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Lyn Expression in Osteoblastic Osteosarcoma Tissues and Its Correlation with Clinicopathologic Factors

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Background : The Src family kinases (SFKs) are involved in multiple aspects of tumorigenesis, such as, proliferation, migration, and angiogenesis, and are involved in the generation and progression of many types of tumors. Furthermore, dasatinib, a general SFKs inhibitor was recently approved for use in chronic myeloid leukemia. This study was performed to evaluate the expression of Lyn, a member of the SFKs, in osteosarcoma tissues. **Methods :** One hundred and sixteen patients with osteoblastic osteosarcoma were selected for Lyn expression analysis. The correlation between Lyn expression in tumor sections and patients' clinicopathologic characteristics and the prognostic significance of Lyn expression were evaluated. **Results :** Lyn was found to be expressed in 52 of the 116 patients (44.8%), and Lyn positive tumor was found to be significantly associated with a lytic tumor pattern on plain radiographs ($p = 0.04$). Furthermore, those positive for Lyn showed longer metastasis free survival (5-year metastasis free survival, 65.2% for Lyn positive and 46.8% for Lyn negative; $p = 0.06$), though this was only marginally significant. **Conclusions :** Lyn was found to be overexpressed in osteosarcoma tissues, and this overexpression was found to be correlated with osteolysis.

Key Words : Osteosarcoma; Lyn protein-tyrosine kinase; Immunohistochemistry

Osteosarcoma is the most commonly encountered primary malignant bone tumor, and shows a predilection for adolescents. Survival rates have improved dramatically during the past three decades, but outcome remains poor for those presenting with metastatic disease.[1] Therefore, there is an urgent need to identify new therapies to treat osteosarcoma.

The Src family kinases (SFKs) mediate signal transduction from several receptor tyrosine kinases, such as, epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR). SFKs are involved in the regulation of cellular processes, such as proliferation, adhesion, motility and survival, and are known to contribute to tumor progression, metastasis, and angiogenesis.[2,3] SFKs are also known to interact with a number of cellular factors that are involved in the degradation of basement membrane, and thus, to facilitate tumor invasion.[4] Furthermore, SFKs activate antiapoptotic pathways in tumor cells that have detached themselves from primary sites, allowing these cells to travel to distant tissues.[5] In addition, SFKs have been reported to be overexpressed in many malignant neoplasms, including, chronic myeloid leukemia, mesothelioma, and numerous epithelial cancers,[6-8] and levels of SFK expression and activation have been reported to be correlated with disease progression,[2-5] which has prompted studies on the effects of inhibiting SFKs in numerous cancers.[6-8]

Dasatinib is the most potent SFK inhibitor currently available in clinical practice. It is a small-molecule tyrosine kinase inhibitor with activity against several signaling proteins, such as, SFKs, bcr-abl, c-kit, PDGFR, and ephrins.[9] Furthermore, dasatinib has

been shown to decrease tumor size, suppress migration and invasion, and reduce metastasis in several tumors.[9,10] Recently, dasatinib was approved for the treatment of imatinib refractory chronic myelogenous leukemia and bcr-abl positive acute lymphoblastic leukemia,[11] and currently phase I and II clinical studies are underway in colorectal cancer, non-small cell lung cancer, prostate cancer, and breast cancer.[12]

Lyn protein-tyrosine kinase is a well known SFK, and is expressed in hematopoietic cells of erythroid/myeloid and B lymphoid origin, neuronal cells, prostate cells, and colon cells,[13] and in prostate cancer, colon cancer, pancreatic cancer, breast cancer, and head and neck squamous cell carcinoma.[14-17] Furthermore, Lyn has been suggested to play an important role in leukemia,[13] and in the development of certain solid tumors.[14-17]

