Disease Markers 30 (2011) 325–333
DOI 10.3233/DMA-2011-0792
IOS Press

Crosstalk between activated and inactivated c-Src in hepatocellular carcinoma

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Abstract. C-Src activity is regulated by tyrosine phosphorylation at two distinct sites, Tyr416 and Tyr527, with opposite effects. However, the clinical roles of these sites in human cancers are not well defined. This study aims to determine whether the alterations and crosstalk of these two sites may contribute to hepatocellular carcinoma (HCC). Specimens from 85 patients who had undergone curative hepatectomy were collected for this study. The patterns of p-Tyr416-Src and p-Tyr527-Src, as well as the non-phosphorylated status for each site, were determined using immunohistochemistry and statistically correlated with clinicopathological characteristics and overall survival rate. The active state of c-Src, p-Tyr416-c-Src, was positively correlated with tumour grade ($P = 0.062$) but inversely correlated with vascular invasion ($P = 0.071$). Its non-phosphorylated status, non-p-Tyr416-c-Src, was positively correlated with tumour stage and grade ($P = 0.041$ and 0.020). The inactive state of c-Src, p-Tyr527-c-Src, was decreased in male patients but increased HCV-infected patients ($P = 0.044$ and 0.033). The Kaplan-Meier survival curve further showed that increased p-Tyr416-c-Src and decreased non-p-Tyr527-c-Src expression were associated with a poor patient survival rate ($P = 0.004$ and 0.025). Interestingly, the expression of non-p-Tyr416-c-Src was positively correlated with that of p-Tyr527-c-Src in the HCC lesions ($P = 0.040$). In addition, the patients with concomitantly low p-Tyr416-c-Src and non-p-Tyr527-c-Src expression had a prolonged overall survival rate ($P = 0.030$). A multivariable COX regression model showed that p-Tyr416-c-Src expression was an effective predictor for patient survival in HCC [OR = 3.78, 95%CI = 1.46–9.76; $P = 0.006$]. Our results suggest that the active state of c-Src, p-Tyr416-c-Src, may serve as an independent prognostic marker of patient survival in HCC. Relative levels of other phosphorylated or non-phosphorylated c-Src kinases may also present different statuses during HCC development and require further investigation.

Keywords: Hepatocellular carcinoma, c-Src, phosphorylation status

1. Introduction

C-Src, a cellular homologue of the Rous sarcoma virus-transforming gene (*v-Src*), is associated with malignant transformation [1,2]. This non-receptor type of tyrosine kinase is expressed ubiquitously in a variety of tissues and, when activated, plays important roles in a variety of signalling networks that regulate growth and proliferation. Using an *in vitro* kinase assay, elevated

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ISSN 0278-0240/11/$27.50

c-Src kinase activity has been observed in mammary carcinoma, colorectal carcinoma, rhabdomyosarcoma and neuroblastoma, and a higher kinase activity has been detected in advanced stages of these cancers [3–7]. However, Sakai et al. have demonstrated that an intense staining of activated c-Src was present in 92% of adenomas, in contrast to a weak staining in 25% of adenocarcinomas [8]. Of note, although higher c-Src kinase activity was detected by an *in vitro* kinase assay in hepatocellular carcinoma (HCC) [9] and c-Src activation has been shown to be involved in the cancerous behaviours of HCC cells [10,11], positive staining for the activated c-Src is more frequently observed in well- or moderately differentiated carcinoma [2].

C-Src activity is regulated by tyrosine phosphorylation at two sites, but their phosphorylation has opposite effects. Phosphorylation of Tyr416 in the activation loop of the kinase domain upregulates enzyme activity, whereas phosphorylation of Tyr527 in the carboxy-terminal tail renders the enzyme less active [12]. In general, an intermolecular autophosphorylation mediated by another Src kinase molecule at Tyr416 promotes kinase activity [13]. The catalytic activity of c-Src is suppressed by phosphorylation on the tyrosine residue Tyr527, which is catalysed by c-terminal Src Kinase (Csk) [14]. Down-regulation of Src kinase activity by adenovirus-mediated *Csk* gene transfer abrogates the highly metastatic phenotype of colon cancer cells [15]. Notably, under basal conditions *in vivo*, 90–95% of Src is phosphorylated at Tyr527 [16]. In addition, the Tyr527Phe Src mutant is more active than the wild-type and can induce anchorage-independent growth *in vitro* and *in vivo* [17,18]. Interestingly, in Drosophila epithelia, the levels of Src signalling determine the cellular outcome of Src activation. Apoptotic cell death is specifically triggered at high Src signalling levels; lower levels direct antiapoptotic signals while promoting proliferation. Furthermore, the expression of kinase-dead Src isoforms do not necessarily act as dominant-negative factors, but can instead increase Src pathway activity [19]. These observations imply that the different phosphorylation statuses of c-Src tyrosine kinase may present crosstalk among signalling networks and have potential utility in monitoring cell fate in cancer. However, information regarding the clinical distribution and implication of phosphorylation of these sites in human cancers is currently unavailable.

