

## Ectopic Expression of the Proto-oncogene Mer in Pediatric T-Cell Acute Lymphoblastic Leukemia

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**Abstract Purpose:** The Mer receptor tyrosine kinase, cloned from a B-lymphoblastoid library, is the mammalian orthologue of the chicken retroviral oncogene *v-eyk* and sends antiapoptotic and transforming signals when activated. To determine if Mer expression is ectopic in T-cell acute lymphoblastic leukemia (ALL) and potentially important in leukemogenesis, we analyzed Mer expression in normal human thymocytes and lymphocytes and in pediatric ALL patient samples. **Experimental Design:** Reverse transcription-PCR, flow cytometry, and immunohistochemistry were used to determine expression of Mer in sorted human thymocyte populations, lymphocytes, and lymphocytes activated by phytohemagglutinin or phorbol 12-myristate 13-acetate/ionophore. Mer expression in 34 T-cell ALL (T-ALL) patient samples was evaluated by reverse transcription-PCR, and Mer protein expression in a separate cohort of 16 patient samples was assayed by flow cytometry and Western blot. **Results:** Mer expression was absent in normal thymocytes or lymphocytes, and in T cells activated with phytohemagglutinin or phorbol 12-myristate 13-acetate/ionophore. In contrast, Jurkat cells and T-ALL patient samples expressed unique 180 to 185 kDa Mer protein glycoforms. Substantial Mer RNA levels were principally observed in a subset of T-ALL patient samples that expressed B220 ( $P = 0.004$ ) but lacked surface expression of CD3 ( $P = 0.02$ ) and CD4 ( $P = 0.006$ ), a phenotypic profile consistent with immature lymphoblasts. In addition, 8 of 16 T-ALL patient samples had Mer protein detected by flow cytometry and Western blot. **Conclusions:** Transforming Mer signals may contribute to T-cell leukemogenesis, and abnormal Mer expression may be a novel therapeutic target in pediatric ALL therapy.

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy. The current 80% 5-year event-free survival rate is a product of national pediatric oncology cooperative group clinical trials that have optimized patient

outcome. Future improvements will rely on a better understanding of the definition and pathogenesis of different ALL subtypes. Specifically, biologically targeted therapy against unique leukemia markers or signaling pathways is being tested in clinical trials and hopefully will complement current treatment regimens. In this study, we report a new potential biological marker in pediatric ALL.

Mer was initially cloned by our group from a human B lymphoblastoid cDNA library, and Mer RNA transcript was also found to be expressed in T-cell ALL (T-ALL) cell lines (1). This was intriguing because Mer RNA was not detected in freshly isolated human (or mouse) T or B lymphocytes. Subsequent work has shown that Mer signaling, via activated Mer receptor tyrosine kinase domain, can be antiapoptotic (32D cells; ref. 2), cytoskeletal regulatory, or frankly transforming [Ba/F3 pro-B-lymphocytes (3) and NIH 3T3 fibroblasts (4)], depending on the cell type. The retrovirally transduced chicken orthologue *v-eyk* was also frankly transforming (5, 6). We now show that Mer expression is absent in normal lymphocytes, regardless of the state of activation, and is not detected in human thymocyte populations. However, Mer is ectopically expressed in lymphoblasts, and unique 180 to 185 kDa Mer protein glycoforms are found in ALL cell lines and patient samples, likely representing underglycosylated proteins. Furthermore, Mer is expressed in the majority of T-ALL patient samples and Mer RNA transcript expression is most significant in a subset derived from early stage of thymocyte differentiation (B220<sup>+</sup>, CD3<sup>-</sup>, and CD4<sup>-</sup>).

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The ectopic expression of Mer protein in T-cell patient samples was confirmed by Western blot and flow cytometry. Abnormal Mer expression in ALL suggests a role for this receptor tyrosine kinase in the pathogenesis of ALL patients and provides a distinctive therapeutic target not found on the surface of nonmalignant lymphocytes.

## Materials and Methods

**Activation of mononuclear cells and T lymphocytes.** Human peripheral blood mononuclear cells (PBMC) and HTCC-1000 (R&D Systems, Minneapolis, MN) column-enriched human T lymphocytes from PBMC were obtained from a healthy donor and stimulated with 10 nmol/L phorbol 12-myristate 13-acetate (Sigma, St. Louis, MO)/100 nmol/L ionomycin or 2 µg/mL phytohemagglutinin (Sigma). Cells ( $2 \times 10^7$ ) were cultured in 100 mm tissue culture Petri dishes for 3, 18, or 48 hours, and then resuspended in a lysing solution [4 mol/L guanidinium isothiocyanate, 25 mmol/L sodium citrate (pH 7.0), 0.5% sarkosyl, and 0.1 mol/L 2-mercaptoethanol]. Total RNA was isolated by the acid phenol procedure (7). Parallel proliferation assays to monitor PBMC or T-lymphocyte activation were conducted in 96-well plates at a concentration of  $5 \times 10^5$  cells per well. Appropriate stimulants were added in triplicate wells, and cell proliferation at 48 hours was quantitated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay (8).

**Immunohistochemistry.** Deidentified human tissue was obtained in accordance with an institutional COMIR B-approved protocol. Human thymus tissue was obtained as discarded human surgical tissue from <12-month-old patients undergoing cardiac surgery at The Children's Hospital, Denver. Normal human kidney used as controls was obtained from excess autopsy tissue. Four-micrometer formalin-fixed tissue sections were deparaffinized in an ethanol series, and sections were incubated with 3% hydrogen peroxide block for 5 minutes to quench endogenous peroxidase activity. Slides were incubated with an affinity-purified goat anti-human Mer antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:25 for 1 hour at room temperature, followed by a secondary biotinylated anti-goat linked antibody (DakoCytomation, Carpinteria, CA) and a streptavidin-horseradish peroxidase reagent (LSAB kit, DakoCytomation) for 30 minutes at room temperature. Diaminobenzidine (DakoCytomation) was used as the chromogen. Slides were counterstained with H&E and mounted.

