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Prognostic Value of Phosphorylated Akt and Survivin Expression in Gastric Adenocarcinoma

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Background : pAkt (the phosphorylated form of the proto-oncogene protein c-akt) and survivin (human BIRC5 protein) are candidate apoptosis-related molecules that may be responsible for cancer progression. The aim of this study was to determine the expression of pAkt and survivin in malignant stomach neoplasm, and their value as prognostic indicators of cancer. **Methods** : The expression of pAkt and survivin in 144 cases of gastric cancer was detected by immunohistochemistry and compared with established clinicopathological parameters and prognosis of this disease. **Results** : Expression of pAkt showed significant correlations with depth of invasion, lymph node and distant metastasis, as well as the stage ($p < 0.05$ for all three correlations), but not with the Lauren classification. Survivin expression closely correlated with histological type, Lauren classification, depth of invasion, metastasis, and stage ($p < 0.05$ for all). The overall survival of patients with pAkt/survivin expression was inferior to that of patients with loss of pAkt/survivin expression. Cox multivariate analysis demonstrated a significant correlation between stage ($p = 0.04$), survivin expression ($p = 0.02$), and prognosis. **Conclusions :** Patients with pAkt/survivin expression in gastric cancer are at increased risk of cancer-related mortality via the apoptosis resistance pathway. Expression of pAkt and survivin could be used as a prognostic indicator for gastric cancer.

Key Words : Proto-oncogene proteins c-akt; BIRC5 protein, human; Stomach neoplasms; Apoptosis

Deregulated apoptosis is essential in carcinogenesis because it aberrantly prolongs cell survival and promotes the accumulation of transforming mutations.[1] Gastric cancers, one of the most common malignancies in the world,[2] use several mechanisms to inhibit apoptosis and prolong cancer cell survival.[3] One emerging key regulator of apoptosis is Akt, a serine/threonine kinase that serves as a central mediator of phosphatidylinositol 3-kinase (PI3K) signaling.

PI3K and Akt are involved in a wide range of responses that drive tumor progression-from cell growth and proliferation to survival and motility.[4] Blockade of the PI3K-Akt pathway has been reported to induce cancer cell apoptosis and tumor suppression in some human cancers such as breast and prostate cancers.[5,6] Akt activation is also associated with enhanced tumor cell invasion. Akt enhances invasiveness of pancreatic carcinoma cells via up-regulation of insulin-like growth factor 1[7] and increases secretion of matrix metalloproteinases 2 and 9 from immortalized mammary epithelial cells and ovarian carcinomas.[8,9] A previous study demonstrated that the constitutive activation of Akt in cancer cells gave rise to the cell-cell detachment and the increased motility required for tissue invasion.[10]

Considering the influence of gastric carcinoma on cancer epidemiology in Asian countries in association with cancer mortality,[2] we hypothesized that the expression status of the active form of Akt, i.e., phosphorylated Akt (pAkt), as detected by immunohistochemistry in histological specimens from patients with gastric carcinoma, could be used to predict the survival time of individual patients. Furthermore, we compared the prognostic potential of pAkt with that of another representative antiapoptotic protein, survivin.

In the present study, we investigated the expression of pAkt and survivin in gastric carcinoma using immunohistochemistry, and their association with clinicopathological parameters and survival. We also evaluated the potential value of the expression of pAkt and survivin as clinically applicable prognostic indicators.

MATERIALS AND METHODS

Patients and tumor samples

Gastric adenocarcinoma specimens were obtained from 144 patients who had undergone curative surgical resection between 1998 and 1999 at Chonnam National University Hospital. The availability of adequate tissue material and clinical follow-up data were the only inclusion criterion. The patients ranged in age from 35 to 84 years (average, 62.0); 101 were men, and 43 were women. No patient had received pre- or post-operative chemotherapy or radiation therapy. Tumor staging was done in accordance with the American Joint Committee on Cancer Staging system.[11] Patients were followed for an average follow-up time of 49.1 months (range, 1 to 80 months) to determine clinical outcomes. Tumors were divided into two histological subgroups: a differentiated type that consisted of papillary and tubular adenocarcinomas, and an undifferentiated type that consisted of poorly differentiated adenocarcinomas, signet ring cell carcinomas, and mucinous adenocarcinomas. This study was approved by the Institutional Review Board of Chonnam National University Hospital.

