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ABCB1 Over-Expression and Drug-Efflux in Acute Lymphoblastic Leukemia Cell Lines With t(17;19) and E2A-HLF Expression

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Background. The t(17;19)(q21;p13), which occurs in a small subset of acute lymphoblastic leukemias (ALLs) and is associated with a dismal prognosis, creates a chimeric E2A-HLF transcription factor with transforming properties. ***Procedure.*** We used representational difference analysis to identify candidate E2A-HLF target genes. Transient transfection assays and an inducible expression model system were then used to evaluate the ability of E2A-HLF to modulate target gene expression. ***Results.*** We identified *ABCB1* (*MDR1*, P-glycoprotein) as a gene differentially expressed in ALL cell lines with and without E2A-HLF expression and demonstrated that t(17;19)+ ALL cell lines expressed high levels of ABCB1 protein and had a drug efflux-positive phenotype. Although *ABCB1* transcription is regulated by C/EBPβ via interaction with a DNA response element that shares significant homology with the optimal E2A-HLF binding site, E2A-HLF did not directly activate transcription of reporter genes under control of *ABCB1* promoter elements in transient transfection assays. However, *ABCB1* expression was induced in a DNA-binding independent manner by E2A-HLF, E2A-PBX1, and truncated E2A polypeptides consisting of those portions of E2A present in leukemic fusion proteins. ***Conclusions.*** E2A-HLF-mediated over-expression of ABCB1 may play a critical role in defining the clinical phenotype of ALLs with a t(17;19), suggesting pharmacologic modulation of ABCB1 activity as a rational therapeutic strategy for this chemotherapy resistant subtype of ALL. Pediatr Blood Cancer 2006;47:757–764. © 2005 Wiley-Liss, Inc.

Key words: ABCB1; E2A-HLF; lymphoblastic leukemia; MDR1; transcription factor; translocation

INTRODUCTION

Genes encoding sequence-specific DNA binding transcription factors are common targets of somatic mutations in human cancer. Chromosome translocations in acute leukemias frequently involve transcription factor genes, often creating chimeric transcription factors with dominant gain-of-function oncogenic properties [1]. In general, oncogenic transcription factors are hypothesized to contribute to malignant transformation by directly activating or repressing transcription of specific target genes, or by interfering, in a dominant negative manner, with the transcriptional activity of endogenous proteins. In addition to playing a critical pathogenetic role during neoplastic transformation, chimeric transcription factors may also modulate the clinical phenotype.

The 19p13.3 *E2A* gene (also termed *TCF3*) is a major target of chromosome translocations in acute lymphoblastic leukemia (ALL) [2]. In about 5% of ALLs, a t(1;19)(q23;p13) disrupts *E2A* and fuses it to the chromosome 1 gene *PBX1*, leading to expression of E2A-PBX1 chimeric proteins [3,4]. Analogous E2A-HLF fusion proteins are produced by a t(17;19)(q21;p13), present in 0.5%–1% of ALLs [5,6]. E2A-HLF expression is associated with disseminated intravascular coagulation [7], and confers a dismal prognosis [2,8].

E2A-HLF and E2A-PBX1 consist of the amino terminal two-thirds of E2A, including two separate transcriptional activation domains (TADs), fused to portions of HLF and PBX1 containing distinct DNA binding domains and protein interaction motifs. HLF contains a basic leucine zipper (bZIP) domain that mediates site-specific DNA binding and protein dimerization [6,9]. E2A-PBX1 includes the PBX1 homeobox and an adjacent region responsible for interaction with a subset of HOX proteins and binds DNA in conjunction with MEIS proteins [3,4,10].

HLF and PBX1 are not normally expressed in lymphocytes. Thus, these translocations have two important consequences: they ectopically express HLF/PBX1 DNA binding domains and fuse them to portions of E2A that contain TADs with potent activity in lymphoid cells, suggesting that aberrant regulation of specific target gene expression plays an important role in leukemogenesis mediated by E2A fusion proteins and may also dictate unique clinical properties of this subtype of ALL.