**Flow cytometry.** Single-cell suspensions of normal human thymocytes and T-cell leukemic lymphoblasts were washed in PBS containing 2% FCS. Cells ( $5 \times 10^5$ ) were stained in 50 µL antibody solution (1:100 of each antibody unless otherwise stated) at 4°C for 30 minutes. The following antibodies were used: PE-CY-7 linked αCD45 (Beckman Coulter, Fullerton, CA); APC-linked αCD8 (Caltag, Burlingame, CA); FITC-linked αCD4 (Caltag); FITC-linked αCD2 (Caltag); and 1:50 concentration of antihuman Mer antibody conjugated with phycoerythrin (R&D Systems). HL60 and Jurkat cell lines were used as negative and positive controls, respectively, for Mer expression. Fluorescence was detected and analyzed using a FC 500 flow cytometer (Beckman Coulter) with CXP data analysis software.

**Western blot analysis.** A monoclonal antibody directed against human Mer extracellular domain was generated using standard hybridoma techniques (9). Briefly, a cDNA encoding the human Mer extracellular and transmembrane domains was cloned into a baculovirus vector (pBlueBac4.5, Invitrogen, Carlsbad, CA) and expressed as a His-tagged protein in Sf9 cells. Recombinant Mer was affinity purified and used to immunize BALB/c mice. Splenocytes from the immunized mice were fused to Fox NY mouse myeloma cells, and the resulting hybridoma clones were screened for interaction with Mer in extracts from several human cell lines by Western blot. Clone A311 expressing an IgG1 antibody specific for human Mer was selected for use.

Diagnostic bone marrow mononuclear cells from T-ALL patients were obtained from The Children's Hospital, Denver, oncology cell repository and analyzed for Mer expression according to an institutionally approved COMIRB protocol. Patient lymphoblasts, Jurkat cells, K562 cells, U937 cells, and A549 cells (all cell lines obtained from American Type Culture Collection, Manassas, VA) were washed in PBS and resuspended in lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA, 10% glycerol, and 1% Triton X-100 and supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals, Basel, Switzerland). Cell lysates were incubated on ice for 10 minutes and then cleared by centrifugation. To remove carbohydrates from the proteins, aliquots of the cell extracts were treated for 3 hours with either PNGase F (New England Biolabs, Ipswich, MA) or a mixture of five glycosidases (Enzymatic CarboRelease kit, QA-Bio, San Mateo, CA). Proteins were resolved by SDS-PAGE, electroblotted onto a nitrocellulose membrane, and Mer was detected using the A311 monoclonal antibody.

**Reverse transcription-PCR analysis of patient samples.** ALL patient samples from the bone marrow or peripheral blood samples were obtained from a Pediatric Oncology Group cell repository of T- and B-ALL patient samples for RNA extraction and reverse transcription-PCR (RT-PCR) analysis. Patient samples were supplied in a deidentified manner with immunophenotype analysis done by Pediatric Oncology Group institutions. For patients B35 and T4, an additional sample was prepared in which the purified lymphoblasts were isolated from contaminating monocytes by flow cytometry on a Becton Dickinson FACSsort Flow cytometer, using the antibody J5 (CD10, Beckman Coulter) or the antibody Leu-9 (CD7, Becton Dickinson, Franklin Lakes, NJ). Total RNA was isolated by the acid phenol procedure as previously described (7). First-strand cDNA synthesis and RT-PCR was done, as previously described, using the Mer primers 3F and 2R (1). To test for the integrity of the RNA samples and for template standardization, amplification by PCR using actin primers HACA-1F and HACA-1R (1) was done for either 24 cycles (phytohemagglutinin and phorbol 12-myristate 13-acetate/ionophore-activated cells) or 29 cycles (ALL patient samples). CD45 amplification was done with parameters identical to Mer amplification. The CD45 primers used (CD452F: bp 140-162, 5'-GTATTTGTGACAGGGCAAAGCC-3'; CD452R: bp 1,146-1,169, 5'-ATGTTGGGTTCAAGGTTTTCTAA-3') span the region of alternative splicing in the extracellular domain and yield products corresponding to eight potential CD45 isoforms. The sizes of the amplification products from the various CD45 isoforms are as follows: 1,029 bp (B220), 831 and 885/888 bp (double exon isoforms), 687/690 and 744 bp (single exon isoforms), and 546 bp (CD45F37). Each PCR reaction contained a reverse transcriptase negative control to rule out any genomic amplification and a no template control. To ensure reproducibility of data, all Mer PCR results were repeated a minimum of three times and at different cycle numbers (35 and 40 cycles).