Immunohistochemistry

We reviewed hematoxylin and eosin stained slides of tumor specimens, and selected tissue blocks that included the edge of the tumor area for formalin-fixing and paraffin-embedding. Antibodies for pAkt (1 : 100, monoclonal, Cell Signaling Tec., MA, USA), and survivin (1 : 800, monoclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for immunohistochemical analysis. Immunostaining was performed using the avidin-biotin complex method. Briefly, representative paraffin blocks were cut consecutively at 4 micron thickness and immunohistochemical staining was carried out using a Microprobe Immuno/DNA stainer (Fisher Scientific, Pittsburgh, PA, USA). Sections were deparaffinized in xylene and treated with 0.3% hydrogen peroxide in methanol for 20 minutes to block endogenous peroxidase activity. For pAkt and survivin, sections were subjected to pressure-cooking for 7 minutes in 10 mM citrate phosphate buffer (pH 6.0) and then incubated with primary antibodies for 120 minutes at room temperature. Anti-mouse immunoglobulin G (Sigma, St. Louis, MO, USA) labeled with biotin was used as a secondary antibody for the detection of primary antibodies; slides were incubated with this secondary antibody for 7 minutes at 45℃. The Vector Elite ABC detection kit (Vector Laboratories, Burlingame, CA, USA) with 3,3′-diaminobenzidine tetrahydrochloride (Sigma) was used as the chromogen. Sections were counterstained with hematoxylin and mounted in Universal Mount (Research Genetics, Huntsville, AL, USA) reagent. Negative controls were treated similarly with the exception that primary antibodies were omitted.

Assessment of pAkt and survivin expression

All immunostained slides were evaluated independently by two independent observers and evaluation was done twice without the evaluator having any knowledge of the clinical details. Sections from a block of infiltrating breast carcinoma were stained as positive controls for pAkt and survivin. Scoring for staining intensity was done as follows: 0, no appreciable staining in tumor cells; 1, barely detectable staining in cytoplasm and/or nucleus as compared with stromal elements; 2, readily appreciable brown staining distinctly marking the tumor cell cytoplasm and/or nucleus; and 3, dark brown staining in tumor cells completely obscuring the cytoplasm and/or nucleus. This scoring system has been reported previously.[12] The percentage of stained tumor cells was scored as 0 (none), 1 (1% to 10%), 2 (11% to 50%), or 3 (51% to 100%). Immunoreactivity was considered low if the sum of the proportion score and the intensity score was between 0 and 3 and high if the sum was between 4 and 6. Immunohistochemical staining was re-evaluated for cases showing disagreement between observers. Two pathologists reviewed those cases together, and reached an agreement for samples that had had inconclusive results.

Statistical analysis

Statistical analysis was done with the SPSS ver. 15.0 (SPSS Inc., Chicago, IL, USA). The relationship between expression of proteins and categorical variables was compared by the χ^2 test, or when appropriate, the Fisher exact probability test. The strength of association between pAkt and survivin was assessed by the Spearman rank correlation test. Survival curves were estimated using the Kaplan-Meier method. The distribution of

survival was comparatively studied using the log-rank test. For multivariate analysis, independent prognostic factors were determined using Cox's proportional hazard model. The level of significance was set at $p < 0.05$.

RESULTS

Clinicopathological features of our cases are summarized in Table 1. High expression of pAkt protein was detected in 56.9% (82 of 144 cases) of gastric cancers. As shown in Fig. 1, pAkt was mainly localized in the nucleus and partially in the cytoplasm of the cancer cells. Results from the analysis of correlation between pAkt expression and various clinicopathological factors are presented in Table 1. High pAkt expression was significantly associated with depth of invasion ($p = 0.022$), regional lymph node metastasis ($p = 0.001$), distant metastasis ($p = 0.012$), and stage ($p < 0.001$).

