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PAX5 Expression in Acute Leukemias: Higher B-Lineage Specificity Than CD79a and Selective Association with t(8;21)-Acute Myelogenous Leukemia

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ABSTRACT

The transcription factor PAX5 plays a key role in the commitment of hematopoietic precursors to the B-cell lineage, but its expression in acute leukemias has not been thoroughly investigated. Hereby, we analyzed routine biopsies from 360 acute leukemias of lymphoid (ALLs) and myeloid (AMLs) origin with a specific anti-PAX5 monoclonal antibody. Blasts from 150 B-cell ALLs showed strong PAX5 nuclear expression, paralleling that of CD79a in the cytoplasm. Conversely, PAX5 was not detected in 50 T-cell ALLs, including 20 cases aberrantly coexpressing CD79a. Among 160 cytogenetically/molecularly characterized AMLs, PAX5 was selectively detected in 15 of 42 cases bearing the t(8;21)/AML1-ETO rearrangement. Real-time reverse transcription-PCR studies in t(8;21)-AML showed a similar up-regulation of PAX5 transcript in all of the 8 tested samples (including 4 cases that were negative at anti-PAX5 immunostaining), suggesting that PAX5 is expressed in t(8;21)-AML more widely than shown by immunohistochemistry. Interestingly, PAX5$^+$ t(8;21)-AML also expressed CD79a and/or CD19 (major transcriptional targets of PAX5 in B-cells) in 10 of 12 evaluable cases. Our results indicate that PAX5 is a more specific marker than CD79a for B-cell ALL diagnosis. Moreover, among AMLs, PAX5 expression selectively clusters with t(8;21), allowing its immunohistochemical recognition in a proportion of cases, and likely explaining a peculiar biological feature of this subset of myeloid leukemias, *i.e.* the aberrant expression of B-cell genes.

INTRODUCTION

PAX5, also named B-cell specific activator protein (BSAP), is a member of the highly conserved paired box (PAX)-domain family of transcription factors (1). Targeted disruption of the *PAX5* gene in mice blocks B-cell development at the earliest B-lineage committed precursor in the fetal liver and at the early pro-B–cell stage in the adult bone marrow (2), thus providing evidence for the key role of PAX5 in the control of early B-lymphopoiesis. In particular, PAX5 fulfills a dual role in the commitment of the bone marrow multipotent progenitor to the B-lymphoid lineage (3), because it favors V_H to D_HJ_H gene rearrangement (2) and activates the expression of B-cell–specific genes [*e.g. CD19* (4), *Blk* (5, 6), *mb-1/CD79a* (7, 8), and *RAG2* (9)], while simultaneously repressing the lineage-promiscuous transcription of genes involved in other hematopoietic differentiation programs [such as *Notch1* (10), *GATA-1*, *perforin,* and *M-CSF-R* (3)]. Moreover, PAX5 maintains the identity and function of B cells during late B-lymphopoiesis (11, 12). Accordingly, the expression of PAX5 is restricted to the B-lineage (13–15), initiating in pro-B cells (2) and reaching the maximum level at the pre-B and mature B-cell differentiation stages (13). Conversely, the PAX5 protein is not detectable in terminally differentiated plasma cells (13, 16). PAX5 expression also occurs in the adult testis and in the mesencephalon and spinal cord during embryogenesis (17), suggesting an important role in the development of these tissues.

Rearrangement of the *PAX5* gene through reciprocal chromosomal translocations has been described in different types of B-cell malignancies (18–23), and, more recently, *PAX5* has also been shown to be targeted by aberrant hypermutation in >50% of diffuse large B-cell lymphomas (24). Immunohistochemical studies with specific polyclonal (14) and monoclonal antibodies (25) have clearly shown that PAX5 expression is restricted to lymphomas of B-cell lineage, including classical Hodgkin's lymphoma (15). The latter finding has been taken as additional evidence for the B-cell origin of Hodgkin's and Reed-Sternberg cells (15), and it has been proposed that immunostaining for PAX5 may be of value in the differential diagnosis between tumor cell-rich Hodgkin's lymphoma (PAX5$^+$) and ALK-negative T/null anaplastic large cell lymphoma (PAX5$^-$; ref. 15).

Despite the extensive studies on lymphomas, only a few data are available on PAX5 expression in acute leukemias that are either based on the detection of PAX5 at the mRNA level [(26) and not at the protein level] or limited to the description of PAX5 protein expression only in B-cell ALL (but not in T-cell ALL nor in AML) and only in comparison to that of CD20 (25) (but not to that of CD79a, which is a far more reliable marker of precursor B-cell neoplasms).

In this article, we used a specific monoclonal antibody (mAb) to investigate the expression of PAX5 in routine biopsies from a large number of acute leukemias of different lineages. The following evidence is provided: (*a*) PAX5 is a more specific marker than CD79a for the diagnosis of B-cell ALL; (*b*) among AMLs, PAX5 expression is specifically associated with the presence of the t(8;21) translocation; and (*c*) PAX5 expression in t(8;21)-AML correlates with that of CD19 and CD79a, which in B-cells are both known to be under the transcriptional control of PAX5 (4, 7, 8).

MATERIALS AND METHODS

Samples. Paraffin blocks from a total of 35 normal and reactive bone marrow samples and 360 leukemia samples were retrieved from the files of the Hematopathology Sections of Perugia and Bologna Universities. The leukemia samples included bone marrow trephine biopsies from 150 B-cell ALLs, 47 T-cell ALLs, and 160 AMLs. Lymph node and skin biopsies from 3 T-cell ALL/lymphoblastic lymphomas, that we previously reported to bear the non-dysulphide linked form of the TCR$\gamma\delta$ (27) and to coexpress T-cell antigens and the CD79a molecule (28, 29), were also available for immunohistochemical studies. AML cases (20 M0, 21 M1, 28 M2, 15 M3, 28 M4, 18 M5, 15 M6, and 10 M7) included bone marrow biopsies from 155 cases enrolled in the GIMEMA LAM99P trial and paraffin blocks from 5 cases of granulocytic sarcomas (bone 3 and skin 2). In all of the cases, histologic examination was done on 3 μm-thick sections that were stained with H&E, Giemsa, and Gomori silver impregnation for reticulin fibers. All of the cases were classified according to the FAB/WHO criteria (30–32) on the basis of the morphologic evaluation of tissue sections and May-Grunwald-Giemsa-stained marrow smears that were integrated by the results of standard cytochemical, immunohistochemical, and flow-cytometric studies.