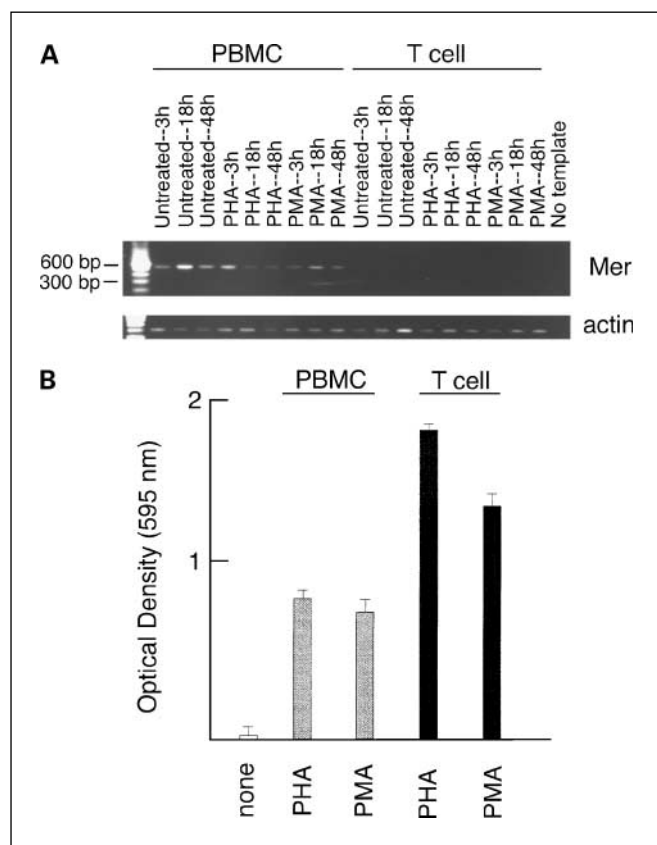
**Statistical methods.** The association between high Mer RNA transcript expression and B220 RNA transcript expression or expression of individual surface markers (CD3, CD4, or CD8) at diagnosis was assessed using the Wilcoxon rank sum test with normal scores. The association between Mer protein expression and the presence of surface CD3 expression was assessed by Fisher's exact test.

## Results

**Mer expression is not induced by activation of PBMC and T-cell populations.** We have previously found Mer RNA transcript to be absent in circulating T and B lymphocytes but present in T-ALL lines (1). To determine whether Mer expression in ALL lines was merely a result of proliferation, Mer was analyzed in PBMC and CD3<sup>+</sup> column enriched T-cell samples treated with phytohemagglutinin or phorbol 12-myristate 13-acetate/ionophore for up to 48 hours. RNA was isolated, and RT-PCR analysis of Mer RNA transcript was done (Fig. 1A). Cell proliferation was

confirmed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay (Fig. 1B). Mer is normally expressed in monocytes/macrophages and these cells account for Mer RNA transcript detected in the PBMC population as previously reported (1). When PBMC were treated with phytohemagglutinin or phorbol 12-myristate 13-acetate/ionophore, there was no significant difference in Mer expression evident in treated versus untreated controls. In the T cell-enriched population, Mer expression was absent in both resting and stimulated T lymphocytes; data are consistent with the absence of Mer RNA transcript in mature, resting T cells (1). Thus, T lymphocyte activation and proliferation do not induce Mer expression.

**Mer is not expressed in normal thymocyte populations.** ALL can be subdivided into early phenotype ALL, intermediate phenotype ALL, and mature phenotype ALL based on surface marker analysis of the lymphoblasts. These subdivisions represent clonal expansion of different stages of thymocyte development. Although we have previously documented that mature lymphocytes do not express Mer (1), we now evaluated Mer expression in human thymocytes to determine if expression in lymphoblasts was truly ectopic. As a positive control for the Mer immunohistochemical staining, Mer expression was



**Fig. 1.** Mer RNA transcript is not induced in stimulated PBMC and T-cell populations. *A*, T cells were isolated from Histopaque-purified PBMC using columns that removed B cells with anti-immunoglobulin-coated beads and removed monocytes based on Fc interactions. The PBMC and T-cell samples were treated with phytohemagglutinin (PHA) or phorbol 12-myristate 13-acetate (PMA) plus ionophore for 48 hours. RT-PCR amplification of Mer shows the presence of a 526 bp Mer fragment in PBMC but not in unstimulated or activated T-cell populations. A 201 bp actin band indicates the relative amounts of cDNA template used in each PCR reaction. *B*, proliferation of PBMC and T-cell samples was quantitated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The average absorbance of triplicate wells was read at 595 nm.

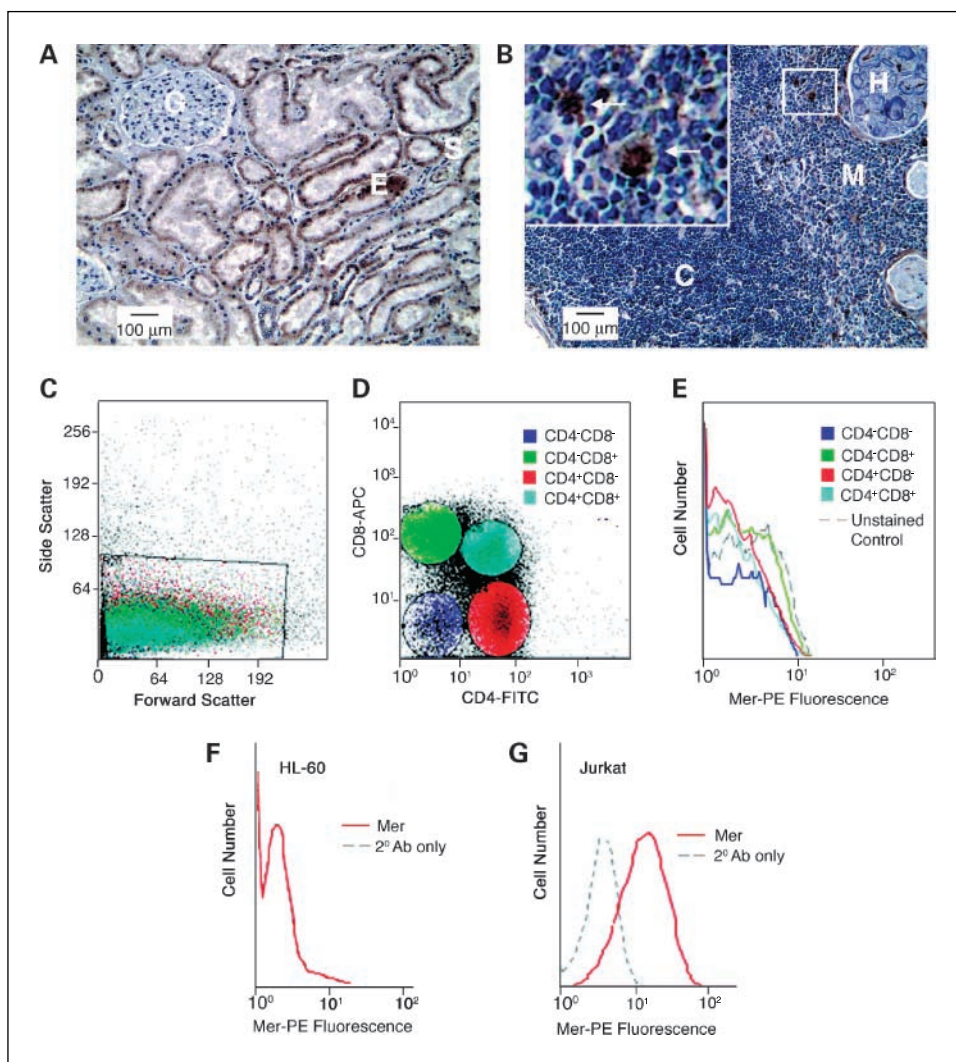
evaluated in normal human kidney, known to express Mer at high levels (1). Epithelial cells of many cell types express Mer, and specific immunoreactivity was noted in the renal tubular epithelium. No Mer staining was detected in the glomerular or stromal components of the kidney (Fig. 2A). In the human thymus, Mer was not detected in the immature thymocytes located in the thymic cortex or in the mature thymocytes of the thymic medulla. Although Mer was not detected in thymocytes, Mer expression was noted as expected in thymic macrophages (Fig. 2B).

