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Differential gene expression, GATA1 target genes, and the chemotherapy sensitivity of Down syndrome megakaryocytic leukemia

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Abstract

Children with Down syndrome (DS) with acute megakaryocytic leukemia (AMkL) have very high survival rates compared with non-DS AMkL patients. Somatic mutations identified in the X-linked transcription factor gene, *GATA1*, in essentially all DS AMkL cases result in the synthesis of a shorter (40 kDa) protein (GATA1s) with altered transactivation activity and may lead to altered expression of GATA1 target genes. Using the Affymetrix U133A microarray chip, we identified 551 differentially expressed genes between DS and non-DS AMkL samples. Transcripts for the bone marrow stromal-cell antigen 2 (*BST2*) gene, encoding a transmembrane glycoprotein potentially involved in interactions between leukemia cells and bone marrow stromal cells, were 7.3-fold higher (validated by real-time polymerase chain reaction) in the non-DS compared with the DS group. Additional studies confirmed GATA1 protein binding and transactivation of the *BST2* promoter; however, stimulation of *BST2* promoter activity by GATA1s was substantially reduced compared with the full-length GATA1. CMK sublines, transfected with the *BST2* cDNA and incubated with HS-5 bone marrow stromal cells, exhibited up to 1.7-fold reduced cytosine arabinoside (ara-C)-induced apoptosis, compared with mock-transfected cells. Our results demonstrate that genes that account for differences in survival between DS and non-DS AMkL cases may be identified by microarray analysis and that differential gene expression may reflect relative transactivation capacities of the GATA1s and full-length GATA1 proteins.

Introduction

Children with Down syndrome (DS) and children without DS (non-DS) with acute megakaryocytic leukemia (AMkL) treated with cytosine arabinoside (ara-C)/anthracycline-based protocols have extremely different treatment outcomes. DS children routinely experience event-free survival (EFS) rates of 80% to 100%,¹ whereas non-DS children have extremely low EFS rates. In contrast, in a series of pediatric patients treated at St Jude Children's Research Hospital, the EFS rate of de novo non-DS AMkL patients treated with chemotherapy alone was 0%, whereas the EFS rate for non-DS AMkL patients on the Children's Cancer Group (CCG) CCG-2861/2891 protocols was 21%, significantly lower than for the DS acute myeloid leukemia (AML) group overall.^{2,3} These results highlight distinct biologic differences between DS and non-DS AMkL cases.

AMkL in DS children has several unique characteristics. For instance, (1) AMkL has been estimated to occur at a 500-fold increased incidence in DS compared with non-DS children.⁴ (2) AMkL is the most common French-American-British (FAB) subtype of DS AML cases, as reported in past studies from the Pediatric Oncology Group (POG), CCG, the Berlin-Frankfurt-Münster (BFM), Nordic Society of Pediatric Hematology and Oncology (NOPHO), Toronto, and Japan groups.³⁻¹⁰ (3) Somatic mutations in exon 2 of the X-linked gene, *GATA1*, which encodes a zinc-finger transcription factor that is essential for normal erythroid and megakaryocytic differentiation, have been detected exclusively and almost uniformly in DS AMkL cases, but not in non-DS AML or non-AMkL DS leukemia cases except for a single case.¹¹⁻¹⁵ This is the most specific abnormality other than trisomy 21 linked exclusively to DS AMkL.

Our recent studies have begun to identify factors that contribute to the significantly higher EFS rates of DS AML patients. DS blast cells generated significantly higher levels of the active intracellular ara-C metabolite, ara-CTP, following in vitro incubations with ³H-ara-C and were 4.5-fold more sensitive to ara-C, compared with a large sample of non-DS leukemia cells.¹⁶ This is reflected in altered expression of genes encoding the ara-C-metabolizing enzymes, cytidine deaminase (CDA; catalyzes the deamination of ara-C to the inactive metabolite ara-U) and deoxycytidine kinase (dCK; catalyzes the rate-limiting phosphorylation of ara-C to its active metabolite, ara-CTP) in DS AML cells.^{17,18} Interestingly, DS blast cells were also more sensitive (~12-fold) to daunorubicin than were non-DS leukemia cells.¹⁶ In all these studies, the DS AML samples were of the AMkL phenotype, suggesting a possible relationship between the AMkL phenotype and increased drug sensitivity. Importantly, the uniform detection of *GATA1* mutations in DS AMkL cases raises the possibility that *GATA1* mutations in DS AMkL blasts in themselves may result in differential regulation of target genes (eg, CDA) that contribute to their extremely high EFS rates.¹⁸

In this report, we examined patterns of differential gene expression between DS and non-DS AMkL cases using microarray technology to identify biologic differences that may account for the extremely high EFS rates of DS AMkL patients and low EFS rates of non-DS patients. Additional results are presented that examine the function of the differentially expressed bone marrow stromal-cell antigen 2 (*BST2*) gene, including its regulation by *GATA1*, and its relationship to chemotherapy sensitivity.

Materials and methods

Clinical samples

Megakaryoblasts from DS and non-DS children diagnosed with AMkL were obtained from the Children's Hospital of Michigan leukemia cell bank and from the POG 9421 study, as previously described.¹⁹ The diagnosis of AMkL was confirmed by flow cytometry detection of the megakaryocytic antigens CD41 and CD61. Mononuclear cells were isolated on Ficoll-Hypaque gradients to obtain highly purified mononuclear-cell fractions consisting mostly of leukemic blasts. Total RNAs were extracted from the samples using TRIzol reagent (Life Technologies, Bethesda, MD). Informed consent was provided according to the Declaration of Helsinki. The research protocol was approved by the Human Investigation Committee of Wayne State University School of Medicine.

Cell culture

The DS AMkL cell line, CMK, was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), and the DS AMkL cell line, CMY (established from a 21-month-old DS boy with AMkL), and the non-DS AMkL cell line, CMS, were gifts from Dr A. Fuse (National Institute of Infectious Diseases, Tokyo, Japan). The non-DS AMkL cell line, Meg-01, and the HS-5 bone marrow stromal cell line were obtained from the American Type Culture Collection (Manassas, VA). The DS and non-DS AMkL cell lines and the HS-5 bone marrow stromal cell line were cultured in RPMI 1640 with 10% fetal bovine serum and antibiotics, as previously described.²⁰ The megakaryocytic phenotypes of the CMK, CMY, CMS, and Meg-01 cell lines were confirmed by flow cytometric analysis. *Drosophila* Mel-2 (*D* Mel-2) cells were purchased from Invitrogen (Carlsbad, CA) and maintained in Schneider insect medium supplemented with 10% fetal bovine serum and 2 mM l-glutamine plus 100 U/mL penicillin and 100 µg/mL streptomycin at 28°C.

MTT drug sensitivity assay

In vitro ara-C and daunorubicin sensitivities of the clinical samples were measured by the 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT) assay, as previously described.¹⁹

Microarray analysis

cRNA synthesis and labeling. First- and second-strand cDNAs were synthesized from 5 to 15 µg total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Life Technologies) and an oligo-dT₂₄-T7 (5'-ggccagtgaattgtaatacgaactcactatagggaggcgg-3') primer according to the manufacturer's instructions. cRNAs were labeled with biotinylated UTP and CTP by in vitro transcription using double-stranded cDNA including a T7 promoter as template and the T7 RNA Transcript Labeling Kit (ENZO Diagnostics, Farmingdale, NY). For labeling, double-stranded cDNAs were washed twice with 70% ethanol and resuspended in 22 µL RNase-free H₂O. The cDNAs were incubated with 4 µL of 10 × reaction buffer, biotin-labeled ribonucleotides, dithiothreitol, RNase inhibitor mix, and 2 µL 20 × T7 RNA polymerase for 5 hours at 37°C. The

labeled cRNAs were separated from unincorporated ribonucleotides by passing through a CHROMA SPIN-100 column (Clontech, Palo Alto, CA) and precipitated at -20°C for 1 hour to overnight.