Candidate E2A-PBX1 and E2A-HLF target genes have been identified, but none have been shown to directly mediate transformation to date. Other data suggest that a critical activity of E2A fusion proteins might be to interfere with the normal function of wild type E2A. Structure–function analyses of E2A-PBX1 revealed that the PBX1 DNA binding domain is not required for transformation of NIH-3T3 cells

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in vitro, or generation of lymphoid tumors in transgenic mice [11–13]. In addition, enforced dimerization of truncated E2A proteins that contained only those portions of E2A present in E2A-PBX1 and E2A-HLF transformed 3T3 cells, suggesting that one critical consequence of the fusion partners might be to dimerize E2A polypeptides lacking the native E2A DNA binding domain [14]. Structure–function studies of E2A-HLF also suggest that E2A chimeras may contribute to transformation via DNA-binding-dependent and -independent mechanisms [15].

We set out to identify genes that were over expressed in leukemia cell lines containing native E2A-HLF fusion proteins and to evaluate their potential role in malignant transformation and/or generation of unique phenotypic properties observed in human leukemias with a t(17;19) and E2A-HLF fusion.

METHODS

ALL Cell Lines

ALL cell lines were grown in RPMI-1640 supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2. Cell lines utilized included B-precursor ALL lines HAL-01, UOC-B1, and YCUB-2 that contain a t(17;19) and E2A-HLF expression, and lines without E2A-HLF including REH (pro-B [16]), RCH-ACV (pre-B [17], SUP-B15 (Ph+ ALL [18]), RS4;11 (biphenotypic [19]), MV4;11 (biphenotypic [20]), HB11;19 [21], NALM-6 (pre-B [22]), CCRF-CEM (T-ALL [23]), and CEM-Dox20 (a doxorubicin resistant CCRF-CEM subclone that over expresses ABCB1).

Molecular Analyses

Standard molecular biology assays including nucleic acid isolation, PCR amplification, and gel electrophoresis were performed as described previously [6,24,25]. Representational difference analysis (RDA) was performed essentially as described by Braun using a protocol kindly provided by Chris Denny [26]. Briefly, cDNA was synthesized using oligo-dT primers and made double-stranded by second-strand synthesis. Tester and driver double-stranded cDNAs were digested with DpnII and ligated to adapters. Amplicons were created by PCR using primers complementary to the ligated adapters. Following amplification, the adapters were removed from tester and driver PCR products by digestion with DpnII and new adapters added only to the tester population. To enrich for differentially expressed mRNAs (tester > driver), the tester pool was hybridized with a molar excess of the driver pool. Fragments present primarily in the tester pool form homodimers with an adapter on both strands, while others will form tester–driver heterodimers with adapters on only one strand or driver–driver homodimers lacking adapters. The hybridization mix was then amplified with primers complementary to the second adapter, which allowed only tester homodimers to be amplified efficiently. In round 1 we used a 1:80 HAL-01:REH ratio, followed by 1:660 round 1 RDA product:REH in round 2, and 1:30,000 round 2 RDA product:REH in round 3. The PCR products were then size fractionated in agarose gel revealing 15–20 discrete bands of approximately 200–1,000 base pairs. This region was excised from the gel, DNA purified, and cloned into a plasmid vector to create a library of differentially expressed DpnII amplicons. We isolated 100 separate clones, purified DNA from these clones, and performed nucleotide sequencing of each end of the clone. DNA sequences were then compared to sequences present in Genbank using the BLAST software [27]. Interpretable data was obtained from 68/100 clones. Reasons for failure included empty vector lacking insert, impure plasmid preparations, and lack of readable DNA sequence.

ABCB1 Expression

Expression of *ABCB1* (P-glycoprotein gene, *MDR1*) was determined in a panel of human ALL cell lines via reverse transcriptase polymerase chain reaction (RT-PCR) as described [28], using forward (GGTGCCTGGCAG-CTGGAAGAC) and reverse primers (GCCAAAATCA-CAAGGGTTAGC) to amplify nucleotides 526–788 of *ABCB1* (Genbank accession number AF016535). To confirm RNA integrity, a portion of *ABL* cDNA was amplified using previously described primers [24].

Expression of ABCB1 protein was measured via fluorescence-activated cell sorter (FACS) analysis using a Coulter (Beckman Coulter; Hialeah, FL) EpicsXL flow cytometer and software. Briefly, 1×10^6 cells were washed in phosphate-buffered saline (PBS) and resuspended in 250 µl of a 1:10 dilution of goat serum (Hyclone Laboratories, Logan, UT) in PBS (PBS/GS), pH 7.4 containing 10 µg of a murine anti-ABCB1 monoclonal antibody (MRK-16; Kamiya Biomedical Company; Seattle, WA). The cells were incubated with antibody for 45 min at 4°C in the dark and then washed with ice-cold PBS and resuspended in 250 µl of a 1:10 dilution of R-phycoerythrin-conjugated goat anti-mouse immunoglobulin in PBS/GS. Samples were then analyzed immediately for orange fluorescence (570 nm) intensity via FACS. Negative controls were performed using an isotype-matched control (mouse IgG_{2a}; DAKO Corporation; Carpinteria, CA).