Mer expression was also evaluated by flow cytometry in defined thymocyte subpopulations. From the forward scatter and side scatter profile (Fig. 2C), a CD45 gate was used to analyze hematopoietic cells in the thymus and exclude epithelial and stromal cells. Four thymocyte subpopulations were sorted: CD4<sup>-</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, and CD4<sup>-</sup>CD8<sup>+</sup> (Fig. 2D). Mer protein expression was not detected on the surface of any of thymocyte subpopulations (Fig. 2E). The HL-60 acute promyelocytic cell line, known to lack expression of Mer RNA transcript (1), was used as a negative control for the Mer antibody (Fig. 2F). The Jurkat T-ALL cell line, known to express Mer RNA transcript (1), was used as a positive control (Fig. 2G). The detection of Mer on the surface of the Jurkat cells confirmed the efficacy of the anti-Mer antibody in flow cytometry analysis. These data suggest that expression of Mer in lymphoblastic leukemia is ectopic, as Mer is not detected in normal thymocytes or mature lymphocytes.

**Unique Mer protein glycoforms are present in T-ALL cell lines and patient samples.** We developed an antihuman Mer monoclonal antibody for use in Western blot analysis and screened several human cell lines and T-ALL patient samples for Mer protein expression. Mer was detected as a 205 kDa protein in the monoblastoid cell line U937, consistent with previous reports of Mer protein size in U937 cells (10). Smaller molecular weight forms of Mer were detected in other cell lines, including a 190 to 195 kDa protein in the chronic myelogenous leukemia cell line K562 and 180 to 185 kDa protein in the Jurkat T-ALL cell line (Fig. 3A). In addition, a second Mer protein is noted at ~140 kDa in the T-ALL patient samples and the U937 cell line. Mer is extensively glycosylated in the extracellular domain due to the presence of 13 potential N-linked glycosylation sites (11), and differential glycosylation could account for the size differences found in different cell types. When Mer protein was treated with protein N-glycosidase F (PNGase F) to remove N-glycosylation, less difference in Mer protein sizes between the cell lines was noted (Fig. 3B). Following treatment with a deglycosylation mixture of five different N-linked and O-linked glycosidases, the Mer protein sizes become similar in Jurkat and K562. The deglycosylated Mer protein in U937 is only slightly larger in molecular weight relative to the Jurkat and K562, likely due to the inability of the glycosidases to completely remove glycosylation. To further confirm that the 180 to 185 kDa Mer protein in T-ALL cell lines and patient samples is due to underglycosylation of the protein rather than large sequence deletions, genomic DNA and cDNA of all Mer exons from several cell lines was sequenced and no deletions were found in Jurkat or U937 cell lines (data not shown).

**Mer RNA transcript is ectopically expressed in the majority of freshly isolated ALL patient samples.** Peripheral blood or bone marrow mononuclear samples from 34 T-ALL patients were

**Fig. 2.** Mer is not expressed in normal thymocytes. *A*, photomicrograph of Mer monoclonal antibody immunostain shows specific immunoreactivity of the renal tubular epithelium (*E*) without staining of the glomerular (*G*) or stromal (*S*) components (original magnification,  $\times 200$ ). *B*, photomicrograph of Mer monoclonal antibody immunostain shows specific immunoreactivity of the thymic macrophages without staining of Hassall's corpuscles (*H*), immature thymocytes located in the thymic cortex (*C*), or mature thymocytes of the thymic medulla (*M*; magnification,  $\times 200$ ). *C*, gating of human thymocytes by forward and side scatter. *D*, thymocytes are stained with conjugated antibodies, CD8-APC and CD4-FITC, and four populations are identified: immature stage CD4<sup>-</sup>CD8<sup>-</sup>, intermediate stage CD4<sup>+</sup>CD8<sup>+</sup>, and mature stages CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>+</sup>. *E*, Mer is not detected on the surface of the four thymocyte populations or unstained control. *F*, the acute promyelocytic leukemia cell line HL-60, used as negative control based on lack of Mer RNA transcript amplification by RT-PCR, did not stain positive with the antihuman Mer antibody. *G*, the Jurkat T-ALL cell line, used a positive control based on Mer RNA transcript amplification by RT-PCR, expressed the Mer protein on its surface.



