Leukemia (2002) 16, 1285–1292

www.nature.com/leu

Non-DNA-binding Ikaros isoform gene expressed in adult B-precursor acute lymphoblastic leukemia

K Nishii[1], N Katayama[1], H Miwa[2], M Shikami[2], E Usui[1], M Masuya[1], H Araki[1], F Lorenzo[1], T Ogawa[3], T Kyo[4], K Nasu[5], H Shiku[1] and K Kita[6]

[1]*The Second Department of Internal Medicine, Mie University School of Medicine, Mie, Japan;* [2]*The Second Department of Internal Medicine, Aichi Medical University School of Medicine, Aichi, Japan;* [3]*Department of Molecular Pathology, Medicinal Biology Research Laboratories, Fujisawa Pharmaceutical Co Ltd, Osaka, Japan;* [4]*Department of Internal Medicine, Hiroshima Red Cross Hospital, Hiroshima, Japan;* [5]*Department of Internal Medicine, Osaka Red Cross Hospital, Osaka, Japan; and* [6]*Tokura Hospital, Kyoto, Japan*

Ikaros, a zinc finger transcription factor, is essential for lymphoid development. Mutant mice expressing dominant-negative Ikaros gene (Ikaros) isoforms develop an aggressive form of lymphoid malignancies. We examined the expression of Ikaros isoforms in 11 leukemic cell lines and adult acute lymphoblastic leukemia cells from 36 patients with B-precursor acute lymphoblastic leukemia (pre-B ALL) and nine with T-precursor acute lymphoblastic leukemia (pre-T ALL), using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. In one pre-B ALL cell line, INC cells, and primary leukemic cells from 16 patients with pre-B ALL, we found the predominant expression of a non-DNA-binding Ikaros isoform, Ik-6. However, Ik-6 was not detected in pre-T ALL cells. All of pre-B ALL cells expressing Ik-6 were CD10$^+$, whereas CD10$^-$ pre-B ALL cells did not express Ik-6. The expression of Ik-6 was not related to karyotype abnormalities such as t(9;22) and t(4;11). Proteins from the cells that expressed Ik-6 alone failed to bind to the Ikaros protein-specific binding sequence in DNA. Ikaros proteins lacking the DNA binding sequences were detected in the cytoplasm but not in the nucleus of the cells. When INC and primary pre-B ALL cells that express Ik-6 alone were irradiated and cultured in the absence of serum, these cells produced functional Ikaros isoforms, Ik-1 and Ik-2. Purified CD19$^+$ CD10$^-$ and CD19$^+$ CD10$^+$ cells from normal human bone marrow did not express Ik-6. The predominant expression of Ik-6, which is the result of post-transcription dysregulation, is characteristic of adult pre-B ALL, especially CD10$^+$ pre-B ALL.
Leukemia (2002) **16,** 1285–1292. doi:10.1038/sj.leu.2402533
Keywords: Ikaros; acute lymphoblastic leukemia; transcription factor; apoptosis; RNA splicing

Introduction

Early B lymphocyte development is characterized by ordered DNA rearrangements of immunoglobulin (Ig) loci. The stages of development can also be distinguished by the expression of specific cell surface markers. Gene targeting experiments in mice have demonstrated that genes such as *Pax–5*,[1,2] *EBF*,[3] *E2A*,[4] *PU.1*,[5] *Oct-2*,[6] *Ikaros*,[7] *RAG-1*[8] and *RAG-2*[9] are required for B cell development. Because B cell development in these mutant mice arrests at an early stage, the precise role of targeted genes is not well understood.

Ikaros, a zinc finger transcription factor, is essential for lymphoid development[7,10,11] and for myeloid cell differentiation.[12] The Ikaros gene (*Ikaros*) encodes, by alternative splicing, at least eight distinct zinc finger isoforms with distinct DNA-binding capabilities, *Ikaros* isoforms, *Ik-1* to *Ik-8*, the expressions of which are restricted to hematopoietic cells.[10] Three of the *Ikaros* isoforms, *Ik-1*, *Ik-2* and *Ik-3*, which contain at least three N-terminal zinc fingers, bind to a sequence of the 4 bp motif GGGA. These DNA-binding isoforms localize in the nucleus. Other isoforms, *Ik-4* to *Ik-8*, which have less than three N-terminal zinc fingers, localize in the cytoplasm and cannot bind to DNA.[10] Mice heterozygous for the *Ikaros* mutation develop lymphoproliferative disorders and ultimately die of lymphoblastic leukemia or lymphoma, a finding which suggests that non-DNA-binding *Ikaros* isoforms may be involved in lymphoid malignancies.[11]

B-precursor acute lymphoblastic leukemia (pre-B ALL) is characterized by clonal proliferation of transformed pre-B cells. Sun *et al*[13] reported that infant pre-B ALL cells with t(4;11) chromosome express dominant-negative and mutant isoforms of Ikaros protein and proposed that Ikaros protein is involved in the leukemogenesis of infant pre-B ALL. Nakase *et al*[14] also showed the expression of dominant-negative Ikaros isoform protein in pre-B ALL. In this study, we also investigated the expression of *Ikaros* isoforms in 11 leukemia cell lines and primary leukemic cells from 45 adult Japanese patients with pre-B or pre-T ALL. The alteration of the expression of *Ikaros* isoforms induced by apoptotic stimulation was also examined.

Materials and methods

Leukemic cell lines

Human pre-B cell lines used include RS(4;11), HPB-null, Reh, INC and NALM6 cells. TSB-2, Jurkat, and MOLT-4 cells were human T cell lines used. Human myeloid cell lines, HL60, Kasumi-1 and U937 cells, were also used. All these lines were maintained at a final concentration of 5×10^5 cells/ml in RPMI 1640 (Nakarai Chemical, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT, USA), 100 U/ml of penicillin–streptomycin, 2 mmol/l L-glutamine, and 5×10^{-5} mol/l 2-mercaptoethanol (2-ME) (Sigma Chemical, St Louis, MO, USA). The cells were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2.