Table 1. Correlation between pAkt/survivin expression and clinicopathological parameters of gastric cancer

	pAkt immunoreactivity			Survivin immunoreactivity		
	Low	High	p-value	Low	High	p-value
Age (yr)						
≤ 62	26	36	0.474	34	28	0.005
> 62	36	46		63	19	
Sex						
Man	38	63	0.034	69	32	0.425
Woman	24	19		28	15	
Tumor size (cm)						
≤ 4.3	37	52	0.388	59	30	0.436
> 4.3	25	30		38	17	
Histologic type						
Differentiated	28	45	0.162	40	33	0.001
Undifferentiated	34	37		57	14	
Lauren classification						
Intestinal	19	36	0.268	28	27	0.028
Diffuse	37	37		60	14	
Mixed	6	9		9	6	
Depth of invasion						
Tis	8	7	0.022	14	1	< 0.001
T1	8	6		12	2	
T2	16	11		23	4	
T3	29	53		45	37	
T4	1	5		3	3	
Lymph node metastasis						
N0	39	29	0.001	56	12	< 0.001
N1	13	26		23	16	
N2	9	15		12	12	
N3	1	11		5	7	
Distant metastasis						
M0	58	64	0.012	88	34	0.003
M1	4	17		8	13	
Stage						
0 & I	28	19	< 0.001	41	6	< 0.001
II	15	14		22	7	
III	14	23		22	15	
IV	5	26		12	19	

We also analyzed the relationship between expression of pAkt and survivin in gastric cancer tissues. The results showed high survivin immunostaining in 32.6% (47 of 144 cases) of the cancers. Fig. 2 displays an expression pattern of survivin that was similar to that of pAkt. High survivin expression was associated with age of the patient ($p = 0.005$), histological type ($p = 0.001$), Lauren classification ($p = 0.028$), depth of invasion ($p < 0.001$), regional lymph node metastasis ($p < 0.001$), distant metastasis ($p = 0.003$), and stage ($p < 0.001$), but was not associated with sex, and tumor size ($p > 0.05$) (Table 1). High pAkt expression significantly correlated with survivin expression ($p = 0.001$, $\rho = 0.276$). When interpretation was limited to a pAkt-low/survivin-low group (51 of 144 cases) and a pAkt-high/survivin-high group (36 of 144 cases), expression status also showed a strong association with all clinicopathological factors except for sex and tumor size (data not shown). However, this apparently additive interaction between pAkt and survivin expression was not proven when validated by Cox's proportional hazard model ($p = 0.584$).

Kaplan-Meier curves were plotted using staining results to determine prognostic benefits of pAkt and survivin expression in gastric cancer. pAkt-low patients survived significantly longer than pAkt-high patients (median survival period, 69.2 months vs 49.7 months, respectively; $p = 0.001$) (Fig. 3). Survivin-low patients also survived longer than survivin-high patients (median survival period, 66.3 months vs 39.6 months, respectively; $p < 0.001$) (Fig. 4).

Table 2 shows the results of a multivariate analysis using Cox proportional hazards. The covariates included in the model were pAkt expression, survivin expression, stage, histological type, Lauren classification, tumor size, age, and sex. The results revealed that stage and survivin expression were independent prognostic factors.

DISCUSSION

The protein kinase Akt has been spotlighted as an important regulator of cell proliferation and survival in recent years.[4] In humans, three members of the Akt family designated *AKT1, AKT2*, and *AKT3,* are located on chromosomes 14q32, 19q13, and 1q44, respectively.[13] Akt activation is explained by a multistep model that requires translocation of Akt to the cell mem-

