Increased Expression of Integrin-Linked Kinase Is Correlated with Melanoma Progression and Poor Patient Survival

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ABSTRACT

Purpose: Integrin-linked kinase (ILK), a key component of the extracellular matrix adhesion, has been studied extensively in recent years. Overexpression of ILK in epithelial cells results in anchorage-independent cell growth with increased cell cycle progression. Furthermore, increased ILK expression is correlated with progression of several human tumor types, including breast, prostate, and colon carcinomas. However, the role of ILK overexpression in human melanoma pathogenesis is not known. To investigate whether ILK plays a role in melanoma progression, we measured ILK expression in primary melanoma biopsies at various stages of invasion and evaluated the prognostic value of ILK expression in human melanoma.

Experimental Design: We used tissue microarray and immunohistochemistry to determine ILK expression in 67 primary melanomas and analyzed the correlation between ILK expression and melanoma progression and 5-year patient survival.

Results: We show that strong ILK expression is significantly associated with melanoma thickness. Strong ILK expression was observed in 0, 22, 33, and 63% in melanoma biopsies ≤0.75, 0.76–1.50, 1.51–3.0, and >3.0 mm in thickness, respectively. Furthermore, strong ILK expression was detected in 83% of the tumors with lymph node invasion compared with only 18% for tumors without lymph node invasion (*P* < 0.01). Strikingly, our data revealed that strong ILK expression is inversely correlated with 5-year patient survival (*P* < 0.05).

Conclusion: ILK expression increases dramatically with melanoma invasion and progression and is inversely correlated with patient survival.

INTRODUCTION

Cell-ECM[2] interactions play an important role in cell survival, growth, differentiation, and migration. ILK, a key component of cell-ECM structures, has been studied extensively since it was cloned in 1996. ILK interacts with the cytoplasmic domain of both β1 and β3 integrins and is activated on extracellular signals via integrins and by certain growth factors (1). The ILK kinase activity is stimulated in a PI3K-dependent manner and negatively regulated by two phosphatases: PTEN, a tumor suppressor lipid phosphatase that dephosphorylates PI 3,4,5-triphosphate to PI 4,5-bisphosphate, and ILKAP, a PP2C protein phosphatase (2–5). ILK can phosphorylate AKT/protein kinase B on Ser-473 and GSK-3 on Ser-9, resulting in an activated AKT and decreased GSK-3 activity, respectively (4, 6). Overexpression of ILK also leads to the loss of cell-cell adhesion because of down-regulation of E-cadherin, possibly by ILK-mediated activation of the E-cadherin repressor Snail and nuclear β-catenin stabilization by ILK-mediated inhibition on GSK-3 activity (6, 7).

Increased ILK expression has been shown in several oncogenesis-related processes. Overexpression of ILK results in anchorage-independent cell cycle progression, cell migration and invasion, and suppression of anoikis (2, 8, 9). ILK overexpression in mammary epithelial cells leads to cell transformation and tumor formation in transgenic mice (10). Inhibition of ILK suppresses activation of AKT and induces cell cycle arrest and apoptosis in PTEN-mutant prostate cancer cells (3). Recently, Graff *et al.* (11) also showed ILK overexpression correlates with prostate tumor progression.

The incidence of cutaneous malignant melanoma is increasing more rapidly than any other tumor (12). Malignant melanoma is associated with one of the highest mortality rates, particularly for advanced disease. Melanoma is a life-threatening disease because of its high capability of invasion and rapid metastasis to other organs. Melanoma is highly radioresistant, and the response rate to single-drug chemotherapy is only approximately 20% (13). Therefore, patients with metastatic melanoma have a poor prognosis, with a median survival of only

[2] The abbreviations used are: ECM, extracellular matrix; ILK, integrin-linked kinase; PI, phosphatidylinositol; PI3K, PI-3-kinase; TMA, tissue microarray; NF-κB, nuclear factor κB; MMP, matrix metalloproteinase; GSK-3, glycogen synthase kinase 3.

