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ILK (β1-integrin-linked protein kinase): a novel immunohistochemical marker for Ewing's sarcoma and primitive neuroectodermal tumour

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Abstract ILK (β1-integrin-linked protein kinase) is a recently identified 59-kDa serine/threonine protein kinase that interacts with the cytoplasmic domain of the β1-integrin containing four ankyrin-like repeats. We have developed a polyclonal antibody against ILK and explored the ILK immunoreactivity in normal human cells and tissues. ILK was mainly expressed in cardiac muscle and skeletal muscles. Surprisingly, ILK expression was observed in Ewing's sarcoma (ES; 100%), primitive neuroectodermal tumour (PNET; 100%), medulloblastoma (100%), and neuroblastoma (33.3%), whereas other small round cell sarcomas were not stained by the anti-ILK antibody. These results suggest that ILK could be a novel marker for tumours with primitive neural differentiation. Our findings support the notion that ES is a tumour that is closely related to PNET and that both originate from the neuroectoderm. ILK may be a sensitive and specific immunohistochemical marker and useful for the positive identification of ES and PNET in formalin-fixed, paraffin-embedded tissue sections.

Key words β1-Integrin-linked protein kinase (ILK) · Ewing's sarcoma · Primitive neuroectodermal tumour · Polyclonal antibody

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Introduction

Among bone and soft tissue neoplasms, ES/PNET (Ewing's sarcoma/primitive neuroectodermal tumour) needs to be distinguished from other small round cell tumours of bone and soft tissue, including neuroblastoma, lymphoma-leukaemia, small cell osteosarcoma, rhabdomyosarcoma, and mesenchymal chondrosarcoma [6, 11, 19, 20, 22]. All these entities share a similar histopathological appearance characterized by poorly differentiated cells with uniform small nuclei and scanty cytoplasm. It is very important to make an accurate and rapid differential diagnosis so as to ensure adequate treatment, particularly in the case of ES/PNET, an extremely aggressive neoplasm whose clinical course is heavily dependent on the adoption of customized therapeutic regimens.

As an adjunct to conventional histopathology for diagnosis of ES/PNET, two innovative approaches aimed at a positive diagnostic characterization of these neoplasms have been developed: immunhistochemical study for the expression of MIC2 (CD99) antigen [1, 7, 10–12, 19, 20, 22], and cytogenetic and molecular study for identification of specific chromosomal aberrations in ES/PNET: t(11;22)(q24;q12) and t(21;22)(q22;q12), respectively [8, 9, 13, 22, 23].

Recently, a gene encoding 59-kDa serine/threonine protein kinase (β1-integrin-linked protein kinase; ILK), located at 11p15, was cloned and appeared to interact with the β1-integrin cytoplasmic domain [15]. Furthermore the 1.8-kb transcript of this gene is widely expressed in various human tissues, such as heart, skeletal muscle, brain, placenta, lung, liver, kidney and pancreas. Although it is proposed that ILK is a receptor-proximal protein kinase regulating integrin-mediated signal transduction, the biological role of this widely expressed protein is not clearly defined. To enhance our understanding of the function of ILK, we first developed a polyclonal antibody against ILK and screened expression of ILK both in the various normal and neoplastic human tissues. In a survey of the distribution of ILK immunoreactivity in the formalin-fixed, paraffin-embedded neoplastic hu-

Table 1 Clinical data on Ewing's sarcoma and primitive neuroectodermal tumour

Case	Sex/age	Location	Characteristics	
1	F/19	Ilium	ES of bone	Primary
2	M/3	Lung	ES	Metastatic
3	M/12	Lung	ES	Metastatic
4	M/22	Scapular	ES of bone	Primary
5	M/4	Lung	ES	Recurrent
6	M/17	Soft tissue, thigh	ES	Primary
7	M/23	Rib	ES of bone	Primary
8	F/23	Soft tissue, Popliteal fossa	ES	Primary
9	M/30	Chest wall	ES	Recurrent
10	F/25	Rib	ES of bone	Primary
1	F/22	Humerus	PNET of bone	Primary
2	M/3	Soft tissue, thigh	PNET	Primary
3	F/3	Lymph node	PNET	Metastatic
4	F/12	Cerebrum	PNET	Primary
5	F/13	Cerebrum	PNET	Primary
6	M/6	Cerebrum	PNET	Primary
7	F/17	Cerebrum	PNET	Primary
8	M/10	Cervical spine	PNET	Primary

man tissues, we noticed strong and consistent immunoreactivity of ES/PNET, in contrast to the lack of immunostaining of the other small round cell sarcomas of bone and soft tissues.