Oligonucleotide array hybridization and analysis. The cRNA pellets were resuspended in 10 μ L RNase-free H₂O and 10 μ g of each sample was fragmented by heat and ion-mediated hydrolysis at 95°C for 35 minutes in 200 mM Tris-acetate, pH 8.1, 500 mM potassium acetate, and 150 mM magnesium acetate. The fragmented cRNAs were hybridized for 16 hours at 45°C to the Affymetrix GeneChip Human Genome U133A microarray, comprising over 39 000 transcript variants, representing 33 000 of the best characterized genes, together with additional probe sets designed to represent expression sequence tagged (EST) sequences. Arrays were washed at 25°C with 6 \times SSPE (0.9 M NaCl, 60 mM NaH₂PO₄, 6 mM EDTA, and 0.01% Tween 20), followed by a stringent wash at 50°C with 100 mM MES, 0.1 M NaCl, and 0.01% Tween 20. The arrays were then stained with phycoerythrin (PE)-conjugated streptavidin (Molecular Probes, Eugene, OR) and the fluorescence intensities were determined using a laser confocal scanner (Hewlett-Packard, Santa Clara, CA). The scanned images were analyzed using Microarray software (Affymetrix, Santa Clara, CA). Sample loading and variations in staining were standardized by scaling the average of the fluorescence intensities of all genes on an array to constant target intensity (ie, 2500) for all arrays used. Data analysis was performed using Microarray Suite 5.0 (Affymetrix), following user guidelines. The signal intensity for each gene was calculated as the average intensity difference, represented by $\Sigma(\text{PM} - \text{MM})/(\text{number of probe pairs})$, where PM and MM denote perfect-match and mismatch probes. Microarray analysis was performed in collaboration with Genome Explorations (Memphis, TN).

Cluster analysis

Microarray data were analyzed using GeneSpring version 7 (Silicon Genetics, Redwood City, CA). All signal values below 0.01 were set to 0.01. Interarray variations were accounted for by dividing the signal of each gene on a given array by the median of all genes on that array. Transcripts detected as present in either the DS AMkL or non-DS AMkL samples were selected using the Affymetrix present, marginal, and absent flags. Only those probes indicated as present or marginal in about 70% of the samples for either sample type were included in subsequent analyses. For DS AMkL, each gene was required to be present or marginal in a minimum of 5 of the 7 samples. For non-DS AMkL, each gene was required to be present or marginal in a minimum of 4 of the 6 samples. Genes that were present in either cell type were further filtered to include genes whose transcripts differed in expression by a minimum of 2-fold between the DS AMkL and non-DS AMkL cases.

Analysis of variance (ANOVA) was applied to the genes selected in the previous filtering to identify differentially expressed genes with a high level of statistical confidence. One-way parametric ANOVA was used with the Benjamini and Hochberg multiple test correction to control the false discovery rate²¹ (FDR) set at 10%. Clustering was performed using the median normalized expression data with a Euclidean distance metric.

Verification of gene expression by real-time PCR

cDNAs were prepared from 1 µg RNA using random hexamer primers and a reverse transcriptase-PCR (RT-PCR) kit (Perkin Elmer, Shelton, CT), and purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Bone marrow stromal-cell antigen 2 (BST2), CD36, dual specificity phosphatase 6 (DUSP6), keratin 18 (KRT18), Rhesus blood group associated-glycoprotein (RHAG), multidrug resistance 1 (MDR1), B-cell CLL/lymphoma 2 (Bcl2), and GATA1 transcripts, along with 18S RNA levels, were quantitated using a LightCycler real time PCR machine (Roche, Indianapolis, IN). PCRs contained 2 µL purified cDNA or standard plasmid, 4 mM MgCl₂, 0.5 µM each of sense and antisense primers, and 2 µL FastStart DNA Master SYBR Green I enzyme-SYBR reaction mix (Roche). Primers are listed in [Table 1](#). PCR conditions consisted of an initial denaturing step of 95°C for 10 minutes, amplification with 35 to 55 cycles of 95°C, 59 to 69°C for 10 seconds, and 72°C for 5 seconds, followed by melting curve analysis from 40°C to 99°C, and a final cooling step to 40°C. External standard curves for the genes of interest were constructed using serial dilutions of linearized standards, prepared by cloning the amplicons synthesized with each primer set in pGEM-T-Easy plasmid (Promega). An 18S RNA plasmid construct was prepared by cloning the 18S RNA amplicon, synthesized from K562 cDNA with commercial primers (Am-bion, Austin, TX), into the pGEM T-Easy vector (Promega) and linearizing with *Apa*I. Serial dilutions were amplified in real time and the data used to prepare the 18S RNA external standard curve. Real-time PCR results were expressed as mean values from 2 to 3 independent experiments using the same cDNA preparation and normalized to 18S RNA.

Preparation of nuclear extracts and electrophoretic mobility shift assays

Nuclear extracts from CMK and Meg-01 cells were prepared as previously described.²⁰ Complementary single-stranded oligonucleotides (upper strand: 5'-gggtgcccttgattatcttcaccatcaagg-3'; lower strand: 5'-ccttgatggtgaagataatcaagggcaccc-3') were annealed (designated BST2+113/+142 probe), end labeled with ³²P-γ-ATP, and purified using Sephadex G-25 quick spin columns (Boehringer-Mannheim, Mannheim, Germany). Nuclear proteins were preincubated in a reaction solution containing 20 mM Tris-HCl, pH 7.9, 2 mM MgCl₂, 1 mM EDTA, 50 mM KCl, 0.5 mM dithiothreitol, 10% glycerol, 0.1% Nonidet P-40, and 2 µg poly(dI-dC). After 10 minutes, the ³²P-end-labeled duplex oligonucleotide (2 × 10⁵ cpm) was added, and the reaction was incubated for another 20 minutes on ice. DNA/protein complexes were separated on 5% non-denaturing polyacrylamide gels in 0.5 × Tris-borate-EDTA (TBE, pH 8.4) at 4°C and 35 mA. The gels were dried and the complexes were visualized by autoradiography.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed in CMK and Meg-01 cells as previously described,²⁰ with GATA1 C-terminus (C-20 antibody, Santa Cruz Biotechnology, Santa Cruz, CA) antibody. Standard PCR for the BST2 promoter region was performed with forward (5'-gtagaagattccagcaccctcccctaa-3') and reverse (5'-gcaggagatgggtgacattgcgacactc-3') primers spanning positions -58 to +211. An unrelated human gene (*GATA1*, exon 3) was also amplified with forward (5'tggagacttgaagacagagcggctgag-3') and reverse (5'-gaagcttgggagaggaataggctgctga-3') primers to validate the specificity of the ChIP assays.

Construction of plasmids, transient transfection, and luciferase assay

The expression vectors, pPacGATA1-L and pPacGATA1-S, corresponding to the full-length and truncated GATA1 forms, respectively, were prepared as previously described.¹⁷ The putative BST2 promoter region, spanning 759 bp upstream of the translation start site and including 211 bp of exon 1, was PCR amplified from CMK gDNA using forward (5'-ttcacgctagcccccttgcagatgaagaaacaggctcaga-3') and reverse (5'-ttcacctcgaggcaggagatgggtgacattgcgacactc-3') primers (underlined sequences in the forward and reverse primers are restriction sites for *NheI* and *XhoI*, respectively). The amplicons were digested with *NheI* and *XhoI* and ligated to the corresponding sites of the reporter gene vector, pGL3Basic, to generate pGL3B-BST2pro. The *D* Mel-2 cells were cotransfected with 1 μ g of the BST2 reporter gene construct and 125 to 500 ng GATA1-L (pPacGATA1) or GATA1-S (pPacGATA1-S), using Fugene 6 reagent (Roche), as described previously.¹⁷ Luciferase activities were assayed using the Single Luciferase Assay System (Promega) and normalized to total cell protein, measured by the Bio-Rad (Hercules, CA) protein assay system. CMK and Meg-01 cells (5×10^6 cells/400 μ L Opti-MEM) were transfected with 20 μ g pGL3B-BST2pro and 1 μ g pRLSV40 by electroporation (200 V and 950 μ F). Cells were harvested and lysates prepared 48 hours after the transfection. Firefly luciferase activity was assayed with a Dual-Luciferase Reporter Assay System (Promega) in a Turner TD2420 luminometer (Sunnyvale, CA) and normalized to *Renilla* luciferase activity.