Rhodamine Efflux Assays

To determine the drug efflux properties of cells, 1×10^6 cells were resuspended in complete media with or without 6 µM verapamil (Sigma-Aldrich, St. Louis, MO) and incubated in 6-well plates at 37°C for 1 hr, pelleted at 1,600 rpm and resuspended with or without 6 µM verapamil in 5 ml of unsupplemented media containing Rhodamine 123 (Sigma) at a final concentration of 1.66 µM. Cells were

incubated for 30 min at room temperature in the dark to absorb Rhodamine, placed on ice for 5 min, and pelleted by centrifugation. To determine Rhodamine content at t = 0, cells were resuspended following centrifugation in 500 μl of PBS and kept on ice. To determine Rhodamine efflux, cells were resuspended in 5 ml of complete media with or without 6 μM verapamil, incubated at 37°C for 30 min, and analyzed for green fluorescence (525 nm) intensity by FACS analysis.

Reporter Gene Assays

The human *ABCB1* promoter region from −1018 to +83 was amplified by PCR from human placental genomic DNA (Clontech, Palo Alto, CA) using primers DC-1 and DC-2 derived from the published *ABCB1* 5′-upstream sequences [29]. Amplified *ABCB1* promoter DNA was ligated upstream of a chloramphenicol acetyl transferase (CAT) reporter gene into the promoterless pCAT3-Enhancer Vector (Promega, Fitchburg, WI) resulting in a test construct termed p1018F-CAT. Deletions of the *ABCB1* promoter were made by PCR using DC-2 and forward primers at positions −190 (DC-27) and −128 (DC-28), and resulted in the corresponding test constructs p190F-CAT and p128F-CAT. The control construct $pHLF_4$-TK-CAT contains four tandem repeats of the HLF consensus binding site 5′-GTTACGTAAT-3′ adjacent to the herpes simplex virus thymidine kinase promoter that drive expression of a CAT gene [25]. The expression construct pCMV-E2A-HLF has been previously described [2,25].

CV-1 cells were cultured in 6-well plates and transfected using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). Two micrograms of the expression plasmid (pCMV-E2A-HLF or empty pCMV vector) were co-transfected with 1.5 μg of the test plasmid (p1018F-CAT, p190F-CAT or p128F-CAT). A reference plasmid expressing the beta-galactosidase gene (pCMV-beta-gal) was used to control for transfection efficiency in all experiments. CAT and beta-galactosidase assays were performed using commercial reagents (Promega). Acetylation of chloramphenicol (^{14}C Cm, PerkinElmer, Boston, MA) was assayed by thin layer chromatography separation of the reaction products. Each experiment was performed in duplicate on at least three separate occasions.

ABCB1 Expression Analysis in REH Cell Lines Containing Inducible E2A Constructs

Generation of the inducible E2A and E2A fusion protein constructs under control of a metal responsive element (MRE) was reported previously [30]. REH [16] ALL cells that were stably transfected with either empty pMRE vector, pMRE-E2A-HLF, pMRE-E2A-PBX1, or pMRE-E2AΔ, which expresses a truncated E2A consisting of that portion of E2A present in E2A-HLF and E2A-PBX1, were grown in RPMI medium (10% FCS, 1% PSG, neomycin 300 μg/ml). Transgene expression was induced by addition of 100 μM $ZnSO_4$ to exponentially growing cultures. Protein expression was confirmed via Western blot using an anti-E2A monoclonal antibody [31] and chemiluminescence detection according to the manufacturer's specifications (Amersham International plc, Amersham, England; data not shown).

Following incubation in $ZnSO_4$ containing medium for 24 or 72 hr, total RNA was isolated from 1×10^7 cells using commercial reagents (RNeasy, Qiagen, Inc., Valencia, CA). cDNA was generated using the Superscript-III first-strand synthesis kit for RT-PCR (Invitrogen) with random hexamer priming. RT-PCR was performed with 2–4 μl cDNA template per 25 μl reaction and a standard PCR protocol, using for *ABCB1* forward 5′-GGTGCC-TGGCAGCTGGAAGAC-3′ and reverse 5′-GCTAACC-CTTGTGATTTTGGC-3′ primers. As a control, *GAPDH* was amplified using proportional amounts of cDNA template (5′-GGTGAAGGTCGGAGTCAACG-3′ forward and 5′-CAAAGTTGTCATGGATGACC-3′ reverse primer).