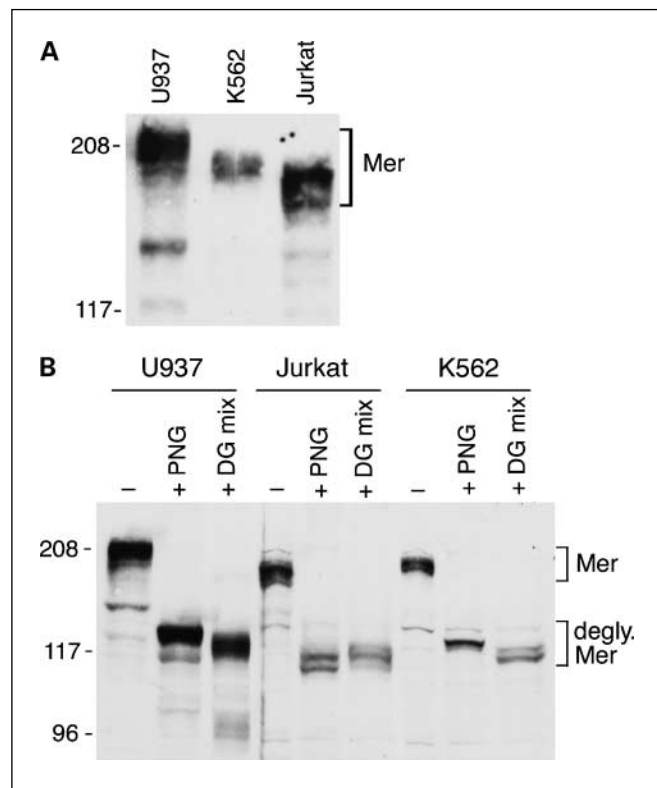
obtained to evaluate Mer expression in freshly isolated lymphoblasts (Fig. 4A). Mer expression in mRNA samples at leukemic diagnosis was normalized to actin and compared with the expression in mononuclear cells from three normal bone marrow and three normal peripheral blood samples. Although cells of the lymphoid lineage in normal peripheral blood and bone marrow do not express Mer, cells of monocytic origin in the normal peripheral blood and bone marrow do express Mer and would be expected to yield a product when analyzed by RT-PCR. Eight of 34 (24%) patient samples (T4, T8, T9, T11, T13, T26, T29, and T33) had significantly higher Mer expression than PBMC or bone marrow; 11 of 34 (32%) patient samples (T3, T5, T12, T15, T17, T18, T19, T20, T31, T32, and T34) had Mer expression comparable with the PBMC and bone marrow; 3 of 34 (9%) patient samples (T7, T14, and T21) expressed Mer at lesser but detectable levels; and 12 of 34 (35%) patient samples (T1, T2, T6, T10, T16, T22, T23, T24, T25, T27, T28, and T30) did not express Mer. In some of the patient samples (T9, T11, T15, T26, and T33), a second larger band of 578 bp was visible above the major product of 526 bp; sequencing included a 52 bp insert in the membrane proximal FNIII domain as previously described (1).

Monocytes were depleted from the mononuclear cell populations of T-ALL patient sample T4, as well as a B-cell ALL patient sample B35, by fluorescence-activated cell sorting. The removal of the monocytes was accomplished by sorting with appropriate antibodies (i.e., CD10 for patient B35 and CD7 for patient T4) based on previous surface phenotype analysis of the leukemic clone. Mer and CD45 expression in these monocyte-depleted samples (Fig. 4B) was identical to the expression profile in the unsorted mononuclear cell samples (Fig. 4A), suggesting that the contribution of monocytes to the Mer expression in these ALL samples is minimal. Because all samples were taken before ALL treatment, the majority of WBC were lymphoblasts, and it is likely that even the low to moderate levels of Mer expression in ALL samples were due to Mer-expressing lymphoblasts.

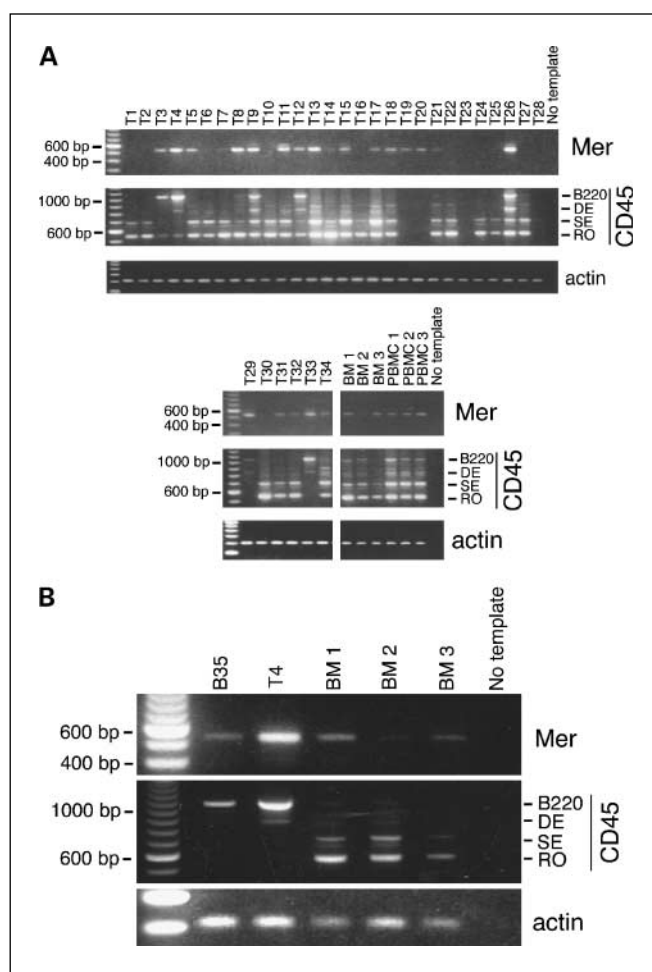
**Expression of CD45 mRNA isoforms.** Because there exists lineage and differentiation state-dependent variation in CD45 isoform expression, we analyzed the CD45 expression profile of these ALL samples. CD45 splice variants arise from alternative splicing of variable exons (4/A, 5/B, 6/C, and 7; refs. 12, 13). B lymphocytes express the high molecular weight isoform (CD45RABC or B220). Although T cells predominantly express

