

Expression of c-Src and phospho-Src in epithelial ovarian carcinoma

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Abstract Abnormal c-Src expression and activation has been observed in a number of tumors. To determine the therapeutic potential of Src inhibitors for ovarian cancer patients, this study aimed to explore the expression patterns of c-Src and phospho-Src in epithelial ovarian cancer. A total of 82 patients with epithelial ovarian cancer treated at Sun Yat-sen University Cancer Center from January 1999 to December 2005 were enrolled along with 25 patients with benign ovarian lesions; 20 normal ovarian tissues served as controls. Expression of c-Src and phospho-Src (Tyr416) was examined using immunohistochemistry. Survival analyses were performed using Kaplan–Meier curves. As compared to

the control group, a significantly greater proportion of ovarian cancer tissues were positive for c-Src and phospho-Src expression ($P < 0.001$). c-Src expression was associated with age, while phospho-Src expression was significantly associated with age, FIGO stage, histology grade, and residual tumor size after surgery (all $P < 0.05$). The mean survival time was associated with phospho-Src expression, but not with c-Src expression. The mean survival times of patients with phospho-Src-positive tumors were significantly greater than those with phospho-Src-negative tumors (87.4 months, 95 % CI = 74.3–100.5 months and 91.5 months, 95 % CI = 84.7–98.2 months, respectively; $P = 0.013$). The increased c-Src expression and activation in epithelial ovarian cancer suggests that ovarian cancer patients may benefit from tyrosine kinase inhibitors such as Dasatinib. Activation of c-Src through phosphorylation at Tyr416 may play a role in the early stages of ovarian cancer development, and evaluation of its expression may be a useful prognostic marker of epithelial ovarian cancer.

Article note Yong-Wen Huang and Chen Chen contributed equally to this work as co-first authors

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Phospho-Src · Prognosis · Immunohistochemistry ·
Tumor development · Association analysis

Abbreviations

VEGF Vascular endothelial growth factor
DAB Diaminobenzidine tetrahydrochloride
FIGO The International Federation of Gynecology and
Obstetrics

Introduction

The mortality rate of ovarian cancer is among the highest of malignant gynecological tumors; standard treatment

methods include resection, followed by platinum-based chemotherapy. Although the majority of tumors are initially responsive, most patients eventually relapse with a median progression-free survival of 18 months [1]. Therefore, identification of new treatment options along with early identification is crucial for improving the clinical outcomes for these patients. For example, bevacizumab, a neutralizing monoclonal antibody specific for vascular endothelial growth factor (VEGF), increased progression-free survival in ovarian cancer patients [2]. In addition, analysis of the efficacy of small molecule inhibitors of Bcr-Abl and Src family kinases, including dasatinib, are presently underway [3–5].

Upregulation of c-Src, a non-receptor protein tyrosine kinase, in cancer has been widely reported [6–8]. Furthermore, in tumor cells harboring activated forms of Src, phosphorylation of numerous c-Src substrates, which have been linked to tumorigenicity and metastasis, has been reported [9]. Src activity is regulated by tyrosine phosphorylation at two sites with opposing effects. Phosphorylation of tyrosine 416 (Tyr416) in the activation loop of the kinase domain upregulates the c-Src activity, and phosphorylation of tyrosine 527 (Tyr527) in the carboxyl-terminal tail reduces enzyme activity [10]. Although Tyr527 is phosphorylated in normal cells, the phosphorylation status of c-Src has not been determined in ovarian cancer.

To evaluate the possible use of Src kinase inhibitors, such as Dasatinib, for ovarian cancer, the expression of c-Src and its phosphorylation status must be assessed. In this study, the expression of c-Src and phospho-Src (Tyr416) was assessed in ovarian tissue derived from patients with epithelial ovarian cancer, benign ovarian lesions, and normal tissue using immunohistochemistry. Correlation between c-Src and phospho-Src expression and clinicopathological parameters, as well as the prognostic significance of c-Src and phospho-Src expression was evaluated. Determination of phosphorylated c-Src expression in ovarian cancer may form the basis for future studies to evaluate its therapeutic and/or prognostic significance.