In the present study, we analysed the distributions of p-Tyr416-Src, non-p-Tyr416-Src, p-Tyr527-Src and non-p-Tyr527-Src in HCC using immunohistochemistry. Furthermore, we analysed their potential interplay/crosstalk and their clinical impact on the prognosis and survival rate of HCC patients. The observations obtained support our speculations that that the different statuses and crosstalk of these sites distinctly contribute to HCC and provide a possibly useful subset of biomarkers in HCC.

2. Materials and methods

2.1. Surgical specimens

Eighty-five patients (67 men and 18 women; mean age 57.07 $\pm$ 11.73, ranging from 30–83 years) with pathologically confirmed HCC who had undergone curative hepatectomy between January 1995 and December 2003 at the Department of Surgery, Kaohsiung Medical University Hospital, were retrospectively collected for this study. The patients were followed for a mean period of 32.1 $\pm$ 2.79 months, ranging from 0.5–111.6 months. The pathological diagnosis and classification of variables were based on the criteria recommended in the General Rules for Clinical and Pathological Study of Primary Liver Cancer [20]. Clinicopathological parameters analysed in this study included gender, histological grade, tumour stage, vascular invasion (including vascular invasion or tumour thrombi in the portal or hepatic vein), cirrhosis, recurrence and viral markers.

2.2. Immunohistochemistry

The detailed protocol for immunohistochemistry was as described previously [21]. In brief, the tissue specimens were fixed with 10% buffered formalin and then dissected, dehydrated and coated with wax. The samples were sliced to a thickness of 4 μm, and then either dyed with hematoxylin and eosin or incubated with the primary antibodies, followed by the Universal LAB+ kit/HRP (Dako) and counterstaining with hematoxylin. The results were captured by a Nikon E-800M microscope and processed by Kodak MGDS330 and Adobe Photoshop 6.0 [21].

Rabbit polyclonal anti-p-Tyr416-Src, anti-non-p-Tyr527-Src, p-Tyr527-Src, and mouse monoclonal anti-non-p-Tyr416-Src were obtained from Cell Signaling Technology. P-Tyr416-Src, non-p-Tyr416-Src, p-Tyr527-Src and non-p-Tyr527-Src were predominantly located in the cytoplasm of cancerous lesions. The intensity of p-Tyr416-Src, non-p-Tyr416-Src, p-Tyr527-Src and non-p-Tyr527-Src immunostaining in HCC le-

Table 1
Clinicopathological characteristics of HCC patients

Characteristics	N	Patients (%)
Tumor stage*		
I	19	22.9
II	42	50.6
III	18	21.7
IV	4	4.8
Histological grade		
I	15	17.6
II	58	68.2
III	12	14.1
Vascular invasion		
Present	26	30.6
Absent	59	69.4
Cirrhosis		
Present	40	52.9
Absent	45	47.1
Recurrence		
Present	34	40.0
Absent	51	60.0
Virus marker		
HBV(+)/HCV(-)	44	51.8
HBV(-)/HCV(+)	23	27.1
HBV(+)/HCV(+)	9	10.6
HBV(-)/HCV(-)	9	10.6

NOTE. *: Undetermined in small cases. HCV, anti-hepatitis C virus antibody; HBV, hepatitis B surface antigen; (+), positive or present; (-), negative or absent.