Table 1 ILK expression and clinicopathological characteristics

	Intensity of ILK staining				Total	P^a
	0	1+	2+	3+		
Age						
≤60	3 (8%)	10 (26%)	19 (50%)	6 (16%)	38	
>60	0 (0%)	7 (24%)	12 (41%)	10 (35%)	29	>0.05
Sex						
Male	0 (0%)	10 (26%)	16 (42%)	12 (32%)	38	
Female	3 (10%)	7 (24%)	15 (52%)	4 (14%)	29	>0.05
Tumor thickness (mm)						
≤0.75	1 (7%)	3 (21%)	10 (71%)	0 (0%)	14	
0.76–1.5	1 (3%)	10 (28%)	17 (47%)	8 (22%)	36	
1.51–3.0	1 (11%)	2 (22%)	3 (33%)	3 (33%)	9	<0.05^b
>3.0	0 (0%)	2 (25%)	1 (13%)	5 (63%)	8	<0.05^c
Lymph node invasion						
Negative	3 (5%)	16 (26%)	31 (51%)	11 (18%)	61	
Positive	0 (0%)	1 (17%)	0 (0%)	5 (83%)	6	<0.01
Tumor subtype^d						
SSM	2 (7%)	6 (19%)	15 (48%)	8 (26%)	31	
LMM	0 (0%)	2 (20%)	7 (70%)	1 (10%)	10	
Other	1 (4%)	9 (35%)	9 (35%)	7 (27%)	26	>0.05
Site^e						
Sun protected	2 (4%)	12 (23%)	24 (45%)	15 (28%)	53	
Sun exposed	1 (7%)	5 (36)	7 (50%)	1 (7%)	14	>0.05

^{a} χ^2 test for low to moderate (0, 1+, 2+) *versus* high ILK expression (3+).
^{b} Tumors ≤1.5 mm *versus* >1.5 mm.
^{c} Tumors ≤3.0 mm *versus* >3.0 mm.
^{d} SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; Other includes desmoplastic melanoma, acrolentigous melanoma, and nodular melanoma.
^{e} Sun-protected sites: trunk, arm, leg, and feet. Sun-exposed sites: head and neck.

5–8 months (14). Because of the fact that ILK overexpression is closely related to tumor cell migration and invasion, we hypothesized that increased ILK expression may be associated with melanoma progression. We, thus, used TMA and immunohistochemistry to evaluate the ILK expression level in primary human melanoma at different stages. Here, we report for the first time that overexpression of ILK is closely correlated with melanoma progression and inversely related to 5-year patient survival.

MATERIALS AND METHODS

TMA Construction. Formalin-fixed, paraffin-embedded tissue blocks containing primary melanoma were obtained from the 1990–1997 archives of the Department of Pathology, Vancouver General Hospital. The most representative tumor area was carefully selected and marked on the H&E-stained slide. The TMAs were assembled using a tissue-array instrument (Beecher Instruments, Silver Spring, MD) consisting of thin-walled stainless steel punches and stylets used to empty and transfer the needle content. The assembly was held in an X-Y position guide equipped with semiautomatic micrometers, with a 1-mm increment between individual samples and 3-mm punch depth stop device. Briefly, the instrument was used to create holes in a recipient block with defined array cores. A solid stylet, which closely fit the needle, was used to transfer the tissue cores into the recipient block. Taking into account the limitations of the representative areas of the tumor, we used duplicate or triplicate 0.6-mm diameter tissue cores from each donor block. Multiple 4-μm sections were cut with a Leica

microtome. Sections were transferred to adhesive-coated slides using routine histology procedures. One section was routinely deparaffinized with standard xylene and hydrated through graded ethanol in water, then stained with H&E and covered with a coverslip. The remaining sections were stored at room temperature for immunohistochemistry staining.

Eighty-seven melanoma primaries and 16 nevi were used for the construction of the array. Because of loss of biopsy cores or lack of tumor, scoring for ILK staining was obtained in 76 melanoma primaries and 12 nevi. In addition, clinical information including patient's gender, age, thickness of tumor (Breslow), tumor location, and histological subtype was available for 67 melanoma patients (Table 1). There were 38 males and 29 females with a mean age of 58 years. Among the 67 patients, information on 5-year disease-specific survival was available for 61 patients.