Patients and methods

Patients and controls

This study was approved by the Institutional Review Board of Sun Yat-sen University, and all enrolled patients signed written informed consents. A total of 82 patients (mean 47.5 years, range 18–67 years) with epithelial ovarian cancer treated with tumor resection at the Cancer Center of Sun Yat-sen University from January 1999 to December 2005 were enrolled in this study. The inclusion criteria for these patients included the following: (1) pathological

diagnosis and initial treatment at the Cancer Center of Sun Yat-sen University; (2) no adjuvant chemotherapy or radiotherapy prior to the surgery; (3) availability of detailed clinical and follow-up data; and (4) the absence of other tumors. In addition, 25 patients with benign ovarian lesions and 20 patients with normal ovarian tissue were enrolled during the same period as controls (mean 36 years, range 17–80 years). Of the patients with benign lesions, pathological diagnosis revealed 15 cases of simple ovarian cyst, 2 cases of corpus luteum cyst, 2 cases of inclusion cyst, 4 cases of follicular cyst, and 2 cases of chronic inflammation. Normal ovarian tissue was obtained from women following surgery for reasons other than ovarian pathology; the presence of normal ovarian epidermis was confirmed upon pathological examination. FIGO staging was performed as described in Heintz et al. [11].

Follow-up was undertaken by contacting the patients by telephone and was completed by October 15, 2010. The frequencies and treatment regimens used in the ovarian cancer group stratified by c-Src and phospho-Src expression groups were summarized in the supplementary Table.

Immunohistochemistry

Immunohistochemistry analysis was carried out using the PV-9000 kit that enhances sensitivity 3–4-fold (Zhongshan Goldenbridge Biotechnology, Beijing, China). Formalin-fixed, paraffin-embedded archived tissues were cut into 4 μ m sections after which they were dewaxed and rehydrated, and endogenous peroxidase was blocked with hydrogen peroxide. Antigen retrieval was undertaken by boiling the sections in a microwave in 10 mM citrate buffer (pH 6.0). After nonspecific antibody binding was blocked with 5 % goat serum, sections were incubated with a rabbit anti-Src antibody (#2108, Cell Signaling Technology, Danvers, MA, USA; dilution of 1:60) or rabbit anti-phospho-Src (Tyr416) antibody (#2101, Cell Signaling Technology dilution of 1:40) overnight at 4 °C, followed by horseradish peroxidase-labeled secondary antibody (Cell Signaling Technology) for 30 min at room temperature. The sections were developed with diaminobenzidine tetrahydrochloride (DAB, Zhongshan Goldenbridge Biotechnology) and counterstained with hematoxylin. The primary antibody was replaced with PBS in the negative control.

A double-blind method was used to evaluate the staining intensity. Two pathologists independently selected ten fields stochastically under a high power objective, and the percentage of positive cells was determined. The results of each pathologist were averaged. The following nomenclature was used to denote the percentage of positive cells: <5 % was classified as (–), 5–30 % were (+), 31–55 % were (++) , 56–80 % were (+++), and >80 % were

(++++). For statistical analysis, (+) to (++++) were considered positive and (–) was considered negative.

Statistical analysis

Subjects' age and the expression rates of c-Src and phospho-Src were summarized as n (%). Differences in subjects' age and the expression rates of c-Src and Phospho-Src between groups were compared using Pearson's Chi square test or Fisher's exact test. Comparisons of the expression rates of c-Src and phospho-Src with clinical characteristics were performed using non-parametric methods, including Mann–Whitney U or Kruskal–Wallis tests. Furthermore, Log-rank tests were used to examine the association of survival time with clinical characteristics. Cumulative survival rates were analyzed using Kaplan–Meier analysis. All statistical assessments were considered significant when $P < 0.05$. Statistical analyses were performed using PASW 18.0 statistics software (SPSS Inc, Chicago, IL, USA).