Recently, Shor *et al*.[18] evaluated the activations of SFKs and the effect of dasatinib in sarcoma, and found that SFKs are overexpressed in osteosarcoma, Ewing sarcoma, leiomyosarcoma, and rhabdomyosarcoma, and that dasatinib blocks the migration and invasion of sarcoma cell lines. Furthermore, dasatinib was found to induce the apoptosis of a subset of osteosarcoma cells and of Ewing sarcoma cells. Interestingly, Lyn-expressing cells in osteosarcoma and Ewing sarcoma show high rates of apoptosis in response to dasatinib, and for this reason, dasatinib is viewed as a potential treatment for these conditions and Lyn as a potential biomarker. In addition, Guan *et al*.[3] found that dasatinib induced Lyn downregulation, suppressed tumor growth, and reduced bone destruction and metastatic potential in Ewing sarcoma.

This study was performed to investigate Lyn expression in osteosarcoma tissues using an immunohistochemical approach. Furthermore, we evaluated correlations between Lyn expression and clinicopathologic characteristics and the prognostic significance of Lyn expression.

MATERIALS AND METHODS

Patients and specimens

One hundred and sixteen osteosarcoma patients treated at the Korea Cancer Center Hospital between 1986 and 2006 were retrospectively selected. The inclusion criteria applied were as follows: 1) extremity osteosarcoma; 2) American Joint Committee on Cancer (AJCC) stage II; 3) scheduled for neo- and adjuvant chemotherapy and wide excision at the Korea Cancer Center Hospital; 4) more than a 5-year follow-up period; 5) osteoblastic subtype; and 6) available formalin-fixed paraffin-embedded tissues for immunohistochemistry.

Plain radiographic films were reviewed. Radiographic patterns were classified as either osteoblastic or mixed and osteolytic. An osteoblastic pattern was defined as one in which more than 80% of the tumor area was denser than the normal cortex, whereas an osteolytic pattern was defined as one in which more than 80% of the tumor area was less dense than the normal cortex. Remaining tumors were defined as having a mixed pattern. Plain radiographic films were independently reviewed by two of the authors; in discrepant cases, images were reviewed by both authors, and decisions were made by consensus.

Patients were also divided into two groups according to tumor growth patterns as observed on magnetic resonance (MR) images. Concentric tumors were defined as extensive extraosseous masses with a maximal tumor width exceeding 1.5 times the affected bone diameter, whereas longitudinal tumors were defined as tumors that fulfilled the following criteria; 1) a tumor length greater than twice the tumor width with 2) no extensive extraosseous mass formation.

All 116 patients underwent two cycles of preoperative chemotherapy followed by four cycles of postoperative chemotherapy. A modified T10 chemotherapy protocol was used in all cases.[19] Each cycle of preoperative chemotherapy consisted of high-dose methotrexate, adriamycin, and cisplatin, administered as follows: 8 to 12 g/m^2 of methotrexate was administered twice (on days 1 and 7); on day 14, 100 mg/m^2 cisplatin was administered over 2 hours, and this was followed by 30 mg/m^2 of adriamycin administered over 18 hours. Scheduled chemotherapy durations ranged from 24 to 36 weeks.

Histological responses to preoperative chemotherapy were evaluated using Huvos criteria. Good responders were defined as those with fewer than 10% viable tumor cells in surgical specimen after preoperative chemotherapy.

Construction of tissue microarray blocks and immunohistochemical staining for Lyn

Formalin-fixed paraffin-embedded tissue specimens prepared from biopsies or resected specimens were chosen. Representative tumor areas were selected to construct tissue microarrays. Single core tissue biopsy specimens of diameter 5 mm were taken from representative regions of paraffin-embedded osteosarcomas (donor blocks) and used to reconstruct new recipient paraffin-embedded blocks.

A section of each block was stained immunohistochemically using Lyn antibody. As external positive controls, sections of

palatine tonsil and appendix were also stained. Negative controls were prepared by substituting non-immune serum for antibody. The Zymed nonbiotin amplification system (Zymed Laboratories Inc., South San Francisco, CA, USA) was used, and primary mouse monoclonal antibody against Lyn (1 : 50, mouse monoclonal antibody sc-7274, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was applied to sections overnight in a moist chamber at 4℃.