Human Cell Lines. PAX5 expression was tested in two human myeloblastic cell lines: SKNO1 (kindly provided by Dr. Francesco Grignani, Institute of

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Internal Medicine, University of Perugia, Perugia, Italy), which bears the *AML1-ETO* gene rearrangement, and U937 (purchased from the American Type Culture Collection, Rockville, MD).

Cytogenetic and Molecular Studies. Cytogenetic and molecular studies were available in all of the 160 AML cases. Banded chromosomes were analyzed with standard methods and identified by GTC banding. At least 20 metaphases were examined for each case. Molecular studies for the major genetic alterations (PML-RARα, AML1/ETO, CBFβ/MYH11, rearrangement of MLL, DEK-CAN, and BCR-ABL) were done as described previously (33–35).

Antibodies for Immunohistochemistry. PAX5 was detected with a specific IgG1 mAb directed against a fixative-resistant epitope located in the octamer/homeodomain regions of the PAX5 protein (Transduction Laboratories, San Diego, CA). Mouse mAbs directed against CD20, CD15, CD68(KP1), glycophorin A, CD34, and polyclonal antibodies against myeloperoxidase and CD3 were all purchased from DakoCytomation (Glostrup, Denmark). The anti-CD79a (clone JCB117) antibody was generously provided by Dr. David Y. Mason (Nuffield Department of Clinical Laboratory Services, John Radcliffe Hospital, Oxford, United Kingdom). The PG-M1 mAb directed against the macrophage-restricted form of the CD68 protein was generated in the laboratory of one of the authors (Brunangelo Falini; ref. 36).

Tissue Processing. Bone marrow biopsies were fixed in B5 for 2 hours, decalcified in EDTA for 4 hours, and routinely processed for paraffin embedding (37). Biopsies from extramedullary sites were fixed in formalin or B5 and embedded in paraffin. Paraffin sections (3-μm thick) were dewaxed in three changes of xylene, attached on silane-coated slides, rehydrated, subjected to microwaving (750 W $\times$ three cycles of 5 minutes each) with 1 mmol/L EDTA buffer (pH 8.0) as antigen retrieval solution (38), and cooled.

Immunostaining Procedure. Immunostaining of antigen-retrieved tissue sections and cytological samples with mAbs was done with the alkaline phosphatase antialkaline phosphatase complex technique (39). Endogenous tissue alkaline phosphatase was inhibited with levamisole (39). Immunostaining with rabbit polyclonal antibodies was done with a four-step procedure (40). Briefly, slides were sequentially incubated with the primary rabbit antibody, followed by a mouse monoclonal antirabbit antibody (Dako A/S, Glostrup, Denmark), a rabbit antimouse polyclonal antibody (Dako A/S, Glostrup, Denmark), and the alkaline phosphatase antialkaline phosphatase complexes. Slides were then counterstained in hematoxylin and mounted in Kayser's gelatin.

A case was considered positive by immunohistochemistry if 10% or more of the leukemic cells were stained by a given antibody. Positive controls were used for each reagent: they included reactive lymph nodes for PAX5, CD3, CD20, CD34, CD68, and CD79a and a spleen with myeloid metaplasia for CD15, myeloperoxidase, and glycophorin A. The same samples without the application of the primary antibody were run as negative controls.

Double Immunoenzymatic Labeling. Antigen-retrieved paraffin sections from normal and leukemic bone marrows were double stained for PAX5 and either CD20, CD79a, or CD3. In the CD20/PAX5 and CD3/PAX5 double stainings, the CD20 and CD3 molecules were detected by a biotin-avidin peroxidase technique with diaminobenzidine/hydrogen peroxide (Sigma-Aldrich, Milan, Italy) as substrate, whereas the PAX5 protein was revealed by the alkaline phosphatase antialkaline phosphatase procedure (39) with naphtol AS-MX plus Fast Blue BB salt (both purchased from Sigma-Aldrich S.r.l.) as substrates (41). In the CD79a/PAX5 double stainings, the PAX5 molecule was detected by the immunoperoxidase technique with diaminobenzidine/hydrogen peroxide as substrate, whereas CD79a was revealed by the alkaline phosphatase antialkaline phosphatase complex procedure with naphtol AS-MX plus Fast Blue BB salt as substrates. Slides were mounted in Kayser's gelatin without counterstain.

Western Blotting Studies. AML cell lines and Ficoll-purified mononuclear cells from a t(8;21)-AML bone marrow sample with >85% blasts and <3% of residual CD20+ mature B cells were lysed with SDS loading buffer. An aliquot of each lysate was loaded onto a 12% SDS-acrylamide gel and electrotransferred to nitrocellulose sheets, following standard procedures. The membrane was subsequently incubated with nonfat dried milk (Bio-Rad, Hercules, CA) for 1 hour at room temperature followed by overnight incubation with the mAb against PAX5 [1:50 dilution in 0.05 mol/L TBS (pH 7.5) plus 0.1% Tween-20] or with a rabbit polyclonal antibody [1:40 dilution in 0.05 mol/L TBS (pH 7.5) plus 0.1% Tween-20] reacting against the Runt domain of AML1 (anti-AML1-RD, Oncogene Research Products, San Diego, CA), which is retained in the AML1-ETO fusion protein. After washing in 0.05 mol/L TBS (pH 7.5) plus 0.1% Tween-20, a secondary goat antimouse-horseradish peroxidase-conjugated or goat antirabbit-horseradish peroxidase-conjugated antibody (Bio-Rad) was applied at 1:10,000 or 1:50,000, respectively, dilution in 0.05 mol/L TBS (pH 7.5) plus 0.1% Tween-20 at room temperature for 40 minutes. After washing in 0.05 mol/L TBS (pH 7.5) plus 0.1% Tween-20, results were visualized by enhanced chemiluminescence detection with ECL Plus reagents (Amersham Pharmacia Biotech, Little Chalfont, England). To check the amount of lysate loaded in each lane of the gel, nitrocellulose sheets were also probed for 2 hours with a goat antiactin polyclonal antibody (sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 in 0.05 mol/L TBS (pH 7.5) plus 0.1% Tween-20 followed by 40 minutes incubation with a secondary mouse antigoat-horseradish peroxidase-conjugated antibody (sc-2354, Santa Cruz Biotechnology) diluted 1:5,000 in 0.05 mol/L TBS (pH 7.5) plus 0.1% Tween-20.