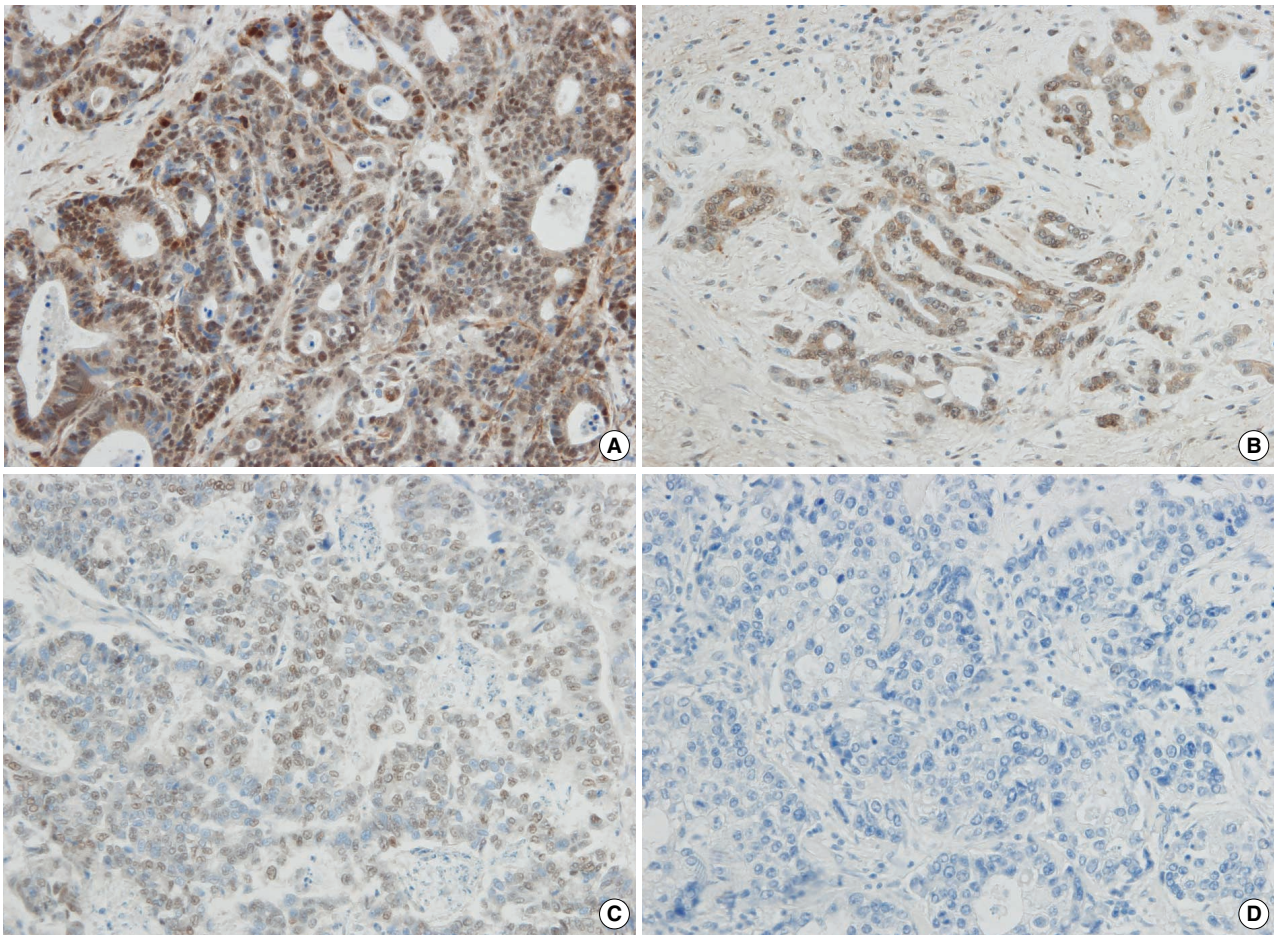

Fig. 1. Assessment of pAkt immunoreactivity according to staining intensity. (A) score 3, (B) score 2, (C) score 1, (D) score 0.