Thus, we designed the current investigation to ascertain the diagnostic reliability of ILK as a novel ES/PNET marker in attempts at diagnosis in formalin-fixed, paraffin-embedded tissue sections.

Materials and methods

Antibody for ILK was raised in Balb/c mice by intraperitoneal injection with an emulsion of 50 μg/100 μl synthetic peptides of ILK (aminoacid residues: 225–239) conjugated with KLH in a mixture of Freund's complete adjuvants (Difco). The same amounts of antigen mixed with Freund's incomplete adjuvant (Difco) were given as a booster injection, on two occasions 2 weeks apart. After final exsanguination by cardiac puncture, sera were isolated and stored at –70° C. Compared with preimmune sera, the quality of ILK antibodies was evaluated in Western blot experiments using total cell lysates of RD-ES cell line derived from Ewing's sarcoma (ATCC, Rockville, Md.). We also used monoclonal antibodies against MIC2, DN16 [17] and O662 [2, 3], a generous gift from Dr. A. Bernard.

Cell line RD-ES, which was initiated from a primary osseous Ewing's sarcoma of the human, was obtained from the American Type Culture Collection (Rockville, Md.). Normal fetal tissues were obtained from autopsy materials of 13 cases (from 16 weeks to 26 weeks gestation), and 10 cases of normal adult tissues obtained from surgical procedure were used to test each organ (Table 1). Ten cases of ES (6 extraosseous and 4 osseous ES), 8 of PNET (7 extraosseous and 1 osseous PNET), 3 of medulloblastoma, 9 of neuroblastoma, 1 of retinoblastoma, 16 of rhabdomyocarcoma, 16 of lymphoblastic lymphoma, 4 of mesendchymal chondrosarcoma, 3 of osteosarcoma and 2 of osteoblastoma, registered with the Department of Pathology, Seoul National University Hospital, Chungbuk National University Hospital, Dongkook University Hospital and Korea Cancer Centre Hospital, were included in this study. Diagnosis of all tumours were reviewed by three experienced pathologists. The clinical data of ES/PNET cases are presented in Table 1. Diagnostic criteria of ES included diffuse growth of undifferentiated small round cells with either cytoplasmic PAS-positive granules or positive immunostaining for MIC2 without evidence of neural differentiation (negative immunostaining for neuron-specific enolase, synaptophysin and glial fibrillary acidic protein). A diagnosis of PNET was recorded when typical undifferentiated small round cells were found with evidence of neural differentiation (rosette formation, positive immunostaining for S-100, neuron-specific enolase, synaptophysin, glial fibrillary acidic protein. Medulloblastoma was diagnosed when a PNET was located in the cerebellum (cerebellar PNET).

The distribution pattern of ILK was evaluated by immunohistochemical analyses using paraffin sections of normal fetal and adult human tissues and all the tumour tissues listed as case material. All samples were immediately fixed in 10% buffered neutral formalin and embedded in paraffin. Some samples of ES, PNET and osteosarcoma were incubated with 10% nitric acid for decalcification. The paraffin blocks were sectioned at 4-μm thickness. After overnight incubation with primary antibodies in a dilution of 1:1,000, the sections were incubated with biotinylated goat anti-mouse immunoglobulins followed by streptavidin–horseradish peroxidase conjugate (Dako, Carpinteria, USA). The final reaction product was visualized with 3,3′-diaminobenzidine (Sigma, 0.5 mg/ml in 50 mM Tris-HCl, pH 7.4). Counter-staining was not done, and serially sectioned and haematoxylin-eosin stained tissues were compared side by side. Sections from normal heart were used as positive control. Negative control was stained with hyperimmune normal mouse sera.