Table 2
Summary of p-tyr416-Src, non-p-tyr416-Src, p-tyr547-Src, and non-p-tyr527-Src in HCC

Characteristics	N	Patients (%)
P-Tyr416-Src*		
Low	31	38.3
Moderate	41	50.6
High	9	11.1
Non-p-Tyr416-Src*		
Low	7	8.4
Moderate	43	51.8
High	33	39.8
P-Tyr527-Src*		
Low	7	8.3
Moderate	63	75.0
High	14	16.7
Non-p-Tyr527-Src*		
Low	20	26.3
Moderate	35	46.1
High	21	27.6

NOTE. *: Undetermined in small cases.

sions was graded by a semi-quantitative system: –/+, low; ++, moderate; +++, high. More than 1000 cells expressed in 3 to 4 different high-power fields (x200) were analysed for each section. The immunostaining for p-Tyr416-Src, non-p-Tyr416-Src, p-Tyr527-Src and non-p-Tyr527-Src were determined separately for each specimen, as estimated by two independent pathologists. The rare cases with discordant scores were re-evaluated and scored on the basis of a consensus opinion [22].

2.3. Statistical analysis

Statistical analyses were performed using the SPSS 10.0 statistical package for PC (SPSS, Inc., Chicago, IL). Groups of patients with different p-Tyr416-Src, non-p-Tyr416-Src, p-Tyr527-Src and non-p-Tyr527-Src expression levels were correlated with gender, histological grade, tumour stage, vascular invasion, cirrhosis, recurrence and viral markers using Spearman's rho coefficient analysis and Fisher's exact test. Survival curves were calculated using the Kaplan-Meier method, and the significance was determined by log rank test. Multivariate OR was calculated using Cox proportional hazards regression. A value of $P \leqslant 0.05$ was considered significant [21,23,24].

3. Results

3.1. Expression profiles of p-Src in HCC lesions

The clinicopathological characteristics of HCC patients are shown in Table 1. The phosphorylated c-Src proteins, p-Tyr416-c-Src (activated c-Src) and p-Tyr527-c-Src (inactivated, were predominantly present in the cytoplasm of HCC lesions and showed a diffuse staining pattern (Fig. 1). The non-phosphorylated c-Src proteins, non-p-Tyr416-c-Src and non-p-Tyr527-c-Src, were predominantly present in the cytoplasm of HCC lesions (Fig. 1). Notably, the staining of non-phosphorylated c-Src was predominantly membrane-associated (Fig. 1). The phosphorylation status of c-Src proteins in HCC lesions is summarised in Table 2.

3.2. Correlation of p-Src expression with clinicopathological parameters in HCC

To further explore their clinical roles in HCC, statistical analyses were performed to examine the correlation of these phosphorylation patterns with clinicopathological characteristics (Table 3). The expression of p-Tyr416-c-Src (activated c-Src) was positively correlated with tumour grade ($P = 0.062$) but inversely correlated with vascular invasion ($P = 0.071$), although though these correlations did not reach significance. Intriguingly, the expression of non-p-Tyr416-c-Src was