Real-Time Reverse Transcription-PCR. In 16 AMLs cases [4 with normal karyotype, 4 with inv(16), and 8 with t(8;21)], total RNA was extracted following standard procedures from Ficoll-purified mononuclear cells deriving from bone marrow samples collected at disease onset and containing >80% blasts and <3% residual mature B-cells. First-strand cDNA synthesis was done with Superscript kit (Invitrogen-Life Technologies, Inc., Carlsbad, CA). In a 20-μl reaction volume, 1 μg RNA and 1 μg of oligo(dT)$_{12-18}$ were mixed to a final volume of 11 μL and incubated 10 minutes at 70°C. Then, 4 μL of 5$\times$ first-strand buffer, 2 μL of DTT 0.1 mol/L, 1 μL of 10 mmol/L deoxynucleoside triphosphate mix, and 2 μL of Superscript reverse transcription were added. The sample was incubated 1 hour at 42°C and then diluted to 100 μL in Tricine KOH 0.5 mol/L (pH 8.5). The subsequent real-time PCR was done in 25 μL volume containing the following reagents: 0.5 μmol/L of each primer, 12.5 μL of 2$\times$ SYBR Green PCR Master MIX, and 100 ng of template. The sequences of primer pairs were as follows: PAX5 forward primer, 5′CTGATCTCCCAGGCAAACAT3′; PAX5 reverse primer, 5′TTGCTCATCAAGGTGTCAGG3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer, 5′ATCAGCAATGCCTCCTGCAC3′; and GAPDH reverse primer, 5′TGGCATGGACTGTGGTCATG3′. Thermal cycling parameters were as follows: 2 minutes at 50°C, then 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C. Each sample was run in triplicate. The mean cycle-threshold (C_T) value for PAX5 and GAPDH across replicates of each sample was calculated (being C_T the cycle number at which the PCR reaction reaches a predetermined fluorescence threshold and set within the linear range of all of the reactions). The amount of gene expression in a given sample was calculated as the difference (ΔC_T) between the mean C_T value of the target mRNA (PAX5) and the mean C_T value of the house-keeping mRNA GAPDH in that sample. Then, the difference ($\Delta\Delta C_T$) between the ΔC_T values of each t(8;21)-AML case and the mean ΔC_T value of the control cases was calculated. Finally, the relative expression value (*i.e.*, the fold-change value) between each test sample and the control samples was calculated as $2^{-\Delta\Delta CT}$.

RESULTS

PAX5 Expression in Normal and Reactive Bone Marrow Samples. We first tested the ability of the anti-PAX5 mAb to detect the protein in B5-fixed/EDTA decalcified paraffin-embedded trephine biopsies from 35 normal and reactive bone marrows. In all of the samples, the antibody strongly labeled a small percentage (ranging from 1 to 5%) of lymphoid looking cells and occasional reactive lymphoid nodules (data not shown). As expected, expression of PAX5 was nuclear. Cells of the erythroid, myeloid, and megakaryocyte cell lineages were consistently PAX5-negative (data not shown) as well as bone marrow plasma cells, which in turn showed cytoplasmic CD79a staining (data not shown).

PAX5 Expression Correlates with That of CD79a in B-Cell ALLs. Blast cells from most B-cell ALL cases studied (142 of 150) coexpressed the CD79a and PAX5 molecules (Fig. 1). PAX5 expression was invariably nuclear and highlighted the nuclear irregularities of leukemic cells, thus facilitating detection of minimal bone marrow infiltrates (especially in samples taken after chemotherapy; data not shown).

A few cases (8 of 150) not coexpressing PAX5 and CD79a were observed: 3 of them were PAX5$^-$/CD79a$^+$ and the remaining 5 were Pax5$^+$/CD79a$^-$. This finding might be, at least in part, explained by denaturation of one or the other antigen related to fixation/embedding procedures. However, in 2 of 5 PAX5$^+$/CD79a$^-$ cases, we were able to show that lack of CD79a expression in PAX5$^+$ leukemic cells was

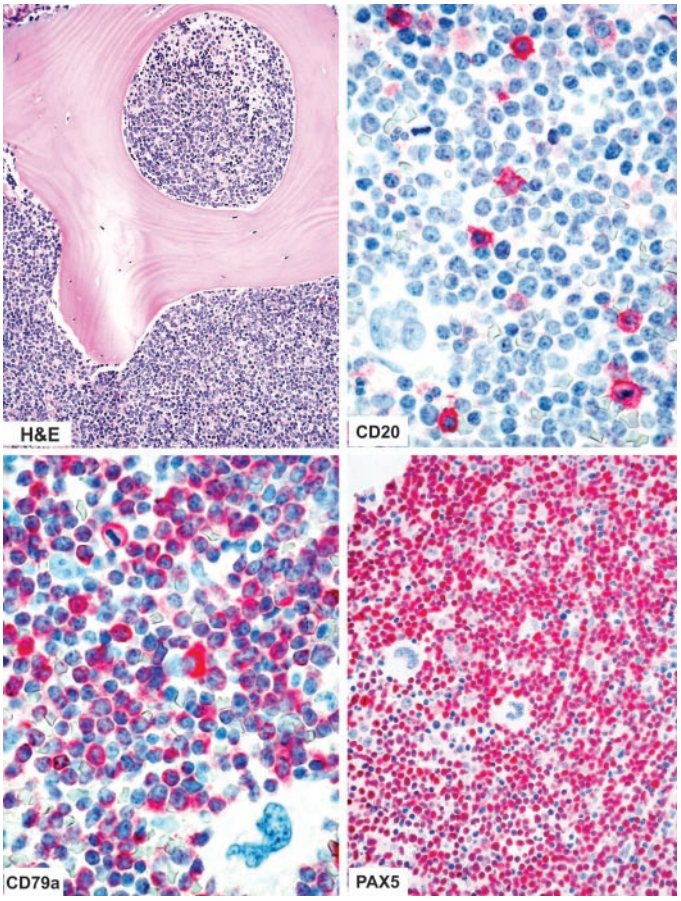

Fig. 1. PAX5 is consistently expressed in B-cell precursor leukemias. Pro-B acute lymphoid leukemia diffusely infiltrating the bone marrow (*top left panel*; H&E, ×100). Leukemic cells are negative for CD20 (*top right panel;* ×800) but show strong positivity for CD79a in the cytoplasm (*bottom left panel*; ×800) and for PAX5 in the nucleus (*bottom right panel*; ×250). *Top right panel* and *bottom panels*: alkaline phosphatase antialkaline phosphatase complex technique and hematoxylin counterstain.

because of true absence of the protein (rather than to antigen denaturation); in fact, nonneoplastic mature B cells expressing CD79a were observed in close proximity to tumor cells and served as an internal positive control for CD79a staining (data not shown).

