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pp60$^{c\text{-}src}$ activation in lung adenocarcinoma

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Abstract

Nine src family members are known including c-Src, c-Yes, c-Lck, c-Fyn, c-Hck, c-Lyn, c-Blk, c-Fgr and c-Yrk. They encode proteins with molecular weights of 55–62 kilodaltons (kDa), which are either cytoplasmic or membrane-associated protein tyrosine kinases. A close correlation exists between an elevated pp60$^{c\text{-}src}$ tyrosine kinase activity and cell transformation. However, the level of activation of pp60$^{c\text{-}src}$ in non-small cell lung cancers (NSCLC) remains obscure. The aim of this study was to examine the level of activity of pp60$^{c\text{-}src}$ in NSCLC. pp60$^{c\text{-}src}$ expression and *in vitro* protein tyrosine kinase activity in lung cancer tissue samples were measured by western blotting and *in vitro* kinase assays and compared with those in the surrounding non-tumour lung tissue from the same patient. pp60$^{c\text{-}src}$ phosphorylation was assessed by two-dimensional tryptic phosphopeptide mapping. The kinase activity of pp60$^{c\text{-}src}$ was significantly activated in NSCLC, especially in adenocarcinomas. In addition, the pp60$^{c\text{-}src}$ kinase activity increased with the size of the adenocarcinoma. Two-dimensional tryptic phosphopeptide mapping showed dephosphorylation of pp60$^{c\text{-}src}$ at Tyr 530 in adenocarcinomas. The proto-oncogene product, pp60$^{c\text{-}src}$, was activated in NSCLC, especially in adenocarcinomas, in part through the dephosphorylation of Tyr 530. Our results suggest that activation of pp60$^{c\text{-}src}$ might play an important role in the progression of lung adenocarcinomas.

Keywords: Src; Kinase activity; Lung cancer

1. Introduction

A large number of cellular protein tyrosine kinase (PTK) genes have been cloned and sequenced. These kinases are classified into two major groups; the first comprises growth factor receptor tyrosine kinases, while the second includes retroviral PTKs and their cellular homologues [1]. The main representatives of the latter group are non-receptor-linked and membrane-associated src-related tyrosine kinases [2]. To date, at least nine src-related tyrosine kinases have been identified. These include the c-Src, c-Yes, c-Lck, c-Fyn, c-Hck, c-Lyn, c-Blk, c-Yrk and c-Fgr proto-oncogene products [3]. All members of the src-family have a molecular mass ranging from 55 to 62 kilodaltons (kDa) and myristoylated glycine residues at the amino terminus [4]. Among these PTKs, the proto-oncogene pp60$^{c\text{-}src}$ is the cellular homologue of the Rous sarcoma transforming gene, *v-src* [5]. Both c-Src and v-Src encode 60 kDa, membrane-associated PTKs. pp60$^{c\text{-}src}$ functions in signalling pathways that regulate diverse cellular functions including proliferation, migration, cytoskeletal organisation, and cellular survival by interacting with and/or phosphorylating specific substrates [6].

The retroviral form of Src (v-Src) has been intensively studied with respect to its ability to induce the malignant transformation of mammalian cells in tissue culture and to induce metastatic tumours in animals [7]. In

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addition, in animal models, considerable evidence has implicated the activation of pp60$^{c\text{-}src}$ as being involved in tumorgenicity. For instance, the association of pp60$^{c\text{-}src}$ with the polyoma middle T antigen leads to increased specific activity of pp60$^{c\text{-}src}$, and is required for the malignant transformation by polyoma virus [8]. Increased pp60$^{c\text{-}src}$ expression and/or its activity have been observed in a number of human cancers [9–16] including our previous reports. The expression of pp60$^{c\text{-}src}$ protein was found to be elevated in half of all lung cancers by western blotting and immunohistochemical studies [9]. However, no analysis of pp60$^{c\text{-}src}$ kinase activity was reported in these studies. Somewhat contrasting results were reported in a study in which 60 human cell lines used by the National Cancer Institute for the random screening of potential anticancer drugs were analysed for pp60$^{c\text{-}src}$ kinase activity [10]. In this study, small cell lung cancer-derived cell lines had a low activity, whereas non-small cell lung carcinoma (NSCLC) cells exhibited activity that was greater than that observed in colon cancer cells, which are considered to have a high pp60$^{c\text{-}src}$ activity. However, the role of pp60$^{c\text{-}src}$ kinase activity in human tissues with lung cancer has not been investigated. We report in this study the level of pp60$^{c\text{-}src}$ kinase activity in human lung cancers.