Lyn immunoreactivity was observed in the cellular membranes and cytoplasm. Lymphocytes were used as internal positive controls. Results were expressed using semi-quantitative scales of membranous and cytoplasmic staining intensities and extents. Intensities were scored as: score 0 (no staining); 1 (weakly positive); 2 (same intensity as the internal control); 3 (strongly positive). Extents were scored as: score 0 (no staining); 1 (1-25% positive cells); 2 (26-50% positive cells); 3 (51-75% positive cells); and 4 (76-100% positive cells). Final scores were calculated by multiplying Lyn immunoreactivity intensity and extent scores. A case was considered positive if the final score was more than 4 (Fig. 1).

Statistical analysis

Data were analyzed using SPSS ver. 11.0 (SPSS Inc., Chicago, IL, USA). Relationships between clinicopathologic variables and Lyn expression were examined using the chi square test. The variables analyzed included age, gender, tumor size and location, plain radiographic pattern, growth pattern by magnetic resonance imaging (MRI), histological response, and final patient outcome. Metastasis-free survival and overall survival rates were calculated using the Kaplan-Meier method, and differences in survival were compared using the log-rank test. Multivariate analyses were performed using the Cox's proportional hazards regression model to identify independent predictors of prognosis. p-values of less

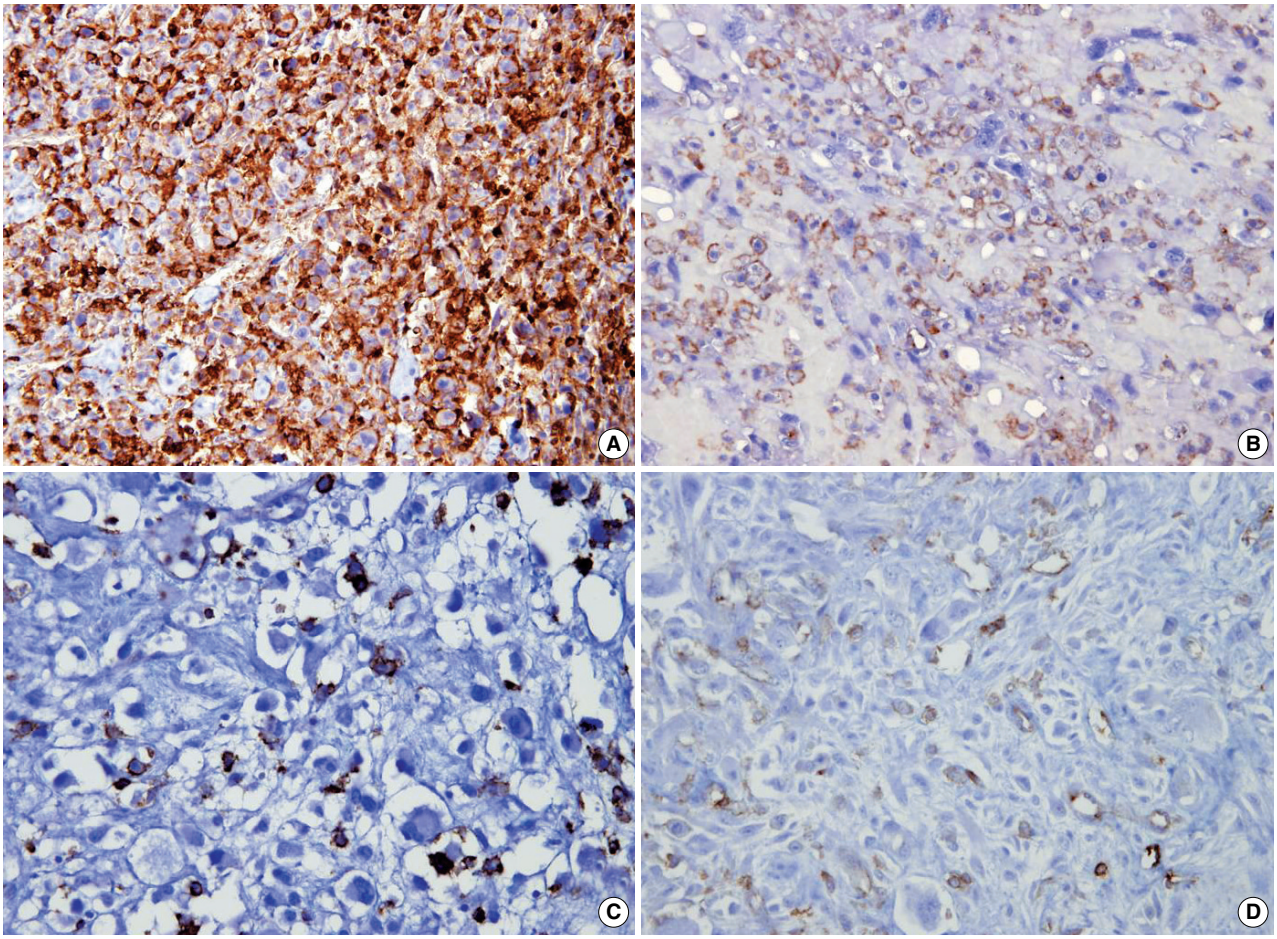

Fig. 1. Immunohistochemical staining for Lyn in osteosarcoma. (A) Positive result (intensity score 2 × frequency score 4 = 8 ≥ 4). (B) Positive result (intensity score 1 × frequency score 4 = 4 ≥ 4). (C) Negative result (intensity score 2 × frequency score 1 = 2 < 4). (D) Negative result (intensity score 1 × frequency score 1 = 1 < 4). Note the membranous and cytoplasmic expressions of Lyn.