the lower molecular weight isoforms, immature thymocytes (lacking surface expression of CD3, CD4, and CD8) may have significant expression of high molecular weight forms of CD45, including B220 (14). We analyzed CD45 RNA transcript expression in the T-ALL patient samples by PCR using primers that span the region of alternative splicing in the CD45 extracellular domain. Seven of 34 (21%) T-ALL patients (T3, T4, T9, T12, T26, T29, and T33) had a CD45 expression profile that included a proportionally high level of B220 (Fig. 4A), consistent with derivation from an immature or early differentiation stage.

**High Mer expression correlates with early thymocyte stage.** T-ALL patient surface phenotype was used to determine whether high Mer expression was correlated with coexpression of surface antigens traditionally used to classify ALL lymphoblasts. Surface marker assays were done and analyzed by Pediatric Oncology Group reference laboratories, and were classified as low/negative (expression detected on 0-20% of cells), moderate (expression detected on 21-50% of cells), or high (expression detected on 51-100% of cells). High Mer RNA transcript expression was defined as the presence of significantly more Mer transcript than was detected in control mononuclear cells. When patient samples were grouped according to stage of thymocyte differentiation (15, 16), there was an association of high Mer expression with the early thymocyte stage phenotype (Table 1). There were 11 patients



**Fig. 3.** Unique Mer glycoforms are present in T-ALL. *A*, protein lysates from U937, K562, and Jurkat cells were analyzed by SDS-PAGE and immunoblotting with anti-Mer antibody. *B*, samples of cell lysates were untreated (-), digested with PNGase F (+PNG), or digested with a mixture of five glycosidases (+DG mix). Immunoblotting these samples with anti-Mer antibody reveals untreated Mer proteins of 180 to 205 kDa and deglycosylated forms migrating at 115 to 125 kDa.



**Fig. 4.** Mer RNA transcript is present in mononuclear cells taken from T-ALL patients at diagnosis. *A*, three bone marrow (BM) mononuclear cell samples and three PBMC samples from normal individuals serve as controls. The ethidium bromide-stained agarose gel indicates the 526 bp Mer fragment. The sizes of the amplification products from the various CD45 isoforms (alternative splicing of exons 4/A, 5/B, and 6/C) are as follows: 1,029 bp (B220), 831 and 885/888 bp (DE, double exon isoforms), 687/690 and 744 bp (SE, single exon isoforms), and 546 bp (CD45RO). Actin amplification indicates the relative amounts of cDNA template used in each PCR reaction. Representative of three independent RT-PCR amplifications. *B*, the removal of monocytes was accomplished in two ALL mononuclear cell samples by sorting for expression of either CD10 (patient B35) or CD7 (patient T4). Three bone marrow mononuclear cell samples from normal individuals serve as controls for Mer expression from monocytic lineage.

with an early-stage phenotype ( $CD3^-CD4^-CD8^-$ ), 5 patients with an intermediate-stage phenotype ( $CD3^+CD4^+CD8^+$ ), and 7 patients with a mature-stage phenotype ( $CD3^+CD4^+CD8^-$  or  $CD3^+CD4^-CD8^+$ ). Eleven patient samples were not easily grouped according to established criteria and are grouped as not classified. As can be seen in Table 1, every patient with high Mer expression can be grouped in the early thymocyte phenotype or unclassified category.

When high Mer expression was analyzed with respect to each surface marker independently, there was a statistically significant association between high Mer expression and lack of surface CD3 ( $P = 0.02$ ) and CD4 ( $P = 0.006$ ). All T-ALL patient samples with the B220 RNA transcript expressed Mer, and six of the seven B220-expressing patient samples were classified as having high Mer expression ( $P = 0.004$ ). Thus, the ectopic expression of Mer expression in T-cell lymphoblasts was most

significant in lymphoblasts that were derived from an early stage of thymocyte development, which has expression of B220 but is CD3<sup>-</sup>CD4<sup>-</sup>. In addition, smaller but detectable amounts of Mer transcript were found in other T-ALL subtypes (Fig. 4; Table 1). It is possible that the even the lower Mer expression in these lymphoblasts should be considered abnormal and could complement transformation.