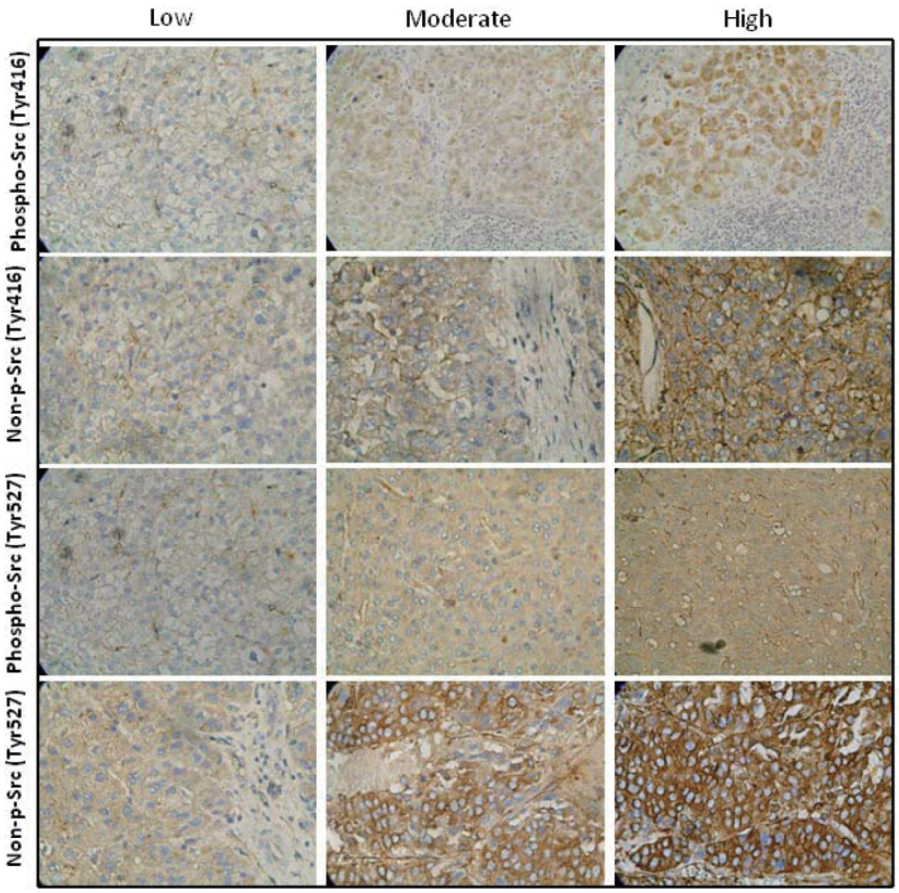

Fig. 1. Scoring systems for p-Tyr416-c-Src, non-p-Tyr416-c-Src, p-Tyr527-c-Src and non-p-Tyr527-c-Src immunostaining in HCC lesions. The cytoplasmic intensity of p-Tyr416-c-Src, non-p-Tyr416-c-Src, p-Tyr527-c-Src and non-p-Tyr527-c-Src in HCC lesions was graded by a semi-quantitative system: low expression (negative and weak staining), moderate expression (moderate staining) and high expression (strong staining). More than 1000 cells expressed in 3–4 different high-power fields (x200) were analysed for each section.

positively correlated with tumour stage and grade in HCC patients ($P = 0.041$ and 0.020). No significant correlation of p-Tyr527-c-Src (inactivated c-Src) and non-p-Tyr527-c-Src with other patient characteristics was present. For unknown reasons, the expression of p-Tyr527-c-Src (inactivated c-Src) was decreased in the lesions of male HCC patients ($P = 0.044$). Additionally, the expression of p-Tyr527-c-Src (inactivated c-Src) was significantly elevated in the lesions of HCV-infected HCC patients ($P = 0.033$).

3.3. *Correlation of p-Src expression with the overall survival of HCC patients*

The associations of the different phosphorylation patterns with survival rate were further analysed using a Kaplan-Meier survival curve. Increased p-Tyr416-c-Src (activated c-Src) expression and decreased non-p-Tyr527-c-Src expression were associated with poor patient survival (Fig. 2A and D, $P = 0.004$ and 0.025). No significant association was observed in the expression subgroups of non-p-Tyr416-c-Src and p-Tyr527-c-Src (inactivated c-Src) in HCC (Fig. 2B and C). A multivariable COX regression model showed that p-Tyr416-c-Src (activated c-Src) expression was an effective determinant for patient survival [OR = 3.78, 95%CI = 1.46–9.76; $P = 0.006$] adjusted by gender, tumour stage, histological grade, vascular invasion, cirrhosis, recurrence, viral marker, and the other statuses of c-Src proteins. Notably, tumour stage [OR = 3.65, 95%CI = 1.60–8.32; $P = 0.002$] and recurrence [OR = 4.78, 95%CI = 1.43–15.71; $P = 0.011$] were two additional significant predictors for patient survival in our cases (Table 4).

Interestingly, the expression of non-p-Tyr416-c-Src was positively correlated with that of p-Tyr527-c-Src

Table 3
Correlation of p-src expression with clinicopathological characteristics

	P-Tyr416-Src				Non-p-Tyr416-Src				P-Tyr527-Src				Non-p-Tyr527-Src			
	Low	Moderate	High	p	Low	Moderate	High	p	Low	Moderate	High	p	Low	Moderate	High	p
Gender				0.958				0.930				0.044				0.291
Male	26	30	9		7	32	27		6	53	8		17	28	15	
Female	5	11	0		0	11	6		1	10	6		3	7	6	
Tumor stage				0.831				0.041				0.483				0.215
I	6	10	2		4	10	4		2	14	2		3	9	3	
II	16	18	5		1	23	17		2	34	6		12	18	9	
III	6	11	1		2	8	8		2	10	6		3	7	6	
IV	2	1	1		0	1	3		1	3	0		1	0	3	
Histological grade				0.062				0.020				0.483				0.992
I	5	6	1		1	11	2		0	12	3		1	8	2	
II	25	27	5		5	29	23		7	40	10		16	23	15	
III	1	8	3		1	3	8		0	11	1		3	4	4	
Vascular invasion				0.071				0.110				0.167				0.669
Present	6	15	4		1	11	13		2	17	7		8	8	7	
Absent	25	26	5		6	32	20		5	46	7		12	27	14	
Cirrhosis				0.203				0.251				0.232				0.887
Present	17	21	2		5	21	14		3	28	9		9	18	10	
Absent	14	20	7		2	22	19		4	35	5		11	17	11	
Recurrence				0.659				0.497				0.676				0.853
Present	12	18	4		4	14	16		2	28	4		8	14	9	
Absent	19	23	5		3	29	17		5	35	10		12	21	12	
Virus marker				0.364				0.819				0.033				0.695
HBV(+)/HCV(-)	19	20	4		2	21	20		4	35	4		9	21	10	
HBV(-)/HCV(+)	5	11	4		1	12	9		1	13	9		5	6	8	
HBV(+)/HCV(+)	4	4	1		2	5	2		1	7	1		4	4	1	
HBV(-)/HCV(-)	3	6	0		2	5	2		1	8	0		2	4	2	