These findings show that the PAX5 expression pattern closely parallels that of CD79a. Moreover, PAX5 can also correctly assign to B-lineage ALL cases unreactive to CD79a staining.

CD79a but not PAX5 Is Expressed in T-Cell ALLs. None of the 50 cases of T-cell ALL/lymphoblastic lymphoma expressed the PAX5 protein, whereas in 40% of cases (20 of 50), a variable percentage of leukemic cells (ranging from 30 to 100%) showed weak to moderate cytoplasmic positivity for the CD79a molecule (Fig. 2).

These results clearly indicate that PAX5 is more specific than CD79a as immunophenotypic marker of B-cell ALLs, because it is not expressed in precursor T-cell malignancies.

Selective Expression of PAX5 in Myeloid Leukemias with t(8;21). Bone marrow trephine biopsies from 160 AML patients, including 5 granulocytic sarcomas and fully characterized by cytogenetics and molecular studies (Table 1), were analyzed for PAX5 expression. In 15 cases (including 2 granulocytic sarcomas), a percentage (ranging from 10 to 50%) of blast cells showed a weak to moderate nuclear labeling for PAX5. All cases expressed the CD68 and myeloperoxidase molecules (Fig. 3) and were classified as M2 according to the FAB/WHO classification (32). Additional correlation with cytogenetic/molecular findings showed that all of the $PAX5^+$ cases displayed the t(8;21) translocation and were positive at reverse transcription-PCR analysis for the AML1/ETO fusion transcript. However, PAX5 positivity was detectable by immunohistochemistry only in 15 of the 42 cases known to bear the t(8;21), among the 160 AML patients investigated, and only in a proportion of blast cells (but not in maturing leukemic cells). These results indicate that among AML, nuclear labeling for PAX5 is specific for the t(8;21) translocation, although only about one-third of cases (15 of 42) expressed the protein.

To provide additional evidence that the nuclear staining observed at immunohistochemistry with the anti-PAX5 mAb in t(8;21)-AML blasts is really due to the presence of the PAX5 protein (and not to a cross-reacting unrelated nuclear protein), we performed Western blotting studies (with the same antibody) on lysates of primary blasts from a $PAX5^+$ t(8;21)-AML patient and of a myeloblastic cell line (SKNO1) bearing the t(8;21) translocation and expressing the AML1-ETO fusion protein: in both of them, we found a specific band of the expected molecular weight (M_r 50,000) of PAX5 (Fig. 4). To additionally study the expression of PAX5 in t(8;21)-AML, we next analyzed by quantitative reverse transcription-PCR the levels of PAX5 mRNA in 8 t(8;21)-AML patients (4 were PAX5-positive and 4 were PAX5-negative at immunohistochemistry) and compared them to 8 control patients devoid of this translocation ([4 of which had inv (16) and 4 had normal karyotype]. Notwithstanding their different PAX5 expression at the protein level, all of the 8 t(8;21)-AML samples showed a similar up-regulation of the PAX5 transcript: in fact, PAX5 mRNA was found to be increased over controls from 6- to 72-fold in the 4 cases that were PAX5-positive at immunohistochemistry and from 9- to 69-fold in the 4 cases that were PAX5-negative at immunohistochemistry (Fig. 5). These findings confirm that PAX5 expression is specific for the t(8;21)-AML and indicate that within this subgroup of myeloid leukemias, the *PAX5* gene is apparently expressed in more cases than it can be appreciated by immunohistochemistry alone.

PAX5 Expression in t(8;21)-AMLs Correlates with That of CD19 and CD79a. Prompted by the fact that the transcription factor PAX5 exerts his master role in B-lymphopoiesis by *trans*-activating B-cell specific genes, we investigated whether the presence of PAX5 in t(8;21)-AML might result in the up-regulation of other B-cell molecules. To this end, we focused on CD79a and CD19 because: (*a*) these two immunologic markers, routinely used for B-cell ALL diagnosis, represent well-known transcriptional targets of PAX5 in B cells (4, 7, 8); (*b*) their expression pattern during B-cell development parallels that of PAX5, including down-regulation at the plasma cell stage; and (*c*) the expression of one of them (*i.e.*, CD19) has been reportedly associated to t(8;21)-AML (42). In 5 of 15 t(8;21)-AML cases displaying PAX5 expression at immunohistology, CD79a expression was detected in the same myeloid blast population expressing PAX5, as shown by double staining experiments (Fig. 3). Flow cytometric data on CD19 expression (no anti-CD19 Ab being suitable for paraffin immunohistochemistry) were available for 3 of these 5 $PAX5^+/CD79a^+$ cases, and 2 of 3 cases were also found to be $CD19^+$. As far as it concerns the remaining 10 cases that were $PAX5^+$ at immunohistology and the 4 cases that were $PAX5^+$ only at real-time reverse transcription-PCR, we were able to retrieve flow cytometric information for 4 of 10 and 3 of 4 of them, respectively, and found CD19 expression in 3 of 4 and 2 of 3, respectively. Taken together, these observations strongly suggest that PAX5 up-regulation in t(8;21)-AML is involved in the expression of CD79a and/or CD19 frequently observed in this subset of acute leukemias (10 of 12 evaluable cases in our study).

DISCUSSION

This article presents new data on PAX5 expression in acute leukemias that are both of biological and clinical value. In fact, we provide evidence that PAX5 expression in B-cell ALL closely parallels that of CD79a, the most useful marker currently available for B-cell ALL diagnosis in routine bone marrow biopsies (43). There are several arguments for