than 0.05 were considered significant.

RESULTS

Clinicopathologic characteristics of the 116 osteosarcoma patients

Patients' characteristics are summarized in Table 1. There were 66 males (57%) and 50 females (43%) of average age 17.0 years (range, 7 to 40 years). Tumor locations were: distal femur in 58 cases (50%), proximal tibia in 28 cases (24%), proximal humerus in 12 cases (10%), fibula in 7 cases (6%), proximal femur in 3 cases (3%), femoral shaft in 4 cases (3%), and others in 4 cases (3%). All cases were of stage IIB according to Enneking's criteria. By the AJCC staging system, 46 cases (40%) were of stage IIA and 70 cases (60%) were of stage IIB. The plain radiographic pattern was lytic in 37 (32%) and blastic or mixed in 79 (68%). Growth patterns by MRI were concentric in 97 (84%) and longitudinal in 19 (16%). There were 82 poor responders (71%) and 34 good responders (29%).

Lyn expression in osteosarcoma

Lyn was detected in cancerous cells but not in noncancerous cells, except for endothelial cells, which were weakly positive. Lyn was expressed in 52 high grade osteosarcoma patients (44.8%). Intensity scores were: 3 in one case (0.9%), 2 in 74 cases (63.8%), 1 in 35 cases (30.2%), and only 6 cases (5.2%) had a score of 0. Extent scores were: 4 in 5 cases (4.3%), 3 in 14 cases (12.1%), 2 in 45 cases (38.8%), and 1 in 45 cases (38.8%). Final scores were: 12 in 1case (0.9%), 8 in 3 cases (2.6%), 6 in 11 cases (9.5%), 4 in 37 cases (31.9%), 3 in 3 cases (2.6%), 2 in 33cases (28.4%), and 1 in 21 cases (18.1%).

Correlations between Lyn expression and clinicopathologic characteristics

Lyn expression showed no correlation with patient or tumor characteristics except for plain radiographic pattern (Table 2). Tumors expressing Lyn more frequently showed a lytic pattern on simple radiographs (p = 0.04).