NOTE. Data are determined by Spearmanfls coefficient test. *: Undetermined in small cases. Abbreviations: HCV, anti-hepatitis C virus antibody; HBV, hepatitis B surface antigen; (+), positive or present; (-), negative or absent.

Table 4
Cox regression multivariate analysis of overall survival for HCC cases

Variables	OR	95% CI	P
Gender (Female/Male)	2.38	0.62–9.12	0.207
Tumor stage (I, II, III, IV)	3.82	1.64–8.88	0.002
Histological grade (I, II, III)	0.59	0.19–1.77	0.334
Vascular invasion (Absent and Present)	1.60	0.51–5.05	0.423
Cirrhosis (Absent and Present)	2.51	0.76–8.31	0.131
Recurrence (Absent and Present)	4.98	1.56–15.96	0.007
Viral marker (-/-, +/-, -/+, +/+)	1.03	0.57–1.88	0.923
P-Tyr416-Src (low, moderate and high)	5.23	1.72–15.88	0.004
Non-p-Tyr416-Src (low, moderate and high)	0.68	0.28–1.63	0.384
P-Tyr527-Src (low, moderate and high)	1.99	0.69–5.71	0.201
Non-p-Tyr527-Src (low, moderate and high)	0.56	0.28–1.12	0.104

NOTE. Data are determined by Cox proportional hazards regression. OR: Odds ratio. CI: Confidence interval. Abbreviations: -/-, HBV(-)/HCV(-). +/-, HBV(+)/HCV(-). -/+, HBV(-)/HCV(+). +/+, HBV(+)/HCV(+). HCV, anti-hepatitis C virus antibody; HBV, hepatitis B surface antigen; (+), positive or present; (-), negative or absent.

(inactivated c-Src) in the HCC lesions (Table 5, $P = 0.040$). However, no direct correlation among these phosphorylated c-Src proteins and non-phosphorylated c-Src proteins was present (Table 5). Furthermore, decreased p-Tyr416-c-Src expression was associated with a poor survival rate in the high non-p-Tyr416-c-Src expression group (Fig. 3A, $P = 0.0001$). In addition, increased p-Tyr416-c-Src (activated c-Src) expression was associated with a poor overall survival rate in the moderate p-Tyr527-c-Src (inactivated c-Src) expression group (Fig. 3B, $P = 0.0021$). The patients with low p-Tyr416-c-Src (activated c-Src) and non-p-Tyr527-c-Src expression had a better overall survival rate (Fig. 3C, $P = 0.030$). Intriguingly, HBV-infected