Patients and leukemic cells

Thirty-six Japanese adults with pre-B-ALL and nine with pre-T ALL were investigated after we obtained written informed consent. Leukemic bone marrow (BM) or peripheral blood (PB) samples were collected. Mononuclear cells (MNC) were separated by Ficoll–Hypaque density gradient centrifugation. Samples stored at −190°C in RPMI 1640 with 20% FCS and 10% dimethylsulfoxide were also used, as needed. In all

Correspondence: Dr K Nishii, The Second Department of Internal Medicine, Mie University School of Medicine, 2–174 Edobashi, Tsu, Mie 514–8507, Japan
Received 2 July 2001; accepted 14 February 2002

cases, over 95% of the isolated cells had leukemic cell-like morphology. Immunostaining for leukemic cells was done, as described.[15] Before immunostaining, MNC were treated with 5% heat-inactivated human AB serum to block non-specific binding of antibodies to receptors for IgG Fc portion. CD19 (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), CD20 (Becton Dickinson Immunocytometry Systems), CD22 (Becton Dickinson Immunocytometry System), and CD10 (DAKO, Glostrup, Denmark) were used as B cell markers. CD2, CD3, CD4, CD5, CD7 and CD8, all from Becton Dickinson Immunocytometry Systems, were used as T cell markers. CD11b (Becton Dickinson Immunocytometry Systems), CD13 (CALTAG Laboratories, Burlingame, CA, USA), CD33 (Becton Dickinson Immunocytometry Systems) and CD34 (Becton Dickinson Immunocytometry Systems) were used as myeloid or stem cell markers. For cytoplasmic-IgM (cIgM) staining, the cells were suspended in staining buffer containing 0.05% saponin (Sigma Chemical) and 10 mmol/l N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes), pH 7.3, and stained as for the cell surface staining using anti-human IgM (DAKO). Throughout staining and washing procedures, 0.05% of saponin was kept in buffer. The stained cells were applied to flow cytometric analysis with FACScan (Becton Dickinson Immunocytometry Systems). Cells present in the blast cell gate defined by light scatter were analyzed. Patients with over 50% of blasts expressing CD19, CD22, and HLA-DR (Becton Dickinson Immunocytometry Systems) and lacking surface immunoglobulins were diagnosed as pre-B ALL. Pre-T ALL was diagnosed when over 30% of the leukemic cells was positive for CD7 and cytoplasmic CD3, over 70% was negative for CD19, and the cells lacked myeloperoxidase (MPO) activity. Thirty-eight adult pre-B ALL samples were also categorized into three immunophenotypically defined subgroups:[15–18] stage I, $CD19^+CD10^-CD20^-$; stage II, $CD19^+CD10^+CD20^-$; stage III, $CD19^+CD10^+CD20^+$.

Enrichment of $CD19^+$cells from normal human BM

After obtaining informed consent, BM cells were obtained from healthy volunteers and MNC were separated by Ficoll–Hypaque density gradient centrifugation. MNC were treated with combinations of antibodies to CD19 conjugated to fluorescein isothiocyanate (FITC) (Becton Dickinson Immunocytometry Systems) and CD10 conjugated to phycoerythrin (PE) (DAKO). $CD19^+CD10^+$ and $CD19^+CD10^-$ cells were sorted, using FACS Vantage (Becton Dickinson Immunocytometry Systems), as described previously.[19]

Expression of Ikaros in cell lines or primary leukemic cells by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

RNA was extracted from cell lines or primary leukemic cells and the expression of *Ikaros* was studied using RT-PCR, as described.[17] PCR was performed for 28 cycles, under the following conditions: 1.5 min at 95°C for denaturating, 2 min at 60°C for annealing, and 2 min at 72°C for extending. The oligonucleotide primers were used as follows: *Ikaros* sense, 5′-CCC CCT GTA AGC GAT ACT CCA GAT–3′; *Ikaros* anti-sense, 5′-GGC TTG GTC CAT CAC GTG GGG A-3′; *β-actin* sense, 5′-GTG GGG CGC CCC AGG CAC CA–3′; *β-actin* anti-sense, 5′-GTC CTT AAT GTC ACG CAC GAT TTC–3′.[20] The PCR products were electrophoresed in a 3% agarose gel, in comparison to the molecular-weight ladder (ϕ174 digested by *Hae*III) and stained with ethidium bromide for viewing under UV illumination.

Cloning and sequencing of PCR products

The sequencing analysis of RT-PCR products was performed as previously described.[21] Briefly, the PCR products were purified by electrophoresis and recovered from the gel, using QIAquick Gel Extraction kits (Qiagen, Chatsworth, CA, USA). The recovered DNA was ligated into the pCR2.1 vector (TA cloning kit; Invitrogen, San Diego, CA, USA). Using the method of blue–white selection with X-Gal (TaKaRa Biochem, Kyoto, Japan), five to 10 white colonies were picked up at random and grown overnight in 2.5 ml of Luria-Bertani (LB) medium. Minipreps of plasmid were prepared from cultures by alkaline lysis and purification of DNA affinity columns (QIAprep Spin Plasmid kit; Qiagen), according to the manufacturer's protocol. The double-stranded plasmid was sequenced in an automatic DNA sequencer (Applied Biosystems 373; Applied Biosystems, Foster City, CA, USA), using the dye terminator cycle sequencing method. For each patient, the procedures of PCR and sequencing were performed independently at least twice.

DNA-binding assay

Proteins were extracted from leukemic cell lines or primary leukemic cells and electrophoretic mobility shift assay (EMSA) was carried out, as described.[22] The following ^{32}P-labeled probes containing Ikaros protein binding sequence were used: sense, 5′-GTT TCT TCA GAG CCT *GGG A*AA CAA GTC-3′; anti-sense, 5′-ATT CTG ACT TGT *TTC CC*A GGC TCG AA-3′. The DNA–protein binding interaction was carried out in 20 μl of 10 mM Tris-Cl, pH 8.0 containing 5 mM $MgCl_2$, 100 mM KCl, 1 mM $CaCl_2$, 2 μg poly dI:dC, 20 000 c.p.m. DNA, and 5 μg nuclear protein or cytoplasmic protein extracts. The mixture was incubated at 25°C for 15 min, reacted with 0.25% bromophenol blue and 39% glycerol, and applied to a 6% native polyacrylamide gel (19:1). Electrophoresis was done at 50 V for 6 h in Tris-glycine buffer and the gel was dried and autoradiographed. After a 100-fold molar excess of the competing non-labeled oligonucleotide had been added to the mixture containing the labeled oligonucleotide, the gel shift competition experiments were done. For supershift experiments, 1 μg of polyclonal antibody against Ikaros protein was added to the binding reaction mixture 20 min prior to the addition of the ^{32}P-labeled wild-type probe. Anti-Ikaros polyclonal antibody was kindly provided by Dr Katia Georgopoulos (Harvard Medical School, Boston, MA, USA).