RESULTS

Identification of Genes Differentially Expressed in Human t(17;19)-Positive Versus t(17;19)-Negative ALL Cell Lines

To identify genes expressed in the normal context of E2A-HLF expression we performed RDA using HAL-01 RNA as the ''tester'' and REH RNA as the ''driver.'' HAL-01 contains a t(17;19) and expresses E2A-HLF, while REH is a phenotypically similar pro-B cell line that does not express E2A-HLF, E2A-PBX1, or HLF (but does express wild type E2A). This strategy was designed to detect genes that are more highly expressed in HAL-01 versus REH. We then sequenced the ends of 100 clones generated from the RDA output and obtained reliable sequence data from 68 clones. Genbank comparisons identified 60 of these clones, representing 8 distinct human genes. Five genes were represented by only one or two clones. Three genes were represented by three or more clones: *CD20* or *MS4A1* (membrane-spanning-4 domains, subfamily A, member 1; 3 clones derived from 3 unique DpnII amplicons), *XIST* [X (inactive)-specific transcript; 22 clones derived from 9 unique DpnII amplicons] and *ABCB1* (ATP-binding cassette, subfamily B, member 1; 28 clones derived from 9 unique DpnII amplicons).

ABCB1 Is Highly Expressed in ALL Cell Lines With a t(17;19)

ABCB1 (also referred to as *MDR1*) encodes a membrane-bound, energy-dependent transporter (P-glycoprotein or P-gp) that can mediate efflux of select chemotherapy agents and has been implicated in chemotherapy resistance in leukemia [32,33]. Previous work has demonstrated that C/EBPβ plays an important role in transcriptional activation of *ABCB1* through interactions with a C/EBP binding site in the *ABCB1* promoter [29]. Hunger et al. [25] have shown previously that

the optimal HLF and C/EBP DNA binding sites share significant similarity and that E2A-HLF can bind to canonical C/EBP binding sites. Thus, we hypothesized that E2A-HLF might directly activate transcription of *ABCB1* through interaction with the C/EBP site present in the *ABCB1* promoter and focused our attention on *ABCB1* as a candidate E2A-HLF target gene.

We performed RT-PCR with HAL-01 and REH RNA and confirmed that *ABCB1* was expressed at much higher levels in HAL-01 than in REH. We extended this analysis to a panel of cell lines with and without a t(17;19) and E2A-HLF expression and found that *ABCB1* transcripts were readily amplified from HAL-01 and UOC-B1, another t(17;19)+ ALL cell line, but were amplified from only one of seven childhood ALL cell lines that lacked a t(17;19) (Fig. 1). *ABCB1* expression was also observed in YCUB-2, another t(17;19)+ ALL cell line (data not shown).

To determine if E2A-HLF+ ALL cell lines also expressed ABCB1 protein, we performed FACS analysis using the MRK-16 monoclonal antibody directed against ABCB1. As a comparison, we used the T-ALL cell line CCRF-CEM and its doxorubicin-resistant derivative CEM-Dox20, which was selected by growth in progressively higher concentrations of doxorubicin and expresses high levels of ABCB1 [32]. UOC-B1 and HAL-01 expressed ABCB1 protein at significantly higher levels than did REH or other control ALL cell lines (Fig. 2A). UOC-B1 expressed ABCB1 at levels comparable to or higher than those present in CEM-Dox20, and HAL-01 expressed ABCB1 at substantially higher levels than either UOC-B1 or CEM-Dox20.

ABCB1 Expression Is Associated With an Efflux-Positive Phenotype in ALL Cell Lines That Express E2A-HLF

To investigate whether ABCB1 over-expression was associated with an efflux-positive phenotype in t(17;19)+ ALL cell lines, we incubated cells in media containing rhodamine 123 in the presence/absence of verapamil and determined intracellular rhodamine content via FACS. Rhodamine 123 is a substrate for ABCB1 and other members of the ABC transporter superfamily implicated in ATP-mediated chemotherapy efflux and drug resistance such as multidrug resistance protein MRP-1 and breast cancer resistance protein BCRP/ABCG2, and verapamil competitively inhibits efflux mediated by ABCB1 and some, but not all, other ABC transporters [34]. In the absence of verapamil, HAL-01 rapidly effluxed rhodamine from the cell, while rhodamine was retained in REH (Fig. 2B). In the presence of verapamil, drug efflux was completely inhibited in HAL-01 and rhodamine was retained intracellular in an identical manner to that present in REH. Similar results were observed with UOC-B1 (data not shown).