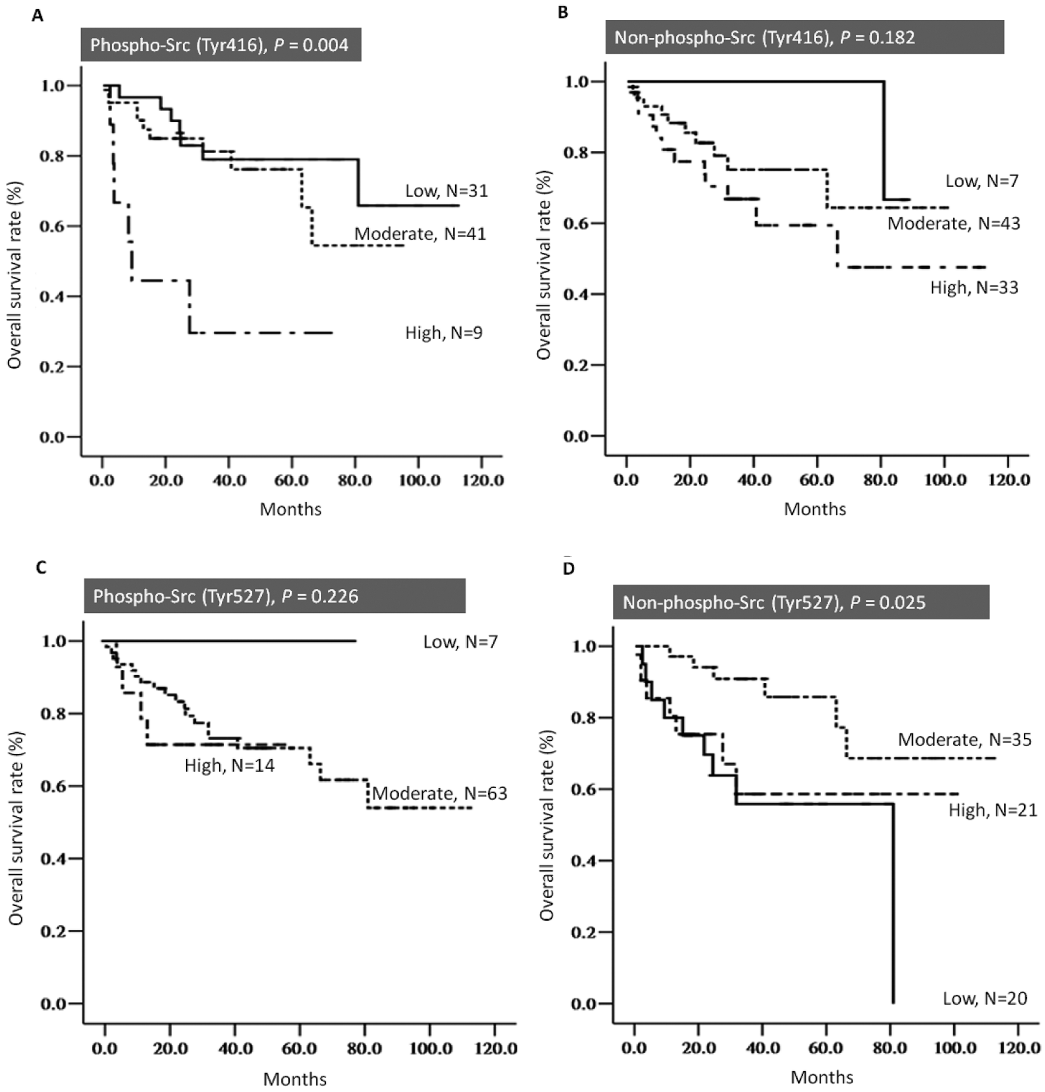

Fig. 2. The Kaplan-Meier survival curve of the HCC patients in the different expression groups: low, moderate and high cytoplasmic intensity of (A) p-Tyr416-c-Src (activated c-Src), (B) non-p-Tyr416-c-Src, (C) p-Tyr527-c-Src (inactivated c-Src) and (D) non-p-Tyr527-c-Src. The significance was determined by log rank test.

patients with either high p-Tyr416-c-Src (activated c-Src) or high p-Tyr527-c-Src (inactivated c-Src) expression had a poor overall survival rate (Fig. 3D and E, $P = 0.002$ and 0.002).

4. Discussion

The non-receptor-type tyrosine kinase c-Src has been associated with human malignancies and is currently being evaluated as a therapeutic target. However, little is known about the distribution and implications of c-Src phosphorylation status in HCC, although they are closely correlated with the protein's activation and/or inactivation state. Increased p-Tyr416-c-Src expression was associated with a poor patient survival using

Table 5
Correlation among p-src expression in HCC

	Variables	r	p
P-Tyr416-Src	vs. Non-p-Tyr416-Src	0.150	0.181
	P-Tyr527-Src	0.174	0.120
	Non-p-Tyr527-Src	0.064	0.585
Non-p-Tyr416-Src	vs. P-Tyr527-Src	0.226	0.040
	Non-p-Tyr527-Src	0.064	0.586
P-Tyr527-Src	vs. Non-p-Tyr527-Src	0.141	0.224

NOTE. Data are determined by Spearmanfls coefficient test.