Subcellular localization studies using confocal laser scanning microscopy

The subcellular localization of Ikaros protein was examined by immunofluorescence and confocal laser scanning microscopy, as described previously.[23] Cells were attached to glass cover slips by a 30-min incubation at room temperature, were washed twice with Ca^{2+}, $Mg2^+$-free phosphate-buffered saline (PBS), and then fixed in methanol for 15 min. To permeabilize the cells and block the nonspecific antibody binding

sites, cells were treated with 10% goat serum in PBS for 30 min. To detect Ikaros protein, the cells were incubated with an affinity-purified polyclonal rabbit anti-Ikaros antibody of 1:200 dilution for 2 h at room temperature. Cells were washed with PBS and then incubated with an Alexa 488 goat anti-rabbit IgG (H+L) conjugate highly cross-adsorbed (Molecular Probes, Eugene, OR, USA) at a 1:40 final dilution for 1 h. Finally, nuclei were stained with 0.5 μg/ml propidium iodide (PI) (Sigma Chemical).

Cell culture and irradiation

Leukemic cell lines and primary leukemic cells were cultured in a 25 cm^2 flask at a final concentration of 5×10^5 cells/ml. Five hundred thousand cells were treated with 300 rad of radiation and then cultured without FCS. RNA was extracted from the cells before and after several hours of re-culture, then, expression of *Ikaros* isoforms in each sample was examined.

Results

Expression of Ikaros and sequencing PCR products in leukemic cell lines

We first investigated the expression of *Ikaros* in several leukemic cell lines, using RT-PCR analysis. As shown in Figure 1 and Table 1, the expression of *Ikaros* was detected in all leukemic cell lines tested. Interestingly, size of the PCR product from INC cells, whose phenotype is $CD19^+CD10^+CD20^+$, was small. As the *Ikaros* encodes, by alternate splicing, at least eight functionally distinct zinc finger proteins,[10] we next determined if these RT-PCR products from leukemic cell lines were the isoforms of *Ikaros*. Sequencing analysis revealed that the long product of *Ikaros* from NALM6 cells was *Ik-1*, while the shortest product of *Ikaros* from INC cells was a non-DNA-binding isoform of *Ikaros*, *Ik-6*. Mutation or deletion sequence of *Ik-6* from INC cells was nil (data not shown). We also confirmed that other RT-PCR products were *Ik-2/3* and *Ik-4*. In our RT-PCR products from leukemic cell lines, *Ikaros* isoforms of *Ik-5*, *Ik-7* and *Ik-8* were not detected.

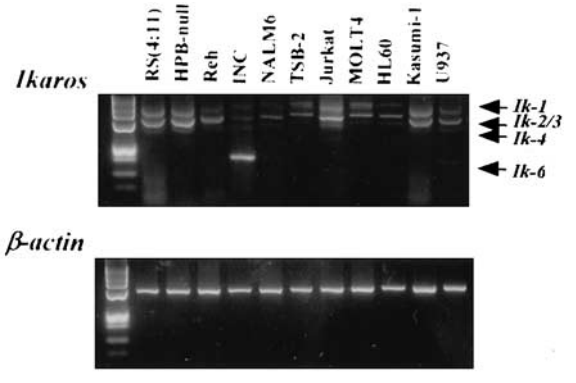

Figure 1 *Ikaros* isoforms are expressed in leukemic cell lines. Expression of *Ikaros* isoforms was examined in leukemic cell lines, using RT-PCR analysis. PCR products were detected by ethidium bromide staining of 3% agarose gels after 28 cycles of amplification. Sequence analysis of the PCR products indicated that the smallest and largest products were *Ik-6* and *Ik-1*, respectively. The arrows indicate the positions of *Ik-1*, *Ik-2/3*, *Ik-4* and *Ik-6*. The expression of *β-actin* served as a control. The left lane is the molecular size marker, ϕ174 digested by *Hae*III.

Table 1 Expression of *Ikaros* isoforms in leukemic cell lines

Cell line	*Subtype*	*Isoforms*			
		Ik-1	*Ik-2/3*	*Ik-4*	*Ik-6*
RS(4;11)	pre-B	+	+	±	−
HPB-null	pre-B	+	+	±	−
Reh	pre-B	+	+	±	−
INC	pre-B	−	−	−	+
NALM6	pre-B	+	+	±	−
TSB-2	pre-T	+	+	±	−
Jurkat	pre-T	+	+	±	−
MOLT4	pre-T	+	+	±	−
HL60	Myeloid	+	+	±	−
Kasumi-1	Myeloid	+	+	±	−
U937	Myeloid	+	+	±	−

Expression of *Ikaros* gene was examined, using RT-PCR analysis. +, expressed; ±, weakly expressed; −, not expressed.

DNA-binding of Ikaros protein in INC and NALM6 cells

DNA-binding capacity of the protein extracts from INC and NALM6 cells was tested, using EMSA (Figure 2a). We used the probe which contained the Ikaros protein binding site.[10] When protein extracts were incubated with the radiolabeled oligonucleotide probe, protein–DNA complex bands were evident in the extracts from NALM6 cells (arrow). In contrast, in the extracts from INC cells only one very weak protein–DNA complex band was found. We cannot confirm whether a small amount of DNA-binding Ikaros protein exists in INC cells or this weak band is caused by nonspecific binding. Binding specificity of the oligonucleotide probe was also examined using a 100-fold molar excess unlabeled oligonucleotide probe. To determine if Ikaros protein was present in the complexes, supershift EMSA analysis was performed using the anti-Ikaros antibody. Upon addition of the anti-Ikaros antibody, specific protein–DNA complexes (Figure 2b, arrow 1) were reduced and the shifted band (arrow 2) was considerably detected in the protein extracts from NALM6. These results suggest that DNA-binding Ikaros proteins are expressed in NALM6 cells but not in INC cells.