Generation of stable BST2 transfectants and apoptosis assay

Human BST2 coding sequence was PCR amplified from CMS cDNA with forward (5'-ttcacggtaccgaattcatggcatctacttcgatga-3') and reverse (5'-ttcacctcgagagatcaagggaatgttcaagcgaaaa-3') primers (underlined sequences in the forward and reverse primers are restriction sites for *BamHI* and *XhoI*, respectively). After digestion with *BamHI* and *XhoI*, the BST2 amplicon was ligated into pcDNA3 expression vector (Invitrogen, Carlsbad, CA) to generate the pcDNA3-BST2 construct. pcDNA3-BST2 was transfected into CMK cells using Lipofectin (Invitrogen), as previously described.¹⁸ Forty-eight hours after transfection, the cells were plated in Noble agar (0.35%) in complete medium plus 10% fetal bovine serum and antibiotics and containing 1 mg/mL G418 (Sigma, St Louis, MO). Single colonies of stably transfected cells were selected after 3 weeks, expanded, and screened for BST2 transcripts by real-time RT-PCR analysis.

Mock- and BST2-transfected CMK cells, seeded at an initial density of 1×10^5 cells/mL, were cocultured in the presence and absence of HS-5 human bone marrow stromal cells (density of 8×10^5 cells/mL). The cells were incubated with 150 nM ara-C for 72 hours at 37°C, after which the cells were trypsinized, washed, and resuspended into a 1-mL volume of incubation media. To differentiate the myeloid leukemia from the stromal-cell populations, CD33-PE antibody (myeloid marker) was added with annexin-fluorescein isothiocyanate (FITC) prior to analysis with an Epics-XL MCL flow cytometer (Fullerton, CA). The individual incubation conditions included (1) culture without the HS-5 cells and no incubation with ara-C, (2) incubation with ara-C without the HS-5 cells, and (3) incubation with ara-C in the presence of HS-5 cells. Absolute viable cell counts (annexin V) in the CD33⁺ gate were corrected by the internal standard and used to establish ara-C sensitivities.

Statistical analysis

Differences in transcript levels between the DS and non-DS patient groups were compared using the nonparametric Mann-Whitney 2-sample test. The nonparametric Spearman rank correlation coefficient was used to analyze transcript levels and their relationship to MTT data. Statistical analyses were performed with StatView (Version 4.5 for Windows).

Results

DS and non-DS megakaryoblasts exhibit different sensitivities to ara-C and daunorubicin

MTT assays were used to analyze in vitro drug sensitivities of megakaryoblasts from newly diagnosed DS patients (mean age, 1.7 years) and non-DS AMkL patients (mean age, 2.3 years). Compared with non-DS megakaryoblasts ($n = 11$), DS megakaryoblasts ($n = 16$) were significantly more sensitive to both ara-C (9.7-fold; median value of 112.6 nM versus 1091 nM; $P = .001$) and daunorubicin (17.6-fold; 6.25 nM versus 109.8 nM; $P = .001$; [Figure 1](#)). Thus, our in vitro drug sensitivity results with megakaryoblast samples appear to be representative of the well-established differences in EFS rates between DS and non-DS AMkL patients. The drug sensitivity results of the DS AMkL samples in [Figure 1](#) have previously been reported.¹⁹

Patterns of differential gene expression between DS and non-DS AMkL

Differences in drug sensitivities between DS and non-DS AMkL cases are likely due to differentially expressed genes that may or may not be localized to chromosome 21. The standard approach for exploring this question is to use microarray technology. Because AMkL represents the predominant DS AML subtype, we restricted our analysis of non-DS samples to the AMkL phenotype so as to control for the clinical and biologic heterogeneity among the different AML FAB subtypes. Accordingly, total RNAs were extracted from megakaryoblasts obtained from DS ($n = 5$) and non-DS ($n = 5$) children and from DS AMkL (CMK and CMY) and non-DS AMkL (CMS) cell lines. cRNAs were prepared from the corresponding cDNAs and hybridized to an Affymetrix GeneChip Human Genome U133A microarray (comprising 22 283 transcripts) to identify differentially expressed genes between DS and non-DS samples.

For the DS group, genes present or marginal were filtered in at least 5 of 7 samples, selecting a total of 6826 genes. In the non-DS group, genes present or marginal were filtered in at least 4 of 6 samples, selecting 9679 genes. Overall, 9820 genes were selected, which were present or marginal in either group. This list of genes was further filtered for those transcripts with a minimum 2-fold change in expression between DS AMkL and non-DS AMkL, providing 1273 genes. ANOVA was applied to these 1273 genes to identify those that are differentially expressed with a high level of statistical confidence. One-way parametric ANOVA was used with the Benjamini and Hochberg multiple test correction to control the FDR.²¹ With the FDR set at 10%, 551 genes were identified as differentially expressed between DS AMkL and non-DS AMkL, all having a minimum 2-fold change in expression. The top 105 probe sets selected for

genes underexpressed and overexpressed in DS AMkL are shown in Tables [Tables22](#) and [and3,3](#), respectively.

When all 22 283 genes were clustered together, the DS and non-DS samples intermingled and could not be distinguished. However, when the 551 differentially expressed genes were clustered, 2 well-separated and distinct clusters were identified between the DS and non-DS AMkL groups, consisting of 105 genes overexpressed in the DS group and 446 genes overexpressed in the non-DS group ([Figure 2A](#)). The 551 genes were widely localized to different chromosomes, predominated by chromosomes 1, 2, 6, and 11, with only 7 genes localized to chromosome 21 ([Figure 2B](#)).

Validation of microarray results by real-time RT-PCR

To more accurately quantitate transcript levels for differentially expressed genes identified by microarrays, real-time RT-PCR was used with RNAs isolated from a larger cohort of DS (n = 15; 71.5% mean blast percentage) and non-DS AMkL (n = 11; 76% mean blast percentage) samples, including the patient samples from the microarray analysis. Several representative genes were selected from the top 105 genes identified in the cluster analysis, which were either underexpressed or overexpressed in the DS specimens relative to the non-DS group ([Table 4](#)). The underexpressed genes included: (1) bone marrow stromal-cell antigen 2 (*BST2*; [NM_004335](#); localized to 19p13.2); (2) dual specificity phosphatase 6 (*DUSP6*; [BC003143](#); localized to 12q22-q23); (3) keratin 18 (*KRT18*; NM_00224; localized to 12q13); and (4) B-cell CLL/lymphoma 2 (*Bcl2*; [NM_000633](#); localized to 18q21.33). The overexpressed genes included (1) CD36 antigen (collagen type I receptor, thrombospondin receptor; [NM_000072](#); localized to 7q11.2), (2) multidrug resistance 1 (*MDR1*; [AF016535](#); localized to 7q21.1); and (3) Rhesus blood group associated-glycoprotein (*RHAG*; [NM_000324](#); localized to 6p21.1-p11). Our results ([Table 4](#)) confirmed that the differences in expression for *BST2*, *DUSP6*, *KRT18*, and *CD36* genes were statistically significant between the 2 groups. However, there were no statistically significant changes in the transcript levels for the *Bcl-2*, *MDR1*, and *RHAG* genes despite appreciable differences in their median values. This is presumably due to the small sample numbers in our patient cohort.