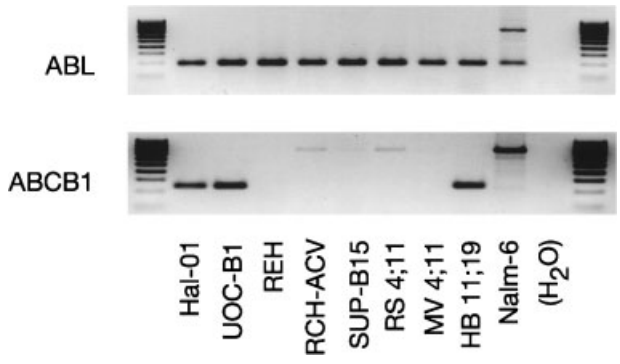

Fig. 1. *ABCB1* transcript expression in ALL cell lines. RT-PCR of *ABCB1* and control *ABL* transcripts in a panel of B-precursor ALL cell lines. The E2A-HLF-positive cell lines HAL-01 and UOC-B1 express *ABCB1* while E2A-HLF-negative lines are negative with the exception of HB(11;19). Higher molecular weight bands are amplified genomic DNA products.

ABCB1 Is not a Direct Transcriptional Target of E2A-HLF

Taken together, our data establish that ABCB1 is expressed at high levels in human ALL cell lines that contain E2A-HLF fusion, and that these lines display an efflux-positive phenotype. To determine if E2A-HLF directly regulated transcription of *ABCB1* through the C/EBP binding element in the *ABCB1* promoter, we generated a series of reporter constructs containing a CAT gene under control of portions of the *ABCB1* promoter regions (Fig. 3A). The full length p1018F-CAT and truncated p190F-CAT constructs contain the C/EBP binding element and are activated by C/EBPβ in transient transfection assays, while p128F-CAT lacks this element and is not activated by C/EBPβ [29]. We co-transfected CV-1 cells with pCMV-E2A-HLF or an empty expression vector along with various reporter constructs and determined CAT activity. E2A-HLF strongly activated $pHLF_4$-TK-CAT reporter gene transcription, but did not activate transcription in any of the *ABCB1* promoter test constructs above background levels (Fig. 3B). Similar results were obtained with beta-galactosidase reporter gene constructs driven by the same *ABCB1* promoter regions (data not shown).

ABCB1 Expression Is Activated in REH Cells Following Expression of E2A Fusion Proteins and Truncated E2A Polypeptides

To further investigate if the ABCB1 expression observed in t(17;19)+ ALL cell lines was related to E2A-HLF expression, we analyzed *ABCB1* RNA levels following inducible expression of E2A-HLF, E2A-PBX1, or E2AΔ constructs in the ALL cell line REH. For these experiments we used REH cells stably transfected with vectors that contain E2A-HLF, E2A-PBX1 or E2AΔ cDNAs under control of a promoter that is activated by addition of $ZnSO_4$ to the media. *ABCB1* RNA levels were determined via RT-PCR before and at several time points following addition of $ZnSO_4$ to the media. In REH subclones containing the metal