brane and phosphorylation of Akt at Thr308 and Ser473.[14] Activated Akt has been reported to regulate the apoptotic process by phosphorylating substrates such as Bcl-2 antagonist of cell death, Forkhead transcription factors, caspase 9, and IκB kinase (IKKα) in order to influence nuclear factor-κB (NF-κB) and glycogen synthase kinase (GSK-3β).[4] The Akt kinases are major downstream mediators of growth factor receptor tyrosine kinases that signal via PI3K.[4]

The present study revealed a significant association between increased pAkt expression and shorter survival time for patients with gastric cancer, suggesting a potential benefit of pAkt as a prognostic marker in patients with gastric carcinoma. Expression of pAkt was also associated with major clinicopathological parameters, as was the expression of survivin. In a previous study, there was no significant correlation between pAkt expression and clinicopathological variables.[15] This discrepancy seems to be due to different criteria being used to define a critical expression level. Murakami *et al*.[15] classified pAkt expression into three categories; negative staining, low expression (1-50% of carcinoma cells were positive), and high expression (> 50% of carcinoma cells were positive). We divided the level of protein expression in the total tumor by the intensity and areas of expression. This difference in methods used to evaluate the amount of protein expression may account for the difference in expression rate between that obtained in this report and previous studies. Cinti *et al*.[16] found results similar to ours-that pAkt expression is inversely correlated with patient survival in gastric cancers. Moreover, they noticed that high expression of nuclear pAkt rather than cytoplasmic pAkt is linked to a low apoptotic index in association with some cell cycle regulators.

Survivin is the smallest member of the mammalian inhibitor of apoptosis family with a molecular weight of 16.5 kDa.[17] Survivin inhibits caspase-3 and -7 in cells, and its overexpression can lead to resistance to cell death caused by various apoptotic stimuli.[18] However, accumulating evidence has suggested that survivin is involved in both caspase-dependent and -independent apoptotic controls and, furthermore, in prolonging cellular lifespan.[19] Survivin is abundantly expressed in proliferating fetal tis-