Role of *GATA1* in the differential expression of *BST2* in DS and non-DS AMkL

The finding that essentially all DS AMkL cases exhibit somatic mutations in exon 2 of the *GATA1* gene that result in synthesis of a shorter (40 kDa) *GATA1* protein with altered transcriptional activity from the full-length (50 kDa) form¹¹⁻¹⁴ provides a possible explanation for the differences in gene expression between DS and non-DS AMkL samples (Tables [\(Tables3,3,4\).4](#)). Indeed, this was exactly the case for CDA, for which our previous study identified a close association between the presence of *GATA1* gene mutations, *GATA1* binding sites in the CDA promoter, and differential patterns of CDA gene expression in DS and non-DS AML.¹⁸

Based on its potential relevance to leukemia biology, the *BST2* gene was selected as a prototype for further studies to identify the mechanism responsible for its differential expression between the non-DS and DS AMkL groups. *BST2* is a novel surface membrane protein expressed in bone marrow stromal cell lines that was originally reported to support the stromal cell-dependent

growth of a murine pre-B-cell line, DW34.²² Notably, BST2 is also expressed in a variety of normal tissues and malignant cells including the non-DS AMkL cell line, Mo7, and multiple myeloma cells.^{22,23} It is interesting that cocubation of leukemia cells with bone marrow stromal cells protects the former from chemotherapy-induced apoptosis, whereas megakaryocytic differentiation of progenitor cells is inhibited by direct contact with bone marrow stromal cells.²⁴⁻²⁶ Collectively, these results suggest a potentially important role of bone marrow stroma in general, and BST2 in particular, in the biology of AMkL in DS and non-DS children. By database analysis (Transfac 3.2, MatInspector V2.2, Gesellschaft für Biotechnologische, Braunschweig, Germany), the BST2 promoter region was found to contain numerous putative *cis* elements including GATA1 (Figure 3).

Experiments were performed to explore the possible role of differences between the truncated and full-length GATA1 in regulating BST2 levels in clinically relevant DS (CMK) and non-DS (Meg-01) cell lines that exhibit similar expression patterns by real-time RT-PCR to those in the primary DS and non-DS AMkL specimens (data not shown). BST2 transcripts were easily detectable in Meg-01 cells by real-time RT-PCR; however, CMK cells lacked detectable BST2 transcripts (Figure 4A upper panel). Likewise, in reporter gene assays with a BST2 promoter-reporter gene construct, there was an 18-fold difference in luciferase activity between the lines (Figure 4A lower panel), consistent with the notion of a regulation at the transcriptional level.

On gel shifts with a double-stranded oligonucleotide probe (BST2+113/+142, including the GATA1-binding site) and nuclear extracts prepared from the CMK and Meg-01 cell lines, 2 major specific DNA-protein complexes were identified in each cell line (lane 2, labeled b and c for the CMK cell line; lanes 7 and 10, labeled a and b for the Meg-01 cell line), one (labeled b) of which was common to both cell lines (Figure 4B). All 3 complexes were competed by a 100-fold molar excess of consensus GATA1 oligonucleotide (lanes 4 and 9). Addition of an antibody (N-6 antibody) that exclusively recognizes the long-form GATA1 protein abolished complex a without an obvious effect on the faster moving complexes b and c (lanes 11 and 5). Moreover, addition of an antibody (C-20 antibody) that recognizes both the long- and short-form GATA1 proteins abolished both complex a in the Meg-01 cells and complex c in the CMK cells without affecting the formation of complex b in both cell lines. These results confirm binding of the long-form GATA1 protein to the BST2 promoter in Meg-01 cells (corresponding to complex a), and of the short GATA1 protein in CMK cells (complex c). Although complex b could be competed by the GATA1 consensus oligonucleotide in both cell lines, the N-6 and C-20 GATA1 antibodies had no effect on its formation, indicating that complex b is non-GATA1 protein. In vivo binding of the long- and short-form GATA1 proteins to the BST2 promoter in both CMK and Meg-01 chromatin extracts was confirmed by ChIP assays (Figure 4C).

To further assess whether GATA1 proteins regulate the transcription of the *BST2* gene in a fashion analogous to CDA,¹⁷ a luciferase reporter gene construct, pGL3B-BST2pro, was cotransfected with GATA1 (pPacGATA1-L or pPacGATA1-S, encoding the long- and short-form GATA1 proteins, respectively) into *D* Mel-2 cells. pPacGATA1-L stimulated the BST2 promoter construct in a dose-dependent manner (Figure 5). Although a dose-dependent transactivation was also seen for pPacGATA1-S, its magnitude was substantially reduced (Figure 5). These results directly establish that BST2 is a bona fide GATA1 target gene.

Relationship between levels of BST2 and GATA1 transcripts in non-DS AMkL patients

Although the results in Figures [Figures44](#) and [and55](#) clearly demonstrate a role for GATA1 protein binding and differential transactivation of the BST2 promoter by short versus long GATA1 proteins, it is important to further validate BST2 as a GATA1 target in primary AMkL specimens. Accordingly, we used real-time RT-PCR to quantitate expression of BST2 and GATA1 transcripts. The focus for this series of experiments was exclusively on non-DS AML cells (11 non-DS AMkL and 17 non-DS AML), because DS megakaryoblasts only express the 40 kDa GATA1 short protein with decreased transactivating potential from that for the full-length form ([Figure 5](#)). As illustrated by [Figure 6](#), there was a statistically significant correlation between GATA1 transcript levels and BST2 transcript levels ($r = 0.67$; $P = .001$) for these 28 non-DS specimens. These results strongly support the notion of an important role for GATA1 in regulating BST2 expression in non-DS AML.

Functional role of the *BST2* gene in determining AML sensitivity to ara-C

There was no statistically significant correlation between BST2 transcript levels and in vitro ara-C sensitivities for primary DS and non-DS AMkL samples (data not shown). However, the finding that coincubation of AML cells with the human bone marrow stromal cells could improve AML-cell survival in vitro, and attenuate ara-C-induced cell killing and apoptosis, [24,25](#) suggested that bone marrow stromal cells play a critical role in regulating proliferation and apoptosis of leukemia cells.

Based on these results, experiments were performed in vitro to directly assess the effects of *BST2* gene expression in clinically relevant AML models on ara-C sensitivities in the presence and absence of HS-5 human bone marrow stromal cells. The DS AMkL cell line, CMK, which does not express BST2, was stably transfected with a full-length BST2 cDNA. Two clones (designated BST2#8 and BST2#11) were isolated that expressed increased (~25- and ~100-fold, respectively) BST2 transcripts over the mock-transfected CMK cells ([Figure 7A](#)). A flow cytometry-based assay (ie, annexin V binding) was used to measure apoptosis in mock-transfected and BST2-transfected sublines coincubated over 72 hours with and without HS-5 cells in the presence of ara-C (150 nM).

As anticipated, in vitro incubation of the mock-transfected and 2 BST2-transfected CMK clones with ara-C in the absence of HS-5 cells did not result in a significant difference in apoptosis (not shown). Similar results were obtained by MTT assay (not shown). However, the BST2#8 and BST2#11 transfectants showed a significantly greater reduction (1.41- and 1.76-fold, respectively) from ara-C-induced apoptosis in the presence of HS-5 bone marrow stromal cells than the mock-transfected CMK cells ([Figure 7B](#)). Due to technical difficulties related to autofluorescence of daunorubicin, daunorubicin-induced apoptosis could not be assessed with the flow cytometry-based apoptosis assay. Although the molecular basis for these results is not entirely clear, they would seem to suggest that BST2 directly mediates the regulatory effects of bone marrow stromal cells on the leukemia cells, leading to leukemia-cell protection from ara-C-induced cytotoxicity.

Discussion

Microarray analysis represents a powerful tool for the simultaneous analysis of expression of thousands of genes that has been used to identify differences between leukemia phenotypes or clinical outcomes.^{27,28} In the present report, we used microarrays to analyze differentially expressed genes between DS and non-DS AMkL patients to identify genes that may be linked to the significantly higher EFS rates of DS AMkL patients or the exceedingly poor outcome of non-DS AMkL patients. Using the Affymetrix U133A microarray, 551 genes were identified by one-way ANOVA that differed at least 2-fold in their expression levels between the DS and non-DS groups and that formed 2 well-separated and distinct clusters. Although it would be expected that a gene copy/gene dosage effect would result in a 1.5-fold higher expression of chromosome 21-localized genes in DS leukemia cells compared with non-DS leukemia cells, we found that the localization of genes identified in the cluster analysis involved a number of chromosomes, with only 7 of the 551 genes being localized to chromosome 21. Because a 2-fold difference in expression was used to filter the differentially expressed genes between DS and non-DS samples, chromosome 21-localized genes with the theoretical 1.5-fold differential expression would be filtered out of the analysis. Of the 551 genes identified by the cluster analysis, 105 genes were overexpressed in the DS group and 447 genes were overexpressed in the non-DS group.