2. Patients and methods

2.1. Patients and specimens

Tumour and resected margin samples were obtained by surgery from 30 patients (24 males and 6 females, mean age ± standard deviation (S.D.) 62.2 ± 10.0 years; median 63.0 years; range 41–80 years). The clinical backgrounds and characteristics of the patients are shown in Table 1. None of the patients had received chemo- or radiation therapy before surgery. 12 of the patients were in stage I, 6 in stage II, 9 in stage IIIA, 1 in stage IIIB and 2 in stage IV according to the International Union Against Cancer (UICC) classification. Adenocarcinomas and squamous cell carcinomas were present in 18 and 12 cases, respectively, according to the

Table 1
Clinical backgrounds and characteristics of the patients[a]

Case	Age (years)	Gender	Histology	Tumour size (cm)	Degree of differentiation	Clinical stage	T/N ratio of c-Src protein	T/N ratio of kinase activity	
								Enolase phosphorylation	c-Src phosphorylation
1	41	M	Ad	7	Poor	I	2.9	1.9	10.3
2	73	M	Ad	7	Poor	IIIA	4.6	1.8	6.1
3	69	M	Ad	5	Mod	IIIA	4.9	2.4	8.6
4	51	M	Ad	5	Poor	IIIA	6.2	2.2	7.8
5	63	M	Ad	5	Poor	IIIB	4.2	1.8	4.8
6	54	M	Ad	4	Mod	I	1.2	1.9	6.2
7	55	F	Ad	4	Mod	II	2.0	1.6	3.9
8	63	M	Ad	4	Mod	II	3.9	1.7	4.6
9	47	F	Ad	3.5	Mod	IV	4.3	1.4	4.3
10	49	M	Ad	3	Mod	IV	5.2	1.9	5.4
11	65	M	Ad	3	Well	IIIA	3.6	1.9	8.9
12	56	F	Ad	3	Poor	IIIA	2.9	1.4	2.9
13	66	M	Ad	3	Poor	IIIA	3.6	1.7	3.1
14	42	M	Ad	2.5	Well	IIIA	1.5	1.3	2.6
15	76	F	Ad	2.5	Well	I	1.8	1.3	2.1
16	72	M	Ad	2	Mod	II	0.8	1.2	1.5
17	62	M	Ad	2	Mod	II	1.0	1.2	1.8
18	59	F	Ad	1.5	Well	I	0.7	0.7	0.7
19	80	M	Sq	6	Poor	II	1.6	1.1	1.4
20	54	M	Sq	6	Poor	IIIA	1.3	1.2	1.9
21	62	M	Sq	6	Mod	I	2.1	1.2	1.9
22	59	M	Sq	6	Mod	I	1.6	1.3	1.3
23	78	M	Sq	4	Mod	I	1.2	1.4	2.3
24	62	M	Sq	4	Poor	IIIA	1.8	1.3	2.1
25	65	M	Sq	4	Poor	II	1.5	1.4	1.8
26	65	M	Sq	3	Mod	I	1.7	1.1	1.6
27	69	M	Sq	2	Mod	I	1.2	0.8	0.7
28	68	F	Sq	2	Mod	I	1.4	0.7	0.9
29	72	M	Sq	2	Mod	I	1.2	1.0	1.2
30	70	M	Sq	1.5	Mod	I	1.0	1.1	1.4

Male, male; F, female; Ad, adenocarcinoma; Sq, squamous cell carcinoma; T/N, tumour/non-tumour tissue; Mod, moderate.
[a] Clinical stages are classified using the UICC TNM classification system

standard World Health Organization (WHO) criteria. In each case, the resected lung tissue was divided visually into tumour (T) and non-tumour (N) regions, which were then histologically confirmed. Tissues were frozen immediately at −80 °C until they were used in the experiments.

2.2. Chemicals

The monoclonal antibody against pp60$^{c\text{-}src}$ (mAb 327) was purchased from Oncogene Science Co., Ltd. (Tokyo, Japan). This antibody reacts with pp60$^{c\text{-}src}$ of mouse, rat and human origins in western blotting and immunoprecipitation analysis, but is not cross-reactive with other src family tyrosine kinases. Other chemicals were purchased from the Sigma Chemical Co. (Tokyo, Japan) or the Wako Pure Chemical Co. (Tokyo, Japan).

2.3. Tissue lysates

The tissue samples were frozen on dry ice within 20 min of collection. The samples were homogenised in 10 mM Tris–HCl (pH 7.5), 1 mM ethlene glycol-bis [β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid (EGTA), 150 mM NaCl, 1 mM Na_3VO_4, 50 mM Na_2MoO_4, 1% Nonidet P-40 and 100 U/ml aprotinin (TNE buffer) centrifuged at 29 000*g* for 15 min at 4 °C. The protein concentration in tissue lysates was measured by the dye binding protein assay [15].

2.4. Gel electrophoresis and western blot

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli [16]. Western blotting was performed according to the method of Towbin and colleagues [17] using a mouse monoclonal antibody to pp60$^{c\text{-}src}$ (Oncogene Laboratory) in a 1:400 dilution, and a horseradish peroxidase-linked secondary antibody in a 1:1000 dilution. Immunoreactive proteins were visualised with an enhanced chemiluminescence detection system (Amersham) on X-ray film.