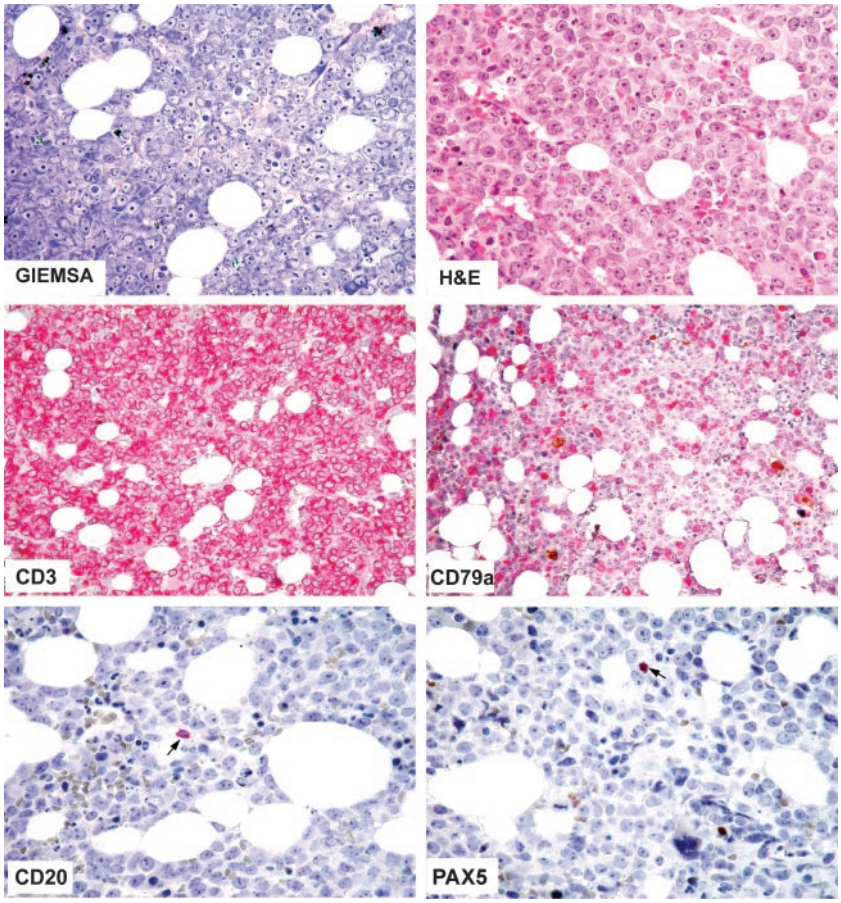

Fig. 2. T-lymphoblastic leukemias do not express PAX5. Diffuse marrow infiltration by T-lymphoid blasts at Giemsa (*top left panel*; ×800) and H&E (*top right panel*; ×800) staining. Leukemic cells are strongly positive for CD3 (*middle left panel*; ×250), and a percentage of them also expresses CD79a (*middle right panel*; ×250). Leukemic cells are negative both for CD20 (*bottom left panel*; the *arrow* points to a residual $CD20^+$ mature B cell; ×800) and PAX5 (*bottom right panel*; the *arrow* points to a residual $PAX5^+$ mature B cell; ×800). *Middle* and *bottom panels*: alkaline phosphatase antialkaline phosphatase complex technique and hematoxylin counterstain.

proposing that PAX5 should be added to CD79a (43, 44) as a new tool for the diagnosis of B-cell ALL. In particular, detection of PAX5 might be of particular aid in correctly diagnosing those B-cell ALL cases, which are unreactive to CD79a due either to denaturation or, more rarely (2 of 150 cases in our series), to true absence of the protein.

Moreover, the results of this study clearly indicate that PAX5 is a more specific marker than CD79a for lineage assignment in ALL. In fact, unlike CD79 that is known to be expressed in about 40% of T-lymphoblastic leukemias/lymphomas (28, 29), the PAX5 protein was not detectable in any of the T-cell ALL cases investigated (including cases $CD3^+$/$CD79a^+$). These immunohistochemical findings are in keeping with the previous observation that among 17 cases of $CD3^+$/$CD79a^+$ T-cell ALL, none was found to be rearranged for *IgH* genes, 12 being only rearranged for *TCRγ* genes, and 5 showing neither *TCRγ* nor *IgH* genes rearrangements (29). Taken together, DNA probe studies (29) and PAX5-immunostaining clearly show that $CD3^+$/$CD79a^+$ T-cell ALL represent cases of T-cell lineage leukemias (regardless of CD79a expression) rather than examples of biphenotypic leukemias.

Surprisingly, leukemic cells from a fraction of AML cases in this study were found to express PAX5 by immunohistochemistry. All cases turned out to bear the t(8;21) translocation that causes the *AML1* gene to fuse with the *ETO* gene (45). Moreover, by extending the analysis of PAX5 expression at the mRNA level, we were able to show a consistent up-regulation of this gene also in cases apparently negative at immunohistology. The reason why only one-third of t(8;21)-AMLs display PAX5 positivity at immunohistochemistry are presently not fully understood, but denaturation of the PAX5 protein (less abundantly expressed in t(8;21)-AML than in B-cell ALL) because of fixation/embedding procedures may, at least in part, explain this finding. It is not yet clear why only a proportion of blast cells (and not maturing leukemic cells) expresses PAX5. Although this might theoretically be due to technical reasons as well, it could also be that a selective expression of PAX5 indeed occurs in a specific subset of blasts possibly characterized by additional genetic evolution.

Table 1 *Immunohistochemical expression of PAX5 in 160 AMLs*

Karyotype	Fusion gene	No. of cases $PAX5^+$/analyzed
t(8;21)	AML1-ETO	15/42
inv(16)-t(16;16)	CBFβ-MYH11	0/21
t(15;17)	PML-RARα	0/17
11q23 rearrangements	MLL/various partners	0/26
t(6;11)	DEK-CAN	0/4
t(9;22)	BCR-ABL	0/4
Other abnormalities		0/16
Normal		0/30

The observation that PAX5, the master regulator of B-lymphopoiesis, is expressed in t(8;21)-AMLs sheds new light on the previously reported association (42) between acute myeloid leukemias bearing the t(8;21) and expression of the *CD19* gene, the transcription of which in B-cell is strictly dependent on PAX5 (4). Importantly, we showed for the first time that CD79a (another major PAX5 transcriptional target in B cells) can also be expressed in t(8;21)-AMLs. However, conversely to previous observations (46) reporting CD79a expression in 9 of 10 acute promyelocytic leukemias with t(15;17), CD79a positivity was never detected in any AML cases (including 17 acute promyelocytic leukemias) other than those with t(8;21). The differences between our results and the results of others (46) are likely due to the reagents used: in fact, we used the mAb JCB117 (43), which is known to be more specific than the mAb HM57 used by other investigators (46).