Immunohistochemistry of TMA. The TMA slides were dewaxed by heating at 55°C for 30 min and by three washes, 5 min each, with xylene. Tissues were rehydrated by a series of 5-min washes in 100, 90, and 70% ethanol and PBS. Antigen retrieval was performed by microwaving the samples for 4 min, 20 s at full power in 250 ml of 10 mM sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 20 min. Nonspecific binding was blocked with goat serum for 30 min. The primary polyclonal rabbit anti-ILK antibody (Stressgen, Victoria, British Columbia, Canada) was diluted 1:100 using goat serum and incubated at room temperature for 1 h. After three washes, 2 min each with PBS, the sections were incubated with a biotinylated goat antirabbit sec-
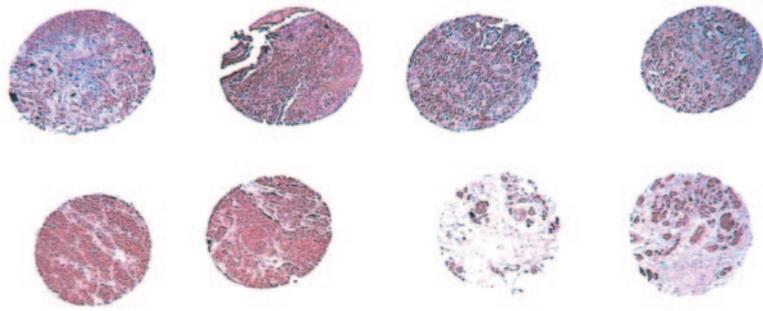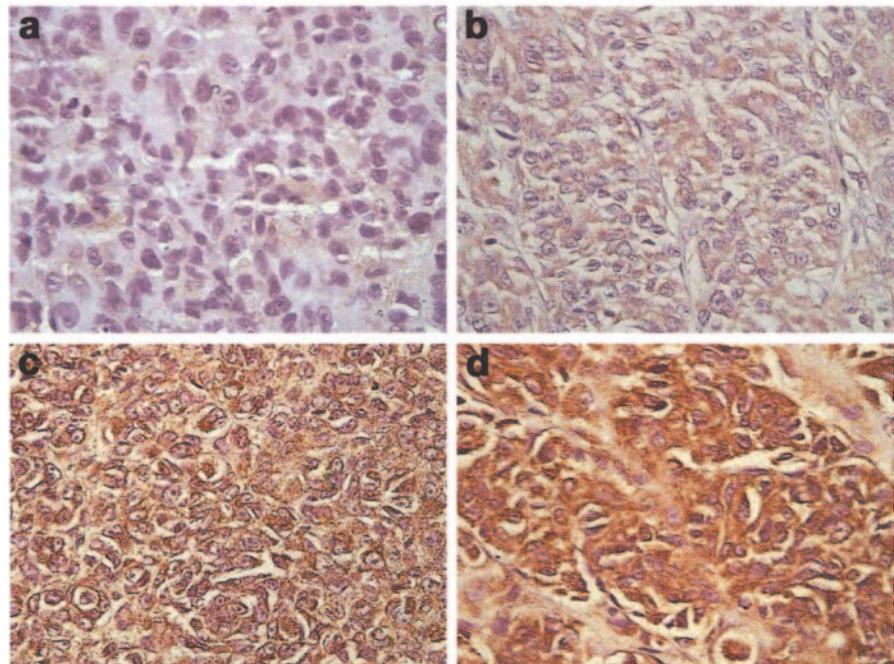
Fig. 1 ILK expression in human melanoma TMA. The *top panel* shows the immunohistochemical staining of representative cores of the TMA (magnification, ×25). The *bottom panels* represent different levels of ILK expression: *a*, negative or no ILK expression (0); *b*, weak ILK expression (1+); *c*, moderate ILK expression (2+); *d*, strong ILK expression (3+). Magnification, ×400.

ondary antibody for 30 min (Santa Cruz Biotechnology, Santa Cruz, CA). After three washes, 2 min each with PBS, horseradish peroxidase-streptavidin (Santa Cruz Biotechnology) was added to the section for 30 min, followed by another three washes, 2 min each with PBS. The samples were developed with 3,3′-diaminobenzidine substrate (Vector Laboratories, Burlington, Ontario, Canada) for 7 min and counterstained with hematoxylin. Then, the slides were dehydrated following a standard procedure and sealed with coverslips. Negative controls were performed by omitting ILK antibody during the primary antibody incubation.