Subcellular localization of Ikaros protein in leukemic cells

We examined the subcellular compartmentalization of Ikaros protein in pre-B ALL cell lines, NALM6 and INC cells, using confocal laser scanning microscopy (Figure 3). Cells were stained with Ikaros antibody (FITC, green) and nuclei were stained with PI (red). In NALM6 cells which expressed *Ik-1* and *Ik-2/3* but not *Ik-6*, Ikaros protein was stained in the nuclei. In contrast, the expression of Ikaros protein was detected in only the cytoplasm of INC cells which expressed *Ik-6* alone. These observations are consistent with previous reports showing nuclear and cytoplasmic localizations of *Ik-1* and *Ik-6* proteins, respectively.[23–25]

Expression of Ikaros isoforms in primary leukemic cells

We next examined the expression of *Ikaros* in adult primary acute leukemic cells, including pre-B ALL and pre-T ALL cells,

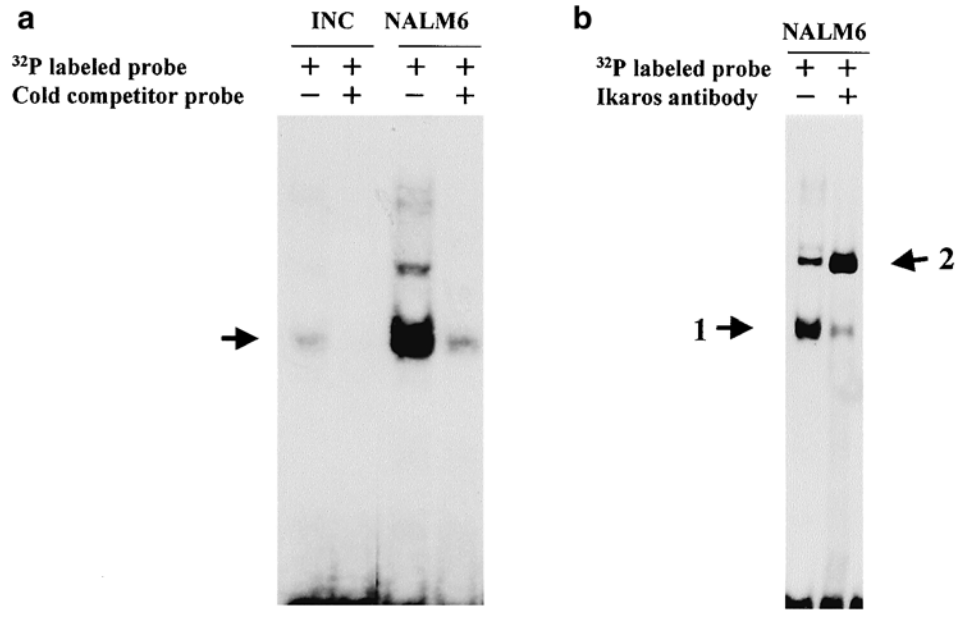

Figure 2 Proteins extracted from INC cells cannot bind to the Ikaros protein-binding site of DNA. EMSA was done, using a ^{32}P-labeled oligonucleotide containing the Ikaros protein-binding sequence. (a) ^{32}P-labeled oligonucleotide was incubated with protein extracts from INC and NALM6 cells. The reaction mixture was also pre-incubated with a 100-fold molar excess of an unlabeled wild-type oligonucleotide. (b) ^{32}P-labeled probe was incubated with proteins from NALM6 cells, and then 1 μg of polyclonal antibody against Ikaros protein was added to the binding reaction mixtures for 20 min.

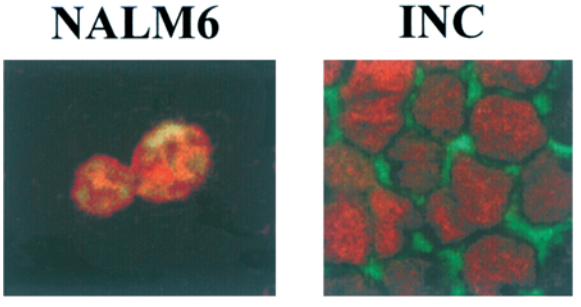

Figure 3 Ikaros protein expression was located in the cytoplasm of the cells expressing *Ik-6* alone. Localization of Ikaros protein in NALM6 and INC cells was analyzed, using confocal laser scanning microscopy. The cells were stained with a rabbit polyclonal antibody against Ikaros protein, followed by a FITC-conjugated anti-rabbit goat antibody (green). The nuclei of these cells were also stained using PI (red). NALM6 cells showed the expression of Ikaros protein only in the nuclei (seen as yellow).

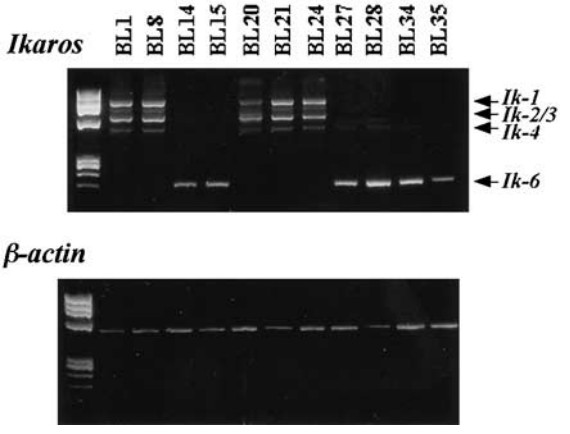

Figure 4 *Ik-6* is expressed in primary adult pre-B ALL cells. Expression of *Ikaros* isoforms in primary adult pre-B ALL cells was examined, using RT-PCR analysis. We used *Ikaros* primers and *β-actin* primers. The bands were detected by ethidium bromide staining. The left lane is the molecular size marker, ϕ174 digested by *Hae*III. Arrows indicate positions of *Ik-1*, *Ik-2/3*, *Ik-4* and *Ik-6*. Case BL1, stage I; cases BL8, BL14 and BL15, stage II; cases BL20, Bl21, BL24, BL27, BL28, BL34 and BL35, stage III. Leukemia samples above the lanes correspond with those in Table 2.

using RT-PCR analysis. All tested pre-B ALL cells expressed several isoforms of *Ikaros*. Predominant expression of *Ik-6* was detected in 16 of 36 of pre-B ALL samples. Interestingly, functional *Ikaros* isoforms, *Ik-1* and *Ik-2/Ik-3*, were not detected in *Ik-6*-expressing cells (Figure 4 and Table 2). Several isoforms of *Ikaros* were also expressed in nine pre-T ALL samples under our PCR condition, however, the expression of *Ik-6* was nil (Figure 5 and Table 3). These results suggest that a non-DNA-binding *Ikaros* isoform, *Ik-6*, was expressed in pre-B ALL but not pre-T ALL in adults. It should be noted that *Ik-6* was frequently expressed in stages II and III pre-B ALL, whereas *Ik-6* was not detected in CD19$^+$CD10$^-$ stage I pre-B ALL cells. Among stage III pre-B ALL, all cIgM$^+$ pre-B ALL cells expressed *Ik-6* (Table 4). The association of *Ik-6* expression with karyotype abnormalities such as t(9;22) or t(4;11) was not observed (Table 2). To determine if chemotherapy would alter the expression of *Ikaros* isoforms, we studied the expression manner of *Ikaros* isoforms in leukemic cells obtained from four pre-B ALL patients, cases BL11, BL12, BL17 and BL19, prior to chemotherapy and after relapse. The expression manner of *Ikaros* isoforms in cases of relapse was the same as that seen prior to chemotherapy (Table 2).