Based on the finding that the same myeloperoxidase-positive myeloid blast population coexpresses PAX5 and CD79a, we propose that the

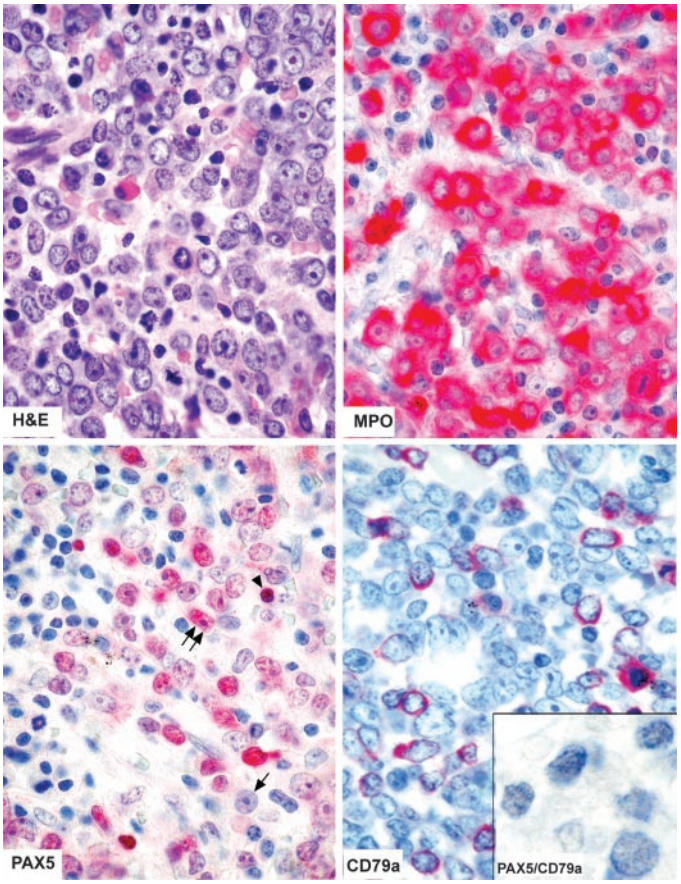

Fig. 3. PAX5 expression in a case of myeloid leukemia bearing the t(8;21) translocation. Diffuse marrow infiltration by t(8;21)-AML blasts (*top left panel*; H&E; ×800) strongly expressing the myeloperoxidase molecule (*top right panel*; ×800). In the *bottom left panel* (×800), a percentage of leukemic cells shows nuclear expression of PAX5 (*double arrow*); the *single arrow* points to a PAX5-negative leukemic cell, and the *arrowhead* points to a PAX5-positive residual mature B lymphocyte. In the *bottom right panel* (×800), a proportion of t(8;21)-leukemic cells shows cytoplasmic positivity for CD79a (×800); three leukemic cells clearly coexpressing PAX5 (*nuclear brown positivity*) and CD79a (*cytoplasmic blue positivity*) are shown in the inset (immunoperoxidase/alkaline phosphatase antialkaline phosphatase complex technique). *Top right panel* and *bottom panels*: alkaline phosphatase antialkaline phosphatase complex technique and hematoxylin counterstain.

aberrant expression of B-cell molecules, such as CD19 and CD79a in t(8;21)-AMLs, is a PAX5-dependent phenomenon. Because AML1 binds *in vitro* to PAX5 through its Runt domain (which is retained in the AML1-ETO fusion protein) and cooperates with PAX5 in the activation of the B cell-specific *blk* gene promoter (47), one could speculate that the interaction of PAX5 and AML1-ETO, which can theoretically occur in t(8;21)-AML blasts, might lead to a functional cooperation of these two proteins in the transcription of PAX5 target genes, such as *CD79a* and *CD19*. Additional studies are warranted to test this hypothesis as well as to clarify the mechanism leading to deregulated expression of PAX5 in t(8;21)-AML.

The finding that CD79a can be expressed both in PAX5-positive t(8;21)-AMLs and in PAX5-negative T-cell ALLs may be, at least in part, explained by the fact that the activity of the *CD79a* gene promoter is positively regulated not only by PAX5 but also by PU.1 (48), an Ets-domain transcription factor involved both in myeloid and lymphoid development (49). Intriguingly, PU.1 has been shown to be expressed in early precursor T cells (50–52) as well as in a subset of T-lineage acute lymphoblastic leukemias (25), and to be inactivated by the AML1-ETO fusion protein in t(8;21)-acute myeloid leukemias (53), thus potentially accounting for the CD79a up-regulation in T-cell ALL but not in t(8;21)-AML.

In addition to raising biological issues, these results are of practical value. Because there are no available mAbs recognizing fixative-resistant epitopes of the CD19 molecule on paraffin sections, labeling for PAX5 is a valuable tool for recognizing the t(8;21) translocation directly on routine bone marrow trephines. Immunostaining for PAX5 may be of particular value for the detection of AML cases with t(8;21) presenting as extramedullary masses (granulocytic sarcoma; refs. 54, 55). In fact, under these circumstances, material for cytogenetic or molecular studies is frequently not available. Although, in the context of our large and representative panel of AMLs, nuclear expression of PAX5 appeared specific for t(8;21), it should be underlined that this finding is only observed in about one-third of cases and that we cannot formally rule out the possibility that PAX5 might be expressed in AML cases bearing rare chromosomal rearrangements that are not included in our series. Therefore, although representing an important clue in the immunohistochemical recognition of t(8;21), PAX5 nuclear labeling must be regarded, in the evaluation of AMLs, as an integration to cytogenetic and molecular studies that, when possible, should always be done.

In conclusion, the results presented in this paper provide the fol-

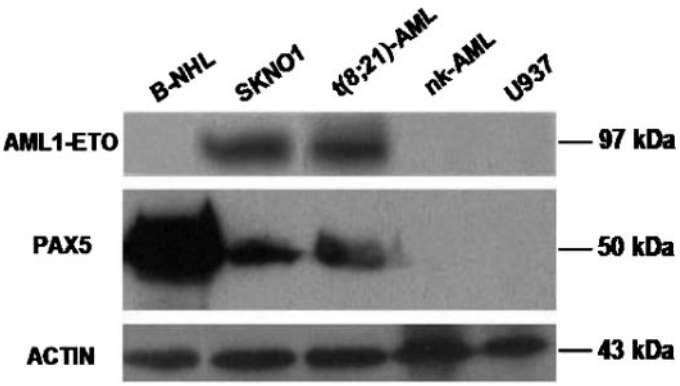

Fig. 4. PAX5 protein expression in Western blots of t(8;21)-myeloid leukemic cells. *Middle panel*: the anti-PAX5 mAb detects the specific M_r 50,000 band corresponding to PAX5 in a B-NHL primary sample (positive control), in the t(8;21)-SKNO1 cell line, and in a t(8;21)-AML primary sample. Conversely, control samples represented by primary leukemic cells from an AML patient with normal karyotype (*nk-AML*) and by the myeloblastic cell line U937, which does not carry the t(8;21) translocation, are both devoid of the PAX5 protein. *Top panel:* as expected, the polyclonal anti-AML1 antibody detects the AML1-ETO fusion protein in the t(8;21)-SKNO1 cell line and in the t(8;21)-AML primary sample but not in the U937 cell line and the nk-AML primary sample. *Bottom panel:* actin (detected by an antiactin polyclonal antibody) is shown as loading control.

