.6%)	129 (54.4%)	
present	388	280 (72.2%)	108 (27.8%)	
Stage				<0.001
I	265	126 (47.5%)	139 (52.5%)	
II	130	98 (75.4%)	32 (24.6%)	
III	135	100 (74.1%)	35 (25.9%)	
IV	95	64 (67.4%)	31 (32.6%)	
Lymphatic invasion				<0.001
absent	441	250 (56.7%)	191 (43.3%)	
present	184	138 (75.0%)	46 (25.0%)	
Vascular invasion				0.534
absent	600	371 (61.8%)	229 (38.2%)	
present	25	17 (68.0%)	8 (32.0%)	

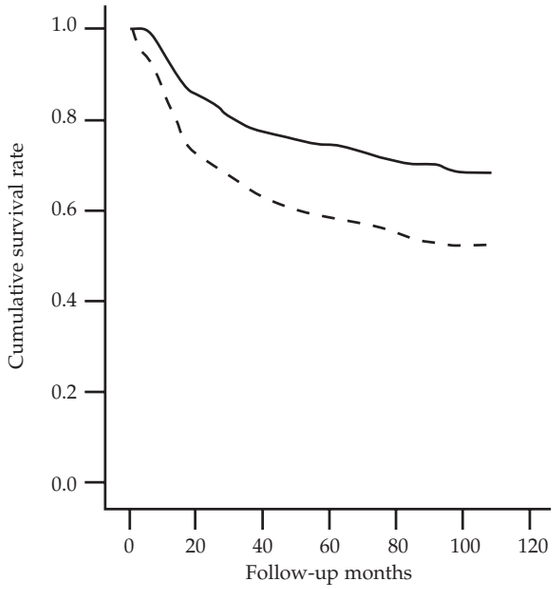

Figure 2. *Kaplan-Meier survival curves showing that c-fos-positive cancers (solid line) are associated with a better prognosis than c-fos-negative ones (broken line) (p<0.001)*

variate analysis adjusted for age and stage, cases with nuclear c-fos expression showed better survival with an odds ratio of 0.851, but the nuclear c-fos expression was not found to be an independent prognostic indicator (p=0.252) (*Table 2*).

Discussion

Our study demonstrates that a loss of c-fos expression is positively correlated with tumor progression, i.e., depth of invasion, lymphatic invasion and lymph node metastasis, although c-fos is not an independent prognostic indicator. These relationships might be due to an association between c-fos and an advanced pTNM stage, and consequently with a poor survival rate. Our report provides the first immunohistochemical evidence that suggests the tumor suppressing activity of c-fos in human clinical tumor tissues.

According to growing evidence from in vitro and in vivo experimental studies, c-fos is best considered as a double-edged sword, which can promote or suppress tumorigenesis. This dual action is probably due to the variable protein compositions of cells and/or their microenvironment; e.g. dimerization partners, promoter architecture, transcription factors and the types of co-activators that act on promoters in these cells.

Although our results are in contrast to mainstream opinion concerning the oncogenic role of c-fos, a number of experimental and clinical reports support our result. In a study comparing two mammary carcinoma cell lines, c-fos expression was observed in non-metastatic cells but not in highly metastatic ones.[20] In another study using murine hepatocytes that conditionally express c-fos, c-fos induction resulted in morphological changes that led to depolarization, inhibition of proliferation and finally apoptosis.[15] In human cancer samples, a Northern blot analysis revealed that FOS mRNA expression was significantly lower in non-small cell lung cancer than in normal lung tissue,[21] and reduced FOS mRNA expression was reported in papillary thyroid carcinomas compared to their normal counterpart.[22]

One of the possible explanations for the tumor suppressing activity of c-fos is that it has a pro-apoptotic function. *fos*$^{-/-}$ *tp53*$^{-/-}$ double-knockout mice develop highly proliferative and invasive rhabdomyosarcomas, a tumor type rarely observed in *tp53*$^{-/-}$ mice. Interestingly, the re-expression of c-fos in an established tumor cell line increased apoptosis, suggesting that c-fos can suppress tumorigenesis by either positively regulating apoptosis-inducing genes or by suppressing survival genes.[23] In another in vivo study using mutant mice in which *fos* and/or *jun* were disrupted, c-fos was found to be essential for light-induced retinal apoptosis, whereas c-jun was dispensable.[24]

Recent observations indicate that c-fos is a mediator of c-myc-induced cell death in human hepatocellular carcinoma cells deprived of growth factors. c-myc can induce apoptotic cell death by upregulating c-fos via the p38 MAP kinase pathway which does not involve p53.[25] A study using UVA-irradiated human keratinocyte cell lines showed that c-fos expression was enhanced via the activation of p38 MAP kinase (MAPK1) and c-jun N-terminal kinase (MAPK8).[26] The authors did not further examine the results of c-fos expression, and concluded that the overexpression of c-fos might be involved in skin carcinogenesis. However, according to another experiment in human hepatocellular carcinoma cells, c-fos expression via the p38 MAP kinase pathway induced apoptosis.[25] There-

Table 2. **Multivariate analysis of c-fos protein expression in gastric carcinomas using the Cox proportional hazard model**

Parameters	*Number of cases*	*Odds ratio (95% CI)*	*p value*
c-fos			0.252
negative	382	1	
positive	236	0.851 (0.645-1.122)	
Age			0.004
0-65 years	492	1	
≥66 years	126	1.495 (1.136-1.996)	
Stage			<0.001
I	264	1	
II	129	3.466 (2.249-5.342)	
III	130	6.826 (4.568-10.199)	
IV	95	15.612 (10.424-23.380)	

fore, it might be reasonable to suspect that c-fos expression induced apoptosis in UVA irradiated keratinocytes. Fas ligand (FASLG or FasL) and the tumor necrosis factor-related apoptosis-inducing ligand (TNFSF10 or TRAIL) are typically associated with cell death and they trigger the transcriptional activation of c-fos in Jurkat cells, a human T-cell leukemia cell line.[27] Perhaps FASLG and TNFSF10 reflect another apoptotic mechanism induced by c-fos overexpression. In addition, FOS mRNA levels were found to be elevated in ceramide-induced neuronal apoptosis, and this phenomenon has been reported in other neuronal apoptosis models such as in models of NMDA or kainic acid excitotoxicity.[28-30]

Recently, *Helicobacter pylori* was placed in the spotlight concerning its role in gastric carcinogenesis, and a number of studies have reported that *H. pylori* infection increases c-fos expression. The upregulation of c-fos protein is one of the most dramatic responses to *H. pylori* infection, and of the results from the activation of the mitogen-activated protein kinase cascade.[31,32] Although this upregulation could also be an initial carcinogenic event caused by *H. pylori*, it might also reflect a protective mechanism initiated by gastric cells designed to induce apoptosis before infected epithelial cells go awry.

As has been discovered in several cell types, c-fos might also have a pro-apoptotic function in human gastric cancer cells. Moreover, the tumor suppressing activity of c-fos, suggested by our study, might be associated with the pro-apoptotic function of c-fos in gastric mucosa. Now, if c-fos is associated with apoptosis in gastric mucosa, loss of c-fos expression could impair the apoptosis of damaged or mutated cells. Consequently, the numbers of mutated genes accumulate, and the relationship between loss of c-fos expression and an advanced stage and a poor prognosis would be established. The apoptotic process might involve proapoptotic signals such as FASLG, TNFSF10, or MAPK1 pathways, but not p53. Moreover, if the above hypothesis proves to be true, then it could be concluded that c-fos could play a protective role in gastric cancer.

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