Results

c-Src and phospho-Src expression in ovarian cancer

A total of 127 participants, including 45 in the control group and 82 in the ovarian cancer group, were enrolled. A significantly greater percentage of participants in the ovarian cancer group was >40 years as compared to the control group ($P = 0.007$; Table 1). As determined by immunohistochemistry analysis, c-Src and phospho-Src (Tyr416) expression was observed within the cell membrane and the cytoplasm (Fig. 1). As shown in Table 1, significant differences in c-Src and phospho-Src

expression were observed between the groups; a significantly greater proportion of patients in the ovarian cancer group had positive c-Src and phospho-Src expression ($P < 0.001$).

Association of c-Src and phospho-Src expression with clinical characteristics

The association of c-Src and phospho-Src (Tyr416) expression with the clinical characteristics in 82 patients with ovarian cancer was next evaluated. Whereas c-Src expression was significantly associated with increased age ($P = 0.027$), phospho-Src expression was significantly associated with increased age, FIGO stage, histology grade, and residual tumor size after surgery ($P = 0.002$, 0.044, <0.001 , and 0.032, respectively; Table 2).

Association of c-Src and phospho-Src expression with patient survival

During the follow-up period, 23 patients (28 %) died, and the mean survival time during the study period was 95.7 months (95 % CI = 84.9–106.5 months). The estimated 6-month, 1, 3, and 5-year survival rates were 97.6, 92.7, 81.3, and 67.4 %, respectively. The overall survival (OS) was significantly associated with FIGO stage II (HR = 6.92, 95 % CI = 2.03–23.66, $P = 0.002$) and residual tumor size (HR = 5.25, 95 % CI = 2.26–12.23, $P < 0.001$; data not shown).

As shown in Fig. 2, the mean survival time was associated with phospho-Src levels, but not c-Src levels. The mean survival times of patients with c-Src-negative and positive tumors were 61.5 months (95 % CI = 0–125.9 months) and 96.2 months (95 % CI = 85.3–107.1 months), respectively ($P = 0.458$). However, the mean survival times of patients

Table 1 c-Src and phospho-Src expression in control and ovarian cancer tissues ($n = 127$)

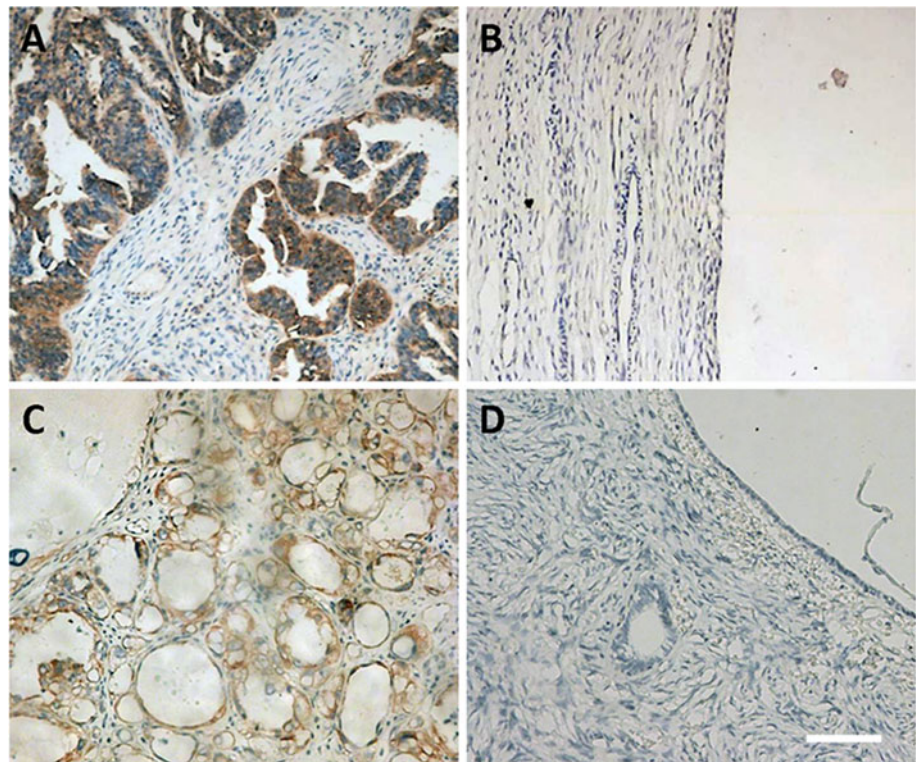
Variables	Total ($n = 127$)	Control ($n = 45$)	Ovarian cancer ($n = 82$)	P value
Age (years)				0.007*
≤40	48 (37.8 %)	35 (53.3 %)	24 (29.3 %)	
>40	79 (62.2 %)	21 (46.7 %)	58 (70.7 %)	
c-Src expression ^a				<0.001*
Positive	39 (30.7 %)	8 (17.8 %)	80 (97.6 %)	
Negative	88 (69.3 %)	37 (82.2 %)	2 (2.4 %)	
Phospho-Src expression ^a				<0.001*
Positive	19 (15 %)	0 (0 %)	19 (23.2 %)	
Negative	108 (85 %)	45 (100 %)	63 (76.8 %)	