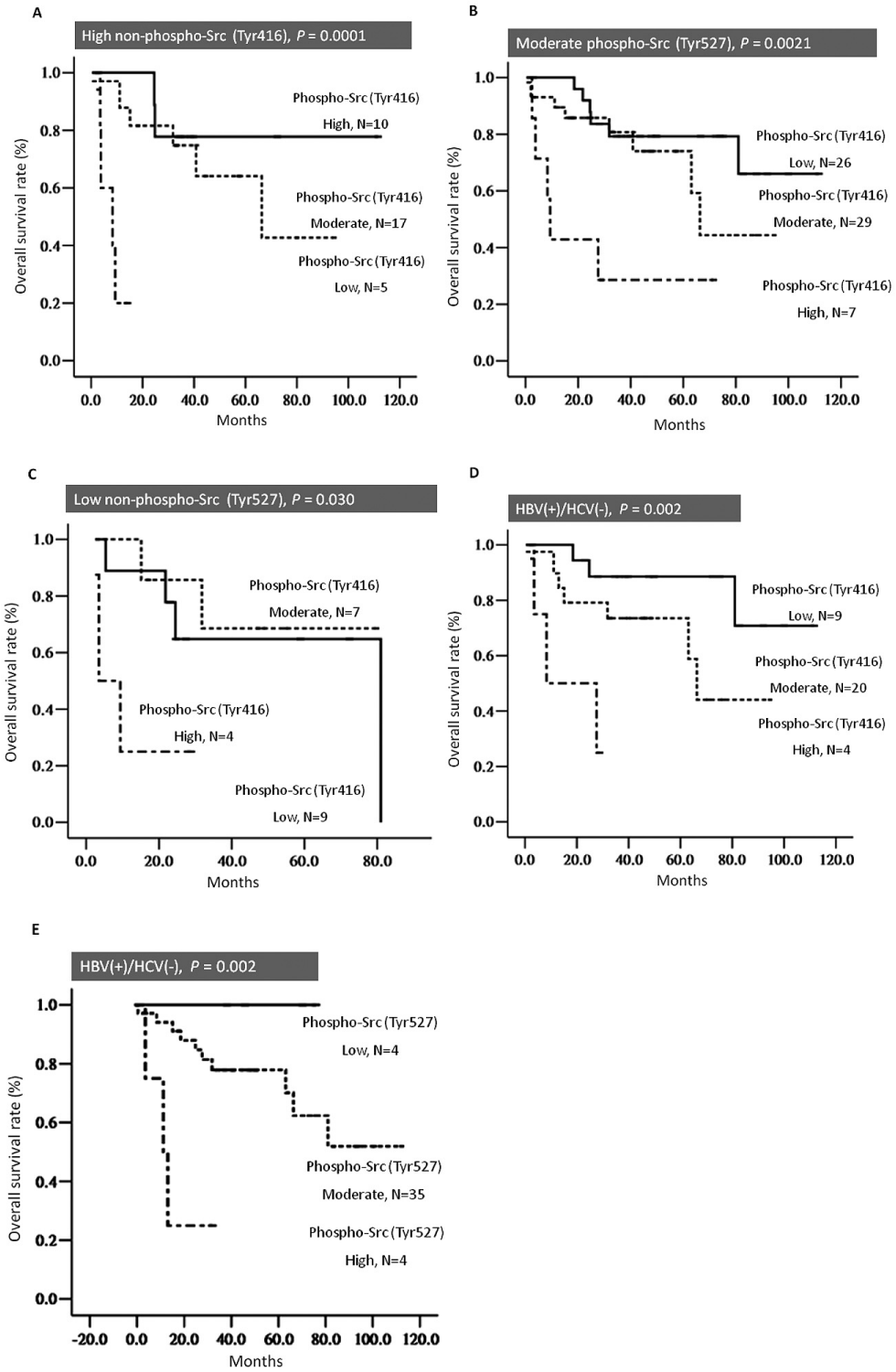

Fig. 3. The Kaplan-Meier survival curve of the HCC patients with low, moderate and high p-Tyr416-c-Src intensity in the (A) high non-p-Tyr416-c-Src expression group, (B) high p-Tyr527-c-Src expression group, (C) low p-Tyr527-c-Src expression group, and (D) HBV-infected patients. (E) *Kaplan-Meier* survival curve of the patients with low, moderate and high p-Tyr527-c-Src intensity in HBV-infected patients. The significance was determined by log rank test.

