Prognostic value of co-expression of STAT3, mTOR and EGFR in gastric cancer

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Abstract. Signal transducer and activator of transcription 3 (STAT3), the mammalian target of rapamycin (mTOR) and epidermal growth factor receptor (EGFR), proteins that mediate intracellular signaling related to cell growth, proliferation and differentiation, have received considerable interest as possible targets for cancer treatment. We examined whether the expression of STAT3, mTOR and EGFR correlates with clinicopathological features and patient outcome in gastric cancer. Tumor samples were obtained from 126 patients with gastric adenocarcinomas who underwent a radical gastrectomy between 1999 and 2002. The expression of phosphorylated STAT3 (p-STAT3), p-mTOR and EGFR was analyzed by immunohistochemical staining. The relations of these to clinicopathological factors and outcomes were assessed. The expression of p-STAT3 p-mTOR and EGFR positively correlated with the following variables related to tumor progression: the depth of tumor invasion (T1 vs. T2-4; p<0.001, p=0.036 and p<0.001, respectively), lymph node involvement (p=0.008, p=0.027 and p=0.007) and tumor stage (I vs. II-IV; p<0.001, p=0.041 and p<0.001). The expression of p-STAT3 and EGFR was significantly related to distant metastasis and recurrence (p=0.001 and p=0.039), as well as significantly poorer disease-specific survival (DSS; p=0.0018 and p=0.026). The expression of p-STAT3 was a marginally non-significant prognostic factor for DSS (hazard ratio=2.0, 95% CI 0.91-4.5, p=0.082). Increasing expression of p-STAT3, p-mTOR and EGFR was associated with progressively worse DSS. Interactions among p-STAT3, p-mTOR and EGFR may play an important role in tumor progression and outcomes in patients with gastric cancer.

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Introduction

Gastric cancer is the second most common cause of cancer-related deaths worldwide (1). Clinical outcomes remain poor in patients with advanced gastric cancer, even after curative resection. Tyrosine kinase receptors, such as epidermal growth factor receptor (EGFR), its homolog c-erb-2 (HER2) and vascular endothelial growth factor (VEGF), have been linked to tumor progression or survival in gastric cancer (2-7). We also previously reported that EGFR or HER3 expression was correlated with tumor progression in gastric cancer (8). Several anticancer drugs designed to inhibit signaling pathways of tyrosine kinases have been evaluated in unresectable or metastatic gastric cancer, and trastuzumab (anti-HER2) has been shown to be effective (9).

The main pathways stimulated by EGFR involve phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinases (MAPK) and signal transducer and activator of transcription 3 (STAT3) (10,11). The mammalian target of rapamycin (mTOR) is one of the targets of activated Akt. Phosphorylated mTOR (p-mTOR) stimulates initiation of translation by two targets: ribosomal p70S6 kinase (S6K1) and eukaryotic translation initiation factor 4E binding protein (4E-BP1). mTOR thus promotes cell growth, proliferation and differentiation (12,13). We previously reported that the expression of cytoplasmic mTOR is associated with tumor progression and poorer outcomes in gastric cancer (14). STAT3 belongs to a family of cytoplasmic latent transcription factors that function as both signal transducers and activators of transcription. EGFR interacts with and activates STAT3. Phosphorylated STAT3 (p-STAT3) translocates to the nucleus and binds to recognition sequences in the promoter region of target genes, thereby regulating their transcription. Thus, aberrant STAT3 signaling has been linked to the promotion of cell cycle progression, cell survival and malignant transformation (15,16). STAT3 is related to tumor progression and worse survival in gastric (17-19), colorectal (20,21), lung (22,23) and breast cancer (24).

The present study examined whether the expression of p-STAT3, p-mTOR and EGFR correlates with clinicopathological features and patient outcome in gastric cancer. The expression was evaluated immunohistochemically.

Patients and methods

Patients. The study group comprised 126 patients with primary invasive (invasion deeper than the muscularis mucosa) gastric adenocarcinomas who underwent gastrectomy from January 1999 through December 2002 at the Department of Esophagogastric Surgery, Tokyo Medical and Dental University, Japan. Each tumor was classified according to the tumor-node-metastasis classification recommended by the International Union against Cancer (UICC). All patients were evaluated for recurrent disease by diagnostic imaging, including computed tomography, ultrasonography and endoscopy, every 3-6 months. The median follow-up time was 73 months (range 2-135). Ten patients who underwent R1 or R2 surgery received S-1 chemotherapy; no other patient received neoadjuvant therapy. Recurrent disease was diagnosed in 28 (24%) of 116 patients who underwent R0 surgery and was the cause of death in these patients.