2.5. Immune complex protein and tyrosine kinase assay for pp60$^{c\text{-}src}$

Aliquots of lysate containing 200 μg of cellular protein were incubated with a 2:1 (vol:vol) ratio of ascites fluid containing a monoclonal antibody (mAb), 327, specific for pp60$^{c\text{-}src}$ [18], and then with 5 μg of affinity-purified rabbit anti-mouse immunoglobulin G as previously described in Ref. [19]. After the immunoprecipitates were adsorbed to immunoglobulin G, they were washed five times with TNE buffer and twice with the kinase assay buffer (50 mM Tris–HCl [pH 7.4], 3 mM $MnCl_2$ and 0.1 mM Na_3VO_4). For the assay, the immunoprecipitate obtained from 200 μg of total protein, 1.0 μg of acid-treated enolase, and 4 nmol/0.74 MBq of [γ-^{32}P] adenosine triphosphate (ATP) were mixed in 25 μl of kinase assay buffer. Phosphorylation was allowed to proceed at 30 °C for 10 min, and phosphoproteins were resolved by SDS–PAGE and visualised using a autoradiography or image analysis using a BAS 2000 system (Fuji Film, Tokyo, Japan). The exposure time in the kinase assay was 30 min at room temperature for all samples.

There are two ways to analyse the kinase activity of pp60$^{c\text{-}src}$ [9–16]. One is by judging the amount of pp60$^{c\text{-}src}$ autophosphorylation, and the other is by judging the amount of phosphorylation of an exogenous substrate, enolase. To calculate the tumour/non-tumour tissue (T/N) ratio of pp60$^{c\text{-}src}$ kinase activity, the density of the phosphorylated band of enolase or pp60$^{c\text{-}src}$ in the tumour tissue was divided by that in the non-tumour tissues from each patient, based on an analysis using a BAS 2000 system. The Bas 2000 system is an image analyser which makes visible the band of the phosphorylated protein obtained from autoradiography, and measures the density the phosphorylated band. To measure pp60$^{c\text{-}src}$ activity, the density of the bands of 32 kDa for enolase phosphorylation and 60 kDa for pp60$^{c\text{-}src}$ autophosphorylation obtained from autoradiography were analysed using the BAS 2000 system. The densitometric ratio of the bands of pp60$^{c\text{-}src}$ autophosphorylation and enolase phosphorylation was expressed by using the level of density in the surrounding non-tumour lung tissue as the reference level (= 1). The kinase activity of pp60$^{c\text{-}src}$ in each sample was obtained by averaging three measurements for each sample. The pp60$^{c\text{-}src}$ specific activity was estimated as the T/N ratio of pp60$^{c\text{-}src}$ protein kinase activity relative to the T/N ratio of the pp60$^{c\text{-}src}$ protein amount.

2.6. Two-dimensional tryptic phosphopeptide mapping

Specimens from lung adenocarcinoma and the adjacent non-tumour lung tissue obtained during surgery were divided into small pieces using a needle and then were incubated for 6 h with [^{32}P] orthophosphate (1 mCi/ml) in phosphate-free Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% dialysed fetal calf serum (FCS). The specimens were homogenised in TNE buffer, and immunoprecipitated with mAb 327. Immunoprecipitates were analysed on a 7% SDS–polyacrylamide gel. pp60$^{c\text{-}src}$ was electro-eluted from the gel bands and digested with trypsin as previously described in Ref. [14]. Peptides were separated in two dimensions on cellulose thin-layer plates by electrophoresis in the first dimension (pH 1.9, 1 kV, 25 min) and chromatography in the second dimension (*n*-butanol/pyridine/acetic acid/H_2O, 75:50:15:60 by volume) [20].

2.7. Statistical analysis

Data were expressed as means ± standard erros of the mean (SEM). The significance of the differences between observations was determined using the one sample t test (null hypothesis, $\mu = 1$). A P value of < 0.05 was considered to indicate a significant difference between the groups.