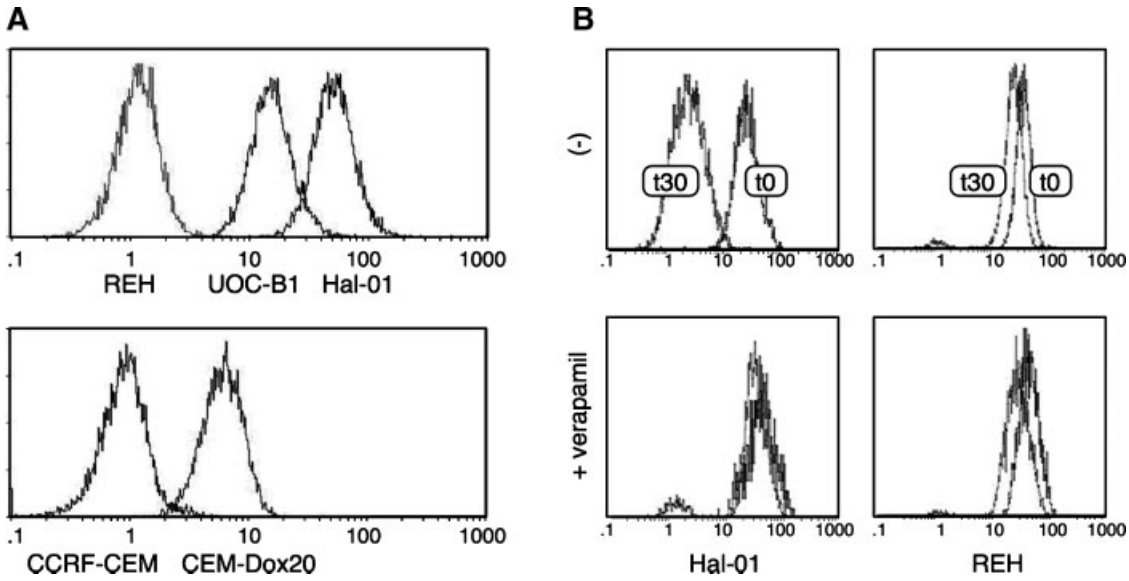

Fig. 2. t(17;19)+ ALL cell lines express high levels of ABCB1 protein and have a drug efflux-positive phenotype. **A**: A high level of ABCB1 expression is shown by FACS in the E2A-HLF-positive cell lines UOC-B1 and HAL-01, compared to the pro-B cell line REH. As comparison, the **lower panel** shows the increased level of ABCB1 in CEM-Dox20 compared to the parental line (CCRF-CEM). **B**: Rhodamine efflux essay. As shown at top left, untreated HAL-01 cells, completely efflux intracellular rhodamine after a 30-min incubation in standard medium (t30) versus initial values (t0). Rhodamine efflux in HAL-01 is almost completely eliminated by verapamil (**bottom left**). In contrast, rhodamine is retained intracellular in the ABCB1 negative REH cells without/with verapamil (**top** and **bottom right**).

inducible E2A-HLF, E2A-PBX1 and E2AΔ constructs, *ABCB1* RNA was expressed by 24 hr following addition of zinc to the media, and continued at least until 96 hr (Fig. 4). In contrast, no *ABCB1* mRNA expression was detected pre- or post-addition of zinc in parental REH or Nalm-6 cells, or in REH cells stably transfected with the empty metal-inducible expression vector (see also Fig. 1).

DISCUSSION

We used RDA as a screen to identify cDNAs expressed at higher levels in human ALL cell lines with and without a t(17;19) and E2A-HLF expression. To prioritize genes for further characterization, we considered both the number of cDNA fragments isolated in our screen and the function of gene products. *XIST* and *ABCB1* accounted for a substantial majority (50/60) of candidates identified by this screen.

XIST is a transcript expressed from the inactive X chromosome that plays a critical role in maintaining X inactivation [35]. We did not investigate potential E2A-HLF regulation of *XIST* further because HAL-01 was derived from a female and has 2 X chromosomes while REH, also derived from a female, has a complex "46,X,-X, . . ." karyotype and has presumably lost the inactive X chromosome [6,36]. Thus, we considered it unlikely that *XIST* played a significant role in mediating either transformation or clinical phenotype.

The *ABCB1* (*MDR1*) gene encodes a transmembrane protein (frequently termed P-glycoprotein or P-gp) that functions as an 170 kDa ATP-dependent drug transporter [37–39]. ABCB1 is expressed at high levels in normal hepatocytes, brain capillary endothelial cells, and renal proximal tubule cells among others and mediates efflux of substrates from the cell [40,41]. *ABCB1* is expressed in a subset of normal hematopoietic cells, with high levels in CD34 positive cells and mature lymphocytes, and low expression in myeloid and lymphoid progenitor cells ([42,43] and expression data on genome.ucsc.edu). ABCB1 mediates efflux of various natural products and chemotherapy agents from cells, and high ABCB1 expression levels are present in many multidrug-resistant mammalian cancer cell lines [44]. Enforced expression of ABCB1 confers the multidrug-resistant phenotype to highly drug-sensitive cell lines [38]. ABCB1 expression has been identified in numerous studies as a strong independent adverse prognostic factor that is predictive of induction failure in adults with AML (reviewed by Mahadevan and List [33]). High ABCB1 expression has been associated with resistance to chemotherapy and an adverse clinical outcome in some [45,46] but not other [47,48] cancers.