Data were summarized as n (%). Differences between the groups were compared using Pearson Chi square test or Fisher's exact test if any cell number was less than five

* Significant difference between groups

^a Positive includes +, ++, +++, and ++++ levels of c-Src and phospho-Src expression

Fig. 1 c-Src and phospho-Src expression in ovarian cancer. c-Src and phospho-Src expression was assessed using immunohistochemistry. Representative results of anti-c-Src staining in ovarian cancer (A) and follicular cyst (B) were shown. Representative results of anti-phospho-Src (Tyr416) staining in ovarian cancer (C) and germinal ovarian epithelium (D) were shown. Scale bar 100 μ m



with phospho-Src-negative tumors were 87.4 months (95 % CI = 74.3–100.5 months) while those with phospho-Src-positive tumors were 91.5 months (95 % CI = 84.7–98.2 months) ($P = 0.013$).

Discussion

Since c-Src is aberrantly expressed and/or activated in a number of tumors [4–6], this study sought to investigate the expression and activation (i.e., phosphorylation at Tyr416) of c-Src in epithelial ovarian cancer. As compared to tissues in the control group, a significantly greater proportion of ovarian cancer tissues expressed c-Src and phospho-Src. Phospho-Src expression was significantly associated with increased age, FIGO stage, histology grade, and residual tumor size after surgery and was associated with the mean survival time. Individuals with phospho-Src-positive tumors had significantly greater mean survival time as compared to those whose tumors were negative.

c-Src is an oncogene that plays a critical role in cell growth and differentiation [10]. Increased expression and activation of c-Src protein has been observed in lung, breast, pancreatic, esophageal, head and neck, and stomach cancers [6–8, 10, 12–15]. In colon cancer, c-Src expression was significantly increased as compared with the surrounding normal tissues, which led to the hypothesis that activation of c-Src was related to colonic carcinogenesis [16, 17]. This hypothesis was

supported by work in an in vivo model in which tumor growth was inhibited through suppressing c-Src expression [18]. In addition, silencing of Src increased docetaxel cytotoxicity and reduced tumor growth and microvessel density in both SKOV3ip1 and HeyA8 cells [18, 19]. Inhibition of Src restored paclitaxel sensitivity to resistant ovarian cancer cells [20], possibly through increasing apoptosis, autophagy, and microtubule stability [21]. In tumors derived from the mouse ovarian cancer cell line, ID8 cells, positive expression of c-Src and phospho-Src (Tyr418) was observed while remaining were absent in the normal ovarian tissues [22]. Furthermore, increased expression and activation (i.e., dephosphorylation at Tyr530) of c-Src in ovarian cancer tissues has been reported [23], which is consistent with the increased c-Src and phospho-Src (Tyr416) expression observed in ovarian cancer patients in the present study.