Real-time PCR was used to validate the expression levels of 7 representative genes in a larger group of DS and non-DS leukemia samples. The *BST2*, *KRT18*, and *DUSP6* transcripts were 7.3-, 18.2-, and 5.3-fold higher, respectively, in the non-DS than in the DS megakaryoblast samples, whereas *CD36* transcripts were increased 3.1-fold in the DS group. Although differences in the median values for *MDR1*, *BCL-2*, and *RHAG* transcripts were also increased in DS AMkLs, these were not statistically significant, possibly due to the modest sample size.

The identification of somatic mutations in exon 2 of the transcription factor gene, *GATA1*, in essentially all DS AMkL cases including the DS samples used in this study (data not shown) suggests that the mutation is synonymous with the DS AMkL phenotype. The resulting synthesis of a shorter (40 kDa) *GATA1* protein, with altered transcriptional activity, may have an impact on transcription of multiple genes, including many of those with differential expression between DS and non-DS AML samples identified herein. *BST2* was selected for further analysis to examine factors that contribute to differentially expressed nonchromosome 21-localized genes between the DS and non-DS AMkL group and the possible relationship between *BST2* expression and the effects of bone marrow stromal cells on AML chemotherapy drug sensitivity. By database analysis, several potential *GATA1*-binding motifs were identified in the *BST2* promoter region and, on gel shifts with nuclear extracts and ChIP assays with chromatin extracts from the DS (CMK) and non-DS (Meg-01) AMkL cell lines, binding of both long- and short-form *GATA1* proteins was demonstrated. Cotransfections of *D* Mel-2 cells with a luciferase *BST2* promoter construct with expression constructs for the long- and short-form *GATA1* proteins confirmed the markedly decreased transactivating potential of short-form *GATA1* compared with the long-form *GATA1* protein. Thus, decreased *BST2* transcripts in DS AMkL cells would appear to result from the exclusive synthesis of short-form *GATA1* protein with a compromised transcriptional activity toward *BST2*. The important role for *GATA1* in *BST2* transcription can be generalized because *GATA1* transcripts closely correlated with *BST2* expression in non-DS AML samples including AMkL and non-AMkL.

The growth and differentiation of many hematopoietic cells in vivo require direct contact with stromal cells that produce a variety of growth factors and, in some cellular systems, direct contact between stromal cells and hematopoietic cells is required for cell growth and differentiation.²⁹ The importance of stromal cells in regulating the proliferation and apoptosis of leukemia cells was previously suggested by the protection from ara-C-induced cytotoxicity/apoptosis when AML cells were coincubated with HS-5 human bone marrow stromal cells.^{24,25} In the present study, we extended these findings to include a critical role for BST2 expression by leukemia cells in facilitating this effect of bone marrow stroma on ara-C chemosensitivity because expression of BST2 was essential for the protection of leukemia cells in these experiments. Hence, low levels of BST2 in DS megakaryoblasts may lead to reduced bone marrow stromal interactions, whereas higher BST2 expression in non-DS leukemia cells likely promotes interactions between leukemia cells and bone marrow stroma, resulting in decreased apoptosis upon treatment with ara-C. Although there are likely to be other molecules on the surface of leukemia cells that interact with bone marrow stroma, because HS-5 stromal cells also provided a significant (albeit reduced) protection of mock-transfected CMK cells from ara-C-induced apoptosis, our results, nonetheless, suggest that BST2 may represent a possible new therapeutic target for AML. Interestingly, BST2 has also been reported to be up-regulated in tamoxifen-resistant breast cancer cells,³⁰ suggesting potential multiple functions for this gene in different cancers.

Two prior studies examined differentially expressed genes between DS transient myeloproliferative disorder (TMD) and DS AMkL cases.^{31,32} We did not observe an overlap between the genes identified in our analysis and from these 2 prior studies. This is not unexpected because the prior studies only examined DS TMD and AMkL blasts, which contain both mutated GATA1 genes and trisomy 21, and, hence, the mechanisms accounting for differentially expressed genes would differ from a comparison group of non-DS AMkL blasts, which lack GATA1 mutations and constitutional trisomy 21.

In conclusion, the results of this study strongly suggest that the factors accounting for the increased chemotherapy sensitivity of DS megakaryoblasts compared with non-DS megakaryoblasts are complex. Our results with BST2 imply that critical genes that contribute to biologic differences related to treatment response may be identified by microarray analysis and that differential transactivation by the long and short forms of the GATA1 protein may account for different patterns of gene expression between the 2 groups. Studies are underway to further explain the patterns of different gene expression between DS and non-DS AMkL groups and to identify additional gene targets linked to the high EFS rates of DS AMkL patients and low EFS rates of non-DS AMkL patients.

Notes

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An Inside *Blood* analysis of this article appears at the front of this issue.