There is no clear consensus on the clinical relevance of ABCB1 expression in childhood ALL, due, at least in part, to use of different methods and reagents in various studies. Olson et al. [49] reported recently that ABCB1 protein is over-expressed in about 30% of childhood ALL cases at initial diagnosis, but functional protein is present in only about 1%. Olson did not find that there was a prognostic impact of ABCB1 protein expression, while others have observed a significant negative prognostic impact for ABCB1 protein expression at initial diagnosis in children with ALL [49,50]. Increased frequency and levels of ABCB1

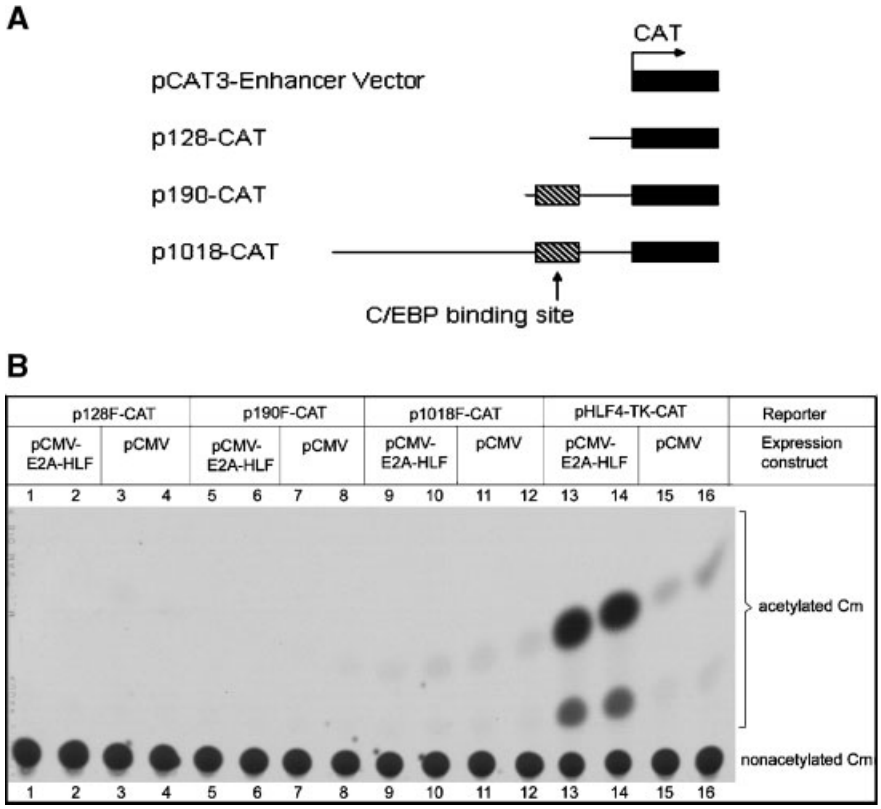

Fig. 3. E2A-HLF is not a direct activator of *ABCB1* gene transcription. **A**: Schematic representation of *ABCB1*-CAT reporter gene constructs. The CAT gene is depicted as a black box and the previously identified C/EBP binding site is shown as a hatched box. **B**: CV-1 cells were transiently transfected with pCMV-E2A-HLF or pCMV vector and the various CAT reporter genes containing *ABCB1* promoter elements. E2A-HLF did not activate transcription of any of the *ABCB1*-CAT constructs but was a strong activator of $pHLF_4$-TK-CAT. Thin layer chromatography of reaction products from a representative experiment is shown with non-acetylated products at the bottom and the upper acetylated products. The expression and reporter constructs are indicated above each lane.

expression have been identified at the time of first or subsequent relapse of childhood ALL [32,51]. Our studies showed expression of ABCB1 RNA via RT-PCR in 3/3 ALL cell lines with versus only 1/7 ALL cell lines without E2A-HLF fusion. We demonstrated that the E2A-HLF-positive cell lines expressed high levels of ABCB1 protein as assessed via flow cytometry using the MRK16 antibody and had a drug efflux-positive phenotype not present in the ABCB1-negative cell lines.