Evaluation of Immunostaining. The ILK staining was examined double-blinded by three independent observers (including one dermatopathologist) simultaneously, and a consensus score was reached for each core. The staining intensity of each core was scored as negative (0), weak staining (1+), moderate staining (2+), and strong staining (3+). There was a high level of consistency of immunohistochemical staining between the duplicate or triplicate cores in the TMAs. Eighty-five percent of the biopsies had uniform staining between different cores. For the other 15% of cases that had one level of difference

in staining between cores, the higher score was used for statistical analysis. The reason for differential staining in some biopsies could be that melanoma is a heterogeneous tumor, so different areas in the tumor may represent different stages of tumor progression.

Statistical Analysis. The relationship between the ILK expression and the clinicopathological parameters, including age, sex, tumor thickness, location, histological subtype, and lymph node invasion, was evaluated by χ^2 test. The relationship between ILK expression and 5-year survival was assessed by F test.

RESULTS

Clinicopathological Findings. For melanoma staging, we used Breslow thickness as our criteria for evaluating ILK expression: ≤0.75 mm, low risk; 0.76–1.5 mm, intermediate risk; 1.51–3.0 mm, high risk; >3.0 mm, very high risk (15). For the 67 cases in which ILK staining and complete clinicopathological information was available, 14 were ≤0.75 mm, 36 were 0.76–1.5 mm, 9 were 1.51–3.0 mm, and 8 were >3.0 mm thick
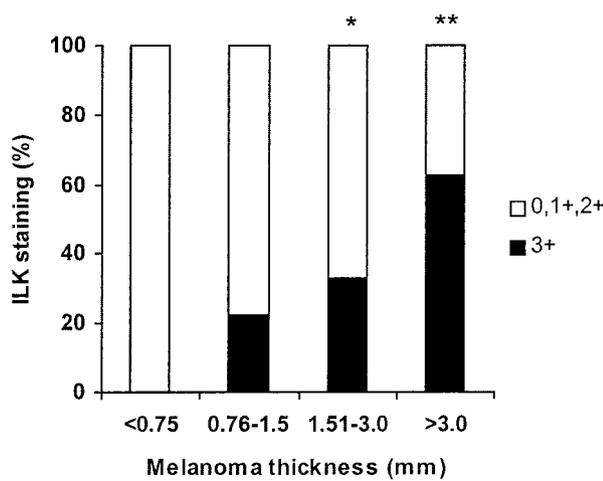
Fig. 2 Correlation between strong ILK expression and melanoma thickness. ∗, $P < 0.05$, comparison of tumors ≤0.75 mm thick *versus* tumors 1.51–3.0 mm thick; ∗∗, $P < 0.01$, comparison of tumors ≤0.75 mm thick *versus* tumors >3.0 mm thick. Analysis was by χ^2 test.
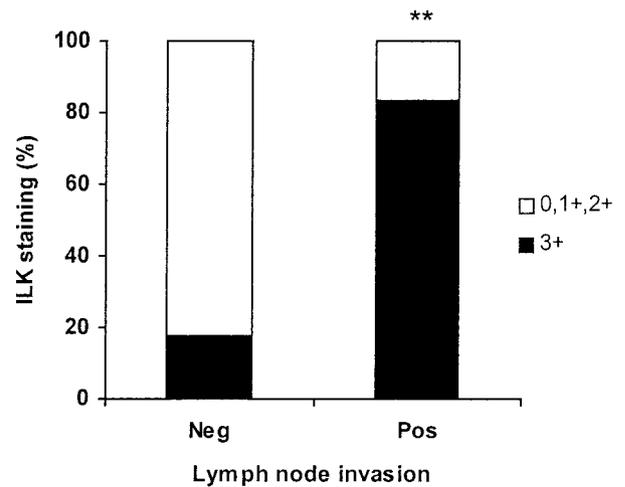

Fig. 3 Correlation between strong ILK expression and lymph node invasion. Tumors with lymph node invasion have a significantly higher percentage of strong ILK expression than tumors without lymph node invasion. ∗∗, $P < 0.01$, χ^2 test.