Immunohistochemical staining. Immunohistochemical staining was performed using the streptavidin-biotin method using a Histofine SAB-PO kit (Nichirei Co., Tokyo, Japan). Polyclonal rabbit anti-human antibodies against p-STAT3 (Thy705) and p-mTOR (Ser2448) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Monoclonal mouse antibodies against EGFR were from Novocastra Laboratories (Newcastle upon Tyne, UK). All available H&E-stained slides of the surgical specimens were reviewed. For each case, representative paraffin blocks were selected for immunohistochemical studies. Four-micrometer sections were cut from formalin-fixed, paraffin-embedded tissue blocks. After deparaffinization and rehydration, antigen retrieval treatment was performed at 121˚C (autoclave) for 5 min in 10 nmol/l sodium citrate buffer (pH 9.0), followed by treatment with 3% hydrogen peroxide for 15 min to quench endogenous peroxidase activity. Non-specific binding was blocked by treating the slides with 5% EzBlock (including 5% normal goat serum and 0.1% Tween-20) for 60 min at room temperature. The slides were incubated with a primary antibody (dilution 1:50) overnight at 4˚C. Immunodetection was performed by the conventional streptavidin-biotin method with a Nichirei SAB-PO kit (Nichirei Co.). The slides were counterstained with 1% Mayer's hematoxylin.

Two independent observers blinded to the patients' clinical information examined all of the slides with an optical microscope. Positive expression was defined as >10% of the cells with nuclear staining for p-STAT3 (17) or >10% of the cells with cytoplasmic staining for p-mTOR, as previously described (14). Positive EGFR expression was defined as >10% of the cells with moderate or strong membrane staining.

Statistical analysis. The Chi-square test was used to test possible associations of the expression of p-STAT3, p-mTOR and EGFR with clinicopathological factors. It was also used to assess correlations between each type of expression. Kaplan-Meier curves were plotted to assess the effects of each type of expression on disease-specific survival (DSS). Survival curves were compared using the log-rank test. P-values of <0.05 were considered to indicate statistical significance. Multivariate proportional Cox models were used to assess the
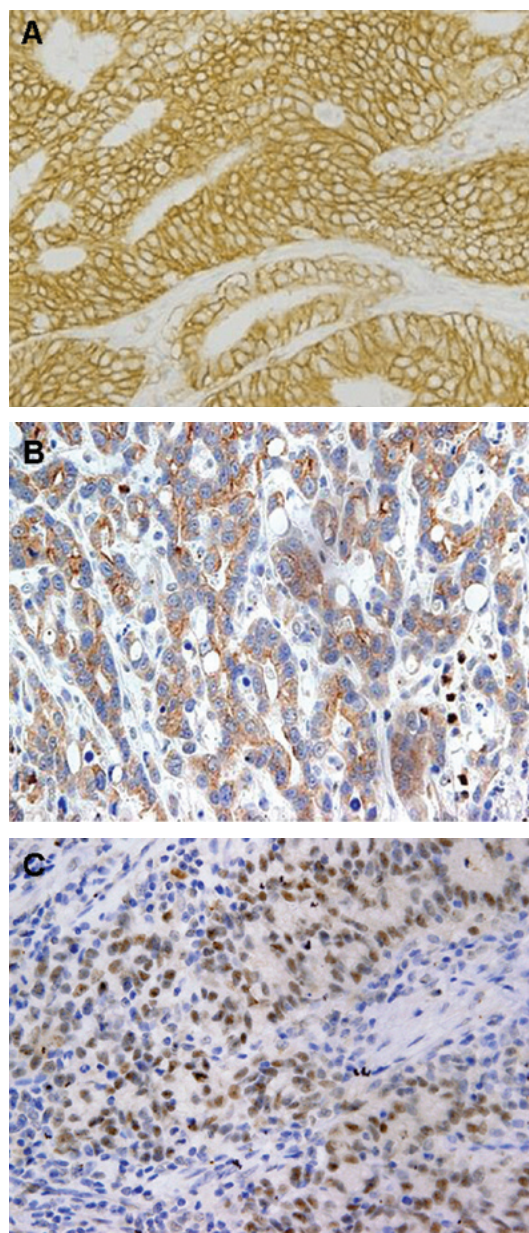

Figure 1. Representative gastric carcinomas showing immunostaining for (A) p-STAT3 predominantly in the nucleus, (B) immunostaining for p-mTOR predominantly in the cytoplasm and (C) immunostaining for EGFR predominantly in the membrane; magnification, x400.

prognostic significance of all expression levels and of several clinicopathological factors. Statistical analysis was performed with the use of SPSS Base, version 11.0 and SPSS Advanced Models, version 11.0 (SPSS Inc., Chicago, IL, USA) software.