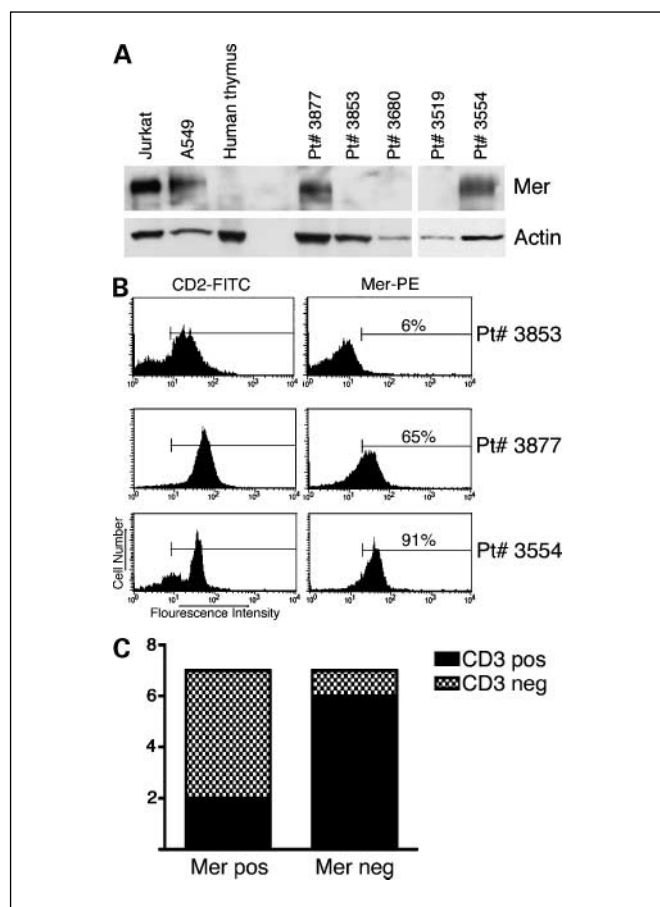
Using a separate cohort of T-ALL patient samples, Mer protein expression was analyzed by Western blot and flow

cytometry (Fig. 5A and B). We found that 8 of 16 T-ALL patient samples had detectable Mer protein by Western blot. Jurkat T-cell leukemia cells and A549 lung carcinoma cells, also known to express Mer (1), were used as positive controls and normal human thymus was used as a negative control for Mer protein expression. Mer expression in patient samples was confirmed by flow cytometry, and CD2 was used in these experiments to gate only on lymphocytes in the ALL patient samples. The ability to exclusively evaluate Mer expression on lymphocytes,

**Table 1.** High Mer expression is associated with an immature T-ALL phenotype

Patient	CD1	CD2	CD3	CD4	CD8	CD5	CD7	CD10	CD19	CD34	CD45	B220	Mer	WBC
I. Early stage														
A. High Mer														
T26	1	97	4	3	2	5	42	1	2	72	99	+++	+++	81
T4	0	94	5	0	8	89	95	4	12	0	3	+++	++	12
T33	13	50	3	4	19	95	97	6	1	1	92	++	++	250
T9	5	95	3	6	2	92	94	1	4	8	2	++	++	22
T29		13	1	1	1	80	87	5	1	6		+	++	100
B. Low/negative Mer														
T20	1	66	3	2	2	3	69	1	2	2	99	0	+	54
T3	4	19	18	5	8	16	70	2	0	2	1	++	+	2
T14		99	18	15	3	99	84	6		75	99	0	+/-	378
T28	0	73	0	1	5	80	82	0	0	0	81	0	0	292
T32		97	3	1	96	95	88				99	0	+	67
T2	9	93	3	7	89	92	61	11	42	3		0	0	192
II. Intermediate stage														
T27	18	96	28	65	94	95	96	90	0	28	97	0	0	110
T1		94	69	23	87	88	95	26	2	2	98	0	0	11
T17	23	74	39	90	94	96	96	96	0	93	95	0	+	37
T18		68	29	54	52	57	64	1	5	5	89	0	+	6
T23	15	97	55	32	25	93	97	0	0	20		0	0	127
III. Mature stage														
T12		48	98	50	1	52	97	1	1	56		++	+	22
T19	0	17	71	54	0	29	97	0	0	7	89	0	+	178
T31	75	60	91	67	11	94	96	1	1	3	94	0	+	130
T21	76	94	23	1	77	88	94	24	0	0	97	0	+/-	49
T10	36	95	74	17	33	94	95	28	12	3	2	0	0	41
T7	4	80	58	5	20	61	79	28	10	1	3	0	+/-	750
T25	13	94	23	17	20	91	93	4	1	51	95	0	0	15
IV. Not classified														
T34	1	94	3	80	3	4	26	6	1	1	86	0	+	10
T22	6	96	72	16	5	96	89	0	0	68	97	0	0	135
T24	0	99	5	87	64	99	99	0	0	0	99	0	0	573
T30		100	8			98	97	0	1	88		0	0	435
T11	31	90	4	8	32	89	90	4	43	3	5	0	++	42
T13	56	94	4	4	50	90	94	1	2			0	++	48
T8		87	11	5	95	94	98	81	2	2	99	0	++	38
T5		88	3	82	83	89	87	72	6		93	0	+	373
T15	84	99	16	34	88	92	93	81	8	4	1	0	+	541
T6	61	3	12	96	53	95	96	90	96	0	2	0	0	445
T16	44	98	18	50	90	96	82	21	2	41	98	0	0	541

NOTE: Surface antigen expression of various markers at time of leukemic diagnosis was determined by flow cytometry and classified as low/negative (expression detected on 0-20% of cells), moderate (expression detected on 21-50% of cells), and high (expression detected on 51-100% of cells). The early stage refers to patients with a CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup> phenotype, intermediate stage refers to patients with a CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>, and the mature stage refers to patients with a CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> or CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup> phenotype. Not classified indicates those patient samples that could not be grouped with any category. RT-PCR results for B220 and Mer are classified as undetectable (0), weak expression but detectable (+/-), low expression (+), and high expression (++ or +++). The WBC count ( $\times 10^3/\mu\text{l}$ ) at time of leukemic diagnosis is indicated.