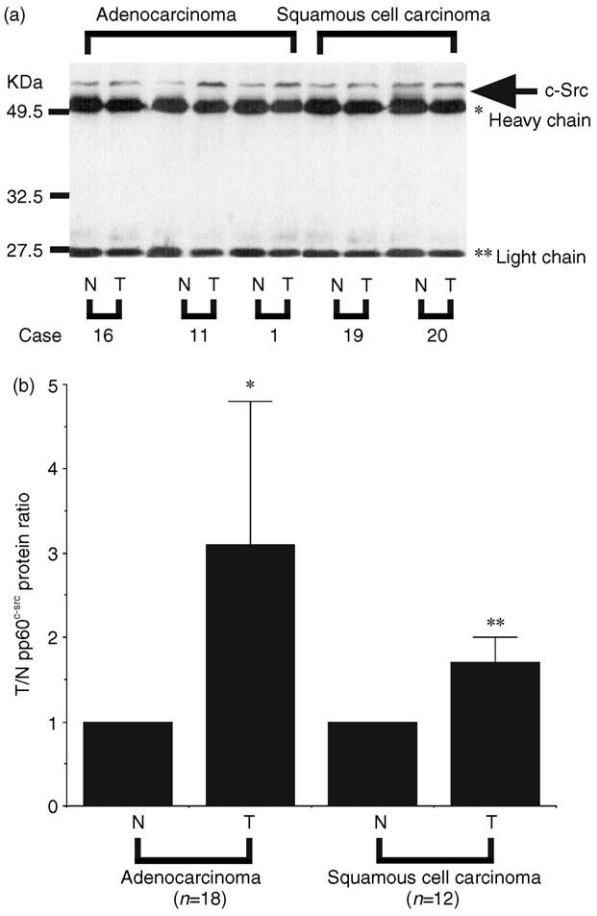

Fig. 1. (a). Level of pp60$^{c\text{-}src}$ protein in non-tumour (N) and tumour (T) portions of lung cancer for each patient. For western blotting analysis, anti-pp60$^{c\text{-}src}$ precipitates were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. The membrane was incubated with excess pp60$^{c\text{-}src}$ antibody and washed. Immunoreactive proteins were then visualised using an enhanced chemiluminescence detection system on X-ray film. Lysate samples (each containing 200 μg of total cellular protein) were prepared and analysed as described in the Methods. Arrow: band corresponding to pp60$^{c\text{-}src}$ protein, *: band representing heavy chain immunoglobulin, ** band representing light chain immunoglobulin, respectively. (b) Relative level of pp60$^{c\text{-}src}$ protein abundance in tumour (T) portions of the lung tissues of patients with lung cancer. For the T/N protein ratio on the y-axis, the level of pp60$^{c\text{-}src}$ in normal tissues has been set at 1.0 for relative comparison with the tumour tissues from each patient $^*P < 0.001$, $^{**}P < 0.001$.

3. Results

3.1. Expression of pp60$^{c\text{-}src}$ protein

The expression of pp60$^{c\text{-}src}$ at the protein level in lung cancer was analysed by western blotting. pp60$^{c\text{-}src}$ immunoprecipitates obtained from the lysates used for protein kinase assays, were resolved by SDS–PAGE before transfer to a nitrocellulose membrane. Representative results of a western blot of pp60$^{c\text{-}src}$ immunoprecipitates from lung cancer samples of five patients are shown in Fig. 1a. Patients 1, 6 and 11 had adenocarcinomas, and the other patients (patients 19 and 20) had squamous cell carcinomas. Three proteins were detected in each gel lane. The upper protein was pp60$^{c\text{-}src}$, the middle one was mAb 327 heavy-chain immunoglobulin, and the lower protein was mAb 327 light-chain immunoglobulin. pp60$^{c\text{-}src}$ expression was detected in all tumour and nontumour tissues tested in this study. The reproducibility of the findings in the individual tumours was confirmed in three experiments without variance. Overall, the amount of pp60$^{c\text{-}src}$ protein in 18 adenocarcinomas and 12 squamous cell carcinomas was 3.1 ± 1.7 (median 3.3; $^*P < 0.001$) and 1.5 ± 0.3 (median 1.5; $^{**}P < 0.001$) times higher, respectively, than that in the surrounding non-tumour lung tissues from the same patient (Fig. 1b, Table 1).

3.2. Protein kinase activity of pp60$^{c\text{-}src}$ in lung cancers

To study the protein kinase activity of pp60$^{c\text{-}src}$ in lung cancers, we prepared lysates of tissue samples, precipitated the protein with a monoclonal antibody specific for pp60$^{c\text{-}src}$, and measured the phosphorylation of pp60$^{c\text{-}src}$ and an exogenous substrate, enolase, using an *in vitro* protein kinase assay. The activity of pp60$^{c\text{-}src}$ in representative adenocarcinomas (patients 1, 6 and 11) and squamous cell carcinomas (patients 19 and 20) is shown in Fig. 2a and b, respectively. On the basis of the electrophoretic mobilities and the results of previous studies including our data [11,12], we consider the upper and lower bands to represent pp60$^{c\text{-}src}$ autophosphorylation (molecular weight 60 kDa) and enolase phosphorylation (molecular weight 42 kDa) products, respectively. The mean pp60$^{c\text{-}src}$ activity in adenocarcinomas was 4.8 ± 2.8 (median 4.5; $^*P < 0.05$) and 1.6 ± 0.41 (median 1.7; $^*P < 0.05$) times higher than that in the surrounding non-tumour lung tissue from the same patient, as measured by pp60$^{c\text{-}src}$ autophosphorylation and enolase phosphorylation, respectively (Fig. 2c, Table 1). The mean pp60$^{c\text{-}src}$ activity in the squamous cell carcinomas was 1.5 ± 0.48 (median 1.5; $^{**}P < 0.001$) and 1.1 ± 0.22 (median 1.2, $^*P < 0.05$) times higher than that in the surrounding normal lung tissue from the same patient as measured by pp60$^{c\text{-}src}$ autophosphorylation and enolase phosphorylation,