We hypothesized that E2A-HLF directly activated *ABCB1* expression through a previously identified C/EBPβ binding element in the *ABCB1* promoter, because optimal C/EBP and HLF DNA binding sites are quite similar and we have previously shown that E2A-HLF can bind to and activate transcription of reporter genes under control of canonical C/EBP binding sites [25]. However, in transient transfection assays we did not detect any evidence that E2A-HLF could activate expression of *ABCB1* reporter gene constructs.

Because experimental data show that E2A fusion proteins can contribute to transformation and deregulated target gene expression via DNA-binding-dependent and -independent mechanisms, we then investigated the possibility that E2A-HLF might activate *ABCB1* expression through alternative mechanisms. We found similar induction of *ABCB1* RNA following inducible expression of E2A-HLF, E2A-PBX1, or E2AΔ, which encodes truncated E2A polypeptides. These data support previous observations that E2A fusion proteins can alter target gene expression via DNA-binding independent and perhaps by dominant negative mechanisms [14,52]. E2A-HLF specific activation of other target genes has been identified using the same metal inducible REH cell lines (Baudis et al., unpublished observations) consistent with dual mechanisms of action for E2A fusion proteins.

It is unlikely that ABCB1 expression contributes to leukemogenesis in ALL cases with a t(17;19). However, high level ABCB1 expression and functional activity in leukemias with E2A-HLF fusion may play a critical role in determining the clinical phenotype of this subset of ALL. While the t(17;19) and E2A-HLF fusion are relatively rare, primary childhood ALL cases with this abnormality have been reported to have a very poor outcome [2,8]. Verapamil, used in our in vitro studies, is a first generation modulator of ABCB1 and other similar transport proteins. Second, third, and fourth generation modulators have been developed and are currently being evaluated in clinical trials in AML and

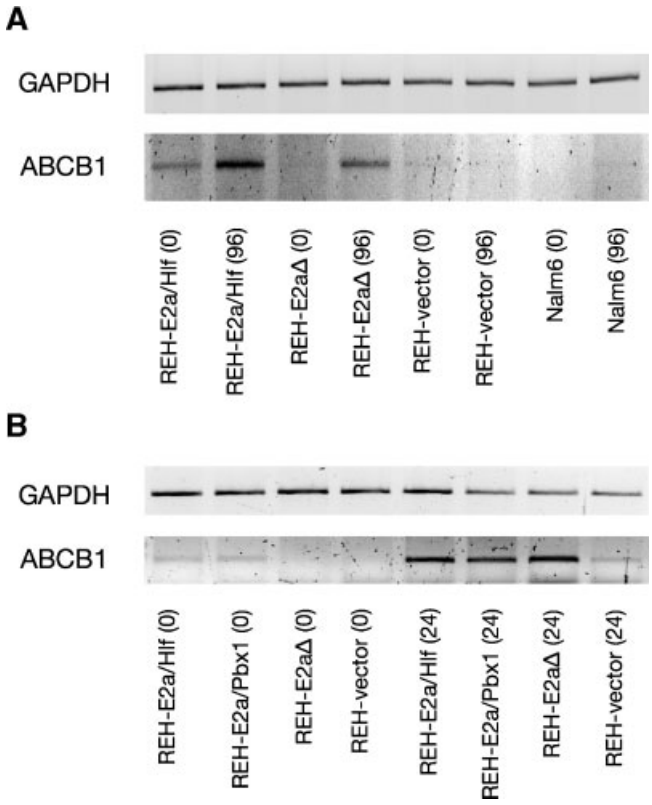

Fig. 4. *ABCB1* mRNA expression is induced following expression of E2A-HLF, E2A-PBX1 or truncated E2A polypeptides. Expression of *ABCB1* RNA was assessed by RT-PCR prior to and following inducible expression of E2A-HLF, E2A-PBX1, and E2AΔ in REH. **A**: Induction of *ABCB1* RNA in REH cells after 96 hr of Zinc activated expression of either E2A-HLF or E2AΔ. In contrast, no ABCB1 RNA is expressed pre- or post-addition of zinc to REH cells stably transfected with the empty vector or in Nalm6 cells. The low level *ABCB1* expression seen prior to induction is variable from experiment to experiment and may partially be due to the known leakiness of this inducible expression vector. **B**: *ABCB1* RNA is induced 24 hr following induction of E2A-HLF, E2A-PBX1, and E2A.

other malignancies [33]. Our observations suggest that pharmacologic inhibition of ABCB1 function might be a rational therapeutic strategy for ALL associated with E2A-HLF fusion.

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