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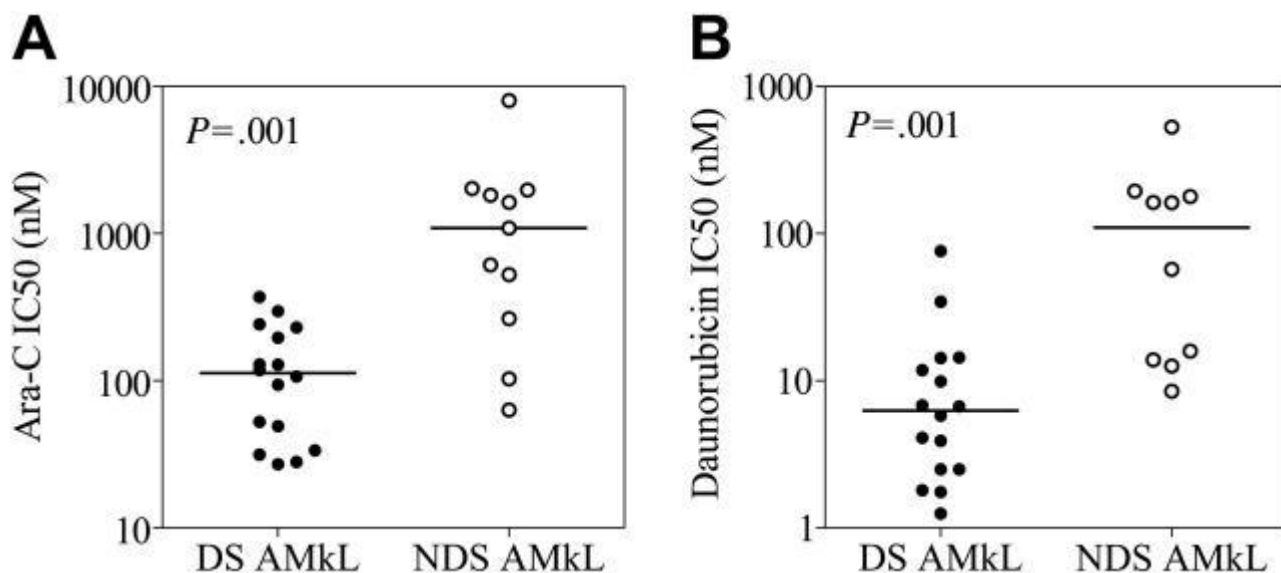
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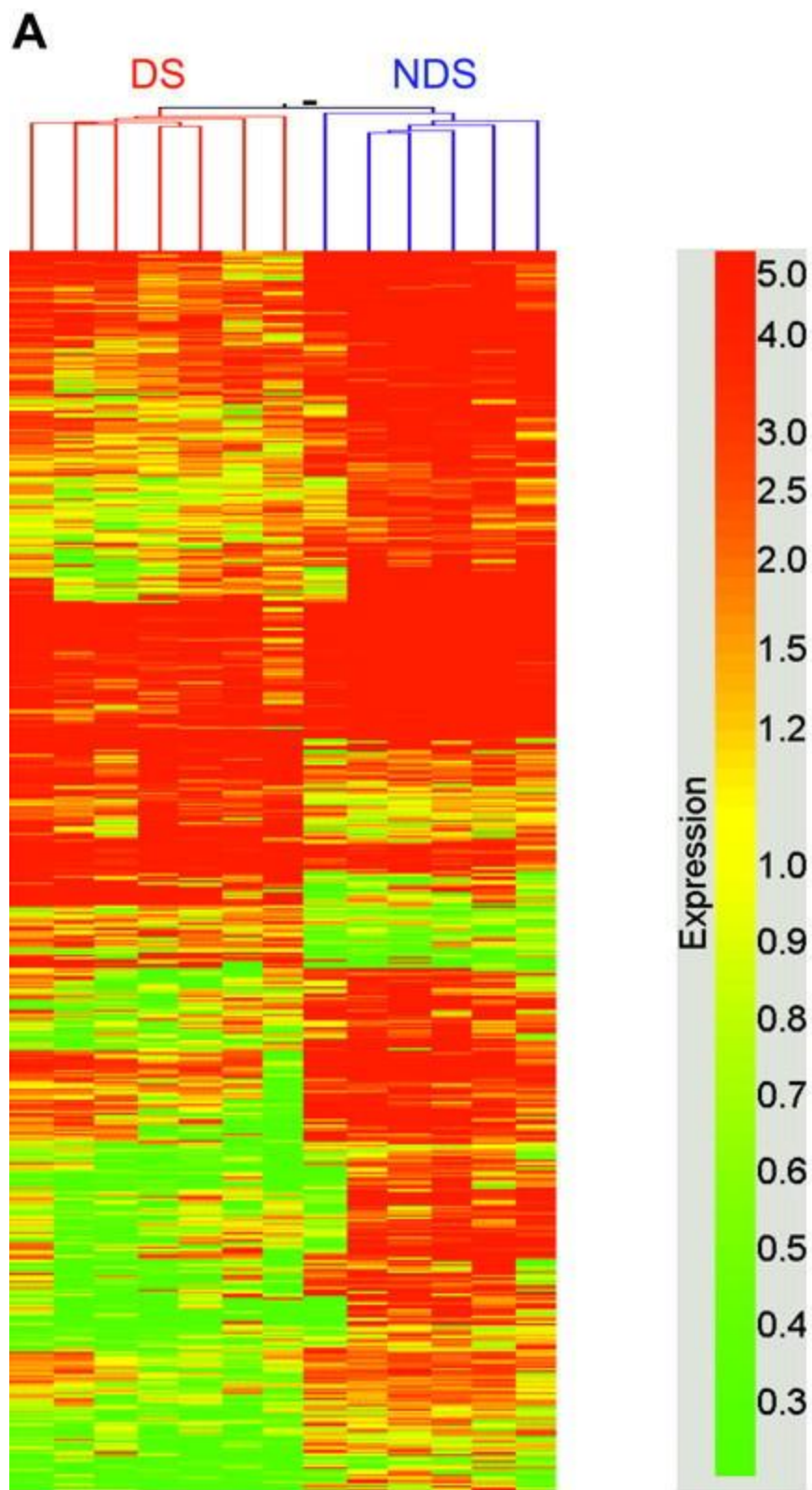
Figures and Tables

Figure 1.



Drug sensitivities of megakaryoblasts from pediatric patients with AMkL. In vitro sensitivities to ara-C (A) and daunorubicin (B) of megakaryoblasts, obtained from newly diagnosed Down syndrome (DS) and non-DS children with AMkL, were measured by the MTT assay. Drug sensitivity results of the DS AMkL samples have been reported previously.¹⁹

Figure 2.



B Physical Distribution of Differentially Expressed Genes



Cluster analysis of differentially expressed genes between DS and non-DS

megakaryoblasts; chromosomal localization of genes in cluster analysis. (A) Microarray analysis using the Affymetrix U133A genechip was performed with DS (n = 5) and non-DS (n = 5) AMkL samples and clinically relevant AMkL cell lines (n = 3). Following selection of genes that were present or marginal in either group and filtered for those transcripts with a minimum 2-fold change in expression between DS AMkL and non-DS AMkL, one-way analysis of variance (ANOVA) was used with the Benjamini and Hochberg multiple test correction to control the FDR at 10%, and identified 551 genes as differentially expressed between DS AMkL and non-DS AMkL. Clustering was performed using the median normalized expression data with a Euclidean distance metric and identified 105 genes overexpressed in the DS group and 447 genes overexpressed in the non-DS group. (B) Chromosome localization of the 551 genes analyzed in the cluster analysis.

Figure 3.