For Western blotting of the tumour cell line (RD-ES; 1×10^8), cells were treated with 1 ml of lysis buffer (1% NP4O in 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 1 mM phenyl methyl sulphonyl fluoride) at 4° C. The lysates were mixed gently by inverting the tubes and incubated at 4° C for 30 min and centrifuged at 13,000 *g* for 15 min to remove the nuclei. The supernatants were subjected to 10% SDS-PAGE under the reduced conditions, with appropriate molecular weight markers. After electrophoretic transfer of proteins, the nitrocellulose paper was blocked with 5% skimmed milk, incubated with anti-ILK antibodies, and followed by peroxidase-conjugated goat anti-mouse IgG (Zymed, Calif.) diluted 1:5000 in blocking solution. After washing with Tris-buffered saline with 1% Tween 20, the antigen-bound peroxidase activity was visualized using the enhanced chemiluminescence (ECL) detection system (Amersham).

Results

In Western blot analysis, ILK antibodies specifically recognized ILK protein as a prominent band (59 kDa) in total lysates of RD-ES cells (Fig. 1).

To explore the expression of ILK in normal human tissues, we screened a number of formalin-fixed, paraf-

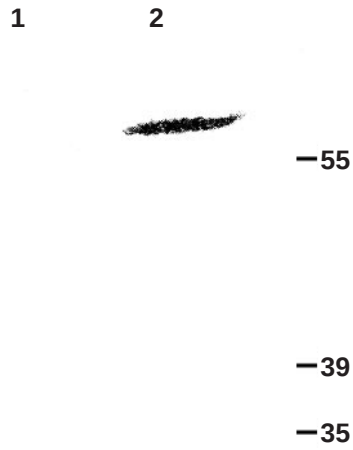

Fig. 1 Western blots of SDS-PAGE-separated RD-ES cell lysates were reacted with anti-ILK polyclonal antibody (*lane 2*). Negative control (*lane 1*). ILK is recognized as a 59-kDa protein in RD-ES cell lysate

Table 2 Expression of ILK (β1-integrin-linked protein kinase) in formalin-fixed, paraffin-embedded sections of various human tissues

Tissue[a]	ILK-immunoreactivity	Cell types
Bone	–	
Cardiac muscle	++	Myofibre
Skeletal muscle	+	Myofibre
Lymph node	–	
Tonsil	–	
Thymus	±	Thymocyte
Kidney	–	
Lung	–	
Thyroid	–	
Adrenal	–	
Liver	–	
Small intestine	–	
Pancreas	–	
Appendix	–	
Ovary	–	
Salpinx	–	
Testis	±	Spermatocyte
Epididymis	–	
Salivary gland	–	
Breast	–	
Placenta	–	
Skin	–	
Brain	–	

[a] Normal fetal tissues from 13 autopsy cases and 10 adult tissues obtained during surgery were used

fin-embedded tissues of the various human fetal and adult organs by immunohistochemical analysis (Table 2). ILK was mainly expressed in the myocardial cells of the heart and skeletal muscle fibres (Fig. 2A). ILK was detected in the cytoplasm of the stained cells along the inner surface of cell membrane. The cortical thymocytes and spermatocytes were weakly stained. Other tissues did not reveal ILK immunoreactivity.

Among the tumours tested, ES and PNET revealed strong ILK immunoreactivity. To evaluate the diagnostic