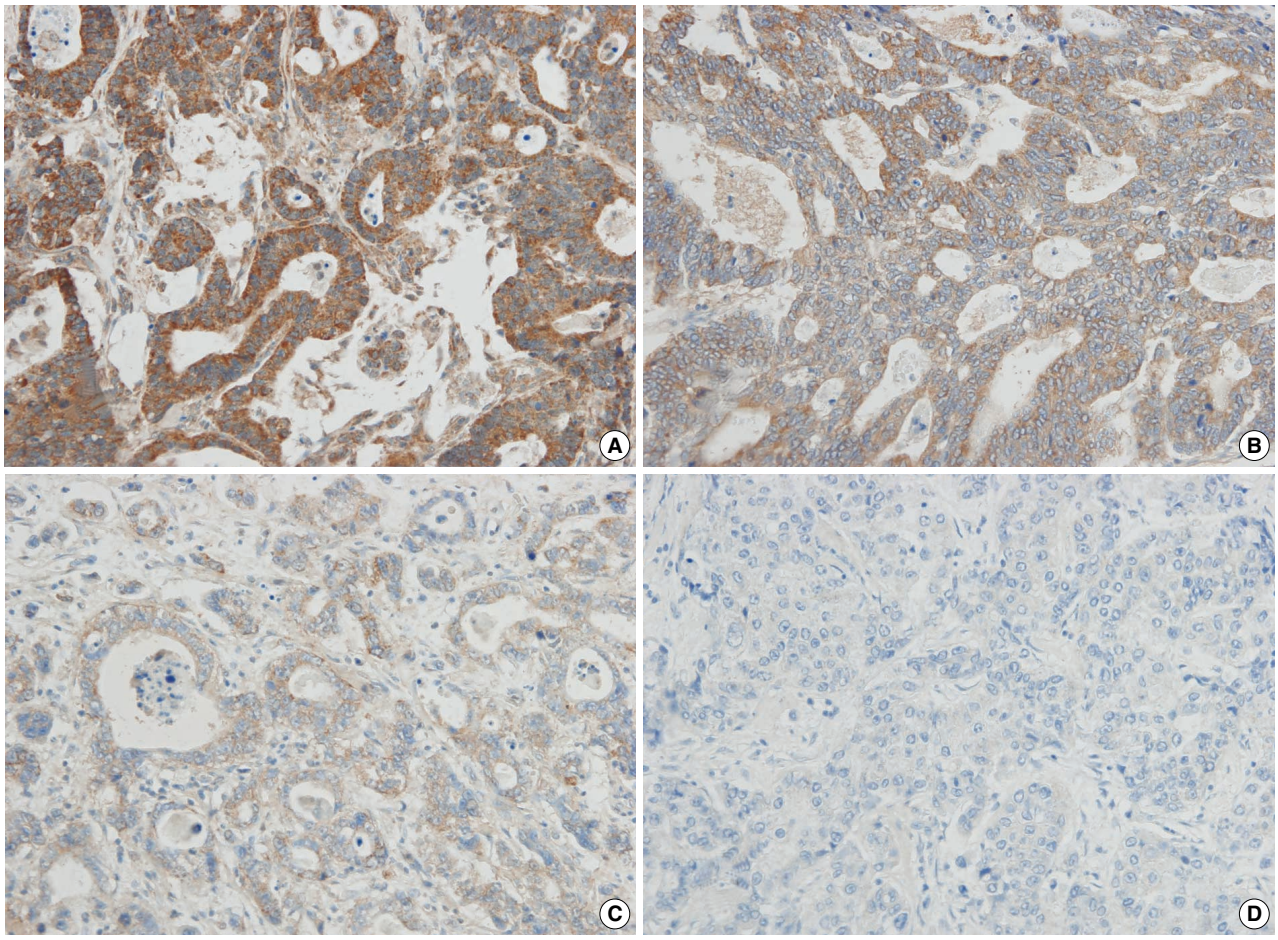

Fig. 2. Assessment of survivin immunoreactivity according to staining intensity. (A) score 3, (B) score 2, (C) score 1, (D) score 0.

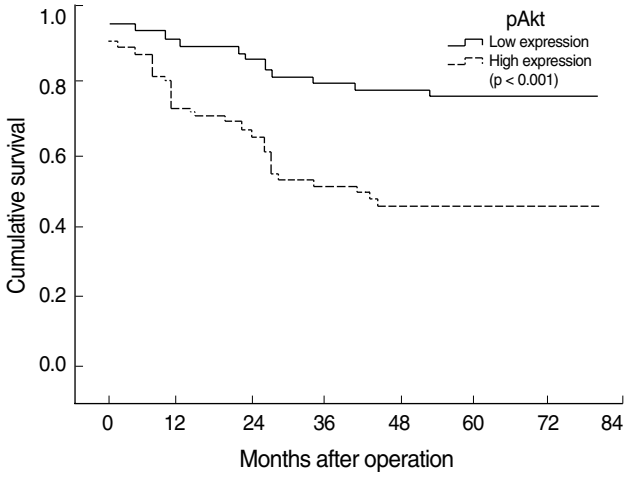

Fig. 3. Kaplan-Meier survival curves correlating disease specific survival with pAkt positive or negative expression.

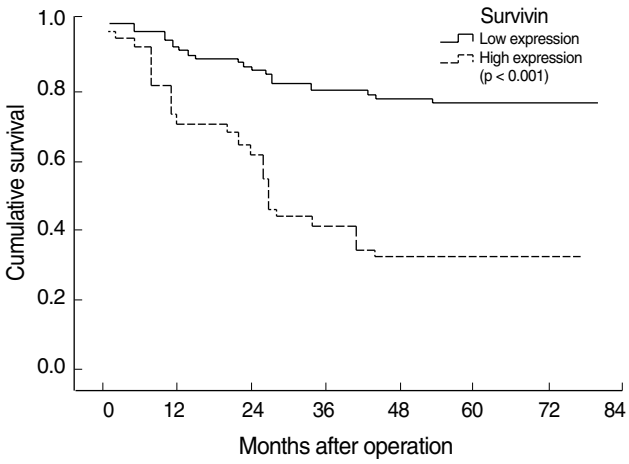

Fig. 4. Kaplan-Meier survival curves correlating disease specific survival with survivin positive or negative expression.