Table 2 Expression of *Ikaros* isoforms in primary pre-B ALL cells

Stage	Case	CD19	CD10	CD20	cIgM	Karyotype	Isoforms			
							Ik-1	Ik-2/3	Ik-4	Ik-6
I	BL1	+	−	−	−	46,XX,t(4;11)(q21;q23)	+	+	±	−
	BL2	+	−	−	−	46,XX	+	+	±	−
	BL3	+	−	−	−	46,XX,t(4;11)(q21;q23)	+	+	±	−
	BL4	+	−	−	−	46,XY	+	+	±	−
	BL5	+	−	−	−	45,XX,dic(11;14)(11pter-cen-11q25; 14q32-cen-14p13)	+	+	±	−
II	BL6	+	+	−	−	46,XX,t(9;22)(q34;q11)	+	+	±	−
	BL7	+	+	−	−	45,XX,t(9;22)(q34;q11),−7	+	+	±	−
	BL8	+	+	−	−	46,XY,t(9;22)(q34;q11)	+	+	±	−
	BL9	+	+	−	−	46,XY,t(9;22)(q34;q11)	+	+	±	−
	BL10	+	+	−	−	46,XX	+	+	±	−
	BL11	+	+	−	−	46,XY,t(9;22)(q34;q11)	+	+	±	−
	(BL11R)	(+)	(+)	(−)	(−)		(+)	(+)	(±)	(−)
	BL12	+	+	−	−	46,XX,	+	+	±	−
	(BL12R)	(+)	(+)	(+)	(−)		(+)	(+)	(±)	(−)
	BL13	+	+	−	−	46,XX,t(9;22)(q34;q11)	+	+	±	−
	BL14	+	+	−	NT	46,XX,t(1;19)	−	−	−	+
	BL15	+	+	−	−	46,XY,t(9;22)(q34;q11)	−	−	−	+
	BL16	+	+	−	+	46,XY,t(9;22)(q34;q11)	−	−	±	+
	BL17	+	+	−	−	46,XX,del 9q22	−	−	−	+
	(BL17R)	(+)	(+)	(−)	(−)		(−)	(−)	(−)	(+)
	BL18	+	+	−	−	46,XX,t(9;22)(q34;q11)	−	−	−	+
III	BL19	+	+	+	−	46,XY,t(9;22)(q34;q11)	+	+	−	−
	(BL19R)	(+)	(+)	(+)	(−)		(+)	(+)	(±)	(−)
	BL20	+	+	+	−	46,XX,t(9;22)(q34;q11)	+	+	±	−
	BL21	+	+	+	−	46,XX,t(9;22)(q34;q11)	+	+	±	−
	BL22	+	+	+	NT	46,XY,t(9;22)(q34;q11)	+	+	±	−
	BL23	+	+	+	−	45,XY,−7	+	+	±	−
	BL24	+	+	+	−	46,XX,t(9;22)(q34;q11)	+	+	±	−
	BL25	+	+	+	−	46,XY,t(9;22)(q34;q11)	+	+	±	−
	BL26	+	+	+	−	46,XY	+	+	±	+
	BL27	+	+	+	NT	46,XY	−	−	±	+
	BL28	+	+	+	+	46,XX	−	−	±	+
	BL29	+	+	+	−	46,XX	−	−	±	+
	BL30	+	+	+	+	46,XY	−	−	±	+
	BL31	+	+	+	NT	46,XX	−	−	−	+
	BL32	+	+	+	+	46,XX	−	−	−	+
	BL33	+	+	+	+	46,XY	−	−	−	+
	BL34	+	+	+	+	46,XY,t(5;9;22),t(7;12),del(9)(p22)	−	−	−	+
	BL35	+	+	+	−	46,XX,t(9;22)(q34;q11)	−	−	−	+
	BL36	+	+	+	+	46,XY	−	−	±	+

Phenotypical analysis of pre-B ALL was done, using flow cytometry. Expression of *Ikaros* isoforms in each sample was examined, using RT-PCR analysis. BL11R, BL12R, BL17R and BL19R were cases in relapse.
BL, pre-B ALL; NT, not tested; +, expressed; ±, weakly expressed; −, not expressed.

Expression of Ikaros in normal human BM pre-B cells

Klug *et al*[12] reported that murine BM progenitor cells expressed *Ik-6*, and also Payne *et al*[26] showed that a small population of the *Ik-6*-expressing cells may exist in normal human BM. To determine if a population of normal human pre-B cells expresses *Ik-6*, $CD19^+CD10^+$ and $CD19^+CD10^-$ cells were enriched from normal human adult BM by sorting. The sorting windows are in Figure 6a. As shown in Figure 6b, *Ik-6* was not detected in sorted $CD19^+CD10^+$ and $CD19^+CD10^-$ pre-B cells in our 28 cycle RT-PCR analysis. These findings suggest that the expression of *Ik-6* detected in primary pre-B ALL cells is not due to contamination of normal BM pre-B cells.

Alteration of Ikaros isoform expression by apoptotic stimulation

As it has been reported that *Ikaros* isoforms are produced by alternative splicing, it is possible that the expression of these isoforms may change in response to environmental factors such as apoptosis-inducing stimulation. Exposure to X-rays and withdrawal of FCS are well known to induce apoptosis on leukemic cells,[27,28] therefore, we next studied the effects of these stimulations on the expression of *Ikaros* isoforms in NALM6 and INC cells (Figure 7). We re-cultured NALM6 and INC cells in the absence of FCS following their exposure to X-rays. RNA was extracted from the cells before and after 4, 6 and 24 h of re-culture and the expression of *Ikaros* isoforms

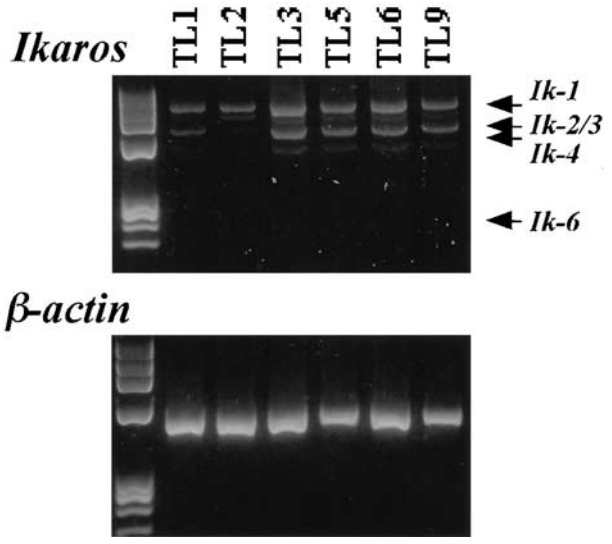

Figure 5 *Ik-6* is not expressed in primary adult pre-T ALL cells. Expression of *Ikaros* isoforms in primary adult pre-T ALL cells was examined, using RT-PCR analysis. We used Ikaros primers and *β-actin* primers. The bands were detected by ethidium bromide staining. The left lane is the molecular size marker, ϕ174 digested by *Hae*III. Arrows indicate the positions of *Ik-1*, *Ik-2/3*, *Ik-4* and *Ik-6*. Leukemia samples the lanes correspond with those in Table 3.