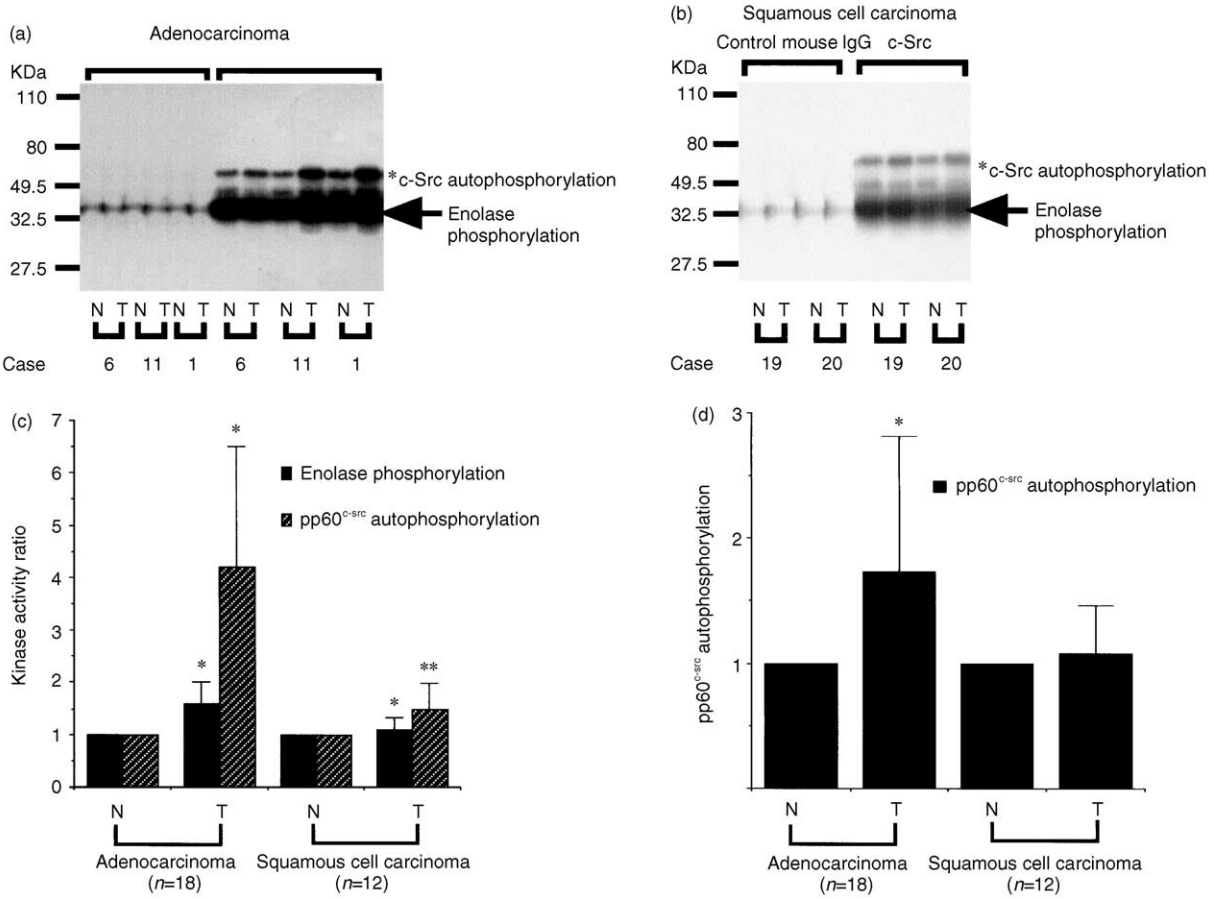

Fig. 2. (a) and (b) *In vitro* protein kinase activity of pp60$^{c\text{-}src}$ in non-tumour (N) and tumour (T) portions of lung adenocarcinomas and squamous cell carcinomas. Assay of tissue samples of the N and T tissues of each patient are shown. Lysate samples containing 200 μg of total cellular protein were prepared as described in the Methods. The proteins were precipitated with excess pp60$^{c\text{-}src}$ antibody, incubated for 10 min at 30 °C with [γ-^{32}P] adenosine triphosphate (ATP) and acid-denatured rabbit muscle enolase, and resolved on 10% sodium dodecyl sulphate (SDS)–polyacrylamide gels. *: band corresponding to pp60$^{c\text{-}src}$ tyrosine phosphorylation, arrows: bands corresponding to enolase. (c) The relative levels of total kinase activities of pp60$^{c\text{-}src}$. The tumour tissue/non-tumour tissue (T/N) kinase activity ratio is shown on the *y*-axis. The level of pp60$^{c\text{-}src}$ in the normal tissues has been set at 1.0 for relative comparison with the tumour tissue from each patient. The total activity of pp60$^{c\text{-}src}$ in the T tissues of the adenocarcinomas and squamous cell carcinomas was significantly higher than that in normal tissues from the same patient when measured by enolase phosphorylation (solid bars) and autophosphorylation (hatched bars). Values represent the mean ± SEM of pp60$^{c\text{-}src}$ activity for each group (**$P < 0.05$, and *$P < 0.001$). (d) The relative levels of specific kinase activity of pp60$^{c\text{-}src}$. The tumour tissue/non-tumour tissue (T/N) specific kinase activity ratio is shown on the y-axis. The level of pp60$^{c\text{-}src}$ in normal tissues has been set at 1.0 for relative comparison with the tumour tissue from each patient. The specific kinase activity of pp60$^{c\text{-}src}$ in the T tissues of lung adenocarcinomas was also significantly higher than that in surrounding normal tissues collected from the same patients when measured by autophosphorylation, while the specific activity in the T tissues of squamous cell carcinomas was not significantly different from that in the surrounding normal tissues collected from the same patients (*$P < 0.01$).