Results

Expression of nuclear p-STAT3, cytoplasmic p-mTOR and membranous EGFR was found in 52 (41%), 81 (64%) and 37 (29%) tumors, respectively (Fig. 1). All three types of expression were observed in 16 (13%) tumors, while 28 (22%) tumors showed no expression. The expression of p-STAT3 significantly correlated with that of mTOR (p=0.035). EGFR expression correlated slightly, but not significantly with p-STAT3 and mTOR expression (p=0.060 and p=0.054).

Table I. Correlation of p-STAT3, p-mTOR and EGFR expression with clinicopathological factors.

	n	Nuclear p-STAT3			Cytoplasmic p-mTOR			Membranous EGFR			Co-expression values				
		Negative	Positive	p-value	Negative	Positive	p-value	Negative	Positive	p-value	0	1	2	3	p-value
Age															
≥70	41	19	22	0.050	12	29	0.290	26	15	0.220	6	13	13	9	0.140
<70	85	55	30		33	52		63	22		22	29	27	7	
Gender															
Male	88	54	34	0.360	31	57	0.860	60	28	0.480	21	27	28	12	0.760
Female	38	20	18		14	24		29	9		7	15	12	4	
Tumor location															
Middle/Lower	103	61	42	>0.990	39	64	0.410	72	31	0.900	23	35	33	12	0.900
Upper	23	13	10		6	17		17	6		5	7	7	4	
Histopathology															
Intestinal	48	32	16	0.160	29	49	0.660	35	13	0.660	10	21	11	6	0.210
Diffuse	78	42	36		16	32		54	24		18	21	29	10	
Depth of invasion															
T1	49	39	10	<0.001	23	26	0.036	45	4	<0.001	19	20	10	0	<0.001
T2/3/4	77	35	42		22	55		44	33		9	22	30	16	
LN metastasis															
Negative (N0)	59	42	17	0.008	27	32	0.027	49	10	0.007	22	19	14	4	0.001
Positive (N1/2/3)	67	32	35		18	49		40	27		6	23	26	12	
Distant metastasis or recurrence															
Negative	88	60	28	0.001	35	53	0.210	67	21	0.039	26	30	24	8	0.008
Positive	38	14	24		10	28		22	16		2	12	16	8	
Stage															
I	63	47	16	<0.001	28	35	0.041	54	9	<0.001	24	21	15	3	<0.001
II/III/IV	63	27	36		17	46		35	28		4	21	25	13	