**Fig. 5.** Mer protein expression in T-cell lymphoblast patient samples is associated with an immature lymphoblast phenotype. **A**, 16 T-ALL patients were analyzed by Western blot and eight patient samples had the presence of Mer protein (two positive and three negative patient samples shown). The Jurkat T-cell leukemia line and A549 lung carcinoma cell line were used as positive controls for Mer expression and normal human thymus was used as a negative control. Actin is shown as a loading control. **B**, flow cytometry analysis of three of the patient samples analyzed by Western blot in (A). CD2-FITC was used to gate on lymphocytes, and Mer antibody conjugated with phycoerythrin (*Mer-PE*) was used to detect Mer surface expression. Patients 3877 and 3544 were positive for Mer by Western blot and flow cytometry and patient 3853 was negative for Mer expression by both assays. **C**, comparison of Mer protein expression relative to known CD3 expression showed Mer expression preferentially detected in CD3 negative (or immature stage) lymphoblasts ( $P = 0.05$ ).

and exclude any Mer expressing monocytes/macrophages, provides supporting data for the ectopic expression of Mer in T-ALL. Out of the 16 T-cell patients evaluated for Mer protein expression, surface CD3 expression was available for 14 of the patients. A comparison of Mer protein expression relative to surface CD3 expression (see Fig. 5C) was consistent with our previous findings and showed a trend for Mer to predominantly be detected in the CD3<sup>-</sup> lymphoblasts ( $P = 0.05$ ).

## Discussion

The Mer receptor tyrosine kinase has transforming properties when abnormally expressed. Abnormal Mer tyrosine kinase activity can transform NIH3T3 cells (4) and allow Ba/F3 pro-B-lymphocyte cells (3) to grow in a cytokine-independent manner. The oncogenic potential of Mer is underscored by the identification of the putative Mer chicken orthologue *eyk* as the

oncogene present in the acute avian retrovirus RPL30, which is responsible for the development of fibrosarcomas, endotheliomas, and visceral lymphomatosis (5, 6). Several human cancers are known to overexpress Mer, including mantle cell lymphomas (17), alveolar rhabdomyosarcomas (18), and pituitary adenomas (19). In gastric cancer (20), Mer is overexpressed and found to be a poor prognostic marker when coexpression was detected with the related protein Axl (21). We now report the abnormal expression of Mer in another human cancer, T-ALL. As Mer expression is not detected in normal lymphocytes or thymocyte subsets, the Mer expression found in T-ALL is truly ectopic.

In addition to our analysis of Mer expression in T-ALL, we have also detected Mer expression in a small number of B-cell ALL patient samples.<sup>7</sup> Additional studies are needed to determine if Mer is normally expressed in B-cell development. Interestingly, Yeoh et al. (22) have used oligonucleotide array analysis to define a B-cell leukemia subtype with a t(1;19) producing the E2A-PBX1 fusion protein (detected in 5% of all childhood leukemias) as a B-cell ALL subset that significantly overexpress Mer.

Mer expression in T-ALL is preferentially, but not entirely, found in T lymphoblasts derived from an early stage of differentiation. Specifically, highest Mer expression was noted in lymphoblasts that lacked surface expression of CD3 and CD4. Expression of other known T-ALL oncogenes is also restricted to early phenotype, intermediate phenotype, or mature phenotype ALL. As we have found with Mer, abnormal expression of the basic helix loop helix (bHLH) transcription factor LYL is associated with early phenotype ALL. In addition, the abnormal expression of HOX11 transcription factor and the TAL1 transcription factors are associated with intermediate phenotype ALL and mature phenotype ALL, respectively (16).

Preliminary studies have reported the prognostic significance of T-ALL oncogenes and the stage of thymocyte differentiation in which they are expressed. An inferior overall survival has been discovered for both early phenotype ALL and mature phenotype ALL relative to intermediate phenotype ALL patients. Specifically, Ferrando et al. reported that pediatric patients with constitutive expression of bHLH oncogenes LYL and TAL1 (associated with early phenotype ALL and mature phenotype ALL respectively) had an overall 5 year survival of  $51 \pm 9\%$  compared with a survival of 100% in patients with constitutive expression of the HOX11 oncogene (associated with the intermediate phenotype ALL; ref. 16). A similar favorable prognosis was found with HOX11-positive adult T-ALL patients (23). The potential prognostic importance of specific ALL subsets, or oncogenes with subset restricted expression, suggests that abnormal Mer expression may have prognostic import in T-ALL, a hypothesis that will need testing with a large sample of clinically annotated samples.

Although Mer overexpression in some cell types is transforming (3, 4), no specific role of Mer has been defined in T-cell leukemogenesis. We have previously found that Mer tyrosine kinase activation leads to downstream activation of antiapoptotic pathways AKT and extracellular signal-regulated kinase 1/2 in an interleukin-3-dependent murine leukemic cell line 32D. Furthermore, Mer activation in the 32D cells dramatically

<sup>7</sup> Unpublished results.

reduced apoptosis initiated by interleukin-3 withdrawal (2). The activation of antiapoptotic signaling pathways may be a means through which Mer contributes to leukemogenesis. It is likely that Mer-expressing T lymphoblasts originating in the bone marrow have Mer activation *in vivo*, as the Mer ligand Gas6 is produced in bone marrow stroma (24). In addition, Gas6 is present in human plasma and could provide continued stimulation to circulating lymphoblasts (25).

Multiple tyrosine kinases have been implicated in leukemogenesis, such as the abnormal activation of the ABL tyrosine kinase by a chromosomal translocation in ALL, acute myelogenous leukemia, and chronic myelogenous leukemia. Abnormal activation of other tyrosine kinases in leukemia include ARG, FLT3, c-FMS, PDGFR $\beta$ , JAK2, and c-KIT (26). Inhibition of tyrosine kinase activity, specifically ABL and FLT3, has led to new therapeutic options in leukemia treatment therapy. The ectopic Mer expression in T-ALL subsets also makes this receptor tyrosine kinase a potential candidate for targeted inhibition in ALL therapy.

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