Although significantly more ovarian cancer tissues were positive for phospho-Src (Tyr416) as compared to control tissues, the positive rate was only 23.2 % in the present study. It is possible that phosphorylation at Tyr416 occurs only at a certain stage of ovarian cancer progression, which is supported by Wiener et al. [23]. Of the nine cases of advanced ovarian cancer, activated c-Src as detected by dephosphorylation at Tyr530 was observed in seven [23]. Thus, activation of c-Src by tyrosine phosphorylation at Tyr416 and dephosphorylation at Tyr527 may occur at different stages of ovarian cancer. Further larger studies will be undertaken to explore this possibility.

Table 2 Association of c-Src and phospho-Src (Tyr416) with the clinical characteristics of ovarian cancer patients ($n = 82$)

Variables	Total ($n = 82$)	c-Src		<i>P</i> value	Phospho-Src		<i>P</i> value
		Negative ($n = 2$)	Positive ^a ($n = 80$)		Negative ($n = 63$)	Positive ^b ($n = 19$)	
Age (years)				0.027*			0.002*
≤ 40	24 (29.3 %)	2 (8.3 %)	22 (91.7 %)		13 (54.2 %)	11 (45.8 %)	
> 40	58 (70.7 %)	0 (0 %)	58 (100 %)		50 (86.2 %)	8 (13.8 %)	
FIGO stage				0.710			0.044*
I	27 (32.9 %)	0 (0 %)	27 (100 %)		17 (63.0 %)	10 (37.0 %)	
II	19 (23.2 %)	1 (5.3 %)	18 (94.7 %)		13 (68.4 %)	6 (31.6 %)	
III	34 (41.5 %)	1 (2.9 %)	33 (97.1 %)		31 (91.2 %)	3 (8.8 %)	
IV	2 (2.4 %)	0 (0 %)	2 (100 %)		2 (100 %)	0 (0 %)	
Pathological type				0.645			0.456
Serous cystadenocarcinoma	45 (54.9 %)	2 (4.4 %)	43 (95.6 %)		35 (77.8 %)	10 (22.2 %)	
Mucous adenocarcinoma	13 (15.39 %)	0 (0 %)	13 (100 %)		8 (61.5 %)	5 (38.5 %)	
Endometrial cancer	2 (2.4 %)	0 (0 %)	2 (100 %)		2 (100 %)	0 (0 %)	
Mixed property cancer	22 (26.8 %)	0 (0 %)	22 (100 %)		18 (81.8 %)	4 (18.2 %)	
Grade of histology				0.399			<0.001*
I	24 (29.3 %)	0 (0 %)	24 (100 %)		10 (41.7 %)	14 (58.3 %)	
II	15 (18.3 %)	0 (0 %)	15 (100 %)		12 (80 %)	3 (20 %)	
III	43 (52.4 %)	2 (4.7 %)	41 (95.3 %)		41 (95.3 %)	2 (4.7 %)	
Size of residual tumor after resection (cm)				0.183			0.032*
≤ 2	69 (84.1 %)	1 (1.4 %)	68 (98.6 %)		50 (72.5 %)	19 (27.5 %)	
> 2	13 (15.9 %)	1 (7.7 %)	12 (92.3 %)		13 (100 %)	0 (0 %)	
Course of chemotherapy (cycles)				0.610			0.070
< 3	21 (25.6 %)	1 (4.8 %)	20 (95.2 %)		15 (71.4 %)	6 (28.6 %)	
3–6	40 (48.8 %)	1 (2.5 %)	39 (97.5 %)		28 (70 %)	12 (30 %)	
> 6 cycles	21 (25.6 %)	0 (0 %)	21 (100 %)		20 (95.2 %)	1 (4.8 %)	