A

B

C
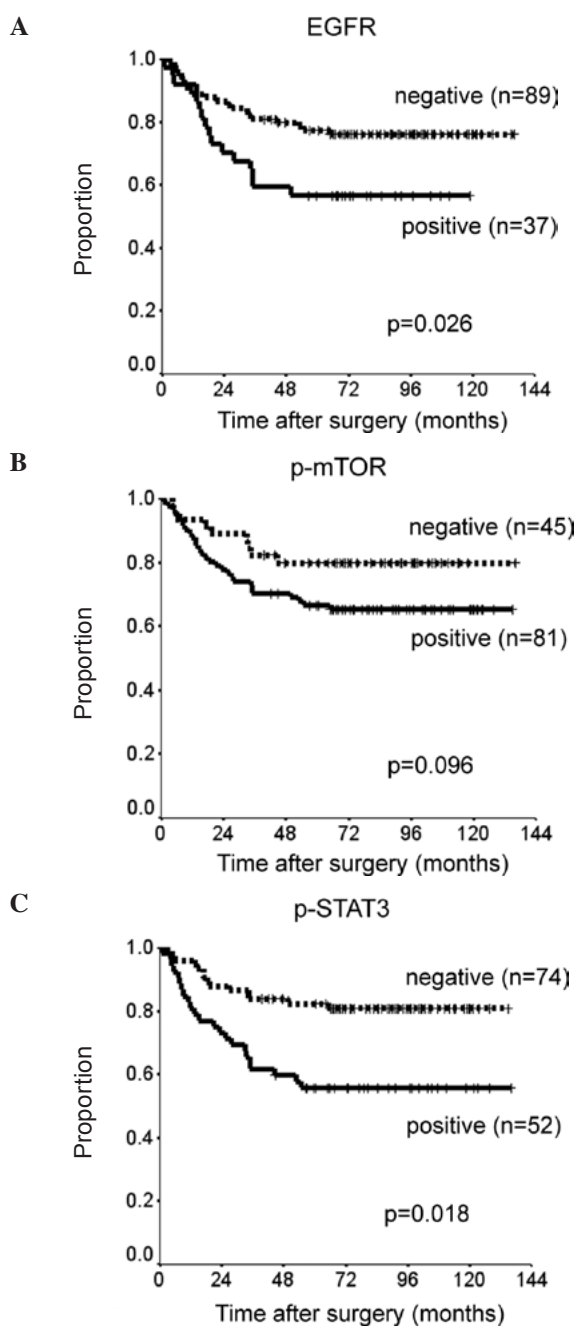

Figure 2. Kaplan-Meier curves comparing disease-specific survival of patients with expression of (A) EGFR, (B) p-mTOR and (C) p-STAT3.

Table II. Prognostic factors in a multivariate Cox proportional hazards regression model.

	DSS		
	HR	95% CI	p-value
Main tumor location			
Middle/Lower	1.00		
Upper	1.40	0.58-3.4	0.450
Histopathology (Lauren)			
Intestinal	1.00		
Diffuse	1.30	0.55-3.0	0.570
Tumor depth			
T1	1.00		
T2-4	3.70	0.75-18	0.110
Lymph node metastasis			
Negative (N0)	1.00		
Positive (N1-3)	1.90	0.67-5.7	0.220
Distant metastasis			
Negative (M0)	1.00		
Positive (M1)	6.40	2.20-19	0.001
EGFR			
Negative	1.00		
Positive	1.70	0.74-3.8	0.220
p-mTOR			
Negative	1.00		
Positive	0.66	0.26-1.7	0.390
p-STAT3			
Negative	1.00		
Positive	2.00	0.91-4.5	0.082

involvement, UICC stage and distant metastasis or recurrence (p<0.001, p=0.001, p<0.001 and p=0.008, respectively) (Table I).

Patients with p-STAT3 or EGFR expression had significantly shorter DSS than did those without such expression (p=0.0018 and p=0.026). There was a slight, but insignificant association between p-mTOR expression and shorter DSS (p=0.096) (Fig. 2). The prognostic relevance of the expression of each protein was assessed using a multivariate proportional hazards model adjusted for several clinical factors (main location of tumor, histological type, depth of tumor, lymph node metastasis and distant metastasis) (Table II). Expression of p-STAT3 was a marginally insignificant prognostic factor for DSS [hazard ratio (HR)=2.0, 95% CI 0.91-4.5, p=0.082]. Distant metastasis was the only independent prognostic factor for DSS (HR=6.4, 95% CI 2.2-19, p=0.001). DSS was reduced with increased co-expression of p-STAT3, p-mTOR and EGFR (Fig. 3). DSS at 5 years was 93% in patients with no expression of the proteins, 76% in patients with positive expression of one type of protein, 60% in those with positive expression of two of the proteins and 50% in those with positive expression of all three proteins. Survival was significantly longer in patients with no positive expression than in those with positive expression of two or all three proteins (none vs. all: p=0.0012; none vs. two: p=0.0047).

Immunoreactivity for p-STAT3, p-mTOR and EGFR was present in advanced cancer. Each type of expression positively correlated with the depth of tumor invasion (T1 vs. T2-4; p<0.001, p=0.036 and p<0.001, respectively) and lymph node involvement (p=0.008, p=0.027 and p=0.007). Each type of expression was also significantly associated with UICC stage (I vs. II-IV; p<0.001, p=0.041 and p<0.001). Expression of p-STAT3 or EGFR was significantly related to distant metastasis or recurrence (p=0.001 and p=0.039), whereas the expression of p-mTOR was not (p=0.21). No type of expression significantly correlated with histological type or gender, although p-STAT3 expression tended to be associated with older age (p=0.050). Co-expression of p-STAT3, p-mTOR and EGFR correlated significantly with tumor depth, lymph node
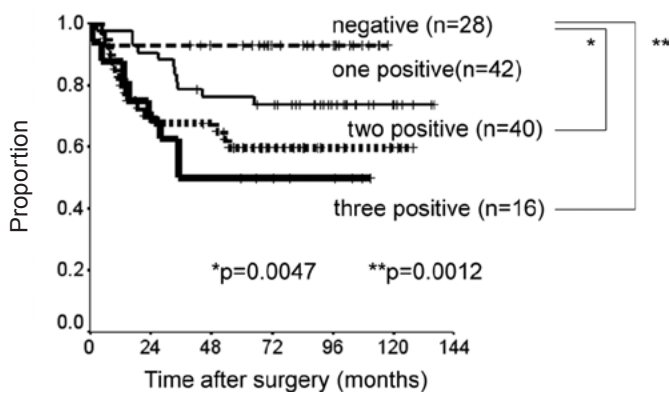
Figure 3. Kaplan-Meier curves of the disease-specific survival of four sub-groups classified according to the co-expression of p-STAT3, p-mTOR and EGFR.