Table 1. Patient and tumor characteristics

Variables		No.	%
Age (yr)	≤ 15	59	51
	> 15 and ≤ 40	57	49
Gender	Male	66	57
	Female	50	43
Tumor diameter (cm)	≤ 8	46	40
	> 8 and ≤ 10	27	23
	> 10	43	37
Location	Distal femur	58	50
	Proximal tibia	28	24
	Proximal humerus	12	10
	Fibula	7	6
	Proximal femur	3	3
	Femoral shaft	4	3
	Other	4	3
Pattern on plain radiograph	Lytic	37	32
	Blastic	79	68
Growth pattern on MRI	Concentric	97	84
	Longitudinal	19	16
Histological response	Good	34	29
	Poor	82	71
Metastasis	Yes	52	45
Final outcome	Alive	80	69
	Dead	36	31
Total		116	100

MRI, magnetic resonance imaging.

Table 2. Correlations between Lyn expression and clinicopathologic characteristics

Variables		Lyn negative (%)	Lyn positive (%)	p-value
Age (yr)	≤ 15	35 (54.7)	24 (46.2)	0.46
	> 15 and ≤ 40	29 (45.3)	28 (53.8)	
Gender	Male	36 (56.3)	30 (57.7)	1.00
	Female	28 (43.7)	22 (42.3)	
Tumor diameter (cm)	≤ 8	27 (42.2)	19 (36.5)	0.22
	> 8 and ≤ 10	11 (17.2)	16 (30.8)	
	> 10	26 (40.6)	17 (32.7)	
Location	Distal femur	31 (48.4)	27 (51.9)	0.48
	Proximal tibia	18 (28.1)	10 (19.2)	
	Proximal humerus	6 (9.4)	6 (11.5)	
	Fibula	4 (6.3)	3 (5.8)	
	Proximal femur	0 (0)	3 (5.8)	
	Femur shaft	2 (3.1)	2 (3.8)	
	Other	3 (4.7)	1 (1.9)	
Pattern on plain radiograph	Lytic	15 (23.4)	22 (42.3)	0.04
	Blastic	49 (76.6)	30 (57.7)	
Growth pattern on MRI	Concentric	53 (82.8)	44 (84.6)	1.00
	Longitudinal	11 (17.2)	8 (15.4)	
Histological response	Good	21 (32.8)	13 (25.0)	0.42
	Poor	43 (67.2)	39 (75.0)	
Metastasis	Yes	34 (53.1)	18 (34.6)	0.06
Final outcome	Alive	42 (65.6)	38 (73.1)	0.43
	Dead	22 (34.4)	14 (26.9)	
Total		64 (55.2)	52 (44.8)	

MRI, magnetic resonance imaging.

Metastasis-free survival

The 5-year metastasis-free survival rate of the 116 patients, as calculated by the Kaplan-Meier method, was 53.9% (95% confidence interval [CI], 49.4% to 58.4%). Metastasis was noted in 52 patients (45%). Locations of tumor metastasis were: lung in 40 patients, bone in 8, lung and bone in 2, and other locations in 2 (scalp and mediastinum). The mean interval from diagnosis to metastasis was 18.3 months (range, 3 to 42 months; median, 16 months). Mean metastasis-free survival for 64 patients was 117.6 months (range, 27 to 251 months; median, 115 months) and that for the entire cohort was 73.1 months (range, 3 to 251 months; median, 46 months).

Patients with Lyn expression showed longer metastasis free survival (65% for Lyn positive vs 47% for Lyn negative) without statistical significance (p = 0.06). Histological response to preoperative chemotherapy was found to have prognostic value with respect to metastasis-free survival by univariate analysis (p < 0.01) (Table 3).