(Table 1). Among the 67 cases, 31 were superficial spreading melanoma, 10 were lentigo maligna melanoma, and other 26 cases consisted of desmoplastic melanoma, acrolentigous melanoma, and nodular melanoma. Fourteen melanomas were located in sun-exposed sites (head and neck), and 53 were located in sun-protected sites (trunk, arm, leg, and feet). Lymph node invasion was observed in six cases.

Correlation between ILK Expression and Melanoma Progression. To assess whether ILK expression is associated with melanoma progression, we examined ILK expression in melanoma primaries at various stages of invasion by immunohistochemistry. Staining for ILK was uniform in 85% of biopsies with duplicate or triplicate cores. Various levels of ILK expression were observed in the cytoplasm in the melanoma biopsies (Fig. 1). ILK expression was negative (0) in 3 (4.5%), weak (1+) in 17 (25.4%), moderate (2+) in 31 (46.3%), and strong (3+) in 16 primary melanomas (23.9%). ILK staining was uniform in the tumor biopsies with more than 75% of cells stained in all three categories (1+, 2+, and 3+). When ILK expression is compared in tumors with different thickness, we find that strong ILK expression (3+) significantly correlates with thicker tumors. As shown in Table 1, strong ILK expression (3+) is significantly more in melanomas >1.5 mm thick (8 of 17, 47%) compared with tumors ≤1.5 mm (8 of 50, 16%; $P < 0.05$, χ^2 test). The percentage of strong ILK expression (3+) is even higher in melanomas over 3 mm thick (5 of 8, 63%) compared with tumors ≤3.0 mm (11 of 59, 19%; $P < 0.05$, χ^2 test). The percentage of strong ILK expression (3+) gradually increases with melanoma thickness: 0% in tumors ≤0.75 mm, 22% in tumors 0.76–1.5 mm, 33% in tumors 1.51–3.0 mm, and 63% in tumors >3.0 mm thick (Fig. 2). Furthermore, strong ILK expression was detected in 83% of the tumors with lymph node invasions compared with only 18% for tumors without lymph node invasions ($P < 0.01$, χ^2 test; Fig. 3 and Table 1). Weak or moderate ILK staining did not correlate with melanoma thickness or lymph node invasion. Tumor ul-

ceration is often considered an indicator for patient survival. However, information on tumor ulceration for these patients was unavailable, so we were unable to compare ILK expression between ulcerated tumors and nonulcerated lesions. In addition, we did not find correlation between ILK expression with age, sex, tumor subtype, or location of tumors (sun protected *versus* sun exposed; Table 1).

For 42 biopsies that contained normal skin, uniform ILK staining was observed in all layers of keratinocytes and hair follicles: 6 weak (1+), 26 moderate (2+), and 10 strong (3+) ILK expression. Little ILK staining was observed in the dermis. In addition, we examined the ILK expression in 12 benign nevi and found that ILK staining was negative in 1 (8.3%), weak (1+) in 4 (33.3%), and moderate (2+) in 7 (58.3%) biopsies. None of the 12 nevi had strong ILK staining.

Survival Analysis. Because strong ILK staining was found in most advanced melanomas, we sought to evaluate whether ILK staining might be related to patient survival. Kaplin-Meier survival curves, constructed using disease-specific survival at 5 years, were evaluated for biopsies that stained negative to moderate (0, 1+, 2+) and those that stained strongly (3+) for ILK expression (Fig. 4). Our data revealed that strong ILK expression was inversely correlated with 5-year patient survival ($P < 0.05$, F test).