respectively (Fig. 2c, Table 1). As control samples, we used the immunoprecipitate products obtained from the tissues using non-immune mouse IgG. Although the phosphorylated enolase band was also seen in control samples, the density was clearly fainter than that produced by the immunoprecipitate product obtained using pp60$^{c\text{-}src}$ monoclonal antibody. No autophosphorylated pp60$^{c\text{-}src}$ band was detected in the control samples. Therefore, the phosphorylated bands were thought to be specific (Fig. 2a and b). We next analysed whether the difference observed in pp60$^{c\text{-}src}$ kinase activity was mainly caused by a difference in the amount of pp60$^{c\text{-}src}$ protein or by the difference in the specific activity of pp60$^{c\text{-}src}$. For this, the pp60$^{c\text{-}src}$ protein levels and its kinase activity were estimated by western blotting and *in vitro* kinase assays, respectively. The specific kinase activity of the adenocarcinomas and squamous cell carcinomas was 1.73 ± 1.08 (median 1.8; *$P < 0.01$) and 1.1 ± 0.38 (median 1.1) times higher than that in surrounding non-tumour lung tissue, respectively (Fig. 2d). Judging from these results, the increase of the pp60$^{c\text{-}src}$ kinase activity observed in squamous cell carcinomas is due to an increase in the pp60$^{c\text{-}src}$ protein level, while the increase of the pp60$^{c\text{-}src}$ kinase activity in adenocarcinomas is caused not only by an increase in pp60$^{c\text{-}src}$ protein, but also by an increase in the specific kinase activity of pp60$^{c\text{-}src}$.

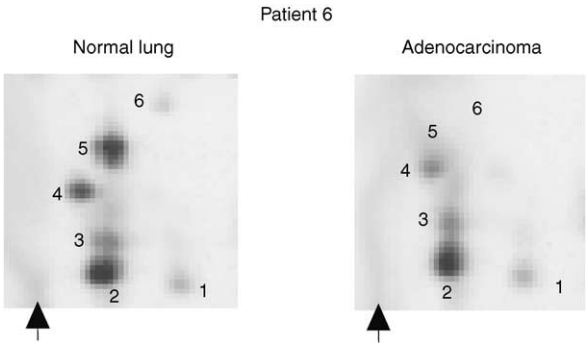

Fig. 3. Comparison of tryptic phosphopeptides of pp60$^{c\text{-}src}$ from adenocarcinoma and the surrounding normal lung tissue collected from patient 6 listed in Table 1. Tissue samples were incubated with ^{32}Pi for 6 h, radiolabelled pp60$^{c\text{-}src}$ was isolated and digested with trypsin, and the phosphopeptides were resolved on thin-layer chromatography plates by electrophoresis at pH 1.9 in the horizontal dimension and by chromatography in the vertical dimension. The origin is indicated by a vertical arrow. Note that phosphopeptides 5 and 6 in the surrounding normal lung tissue were not present in the lung adenocarcinoma.

3.3. Two-dimensional tryptic phosphopeptide mapping

The major site of tyrosine phosphorylation on pp60$^{c\text{-}src}$ in humans is Tyr 530. Dephosphorylation of this site activates pp60$^{c\text{-}src}$. To examine whether such dephosphorylation is responsible for the pp60$^{c\text{-}src}$ activation in adenocarcinoma, we compared the phosphorylation sites on pp60$^{c\text{-}src}$ obtained from adenocarcinoma with those in adjacent non-tumour lung tissue from patient 6 (Table 1) by two-dimensional tryptic phosphopeptide mapping (Fig. 3). Tryptic digestion of pp60$^{c\text{-}src}$ from *ex-vivo* $^{32}PO_4$-labelled normal lung generated six major phosphopeptides (left panel). Peptide 5 is known to be phosphorylated on Tyr 530 [21] by C-terminal src kinase (Csk) [22] and peptides 1 and 2 on Ser 17 by protein kinase A [23], and peptide 3 on the amino-terminal serine residue [24]. The identity of peptide 6 is unknown. Tryptic digestion of pp60$^{c\text{-}src}$ from lung adenocarcinoma generated four major phosphopeptides (right panel), which were similar to peptides 1–4 of pp60$^{c\text{-}src}$ generated from the non-tumour lung tissue. Phosphopeptides 5 and 6 present in the normal lung tissue were not present in the lung adenocarcinomas. Thus, pp60$^{c\text{-}src}$ appeared to be dephosphorylated at Tyr 530 in lung adenocarcinomas compared with the adjacent non-tumour lung tissues.

3.4. Relationship between pp60$^{c\text{-}src}$ activity and clinicohistopathological characteristics of the adenocarcinomas

The tumour/non-tumour (T/N) ratio of pp60$^{c\text{-}src}$ kinase activity increased with the tumour size of the adenocarcinomas ($r=0.76$, $P<0.001$). In the adenocarcinomas with a tumour size of $\geqslant 3$ cm, the increased T/N ratio of pp60$^{c\text{-}src}$ activity was significantly higher than that in tumours with a size of <3 cm (1.1 ± 0.25 versus 1.8 ± 0.28 ($P<0.01$), 1.7 ± 0.71 versus 5.9 ± 2.3 ($P<0.01$), as measured by enolase phosphorylation and pp60$^{c\text{-}src}$ autophosphorylation, respectively). The T/N ratio of pp60$^{c\text{-}src}$ activity in squamous cell carcinomas with a tumour size of $\geqslant$3cm was as high as in tumours with a size of <3 cm (1.3 ± 0.12 versus 0.9 ± 0.18 and 1.8 ± 0.34 vs. 1.1 ± 0.31, as measured by enolase phosphorylation and pp60$^{c\text{-}src}$ autophosphorylation, respectively) (Fig. 4). However, the T/N ratio of pp60$^{c\text{-}src}$ kinase activity was not correlated with tumour stage or classification of adenocarcinomas. The T/N ratio of pp60$^{c\text{-}src}$ activity was not significantly correlated with the degree of cell differentiation of adenocarcinoma, although there was a tendency for the T/N ratio to increase in poorly differentiated adenocarcinomas. In squamous cell carcinomas, the T/N ratio of pp60$^{c\text{-}src}$ kinase activity was not associated with the degree of differentiation or stage of the tumour.