Table 3. Five-year metastasis-free survival of the 116 patients with AJCC stage II osteosarcoma

Variables		No.	Univariate	
			5y-MFSR (%)	p-value
Age (yr)	≤ 15	59	49.0 ± 6.5	0.09
	> 15	57	61.3 ± 6.5	
Gender	Male	66	53.0 ± 6.2	0.52
	Female	50	57.7 ± 7.0	
Tumor diameter (cm)	≤ 8	46	67.3 ± 6.9	0.05
	> 8 and ≤ 10	27	55.6 ± 9.6	
	> 10	43	41.5 ± 7.6	
Location	Other	104	57.6 ± 4.9	0.06
	Proximal humerus	12	33.3 ± 13.6	
Pattern on plain radiograph	Lytic	37	56.6 ± 8.2	0.86
	Blastic	79	54.3 ± 5.6	
Growth pattern on MRI	Concentric	97	52.5 ± 5.1	0.15
	Longitudinal	19	68.0 ± 10.8	
Histological response	Good	34	79.2 ± 7.0	< 0.01
	Poor	82	45.0 ± 5.5	
Lyn expression	Negative	64	46.8 ± 6.3	0.06
	Positive	52	65.2 ± 6.6	
Total			53.9 ± 4.5	

AJCC, American Joint Committee on Cancer; 5y-MFSR, 5 year metastasis-free survival rate; MRI, magnetic resonance imaging.

Overall survival

The 5-year overall survival rate of the 116 patients, as calculated by the Kaplan-Meier method, was 71.8% (95% CI, 67.5% to 76.1%). Of the 116 patients, 31 succumbed to the disease, 64 were disease free, 16 showed no evidence of disease, and 5 remained alive with disease. The mean follow up duration was 88.0 months (range, 6 to 251 months; median, 88 months).

Table 4. Five-year overall survival of the 116 patients with AJCC stage II osteosarcoma

Variables		No.	Univariate		Multivariate		
			5y-OSR (%)	p-value	RR	95% CI	p-value
Age (yr)	≤ 15	59	63.7 ± 6.6	0.03	1		
	> 15	57	79.9 ± 5.4		0.45	0.21-0.94	0.03
Gender	Male	66	69.6 ± 5.9	0.50	ND	ND	ND
	Female	50	74.8 ± 6.3				
Tumor diameter (cm)	≤ 8	46	82.2 ± 5.7	0.08	ND	ND	ND
	> 8 and ≤ 10	27	72.4 ± 8.9				
	> 10	43	60.3 ± 7.8				
Location	Other	104	74.1 ± 4.4	0.13	ND	ND	ND
	Proximal humerus	12	37.0 ± 19.6				
Pattern on plain radiograph	Lytic	37	66.3 ± 8.0	0.39	ND	ND	ND
	Blastic	79	74.4 ± 4.4				
Growth pattern on MRI	Concentric	97	69.7 ± 4.8	0.26	ND	ND	ND
	Longitudinal	19	83.1 ± 9.0				
Histological response	Good	34	87.7 ± 5.8	0.02	1		
	Poor	82	65.0 ± 5.5		3.28	1.15-9.39	0.01
Lyn expression	Negative	64	70.7 ± 5.8	0.60	ND	ND	ND
	Positive	52	73.1 ± 6.4				
Total			71.8 ± 4.3				

AJCC, American Joint Committee on Cancer; 5y-OSR, 5 year overall survival rate; RR, relative risk; CI, confidence interval; ND, not done; MRI, magnetic resonance imaging.

Age (p = 0.03) and histological response (p = 0.02) were found to be prognostically relevant in terms of overall survival by univariate analysis. However, Lyn expression showed no correlation with overall survival (p = 0.60). Multivariate analysis revealed that a young age (p = 0.03) and a poor histological response to preoperative chemotherapy (p = 0.01) independently predicted poorer overall survival (Table 4).

DISCUSSION

This study presents immunohistochemical results for Lyn in high-grade osteoblastic osteosarcoma tumors in 116 patients. A few previous reports have evaluated the expressions of SFKs using an immunohistochemical approach.[6,8,18,20-22] In these studies, SFKs were examined in colorectal carcinoma, malignant pleural mesothelioma, hepatocellular carcinoma (HCC), glioblastoma and sarcoma including leiomyosarcoma, high-grade osteosarcoma and liposarcoma. Src was studied in each of the above tumors, focal adhesion kinase was studied in HCC, Lyn and Fyn were studied in glioblastoma.