multivariable COX regression model in our cases, suggesting that p-Tyr416-c-Src expression is an effective predictor for patient survival. Of note, increased p-Tyr416-c-Src expression was also significantly associated with disease-free survival ($p < 0.001$), whereas other phosphorylated and non-phosphorylated forms of c-Src were not significantly associated with disease-free survival. It is well-known that the phosphorylation of Tyr416 in the activation loop of the kinase domain upregulates its enzyme activity [12,13]. Thus, an increased p-Tyr416-c-Src expression may assist further HCC development and is thus associated with poor patient survival. Intriguingly, HBV-infected, but not HCV-infected, patients with high p-Tyr416-c-Src expression had a poor overall survival rate. c-Src has been demonstrated to be involved in the replication of hepatitis B virus, and the hepatitis B virus HBx protein can further activate the c-Src kinase [25]. Thus, a positive loop may exist among HBx protein-Src kinase-hepatitis B virus replication, and HBV-infection may result in constitutive activation of c-Src, leading to the progressive development of HCC. Further investigations are required to address these possibilities.

The expression of non-p-Tyr416-c-Src was positively correlated with that of p-Tyr527-c-Src in the HCC lesions. The catalytic activity of c-Src has been noted to be suppressed by phosphorylation on the Tyr527 residue [13,14], providing the rationale for their correlation. However, the expression of p-Tyr527-c-Src was not directly correlated with any patient characteristic. This may be explained by the observation that under basal conditions *in vivo*, 90–95% of c-Src is phosphorylated at Tyr527 [26], and therefore, we could not find any significant correlation to distinguish the contribution of p-Tyr527-c-Src. For unknown reasons, the expression of p-Tyr527-c-Src (inactivated c-Src) was decreased in the lesions of male HCC patients. Additionally, the expression of p-Tyr527-c-Src was significantly elevated in the lesions of HCV-infected HCC patients. The mechanisms underlying these expression patterns require further investigation. In addition, decreased non-p-Tyr527-c-Src expression was independently associated with poor patient survival. Furthermore, the patients with concomitantly low p-Tyr416-c-Src and non-p-Tyr527-c-Src expression had a better overall survival rate. These results suggest that decreased non-p-Tyr527-c-Src may reflect an increase in activated c-Src and thus contribute to patient survival. Indeed, we found a significant association between increased p-Tyr416-c-Src (activated c-Src) expression and a poor survival rate.

In Drosophila epithelia *in situ*, apoptosis is specifically triggered at high c-Src signalling levels; lower levels direct antiapoptotic signals while promoting proliferation, suggesting that levels of c-Src activation or phosphorylation statuses may determine the cellular outcome [19] We consistently observed that decreased p-Tyr416-c-Src expression was associated with a poor survival rate in the high non-p-Tyr416-c-Src expression group, and the patients with an increased p-Tyr416-c-Src expression had a poor outcome in the moderate p-Tyr527-c-Src expression group. Sakai et al. have demonstrated that a weak staining of activated c-Src is present in adenocarcinoma [8] and that activated c-Src is more frequently observed in well- or moderately differentiated carcinoma [2]. In addition, hepatocyte growth factor (HGF) activates c-Src and reduces spontaneous migration and specific chemoinvasion towards CXCL12 in highly invasive and metastatic MDA-MB231 cells [27]. In the present study, the expression of non-p-Tyr416-c-Src was positively correlated with tumour stage and grade in HCC patients, suggesting that activated c-Src, p-Tyr416-c-Src, may have additional benefits in disrupting the further development of HCC. Notably, p-Tyr416-c-Src (activated c-Src) expression was inversely correlated with vascular invasion in our cases. The contribution of activated c-Src to cell fate even in cancer cells may depend on disease staging, differentiation state or tissue types.

In the present study, we showed, for the first time, the distribution and clinical impact of the different phosphorylated and/or non-phosphorylated status of two c-Src residues in HCC. Our results suggest that these statuses may present crosstalk among signalling networks and have potential utility in monitoring cell fate in HCC. Most importantly, the expression of p-Tyr416-c-Src may serve as an independent determinant of patient survival. Strikingly, non-phosphorylated c-Src, including non-p-Tyr416-c-Src and non-p-Tyr527-c-Src, as well as inactivated c-Src, p-Tyr527-c-Src, cannot be generally regarded as functionally dead c-Src proteins in HCC. Further efforts are needed to address the possibility that non-phosphorylated c-Src or inactivated c-Src may promote oncogenic signalling or behaviours in HCC.

Acknowledgment

This study was partly supported by NSC grants (NSC 97-2320-B-242-001-MY3 to YT Yeh and 96-2314-B-037-006-MY3 to SN Wang).

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