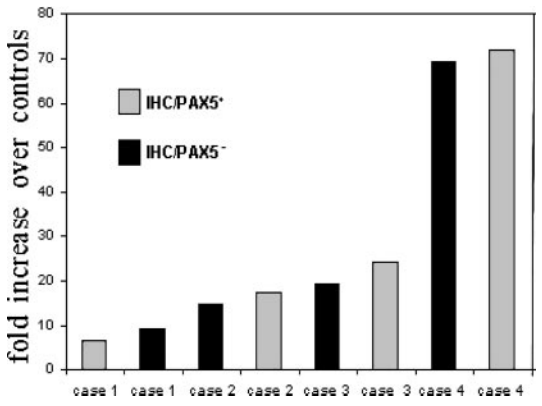

Fig. 5. PAX5 mRNA expression in t(8;21)-AML. The levels of PAX5 transcript (shown in the *Y* axis) were measured by real-time reverse transcription-PCR in 8 t(8;21)-AML patients (shown in the *X* axis), including 4 PAX5$^+$ (□) and 4 PAX5$^-$ (■) at immunohistochemistry, as compared with 8 control AML patients devoid of t(8;21) [see Materials and Methods]. Irrespective of their different PAX5 expression at immunohistochemistry (IHC/PAX5$^+$ and IHC/PAX5$^-$), all of the 8 t(8;21)-AMLs displayed a similar pattern of PAX5 up-regulation, with fold increases over controls ranging from 6 to 72 in the IHC/PAX5$^+$ group and from 9 to 69 in the IHC/PAX5$^-$ group.

lowing evidence: (*a*) PAX5 is a more specific marker than CD79a for immunophenotyping of ALL, and it can also be used to correctly assign to the B-lineage CD79a-negative cases; and (*b*) PAX5 expression in AML selectively associates with t(8;21) translocation, serving as a new tool for the recognition of this translocation in routine biopsies and likely accounting for the expression of other B-cell genes (CD19 and CD79a) in this myeloid neoplasm.