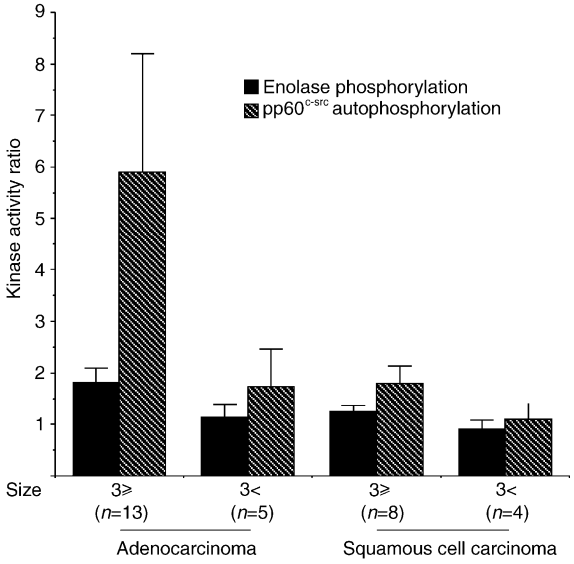

Fig. 4. Comparison of the tumour tissue/non-tumour tissue (T/N) kinase activity ratio of pp60$^{c\text{-}src}$ for adenocarcinomas and squamous cell carcinomas in two groups divided according to tumour size $\geqslant 3$ cm and <3 cm. The difference in the two groups of adenocarcinomas was significant ($^{*}P<0.01$). Values represent the mean$\pm$standard deviation (S.D.) of pp60$^{c\text{-}src}$ kinase activity ratio for each group.

4. Discussion

Aberrant expression of individual proto-oncogenes has been detected in several human tumours [9–13]. Although little is known about the biochemical function of most proto-oncogene products, several of these products are known to possess tyrosine-specific protein kinase activity. The most extensively studied protein kinase is pp60$^{c\text{-}src}$. Previous reports, including ours, [9–13] demonstrated that pp60$^{c\text{-}src}$ kinase activity is significantly elevated in several types of human cancer. Regarding lung cancer, pp60$^{c\text{-}src}$ protein was found to

be elevated in 60% of all lung cancers when biopsy materials of tumours were analysed by immunoblotting and immunohistochemistry [9,10]. However, no analysis of pp60$^{c\text{-}src}$ kinase activity in human lung cancers has been reported.

In general, pp60$^{c\text{-}src}$ activity has been measured by two methods in previous reports. One is a score based on pp60$^{c\text{-}src}$ autophosphorylation, and the other is a score based on the phosphorylation of an exogenous substrate, enolase. In the present study, the T/N ratio of kinase activity for enolase phosphorylation was smaller than for pp60$^{c\text{-}src}$ autophosphorylation. Previous reports have been also supported these findings [11,14,15]. Considering these previous reports and our results in the present study, the sensitivity of enolase phosphorylation due to pp60$^{c\text{-}src}$ activation might be very low compared with pp60$^{c\text{-}src}$ autophosphorylation.

The major finding of the present study was that the protein level and activity of pp60$^{c\text{-}src}$ were elevated in malignant lung tissues, especially in adenocarcinomas, compared with surrounding non-tumour lung tissues from the same patients (Figs. 1 and 2). These data suggest that the activation of pp60$^{c\text{-}src}$ kinase may play a role in the tumorigenesis of lung cancers. In addition, we showed that the enhanced pp60$^{c\text{-}src}$ kinase activity in lung adenocarcinomas was probably due to both an increased amount of pp60$^{c\text{-}src}$ protein and higher specific activity of the pp60$^{c\text{-}src}$ kinase. These results strongly suggest that the malignancy of some lung adenocarcinomas is not only critically dependent on the level of pp60$^{c\text{-}src}$ protein synthesis, but also on the activity of the pp60$^{c\text{-}src}$ protein tyrosine kinase.

The relationship between pp60$^{c\text{-}src}$ kinase activation and the clinicopathological features of lung cancer was then evaluated. pp60$^{c\text{-}src}$ kinase activity increased with tumour size, and was significantly higher in adenocarcinomas with a diameter $\geqslant$ 3 cm than in those with a diameter $<$ 3 cm. This finding supports the possibility that activation of the pp60$^{c\text{-}src}$ kinase is related to the development of adenocarcinomas.