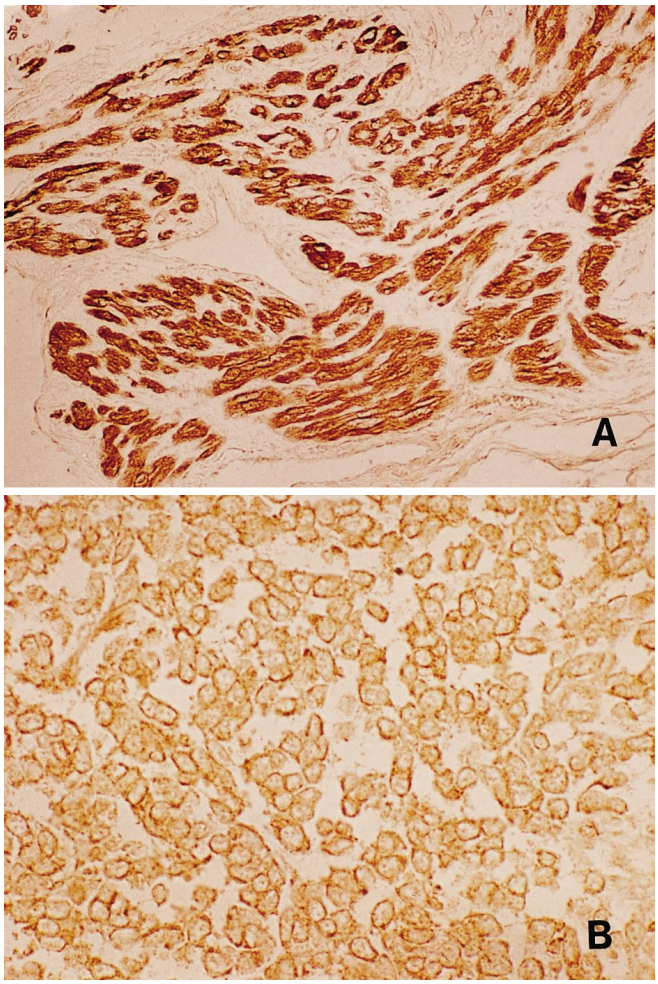

Fig. 2 Strong immunoreactivity for anti-ILK on **A** normal cardiac muscle cells and **B** a Ewing's sarcoma (anti-ILK polyclonal antibody. Streptavidin–biotin complex stain, 1:1,000 dilution, original magnification **A** ×200, **B** ×400)

usefulness of ILK in differentiation of ES/PNET from other small round cell sarcomas, we analysed the expression of ILK in the various types of small round cell sarcomas (Table 3). We also included the other bony lesions that should be differentiated from ES/PNET, including mesenchymal chondrosarcoma, osteoblastoma and osteosarcoma [22].

All 10 ESs, 8 PNETs and 3 medulloblastomas were positive for ILK (Fig. 2B). The staining pattern was intense cell surface membrane staining. Three cases of neuroblastoma (33.3%) were also positive for ILK. This expression pattern suggested a value for ILK as a specific diagnostic marker on tumours with primitive neural differentiation. In ILK-positive cases, most of the tumour cells were strongly stained. In cases of rhabdomyosarcoma, ILK positivitiy was only noted in the cytoplasm of well-differentiated, rhabdomyoblast-like tumour cells, whereas the majority of the undifferentiated small round tumour cells were negative for ILK.

For the comparison between ILK and MIC2 immunoreactivity, a well-known tumour marker of ES/PNET, we

Table 3 Expression of ILK in various types of human small round cell sarcomas in formalin-fixed, paraffin-embedded tissue sections and comparison with expression of MIC2

Tumour types	No. of cases	ILK immunoreactivity	MIC2 immunoreactivity[a]
Ewing's sarcoma	10	10 (100%)	10 (100%)
PNET	8	8 (100%)	7 (87.5%)
Medulloblastoma	3	3 (100%)	1 (33.3%)
Neuroblastoma	9	3 (33.3%)	2 (22.2%)
Retinoblastoma	1	0 (0%)	0 (0%)
Rhabdomyosarcoma	16	0 (0%)	0 (0%)
Lymphoblastic lymphoma	16	0 (0%)	16 (100%)
Mesenchymal chondrosarcoma	4	0 (0%)	0 (0%)
Osteosarcoma	3	0 (0%)	0 (0%)
Osteoblastoma	2	0 (0%)	0 (0%)

[a] Positivity either with O662 or DN16 monoclonal antibody

also stained the small round cell sarcomas using monoclonal antibodies against MIC2, DN16 and O662 (Table 3). MIC2 was expressed in all cases of ES, 7 cases (87.5%) of PNET and 1 case (33.3%) of medulloblastoma. All 16 cases (100%) of lymphoblastic lymphoma were positive for MIC2. These results suggest that ILK is a more specific tumour marker of ES/PNET than MIC2 in the differential diagnosis of ES/PNET from other small round cell tumours, especially lymphoblastic lymphoma.

Discussion

For identification and diagnosis of ES and PNET, it would be useful to have a specific immunohistochemical probe that can be used with formalin-fixed, paraffin-embedded tissues [1, 5–7, 18, 21]. In recent years, two innovative approaches to the positive identification of ES/PNET have been developed and characterized: immunohistochemical study for the expression of MIC2 antigen [10–12, 19, 20] and cytogenetic study for the identification of specific chromosomal aberrations [8, 9, 13, 22, 23].

t(11;22)(q24;q12) is known to be present in 85% of ES/PNET and t(21;22)(q22;q12), in a small proportion of ES/PNET [8, 9, 13, 22, 23]. The diagnostic usefulness of these chromosomal aberrations is considerable, and they can be determined cytogenetically or by way of the detection of translocation transcript by RT-PCR, using RNA extracted from paraffin-embedded tissue samples.