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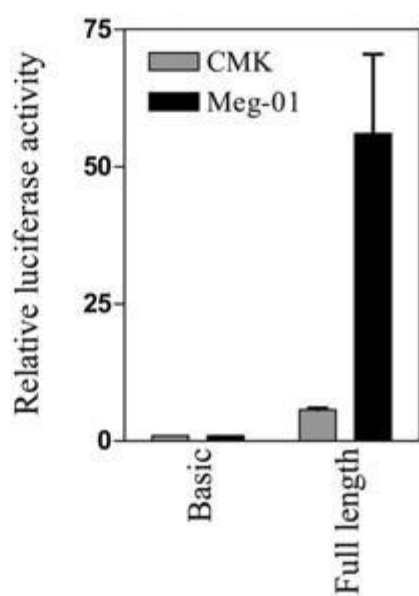
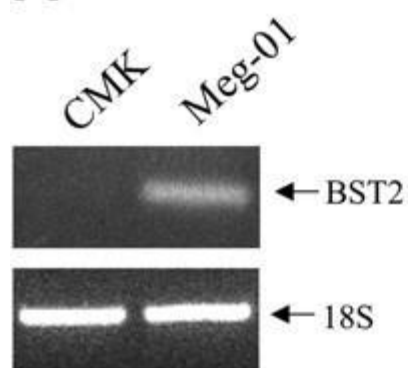
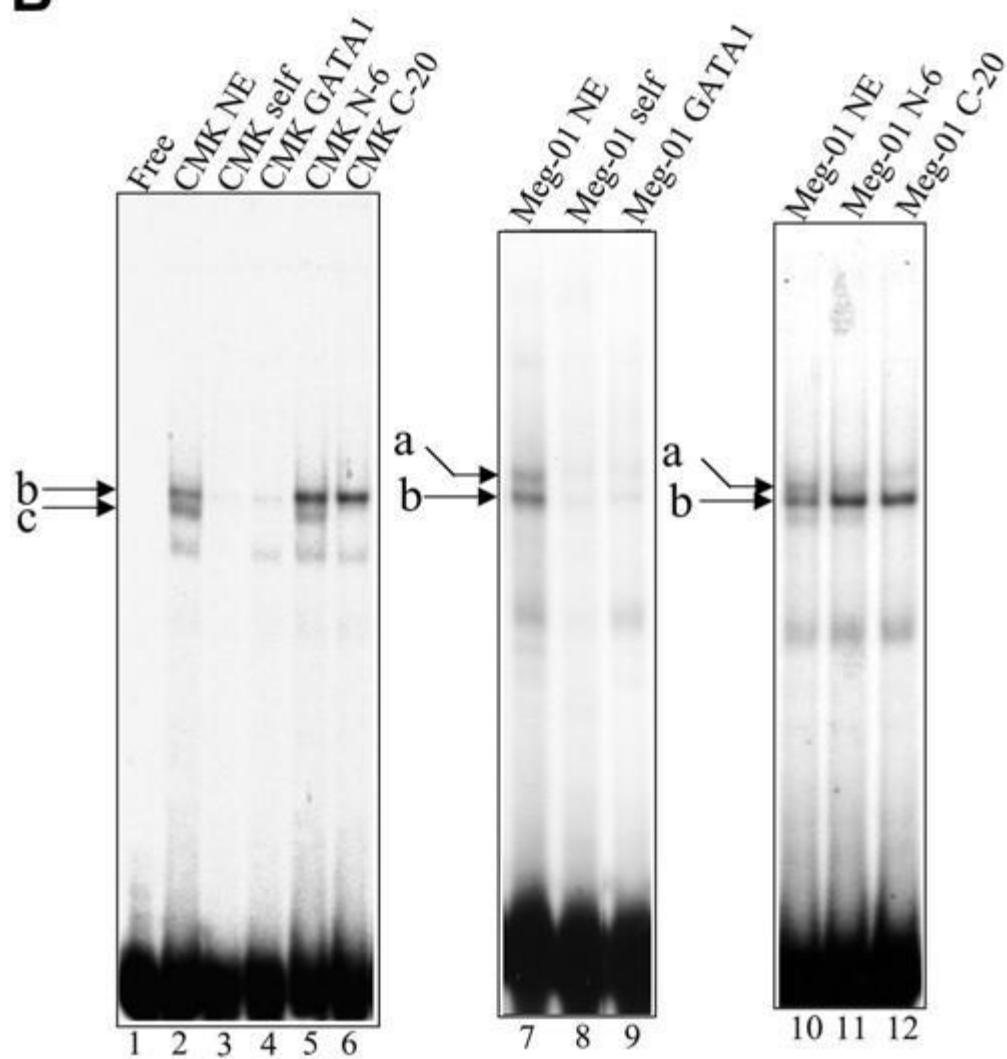
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                                     GATA1 (-)
GC ACTTTTCTGGAAGTAGGAGCTTGTTCCTGCAGGTGCCAAGACAGAGACCGACATTG -640
TT TGTGGCTGGGTGGTCTCCAGTTTTTCAGCTGGCTCCAGTCTCACC TGTTGCTCACA -580
CACCCCTCATGTCTCCCATAGTCCCCTCGGTGGGGACAGAGGCACTGGA TGAAGCCCTGC -520
TC GTCACCACAGAGACACCTGAACACAAAACCAGTCCCTGGGGTCAGACCCAGGCCCCG -460
                                     GC (-)
CCCCAGACCCAGGCCCTGCCCTCACTCCACCACGCAACTGTGCAACCTCAGTTTCCCCA -400
GG TGGAGACCGGACCAACAGTGATGGCCTCTGCCTCTTCAGGT CATAGTACAGATGAATA -340
CA GGCTGGCACGGCCTAGGCACTCAGTAACACACAGCAGAGGCACAGGGACTTAAGATGG -280
AG TGTCCAGGCAGCCACAGTTGGCTGGCACCCAGCTGGGAAGGGCCCAAGGGCTTTTAA -220
AG CAGGGTGAAAAAAAGCCCACCTCCTTTCTGGGAAACTGAAACTGAA AACCTAATTAA -160
                                     STAT1 (-)
TC CTCTGCCTGTAGGTGCCTCATGCAAGAGCTGCTGGTCAGAGCACTTTCCTGGAAC TTGC -100
                                     STAT1 (+)
TATTGGTCAGGACGTTTCCTATGCTAATAAAGGGGTGGCCCGTAGAAGATTCCAGCACCC -40
CCAAT (-)                               +1
TC CCCTAACTCCAGGCCAGACTCTAAAGGGGAGATCTGGatgcatctacttcgtatgac +21
tattgcagagtgcccatggaagacggggataagcgctgtaagcttctgctggggatagga +81
                                     GATA1 (+)                               GATA1 (+)
At tctggtgctcctgateatcgatgattctgggggtgcccctgat tatictcaacctcaag +141
                                     GATA1 (-)
gc caacagcgaggcctgccgggacggccttcgggcagtgatggagtgtcgcaatgtcacc +201
Catctcctgc +211

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Schematic of the promoter region of the bone marrow stromal-cell antigen 2 gene. Putative promoter region, spanning 759 bp upstream of the translation start site ATG including 211 bp of exon 1 of the *BST2* gene. Numbering is relative to the translation start site (+1). Potential *cis*-

regulatory elements on the plus (+) and minus (-) DNA strands (determined by Transfac 3.2, MatInspector V2.2) are underlined and shown in bold.

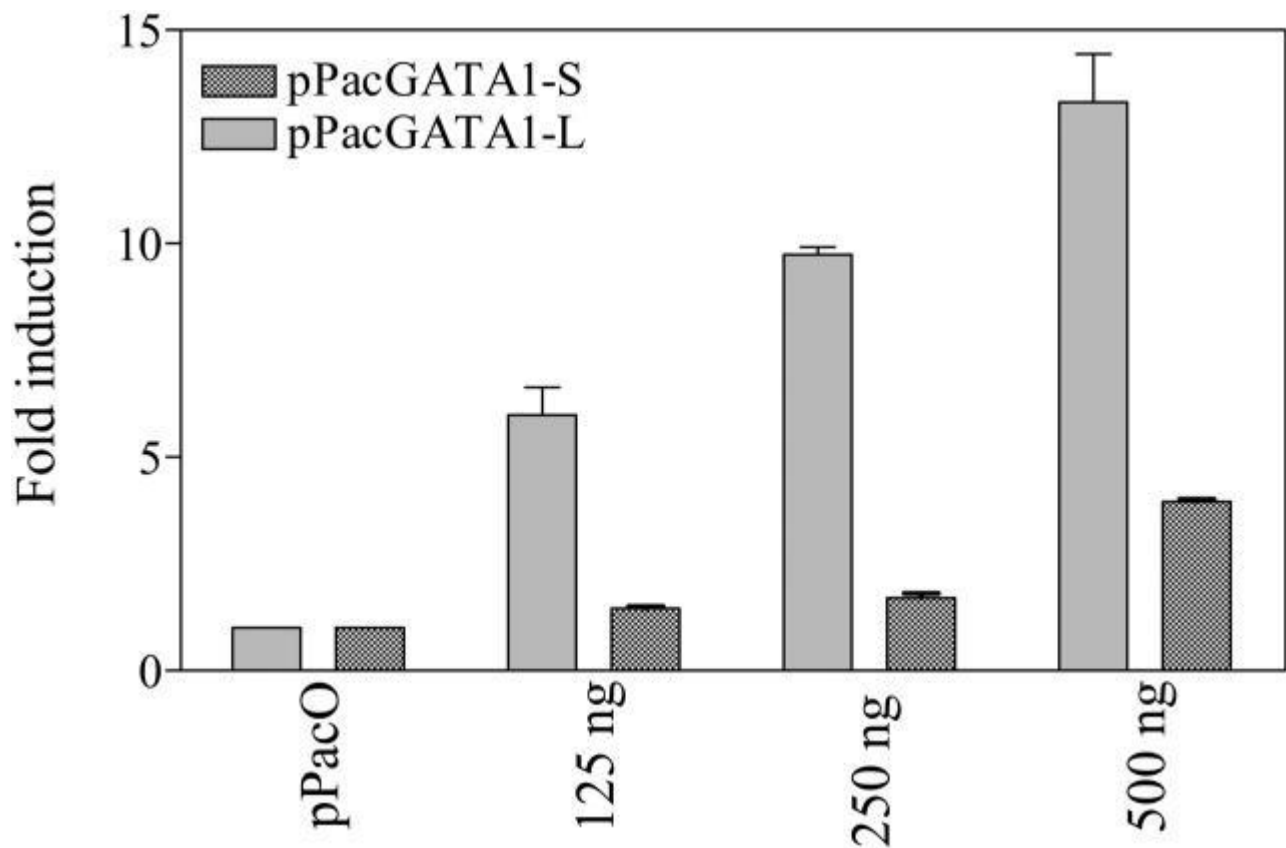
Figure 4.