was examined, using RT-PCR analysis. Whereas the expression of *Ikaros* isoforms in NALM6 cells was not modulated by exposure to X-rays and FCS withdrawal, INC cells, in which *Ik-6* alone was detected before the apoptotic stimulation, did produce *Ik-1*. Interestingly, when FCS was added again to the culture of INC cells which expressed *Ik-1* after irradiation and FCS withdrawal, the expression of *Ik-1* decreased. The effects of apoptotic stimulation on *Ikaros* isoform expression of primary pre-B ALL cells were also examined. We selected cases BL12 and BL19 as representatives of pre-B ALL cells which express *Ik-1*, and cases BL31 and BL35 as representative of *Ik-6*-expressing cells. The expression of *Ikaros* isoforms in pre-B ALL cells from cases BL12 and BL19 did not alter. Similar to the observations with INC cells, pre-B ALL cells from cases BL31 and BL35 did produce *Ik-1* in response to apoptosis-inducing stimulation. These results indicate that primary leukemic cells expressing *Ik-6* alone have the potential to produce *Ik-1*.

Discussion

Mice homozygous for the mutation of *Ikaros* lack lymphocytes, NK cells and their progenitors because of a complete block in early lymphocyte development.[29] Sun *et al*[13] reported the non-DNA-binding expression of *Ikaros* in infant pre-B ALL cells and proposed the involvement of non-DNA-binding *Ikaros* isoforms in the leukemogenesis of infant pre-B ALL. Their infant pre-B ALL cells with a non-DNA-binding *Ikaros* isoform had t(4;11) karyotype and $CD19^+CD10^-CD20^-$ phenotype.[13] Nakayama *et al*[30] showed that a non-DNA-binding *Ikaros* isoform, *Ik-6*, is expressed in the cells of lymphoid crisis in chronic myelogenous leukemia. In our adult pre-B ALL cases, no relationship between the expression of *Ik-6* and karyotypes such as t(4;11) or t(9;22) was found. Indeed, adult pre-B ALL cells of cases BL1 and BL3 with the t(4;11) karyotype did not express *Ik-6*. Surprisingly, the expression of *Ik-6* was detected in $CD19^+CD10^+$ but not in $CD19^+CD10^-$ pre-B ALL cells, suggesting that the expression of *Ik-6* in pre-B ALL may be restricted to $CD10^+$ pre-B ALL cells in adults. These findings imply that the characteristics of pre-B ALL, including leukemogenesis, differ between adult and infant patients. It was reported that infant pre-B ALL may originate from an *in utero* initiating event.[31,32] Exploration of the origin of adult pre-B ALL will aid in understanding the differences between adult and infant pre-B ALL. Our finding that expression of *Ik-6* was frequently detected in $cIgM^+$ pre-B ALL cells but not in normal BM pre-B cells suggests that the expression of non-DNA-binding *Ikaros, Ik-6,* may play an important role in the characteristics of adults pre-B ALL. Further studies are necessary to address this issue.

Mice heterozygous for the mutation of the *Ikaros* develop

Table 4 Expression of *Ik-6* in primary pre-B ALL

Stage	*% Positively of Ik-6*
I	0 (0/5)
II	38.5 (5/13)
III	61.1 (11/18)
$cIgM^-$	33.3 (3/9)
$cIgM^+$	100 (6/6)

Numbers in parentheses depict the number of *Ik-6*-expressing samples per total number of samples tested.

Table 3 Expression of *Ikaros* isoforms in primary pre-T ALL cells

Case	*CD7*	*CD3*	*CD4*	*CD8*	*Karyotype*	*Isoforms*			
						Ik-1	*Ik-2/3*	*Ik-4*	*Ik-6*
TL1	+	−	−	−	47,XY,+5	+	+	−	−
TL2	+	−	−	−	NT	+	±	−	−
TL3	+	−	−	−	NT	+	+	±	−
TL4	+	−	−	−	44,X,-Y,del(6)8(q15;q23), −10,+11,−14,−16	+	+	±	−
TL5	+	+	−	−	46,XY	+	+	±	−
TL6	+	+	−	−	NT	+	+	±	−
TL7	+	+	+	+	46,XY	+	+	−	−
TL8	+	+	+	+	46,XY,t(11;14)(p13;q11)	+	+	±	−
TL9	+	−	+	−	46,XX	+	+	±	−

Phenotypical analysis of pre-T ALL cells was done, using flow cytometry. Expression of *Ikaros* isoforms in each sample was examined using RT-PCR analysis.
TL, pre-T ALL; NT, not tested; +, expressed; ±, weakly expressed; −, not expressed.