The upregulation of pp60$^{c\text{-}src}$ kinase activity in lung adenocarcinomas was higher than the elevation of the pp60$^{c\text{-}src}$ protein. These findings suggest that the high activity of pp60$^{c\text{-}src}$ was not only a result of increased amounts of the pp60$^{c\text{-}src}$ protein, but also a reasult of an increase in the specific kinase activity of the pp60$^{c\text{-}src}$ kinase. What are the mechanisms by which the specific kinase of pp60$^{c\text{-}src}$ is activated in lung adenocarcinoma? pp60$^{c\text{-}src}$ activation may occur as a result of one of several regulatory mechanisms, such as shifts in the subcellular localisation of pp60$^{c\text{-}src}$, alteration in the regulation of pp60$^{c\text{-}src}$ via phosphorylation/dephosphorylation, association of pp60$^{c\text{-}src}$ with other cellular proteins, or genetic mutations. Recent reports have shown that the kinase active form of pp60$^{c\text{-}src}$ is associated with the insoluble cytoskeletal fraction [25]. To provide further insight into possible mechanisms in lung cancers, we tested the hypothesis that increased pp60$^{c\text{-}src}$ specific kinase activity in lung adenocarcinomas was caused by altered tyrosine phosphorylation. Among the sites of pp60$^{c\text{-}src}$ tyrosine phosphorylation, it is known that phosphorylation of Tyr 530 decreases its kinase activity, whereas dephosphorylation of Tyr 530 activates its kinase activity. We demonstrated, by two-dimensional tryptic phosphopeptide mapping, that Tyr 530 on pp60$^{c\text{-}src}$ is dephosphorylated in lung adenocarcinomas. Because dephosphorylation of Tyr 530 is one of the major mechanisms involved in the upregulation of the pp60$^{c\text{-}src}$-specific kinase, these results suggest that this mechanism may be responsible (at least in part) for the activation of pp60$^{c\text{-}src}$ in lung adenocarcinomas. Another possible mechanism of upregulation of pp60$^{c\text{-}src}$ kinase in lung cancers is the genetic mutation of pp60$^{c\text{-}src}$. Irby and colleagues [26] detected truncating mutations at codon 531 of the pp60$^{c\text{-}src}$ gene in 12% of the advanced colon cancers they tested and also demonstrated that this change was activating, transforming, tumorigenic, and metastasis-promoting. However, three subsequent studies failed to detect truncating mutations at codon 531 of pp60$^{c\text{-}src}$ in colorectal cancers from Italians [27], North Europeans and Japanese [28], as well as Chinese [29] patients. In addition, activated expression of pp60$^{c\text{-}src}$ was shown to occur in the colon, breast, liver, pancreas, or head and neck samples in the absence of any genetic changes [13–16]. These previous findings suggest that pp60$^{c\text{-}src}$ activation due to mutation at codon 531 of pp60$^{c\text{-}src}$ is probably not a major mechanism in lung cancers.

Csk is a novel cytoplasmic protein tyrosine kinase that inactivates members of the Src family, including pp60$^{c\text{-}src}$, *in vitro* and *in vivo*. It negatively regulates pp60$^{c\text{-}src}$ activity by specifically phosphorylating Tyr-530 of pp60$^{c\text{-}src}$ [23,33]. This suggests that the dephosphorylation of Tyr 530 in some lung adenocarcinomas might be due to a reduction in Csk activity. Regarding other human cancers, we have reported that reduced Csk activity is detected in human hepatocellular carcinomas [33] and colorectal cancers [12]. Taken collectively, these data suggest that a reduced Csk activity might occur in human lung cancer, but this will need to be tested in future studies.

The development of a vascular network, a process known as angiogenesis, has been shown to be important in the growth of several human solid tumours [34–36], including lung cancers. Vascular endothelial growth factor (VEGF) is a very effective factor for inducing the formation of new blood vessels, and acts specifically on endothelial cells [38]. VEGF is a homodimeric 34–42 kDa heparin-binding glycoprotein that is expressed by almost all human solid tumours [39]. A report has shown that VEGF mRNA expression is higher in NSCLC tissue than in healthy lung tissues [36]. In

addition, some studies have also reported that a high VEGF expression was associated with an advanced clinical stage, larger tumour size, lymph node metastasis, shorter patient survival and early relapse [40]. Furthermore, some studies have shown that a decreased pp60$^{c\text{-}src}$ activity in colon cancer cells contributes to a decrease in the expression of VEGF [41], suggesting that pp60$^{c\text{-}src}$ activity regulates this expression. In the present study, we have shown that the level of pp60$^{c\text{-}src}$ kinase was related to tumour size. Thus, these data suggest that VEGF might be induced in larger lung adenocarcinomas with an increased pp60$^{c\text{-}src}$ kinase activity.

In conclusion, activation of the proto-oncogene product pp60$^{c\text{-}src}$ may play an important role in the malignant transformation of lung adenocarcinomas, and is closely related to the progression of lung adenocarcinomas. Therefore, lung cancer is an appropriate model for studying the function of pp60$^{c\text{-}src}$ in malignant transformation. The suppression of pp60$^{c\text{-}src}$ kinase activity may offer a novel strategy for overcoming the development and invasion of lung cancers. Further studies are necessary to investigate such processes.

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