We have found that a polyclonal antibody to ILK consistently immunostains ES/PNET in formalin-fixed, paraffin-embedded tissue sections. In this survey of the distribution of ILK immunoreactivity in various human small round cell sarcomas, all ESs, PNETs and medulloblastomas (cerebellar PNETs) were immunohistochemically positive for ILK, revealing intense cell membrane staining (Table 3, Fig. 2B). This is a remarkable finding, which was comparable to known MIC2 immunoreactivity for positive identification of ES/PNET. Although MIC2 positivity by O662 monoclonal antibody was also noted in all cases of ES/PNET tested except 1 case of PNET, anti-ILK antibody seems to recognize the more stable and constant epitope for positive identification of ES/PNET. One case of MIC2-negative PNET (case 3) showed undifferentiated small cells with positive immunostaining for neuron-specific enolase and revealed ILK positivity. Sixteen cases of lymphoblastic lymphomas were negative for ILK, but all were positive for MIC2. In rhabdomyosarcoma, the majority of undifferentiated tumour cells were ILK negative, although the only plump well-differentiated tumour cells showed positive cytoplasmic staining. Considering the negative ILK immunoreactivity for lymphoblastic lymphomas, these results suggest that ILK can be used as a more specific and sensitive marker for identification of ES/PNET than MIC2. We also noted the ILK immunoreactivity of some (33.3%) neuroblastomas (Table 3), revealing ILK as a possible marker of primitive neural differentiation. The findings that ILK was highly expressed on the tumour cells of both ES and PNET support the notion that Es is a tumour that is closely related to PNET and that both originate from the neuroectoderm [24].

ILK is a recently identified, 59-kDa serine/threonine protein kinase containing an ankyrin-like repeat that interacts with the cytoplasmic domain of the β1-integrin; the mRNA of ILK is widely expressed in various human tissues [15]. Overexpression of ILK disrupts epithelial cell architecture and inhibits adhesion to integrin substrates, while inducing anchorage-independent growth. ILK is therefore thought to be a receptor-proximal protein kinase regulating integrin-mediated signal transduction [15]. We do not know the significance of the relationship of the ILK gene locus (11p15) to the common chromosomal translocation site (11q24) of ES/PNET or its potential role in tumour growth. Although ILK is known to be expressed in a wide spectrum of human tissues at the mRNA level [15], we found, by screening for immunoreactivity for ILK in formalin-fixed, paraffin-embedded tissues, that ILK immunoreactivity was detected only in normal skeletal and cardiac muscle cells (Table 2, Fig. 2A) and that most of the ES/PNET expressed ILK at a consistently high level. This discrepancy in the distribution or expression pattern between mRNA and immunoreactivity could be explained by the difference in the amount of the protein expressed in cells or tissues. Although the biological significance of the unusually high expression of ILK in ES/PNET is currently unknown, we can take advantage of its restricted expression in the differential diagnosis of small round cell sarcomas.

The product of MIC2 gene is a cell surface glycoprotein with a molecular weight of 30 or 32 kDa, often referred to as p30/32 [2, 10, 12, 17]. This glycoprotein locates on the cell surface and appears to be involved in cell adhesion processes [4, 14]. Several antibodies that react with this protein have been described, each recognizing a different epitope on the protein [2, 3, 10, 12, 16, 19]. Although several studies have previously supported the diagnostic usefulness of immunodetection of MIC2 for the identification of ES/PNET, the specificity of this method has recently been criticized. Scotlandi et al. reported that MIC2 was highly expressed in ES/PNET but was also present in a number of small round cell tumours of other cell types [22]. This broad spectrum of positivity for MIC2 in small round cell tumours limits the diagnostic usefulness of this method for differentiation of ES/PNET from other small round cell tumours. Further, activated lymphocytes and several leukaemic cell lines express MIC2 very strongly (unpublished data), and we observed that MIC2 was detected in all cases of lymphoblastic lymphoma by using O662 and DN16 monolconal antibody (Table 3).

This study documented the diagnostic reliability of ILK immunostaining for positive identification of ES/PNET in formalin-fixed and paraffin-embedded tissue sections.

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