Discussion

The present study showed that the co-expression of p-STAT3, p-mTOR and EGFR, members of the EGFR signaling pathway, was significantly associated with tumor progression and less favorable survival in gastric cancer. The expression levels of these proteins appeared to be related to clinical outcomes, but were not independent prognostic factors. In addition, the co-expression of these proteins was associated with poorer DDS.

STAT3 translocates to the nucleus, where it activates gene transcription and modulates cell proliferation, apoptosis and differentiation. Therefore, nuclear expression of p-STAT3, the activated form of STAT3, may be more intimately related to tumor progression than its cytoplasmic expression. Immunohistochemically, STAT3 is detected much more frequently in gastric cancer than in surrounding normal tissue (18). In the present study, p-STAT3 expression was rarely detected in normal gastric mucosa. Nuclear expression of p-STAT3 was significantly associated with tumor progression and clinical outcomes in gastric cancer, whereas cytoplasmic expression of p-STAT3 was not (data not shown). Several studies showed that immunohistochemical staining for p-STAT3 or STAT3 was related to poor survival in gastric cancer, without specifying the site of staining. Park *et al* reported that p-STAT3 was expressed predominantly in the nucleus in colon cancer. However, when they analyzed nuclear and cytoplasmic expression together, p-STAT3 was found to be associated with clinical stage (21). In breast cancer, STAT3 was detected mainly in the cytoplasm and its expression was related to the overall 5-year survival rate (24). This discrepancy in the location of staining may be attributed to differences in the type of cancer or in the antibodies used for immunostaining. Lee *et al* found that immunohistochemical staining for nuclear p-STAT3 was an independent prognostic factor in gastric cancer. The prognostic value appeared to be enhanced when p-STAT3 and matrix metalloproteinase-10 (MMP-10) were combined (19). STAT3 activation is implicated in the modulation of MMP-10 activity. Several molecular targets involving the STAT3 signaling pathway may have a substantial effect on patient outcome in gastric cancer.

EGFR expression has been considered a prognostic factor for patients with gastric cancer (2,3). In the present study, EGFR expression was significantly associated with tumor progression and poor survival in gastric cancer, but was not an independent prognostic factor. EGFR consists of an extracellular ligand-binding domain, a transmembrane domain and an intracytoplasmic tyrosine kinase domain. EGFR expression is detected mainly in the membrane of cancer cells, but has also been detected in the nucleus in several studies (25,26). The EGFR antibodies used in the present study were rarely detected in the cytoplasm or nucleus. EGFR interacts with STAT3 in the nucleus, leading to transcriptional activation of inducible nitric oxide synthases (26). Several inhibitors of EGFR have been used to treat unresectable or recurrent gastric cancer, but provided no benefit in such patients or in those with colorectal or lung cancer (27). Inhibition of EGFR alone may be inadequate and synchronous inhibition of downstream substrates, such as mTOR and STAT3, may be essential for improving outcomes in gastric cancer.

We previously reported that the cytoplasmic expression of p-mTOR positively correlated with tumor progression and survival in gastric cancer. However, few studies have demonstrated the impact of mTOR expression on survival in patients with cancer (28). Akt, which is an upstream regulator of mTOR, was not associated with outcomes in patients with several types of cancer in previous studies, including ours (14,29,30). Therefore, activation of Akt/mTOR alone may not have an impact on tumor growth or patient survival. Ma *et al* demonstrated that mTOR activates STAT3/p63/Jagged signal cascade *in vitro* (31). In the present study, p-mTOR expression correlated significantly with p-STAT3 expression. Activation of STAT3, by not only EGFR/STAT3 but also mTOR/STAT3 signaling, may lead to poor survival in gastric cancer.

In conclusion, co-expression of p-STAT3, p-mTOR and EGFR may play an important role in tumor progression and clinical outcomes in patients with gastric cancer.

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