sues but barely detectable in terminally differentiated adult tissues.[20] It is prominently re-expressed in transformed cell lines and human cancers.[17] Furthermore, the expression of survivin in the vast majority of human cancers including gastric cancer is enhanced and associated with a more progressive disease and with unfavorable outcomes.[21-23] It is also involved in the resistance

Table 2. Multivariate analyses of prognostic variables

	Hazard ratio	95% Confidence interval	p-value
pAkt	1.55	0.76-3.19	0.23
Survivin	2.00	1.10-3.64	0.02
Stage			
Stage (1)	9.41	1.12-78.97	0.04
Stage (2)	28.38	3.75-214.68	0.00
Stage (3)	53.25	6.90-410.71	0.00
Lauren classification	0.79	0.42-1.51	0.23
Tumor size	1.02	0.92-1.13	0.78
Age	1.02	0.99-1.06	0.23
Sex	0.76	0.38-1.50	0.42

to chemotherapy observed in certain tumors.[24,25]

The results of multivariate analysis demonstrated a significant potential for survivin expression as an independent prognostic factor. In the current study survivin expression as a prognostic indicator was as potent as stage was. Its usefulness may be strengthened by combined analysis with pAkt expression considering the correlation between them. One of the limitations of our study was our use of immunohistochemical staining to detect expression of pAkt and survivin, which was more qualitative than quantitative. The precise determination of the role of pAkt with quantitative methods and correlation with survivin expression are warranted in future studies.

Survivin has been reported to be a target of Akt in several cell types. Akt was first identified as a positive regulator of survivin expression in endothelial cells,[26] while other studies designated survivin as an anti-apoptotic target responsive to cytokines and other pro-survival factors in leukemia and prostate cancer.[27,28] A requirement of the PI3K-Akt pathway for survivin expression has been shown in myeloma cells, in which apoptosis induced by a specific Akt inhibitor was counteracted by survivin expression via abrogation of NF-kB transcriptional activity.[29]

As stated above, there is strong evidence that survivin is a crucial downstream target of Akt and is likely to be responsible for many of the apoptosis-related consequences of Akt activation. In contrast, blockade of the PI3K/Akt pathway did not affect survivin expression.[30] Given the inconsistent results on the relationship between pAkt and survivin expression, it might be inferred that pAkt has influences on apoptosis by both surivivin-dependent and survivin-independent mechanisms. The results of the present study showed that the greatest prognostic benefits could be obtained with combined pAkt and survivin expression, implying that targeted therapy focusing on the pAkt-survivin pathway would be more effective than that focusing on other possible mechanisms. Nonetheless, it is still unclear how or whether Akt activation is a causal mechanism for increased expression of survivin. Further investigation of the relationship between the PI3K/Akt and survivin pathways is required to fully understand the effects of expression of these apoptosis-related proteins.

The absence of an appropriate adjuvant therapy other than radical gastrectomy remains the main obstacle to improving survival for patients with advanced gastric cancer. Developing an advanced therapeutic approach to a chemoresistant cancer such as gastric cancer poses a major challenge. If overcome, it might lead to significant advances in treatment. Understanding the molecular mechanisms involved in the chemoresistant feature of gastric cancer is critical for a basic understanding of gastric cancer biology, as well as for designing targeted therapies. A better understanding of these details will undoubtedly provide new insights and opportunities for pharmacological intervention in PI3K-Akt-pathway-driven cancers.

In summary, we investigated the expression of activated Akt in association with survivin expression in 144 human gastric cancers using immunohistochemistry. We also showed that the status of pAkt and survivin expression is related to the outcome of patients with gastric cancer and to the clinically important parameters. In this regard, the present study provides some basis for the suggestion that pAkt-survivin signaling pathway is a promising therapeutic target in gastric cancer.

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