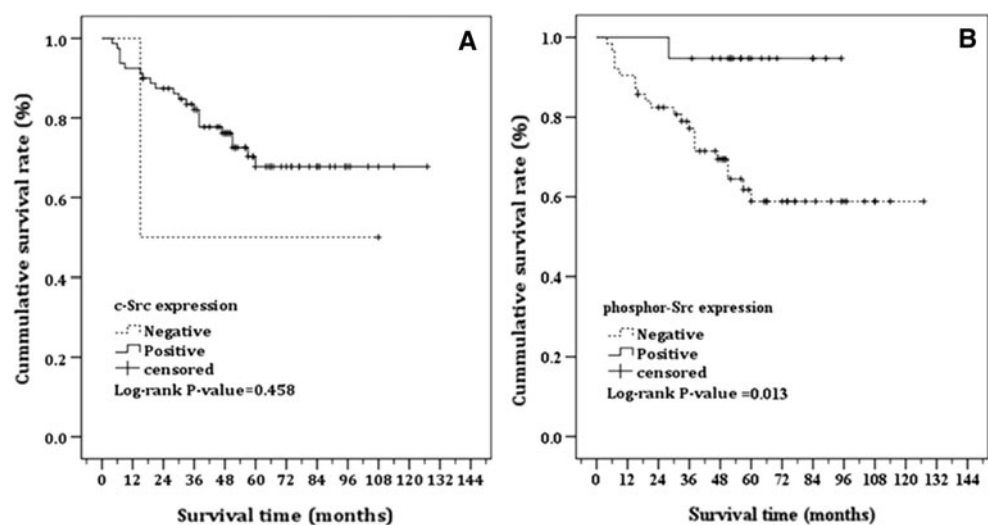
Clinical characteristics were presented as n (%). Comparisons between the groups between clinical characteristics were performed using the non-parametric methods, Mann–Whitney U or Kruskal–Wallis tests

* Significant association

^a Positive includes +, ++, +++, and ++++ levels of c-Src expression

^b Positive includes +, ++, +++, and ++++ levels of phospho-Src expression

Fig. 2 Association of c-Src and phospho-Src expression with patient survival. Patient survival was determined using Kaplan–Meier survival curves by c-Src (A) and phospho-Src (B) expression levels. Differences between expression groups were compared using a Log-rank test



The present study was the first to assess the possible correlation of c-Src and phospho-Src with patient clinicopathological characteristics and evaluate their prognostic significance. The expression of c-Src was not significantly related to pathological type, histology grade, and clinical stage of ovarian cancer; however, its expression was significantly greater in patients >40 years of age. The relevance of c-Src and the clinicopathological characteristics may be underestimated due to the limited number of cases analyzed, requiring further larger studies. Although c-Src was not correlated with most patient characteristics analyzed, phospho-Src (Tyr416) was related to clinical stage, histology grade, and size of residual tumor following surgery. The percentage of early stage tumors positive for phospho-Src expression was greater than that observed for late-stage tumors, suggesting that c-Src phosphorylation at Tyr416 may occur early in ovarian cancer development. In addition, a greater percentage of moderate to well-differentiated tumors expressed phospho-Src (Tyr416). These results are consistent with Weber et al. [24] in which the highest levels of c-Src activity in human colon tumors were observed in moderately-to-well-differentiated tumors. In contrast, Src kinase activation was not observed in the endometrial cancer progression [25]. Although it remains possible that activation of c-Src by Tyr416 phosphorylation may promote cell differentiation as well as cell survival, proliferation, and metastasis [23], the influence of c-Src overexpression and/or activation on ovarian tumor cell growth and differentiation requires further analysis.

In the present study, the 5-year OS rate was higher in patients with phospho-Src-positive tumors as compared to those whose tumors were negative, suggesting that c-Src protein phosphorylation at Tyr416 may be related to better prognosis in ovarian cancer patients. These results are inconsistent with those reported for tongue cancer in which an inverse correlation between phospho-Src (Tyr416) expression and patient prognosis was observed [26]. Although the effects of phospho-Src (Tyr416) expression may be cancer type-specific, further larger studies are necessary to determine the prognostic value of examining phospho-Src (Tyr416) in ovarian cancer.

In summary, activation of c-Src protein through phosphorylation at Tyr416 may play a role in the early stages of ovarian cancer development. Since phospho-Src (Tyr416) expression was related to better survival in ovarian patients, it might represent a new prognostic marker for ovarian cancer. Furthermore, the results of the present study offer a theoretical basis for the use of c-Src inhibitors, including Dasatinib, for treating ovarian cancer.

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Conflicts of interest None.

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