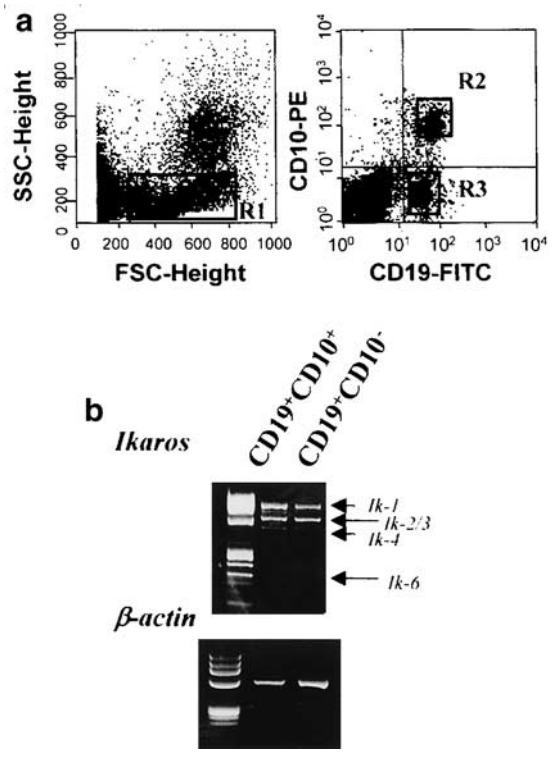

Figure 6 *Ik-6* is not expressed in normal BM pre-B cells. (a) Bone marrow MNC were stained with FITC-conjugated anti-CD19 and PE-conjugated anti-CD10 antibodies. The lymphoblastic region (R1) was gated, based on FSC and SSC heights. Cells in the R2 and R3 regions were sorted as $CD19^+CD10^+$ and $CD19^+CD10^-$ cells, respectivley, using an FACS Vantage. Purity of the sorted cells in each fraction exceeded 99%. (b) Expression of *Ikaros* isoforms in $CD19^+CD10^+$ and $CD19^+CD10^-$ cells was examined by RT-PCR analysis. We also used *β-actin* primers as a control. The bands were detected by ethidium bromide staining. The left lane is the molecular size marker, ϕ174 digested by *Hae*III.

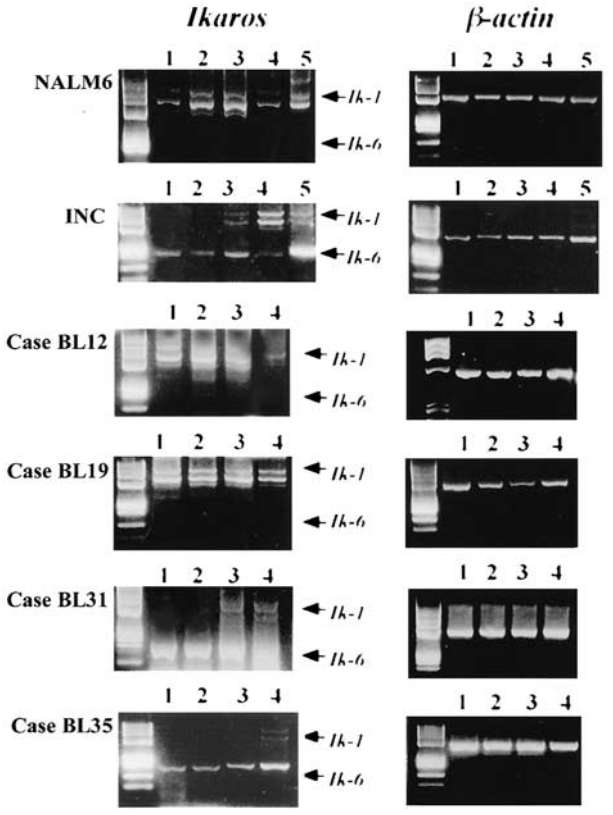

Figure 7 Expression of *Ikaros* isoforms is modulated by apoptosis-inducing stimulation. Effects of FCS withdrawal following exposure to X-rays on the expression of *Ikaros* isofroms were examined in NALM6, INC and primary pre-B ALL cells from cases BL12, BL19, BL31 and BL35, using RT-PCR analysis. We used *Ikaros* primers and *β-actin* primers. The bands were detected by ethidium bromide staining. Lane 1, before; lane 2, 4 h, lane 3, 6 h, lane 4, 24 h after FCS withdrawal following exposure to X-rays; lane 5, FCS was added again to the culture medium 24 h after exposure to X-rays, then, RNA was isolated 48 h later. The results are representative of three independent experiments.

T cell leukemia and/or lymphoma.[11] In humans, the expression of *Ik-6* and/or an inframe deletion of *Ikaros* was detected in childhood pre-T ALL.[23] In the present study, the expression of *Ik-6* was not detected in adult pre-T ALL samples and mutation or deletion in *Ik-1* in these samples was nil. This discrepancy may also result from differences between adult and childhood pre-T ALL. It can be speculated that the abnormal expression of *Ikaros* isoforms may occur in B cell precursors but not in T cell precursors in adults.

We observed that INC and primary pre-B ALL cells which express *Ik-6* alone led to the production of *Ik-1* in response to apoptotic stimulation, including irradiation and starvation. Moreover, when the cells were re-exposed to FCS, the level of *Ik-1* decreased. These results suggest that *Ik-6*-expressing cells have the potential to produce other *Ikaros* isoforms in response to their environmental alteration. Signals which are antagonized by apoptotic stimulation induced by irradiation and starvation may give rise to the predominant expression of *Ik-6* in adult pre-B ALL. Further studies on the mechanism(s) of predominant expression of *Ik-6* are expected to determine the clinical relevance of post-transcriptional dysregulation of *Ikaros* isoforms in adult pre-B ALL.

Acknowledgements

We thank Professor MF Greaves and Dr T Ford (Leukemia Research Fund Centre at the Institute of Cancer Research, London, UK) for helpful discussion. We also thank Professor K Georgopoulos (Massachusetts General Hospital, Harvard Medical School, Boston, MA) for providing the anti-Ikaros antibody and we thank Ms C Atsumaru (Fujisawa Pharmaceutical Co Ltd, Osaka, Japan) for providing technical advice. This work was supported by research grants from the Ministry of Education, Culture, Sports, Science, and Technology and from the Ministry of Health, Labour, and Welfare, Japan and a Grant-in-Aid from the Mie Medical Research Foundation.

References

1 Kozmik Z, Wang S, Dorfler P, Adams B, Busslinger M. The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. *Mol Cell Biol* 1992; **12**: 2662–2672.
2 Urbánek P, Wang Z-Q, Fetka I, Wagner EF, Busslinger M. Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell* 1994; **79**: 901–912.