DISCUSSION

Melanoma is one of the most lethal malignancies among human cancers because of its highly metastatic character and resistance to conventional therapies. Patients with melanoma have a dismal prognosis, and there is still no reliable prognostic marker for this disease. Although ILK has been shown to play an important role in tumorigenesis of a number of human cancers (7, 8, 16–18), most data are derived from *in vitro* studies or animal models. The purpose of this study was to gain information on the role of ILK in melanoma progression. We used TMA technology and immunohistochemistry to investigate ILK
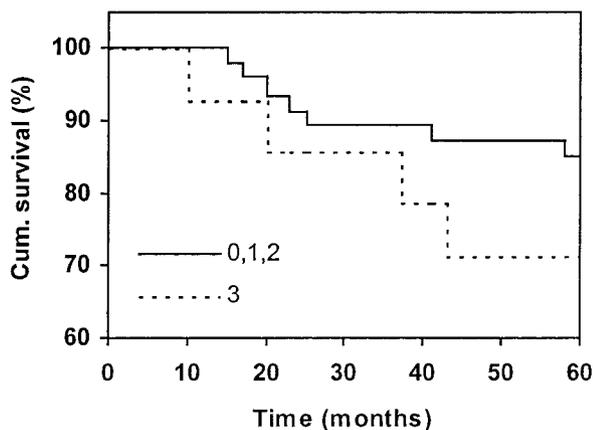
Fig. 4 Kaplan-Meier survival curve according to ILK expression. Patients with negative to moderate ILK expression (0, 1+, 2+; $n = 47$) have a significantly better 5-year survival than those with strong ILK expression (3+; $n = 14$). $P < 0.05$, F test.

expression in primary melanoma biopsies. Our results for the first time demonstrate that ILK expression increases as melanoma progresses (Fig. 2). In addition, strong ILK expression is also correlated with lymph node invasion (Fig. 3) and inversely related to 5-year survival (Fig. 4). Our data are concordant with the published association of ILK expression with prostate cancer progression (11).

The overexpression of ILK in advanced melanoma could be explained by the involvement of ILK in cell survival and death pathways. Previous studies indicate that ILK functions as an effector of the PI3K/Akt cell survival pathway. ILK positively regulates Akt activity and negatively regulates GSK-3 in a PI3K-dependent manner (4, 6). ILK directly phosphorylates Akt on Ser-473, which, together with Thr-308 phosphorylation by phosphoinositide-dependent kinase, activates Akt (4, 6). Akt then activates IKK, the IκB kinase that phosphorylates IκB, leading to its degradation and subsequent activation of NF-κB (19), leading to suppression of apoptosis. Not surprisingly, a recent study by Dhawan *et al.* (20) showed Akt is constitutively activated in melanoma, which leads to up-regulation of NF-κB and tumor progression. It is conceivable that high expression of ILK in melanoma, as we found in this study, would result in constitutive activation of Akt. Activation of Akt can also up-regulate vascular endothelial growth factor expression (21), a key component for angiogenesis, thus stimulating tumor invasion. Indeed, Segrelles *et al.* (22) showed that Akt signaling plays an important role in skin tumorigenesis.

In addition to its involvement in PI3K/Akt/NF-κB signaling, ILK is believed to play a role in *Wnt* and growth factor signaling pathways. ILK directly phosphorylates GSK-3 at Ser-9 (6), resulting in reduced GSK-3 activity. ILK-mediated inhibition of GSK-3 activity may lead to down-regulation of E-cadherin expression, nuclear translocation of β-catenin, and activation of the transcription factor AP-1 (2, 23). Stabilization of β-catenin may be responsible for ILK-mediated up-regulation of cyclin D1 (24). Up-regulation of AP-1 and cyclin D1, thus, leads to cell cycle progression. In fact, a recent study by Kielhorn *et al.* (25) demonstrated that nuclear phospho-β-catenin

expression was common in metastatic melanoma and significantly associated with poor overall survival. The increased nuclear phospho-β-catenin in melanoma could be caused by high expression of ILK in these tumors.

Another ILK-mediated pathway that may enhance tumor progression is its regulation on MMP expression. During tumor progression, MMPs facilitate the pathological processes of tumor invasion, angiogenesis, and metastasis by breaking down the ECM (26). It has been shown that overexpression of ILK results in increased MMP-9 expression (27), which is in agreement with our observation that melanoma biopsies with lymph node invasion expressed a significantly higher amount of ILK compared with those without lymph node invasion.

Overexpression of ILK induces invasive phenotypes *in vitro*, and inhibition of ILK can suppress the growth of colon carcinoma xenografts (7, 27). However, limited information is available for ILK expression in human tumor biopsies. Given the fact that there is still no reliable prognostic marker available for melanoma and that increased ILK expression is significantly associated with melanoma progression and inversely correlated with patient survival, ILK may serve as a promising prognostic marker and therapeutic target for malignant melanoma.

ACKNOWLEDGMENTS

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