In this study, Lyn was found to be expressed in 52 patients with osteosarcoma (44.8%), which suggests that SFKs inhibitors might be a therapeutic option for the treatment of osteosarcoma. In fact, dasatinib has already shown bone-specific inhibitory effects in a preclinical study.[18] A larger study is needed to prove the hypothesis that SFKs inhibitors might be a therapeutic option for the treatment of osteosarcoma.

This study shows that tumors expressing Lyn significantly more frequently have a lytic pattern on simple radiographs (p = 0.04). Although the underlying mechanisms of tumor-related bone resorption are complex and multifactorial, this finding is consistent with the known role of SFKs in osteolysis, as SFKs play a central role in normal, healthy bone turnover by positively regulating osteoclasts and negatively regulating osteoblasts.[23] Furthermore, the activities of SFKs are essential for osteoclast cytoskeleton organization and bone resorbing activity.[24] In addition, the targeted disruption of SFKs in mice was found to cause a defect in osteoclast-mediated bone resorption,[25] and the suppression of SFKs was found to interfere with ion transport, which is required to solubilize bone mineral during bone resorption by osteoclasts.[26] Lyn also shows these features of SFK.[3,27]

In the present study, patients with tumors expressing Lyn showed longer metastasis free survival (p = 0.06), which apparently contradicts the known tumor-progressive effects of SFKs. This contradiction may be due to the fact that the present study involved a comparatively small sample size, and was conducted retrospectively. Because resected specimens were included, higher proportions of poor responders and patients with a higher metastatic rate were probably enrolled than are found in the general patient population. In addition, the evaluation of phosphorylated antibodies in archived paraffin tumors proved to be problematic, as the time lapsed between tumor removal and evaluation can affect receptor phosphorylation status. In fact, the archived tissues used in this study were collected over twenty years, and the amount of time that lapsed between tumor removal and fixation was undeterminable.

However, some studies concur with the present study. Ito *et al*.[8] identified Src levels by immunohistochemistry on formalin-fixed paraffin-embedded tissues obtained from 87 Japanese HCC patients and detected Src in 40 (46%) cases. In addition, they observed that elevated Src expression was more common in well or moderately differentiated carcinomas than in poorly differentiated carcinomas, and that Src expression was inversely correlated with Ki-67 expression, intrahepatic metastasis, tumor-node-metastases staging, alpha-fetoprotein, and EGFR expression. Lau *et al*.[6] also showed that Src expression levels were elevated in HCC by immunohistochemistry and that levels of SRC overexpression were not correlated with most clinical, pathological or phenotypic parameters. Tsao *et al*.[21] used immunohistochemical analysis to determine Src expressional levels in malignant pleural mesothelioma, and found that Src protein was highly expressed in tumor cells, but that its expression was not correlated with survival.

It is interesting to note that all previous studies, including the present study, that have used an immunohistochemical approach and formalin fixed paraffin-embedded tissues, found no or an inverse correlation between SFK expression and survival. Iravani *et al*.[28] also reported a difference between the results obtained by tissue staining and *in vitro* kinase assays, which may help explain these discrepancies.

Despite the limitations mentioned above, it was interesting to find that Lyn was expressed in a large proportion of the osteosarcoma tumors examined, suggesting that Lyn may be an attractive therapeutic target. Lyn was found to be expressed in 52 patients (44.8%), and tumors expressing Lyn more frequently showed a lytic pattern on simple radiographs (p = 0.04). Furthermore, patients with tumors expressing Lyn showed longer metastasis free survival (p = 0.06). Further studies are needed to elucidate how Lyn activation contributes to osteosarcoma progression, and prospective study is required to confirm the prognostic value of Lyn in a large osteosarcoma cohort.

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