3 Lin H, Grosschedl R. Failure of B-cell differentiation in mice lacking the transcription factor EBF. *Nature* 1995; **376**: 263–267.
4 Zhuang Y, Soriano P, Weintraub H. The helix–loop–helix gene E2A is required for B cell formation. *Cell* 1994; **79**: 875–884.
5 Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 1994; **265**: 1573–1577.
6 Thévenin C, Lucas BP, Kozlow EJ, Kehrl JH. Cell type- and stage-specific expression of the CD20/B1 antigen correlates with the activity of a diverged octamer DNA motif present in its promoter. *J Biol Chem* 1993; **268**: 5949–5956.
7 Georgopoulos K, Moore DD, Derfler B. Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. *Science* 1992; **258**: 808–812.
8 Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 1992; **68**: 869–877.
9 Shinkai Y, Rathbun G, Lam K-P, Oltz EM, Stewart V, Mendelsohn M, Charron J, Datta M, Young F, Stall AM, Alt FW. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 1992; **68**: 855–867.
10 Molnár Á, Georgopoulos K. The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. *Mol Cell Biol* 1994; **14**: 8292–8303.
11 Winandy S, Wu P, Georgopoulos K. A dominant mutation in the Ikaros gene leads to rapid development of leukemia and lymphoma. *Cell* 1995; **83**: 289–299.
12 Klug CA, Morrison SJ, Masek M, Hahm K, Smale ST, Weissman IL. Hematopoietic stem cells and lymphoid progenitors express different Ikaros isoforms, and Ikaros is localized to heterochromatin in immature lymphocytes. *Proc Natl Acad Sci USA* 1998; **95**: 657–662.
13 Sun L, Heerema N, Crotty L, Wu X, Navara C, Vassilev A, Sensel M, Reaman GH, Uckun FM. Expression of dominant-negative and mutant isoforms of the antileukemic transcription factor Ikaros in infant acute lymphoblastic leukemia. *Proc Natl Acad Sci USA* 1999; **96**: 680–685.
14 Nakase K, Ishimaru F, Avitahl N, Dansako H, Matsuo K, Fujii K, Sezaki N, Nakayama H, Yano T, Fukuda S, Imajoh K, Takeuchi M, Miyata A, Hara M, Yasukawa M, Takahashi I, Taguchi H, Matsue K, Nakao S, Niho Y, Takenaka K, Shinagawa K, Ikeda K, Niiya K, Harada M. Dominant negative isoform of the Ikaros gene in patients with adult B-cell acute lymphoblastic leukemia. *Cancer Res* 2000; **60**: 4062–4065.
15 Nishii K, Katayama N, Miwa H, Shikami M, Masuya M, Shiku H, Kita K. Survival of human leukaemic B-cell precursors is supported by stromal cells and cytokines: association with the expression of bcl-2 protein. *Br J Haematol* 1999; **105**: 701–710.
16 Foon KA, Todd RF III. Immunologic classification of leukemia and lymphoma. *Blood* 1986; **68**: 1–31.
17 Nishii K, Kita K, Miwa H, Shikami H, Taniguchi M, Usui E, Katayama N, Shiku H. Expression of B-cell associated transcription factors in precursor B-cell acute lymphoblastic leukemia cells: association with PU.1 expression, phenotype, and immunogenotype. *Int J Hematol* 2000; **71**: 372–378.
18 Lemmers B, Arnoulet C, Fossat C, Chambost H, Sainty D, Gabert J, Schiff C. Fine characterization of childhood and adult acute lymphoblastic leukemia (ALL) by a proB and preB surrogate light chain-specific mAb and a proposal for a new B cell ALL classification. *Leukemia* 2000, **14**: 2103–2111.
19 Ohishi K, Katayama N, Mitani H, Araki H, Masuya M, Suzuki H, Hoshino N, Miyashita H, Nishii K, Kageyama S-I, Minami N, Shiku H. Efficient *ex vivo* generation of human dendritic cells from mobilized CD34+ peripheral blood progenitors. *Int J Hematol* 2001; **74**: 287–296.
20 Molnár Á, Wu P, Largespada DA, Vortkamp A, Scherer S, Copeland NG, Jenkins NA, Bruns G, Georgopoulos K. The Ikaros gene encodes a family of lymphocyte-restricted zinc finger DNA binding proteins, highly conserved in human and mouse. *J Immunol* 1996; **156**: 585–592.
21 Taniguchi M, Oka K, Hiasa A, Yamaguchi M, Ohno T, Kita K, Shiku H. *De novo* CD5+ diffuse large B-cell lymphomas express VH genes with somatic mutation. *Blood* 1998; **91**: 1145–1151.
22 Shikami M, Miwa H, Nishii K, Takahashi T, Sekine T, Mahmud N, Nishikawa M, Shiku H, Kamada N, Kita K. Low BCL-2 expression in acute leukemia with t(8;21) chromosomal abnormality. *Leukemia* 1999; **13**: 358–368.
23 Sun L, Goodman PA, Wood CM, Crotty M-L, Sensel M, Sather H, Navara C, Nachman J, Steinherz PG, Gaynon PS, Seilbel N, Vassilev A, Juran BD, Reaman GH, Uckun FM. Expression of aberrantly spliced oncogenic Ikaros isoforms in childhood acute lymphoblastic leukemia. *J Clin Oncol* 1999; **17**: 3753–3766.
24 Sun L, Liu A, Georgopoulos K. Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development. *EMBO J* 1996; **15**: 5358–5369.
25 Sun L, Crotty M-L, Sensel M, Sather H, Navara C, Nachman J, Steinherz PG, Gaynon PS, Seibel N, Mao C, Vassilev A, Reaman GH, Uckun FM. Expression of dominant-negative Ikaros isoforms in T-cell acute lymphoblastic leukemia. *Clin Cancer Res* 1999; **7**: 2112–2120.
26 Payne KJ, Nicolas J-H, Zhu JY, Barsky LW, Crooks GM. Cutting edge: predominant expression of a novel Ikaros isoform in normal human hemopoiesis. *J Immunol* 2001; **167**: 1867–1870.
27 Daniel PT. Dissecting the pathway to death. *Leukemia* 2000; **14**: 2035–2044.
28 O'Gorman DM, Cotter TG. Molecular signals in anti-apoptotic pathways. *Leukemia* 2001; **15**: 21–34.
29 Georgopoulos K, Bigby M, Wang J-H, Molnar A, Wu P, Winandy S, Sharpe A. The Ikaros gene is required for the development of all lymphoid lineages. *Cell* 1994; **79**: 143–156.
30 Nakayama H, Ishimaru F, Avitahl N, Sezaki N, Fujii N, Nakase K, Ninomiya Y, Harashima A, Minowada J, Tsuchiyama J, Imajoh K, Tsubota T, Fukuda S, Sezaki T, Kojima K, Hara M, Takimoto H, Yorimitsu S, Takahashi I, Miyata A, Taniguchi S, Tokunaga Y, Gondo H, Niho Y, Nakao S, Kyo T, Dohy H, Kamada N, Harada M. Decreases in Ikaros activity correlate with blast crisis in patients with chronic myelogenous leukemia. *Cancer Res* 1999; **59**: 3931–3934.
31 Greaves M. Molecular genetics, natural history and the demise of childhood leukaemia. *Eur J Cancer* 1999; **35**: 173–185.
32 Wiemels JL, Ford AM, van Wering ER, Postma A, Greaves M. Protracted and variable latency of acute lymphoblastic leukemia after TEL-AML1 gene fusion *in utero*. *Blood* 1999; **94**: 1057–1062.