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REFERENCES

1. Busslinger M, Urbanek P. The role of BSAP (Pax-5) in B-cell development. Curr Opin Genet Dev 1995;5:595–601.
2. Nutt SL, Thevenin C, Busslinger M. Essential functions of Pax-5 (BSAP) in pro-B cell development. Immunobiology 1997;198:227–35.
3. Nutt SL, Heavey B, Rolink AG, Busslinger M. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. Nature (Lond) 1999;401:556–62.
4. 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;12:2662–72.
5. Zwollo P, Desiderio S. Specific recognition of the blk promoter by the B-lymphoid transcription factor B-cell-specific activator protein. J Biol Chem 1994;269:15310–7.
6. Zwollo P, Rao S, Wallin JJ, Gackstetter ER, Koshland ME. The transcription factor NF-kappaB/p50 interacts with the blk gene during B cell activation. J Biol Chem 1998;273:18647–55.
7. Nutt SL, Morrison AM, Dorfler P, Rolink A, Busslinger M. Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. EMBO J 1998;17:2319–33.
8. Fitzsimmons D, Hodsdon W, Wheat W, et al. Pax-5 (BSAP) recruits Ets proto-oncogene family proteins to form functional ternary complexes on a B-cell-specific promoter. Genes Dev 1996;10:2198–211.
9. Kishi H, Wei XC, Jin ZX, et al. Lineage-specific regulation of the murine RAG-2 promoter: GATA-3 in T cells and Pax-5 in B cells. Blood 2000;95:3845–52.
10. Souabni A, Cobaleda C, Schebesta M, Busslinger M. Pax5 promotes B lymphopoiesis and blocks T cell development by repressing Notch1. Immunity 2002;17:781–93.
11. Horcher M, Souabni A, Busslinger M. Pax5/BSAP maintains the identity of B cells in late B lymphopoiesis. Immunity 2001;14:779–90.
12. Gonda H, Sugai M, Nambu Y, et al. The balance between Pax5 and Id2 activities is the key to AID gene expression. J Exp Med 2003;198:1427–37.
13. Kehrl JH, Riva A, Wilson GL, Thevenin C. Molecular mechanisms regulating CD19, CD20 and CD22 gene expression. Immunol Today 1994;15:432–6.
14. Krenacs L, Himmelmann AW, Quintanilla-Martinez L, et al. Transcription factor B-cell-specific activator protein (BSAP) is differentially expressed in B cells and in subsets of B-cell lymphomas. Blood 1998;92:1308–16.
15. Foss HD, Reusch R, Demel G, et al. Frequent expression of the B-cell-specific activator protein in Reed-Sternberg cells of classical Hodgkin's disease provides additional evidence for its B-cell origin. Blood 1999;94:3108–13.
16. Usui T, Wakatsuki Y, Matsunaga Y, et al. Overexpression of B cell-specific activator protein (BSAP/Pax-5) in a late B cell is sufficient to suppress differentiation to an Ig high producer cell with plasma cell phenotype. J Immunol 1997;158:3197–204.
17. Adams B, Dorfler P, Aguzzi A, et al. Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. Genes Dev 1992;6:1589–607.
18. Iida S, Rao PH, Nallasivam P, et al. The t(9;14)(p13;q32) chromosomal translocation associated with lymphoplasmacytoid lymphoma involves the PAX-5 gene. Blood 1996;88:4110–7.
19. Hamada T, Yonetani N, Ueda C, et al. Expression of the PAX5/BSAP transcription factor in haematological tumour cells and further molecular characterization of the t(9;14)(p13;q32) translocation in B-cell non-Hodgkin's lymphoma. Br J Haematol 1998;102:691–700.
20. Amakawa R, Ohno H, Fukuhara S. t(9;14)(p13;q32) involving the PAX-5 gene: a unique subtype of 14q32 translocation in B cell non-Hodgkin's lymphoma. Int J Hematol 1999;69:65–9.
21. Morrison AM, Jager U, Chott A, et al. Deregulated PAX-5 transcription from a translocated IgH promoter in marginal zone lymphoma. Blood 1998;92:3865–78.
22. Ichinohasama R, Miura I, Kobayashi N, et al. Herpes virus type 8-negative primary effusion lymphoma associated with PAX-5 gene rearrangement and hepatitis C virus: a case report and review of the literature. Am J Surg Pathol 1998;22:1528–37.
23. Cazzaniga G, Daniotti M, Tosi S, et al. The paired box domain gene PAX5 is fused to ETV6/TEL in an acute lymphoblastic leukemia case. Cancer Res 2001;61:4666–70.
24. Pasqualucci L, Neumeister P, Goossens T, et al. Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. Nature (Lond) 2001;412:341–6.
25. Torlakovic E, Torlakovic G, Nguyen PL, Brunning RD, Delabie J. The value of anti-pax-5 immunostaining in routinely fixed and paraffin-embedded sections: a novel pan pre-B and B-cell marker. Am J Surg Pathol 2002;26:1343–50.
26. Nishii K, Kita K, Miwa H, et al. Expression of B cell-associated transcription factors in B-cell precursor acute lymphoblastic leukemia cells: association with PU.1 expression, phenotype, and immunogenotype. Int J Hematol 2000;71:372–8.
27. Falini B, Flenghi L, Fagioli M, et al. T-lymphoblastic lymphomas expressing the non-disulfide-linked form of the T-cell receptor gamma/delta: characterization with monoclonal antibodies and genotypic analysis. Blood 1989;74:2501–7.
28. Pilozzi E, Pulford K, Jones M, et al. Co-expression of CD79a (JCB117) and CD3 by lymphoblastic lymphoma. J Pathol 1998;186:140–3.
29. Pilozzi E, Muller-Hermelink HK, Falini B, et al. Gene rearrangements in T-cell lymphoblastic lymphoma. J Pathol 1999;188:267–70.
30. Bennett JM, Catovsky D, Daniel MT, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann Intern Med 1985;103:620–5.
31. Brunning RD, McKenna RW. Tumors of the bone marrow. Atlas of Tumor Pathology, 3rd series, Fascicle 9. Washington: AFIP; 1994.
32. Jaffe ES, Harris, NL, Stein H, Vardiman JW. Tumors of haematopoietic and lymphoid tissues. Lyon: IARC Press; 2001.
33. van Dongen JJ, Macintyre EA, Gabert JA, et al. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia (Baltimore) 1999;13:1901–28.
34. Cimino G, Rapanotti MC, Elia L, et al. ALL-1 gene rearrangements in acute myeloid leukemia: association with M4–M5 French-American-British classification subtypes and young age. Cancer Res 1995;55:1625–8.
35. Soekarman D, von Lindern M, Daenen S, et al. The translocation (6;9) (p23;q34) shows consistent rearrangement of two genes and defines a myeloproliferative disorder with specific clinical features. Blood 1992;79:2990–7.
36. Falini B, Flenghi L, Pileri S, et al. PG-M1: a new monoclonal antibody directed against a fixative-resistant epitope on the macrophage-restricted form of the CD68 molecule. Am J Pathol 1993;142:1359–72.
37. Pileri SA, Ascani S, Milani M, et al. Acute leukaemia immunophenotyping in bone-marrow routine sections. Br J Haematol 1999;105:394–401.
38. Pileri SA, Roncador G, Ceccarelli C, et al. Antigen retrieval techniques in immunohistochemistry: comparison of different methods. J Pathol 1997;183:116–23.
39. Cordell JL, Falini B, Erber WN, et al. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). J Histochem Cytochem 1984;32:219–29.
40. Falini B, Fizzotti M, Pucciarini A, et al. A monoclonal antibody (MUM1p) detects expression of the MUM1/IRF4 protein in a subset of germinal center B cells, plasma cells, and activated T cells. Blood 2000;95:2084–92.
41. Falini B, Abdulaziz Z, Gerdes J, et al. Description of a sequential staining procedure for double immunoenzymatic staining of pairs of antigens using monoclonal antibodies. J Immunol Methods 1986;93:265–73.
42. Ferrara F, Di Noto R, Annunziata M, et al. Immunophenotypic analysis enables the correct prediction of t(8;21) in acute myeloid leukaemia. Br J Haematol 1998;102:444–8.
43. Mason DY, Cordell JL, Brown MH, et al. CD79a: a novel marker for B-cell neoplasms in routinely processed tissue samples. Blood 1995;86:1453–9.
44. Mason DY, Cordell JL, Tse AG, et al. The IgM-associated protein mb-1 as a marker of normal and neoplastic B cells. J Immunol 1991;147:2474–82.
45. Erickson P, Gao J, Chang KS, et al. Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to Drosophila segmentation gene, runt. Blood 1992;80:1825–31.
46. Arber DA, Jenkins KA, Slovak ML. CD79 alpha expression in acute myeloid leukemia. High frequency of expression in acute promyelocytic leukemia. Am J Pathol 1996;149:1105–10.
47. Libermann TA, Pan Z, Akbarali Y, et al. AML1 (CBFalpha2) cooperates with B cell-specific activating protein (BSAP/PAX5) in activation of the B cell-specific BLK gene promoter. J Biol Chem 1999;274:24671–6.
48. Hagman J, Grosschedl R. An inhibitory carboxyl-terminal domain in Ets-1 and Ets-2 mediates differential binding of ETS family factors to promoter sequences of the mb-1 gene. Proc Natl Acad Sci USA 1992;89:8889–93.
49. Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science (Wash DC) 1994;265:1573–7.
50. Anderson MK, Hernandez-Hoyos G, Diamond RA, Rothenberg EV. Precise developmental regulation of Ets family transcription factors during specification and commitment to the T cell lineage. Development (Camb) 1999;126:3131–48.
51. Spain LM, Guerriero A, Kunjibettu S, Scott EW. T cell development in PU.1-deficient mice. J Immunol 1999;163:2681–7.
52. Anderson MK, Hernandez-Hoyos G, Dionne CJ, et al. Definition of regulatory network elements for T cell development by perturbation analysis with PU.1 and GATA-3. Dev Biol 2002;246:103–21.
53. Vangala RK, Heiss-Neumann MS, Rangatia JS, et al. The myeloid master regulator transcription factor PU.1 is inactivated by AML1-ETO in t(8;21) myeloid leukemia. Blood 2003;101:270–7.
54. Byrd JC, Weiss RB. Recurrent granulocytic sarcoma. An unusual variation of acute myelogenous leukemia associated with 8;21 chromosomal translocation and blast expression of the neural cell adhesion molecule. Cancer (Phila) 1994;73:2107–12.
55. Kita K, Nakase K, Miwa H, et al. Phenotypical characteristics of acute myelocytic leukemia associated with the t(8;21)(q22;q22) chromosomal abnormality: frequent expression of immature B-cell antigen CD19 together with stem cell antigen CD34. Blood 1992;80:470–7.

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