A**B****C**

ID: 00110111

BST2 transcripts and promoter activity in AMkL cell lines; gel shifts with the +39/+65 BST2 probe. (A) Transcript levels of BST2 in the DS AMkL cell line, CMK, and non-DS AMkL cell line, Meg-01 (top panel), measured by real-time PCR, and BST2 promoter activities in CMK and Meg-01 cells (bottom panel), measured by relative luciferase activity following transient transfection of the cell lines with the BST2 reporter gene construct, pGL3B-BST2pro. Error bars indicate standard error of measurement of 3 independent experiments. (B) Gel shift assays were performed with CMK and Meg-01 nuclear extracts and the ³²P-labeled +39/+65 BST2 oligonucleotide probe in the absence and presence of 100-fold molar excess commercial consensus GATA1 oligonucleotide. The specific DNA/protein complexes are indicated by lowercase letters. For the supershifts, GATA1 antibodies were added to the reaction mixtures and incubated for 30 minutes prior to separating the DNA/protein complexes. (C) In vivo binding of the long- and short-form GATA1 proteins to the BST2 promoter was confirmed by ChIP assays, as described in “Materials and methods.”

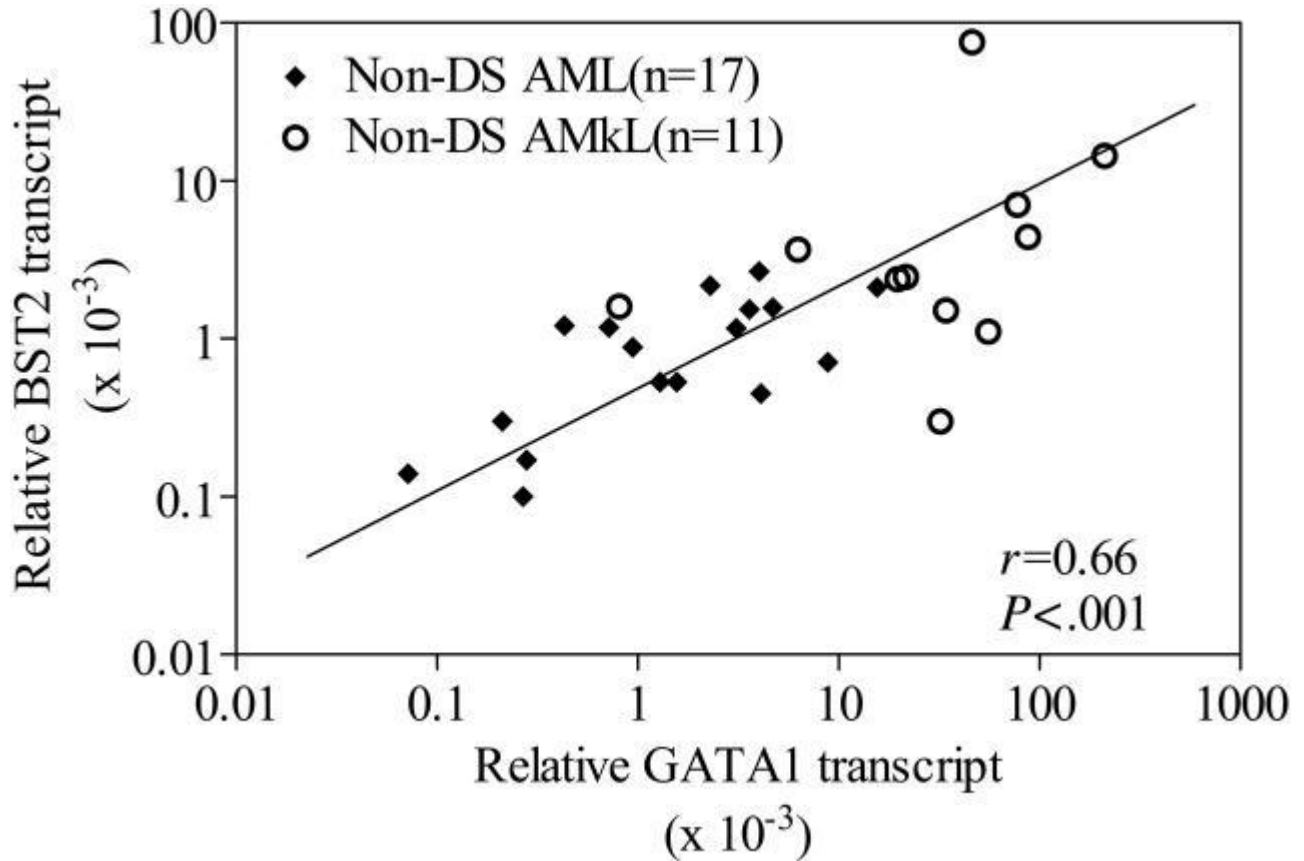
Figure 5.



Differential activation of the BST2 promoter by long- and short-form GATA1. Activation of the BST2 promoter by the long- and short-form GATA1 in *Drosophila Mel-2* cells. *Drosophila Mel-2* cells were cotransfected with 1 µg of the BST2 reporter gene construct (pGL3B-BST2pro) and 125 to 500 ng pPacGATA1-L or pPacGATA1-S. For all transfections, constant plasmid was maintained (at 500 ng of the pPac series). The results represent data from 3 experiments as the

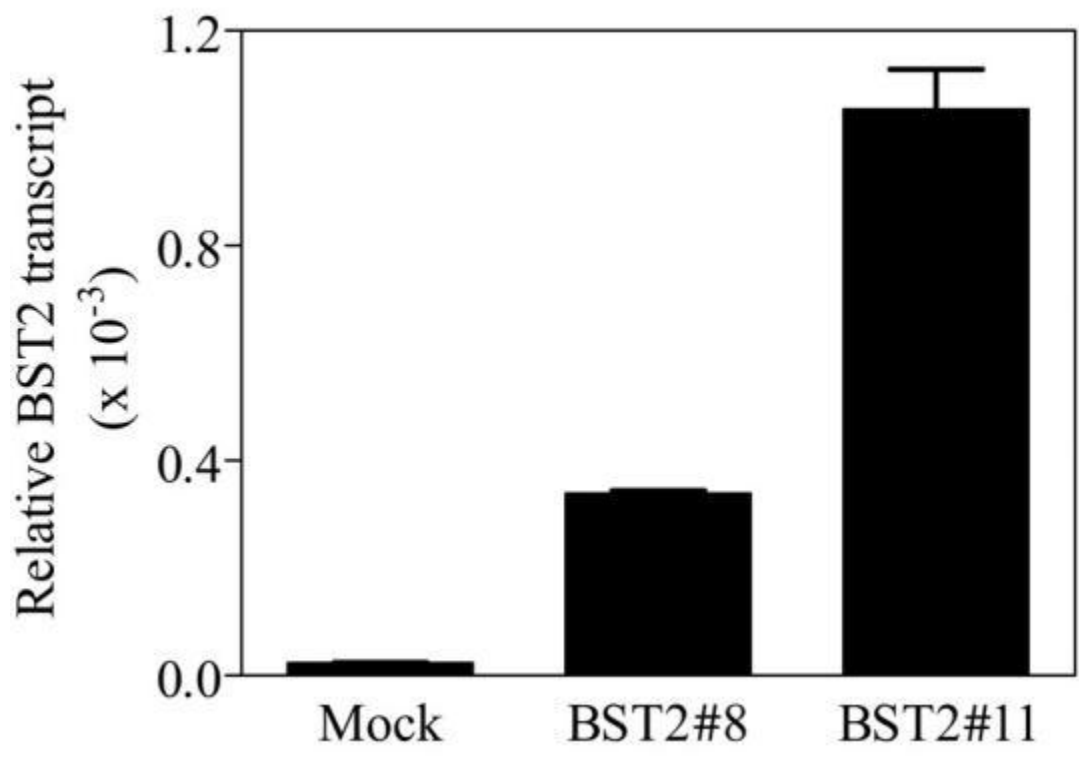
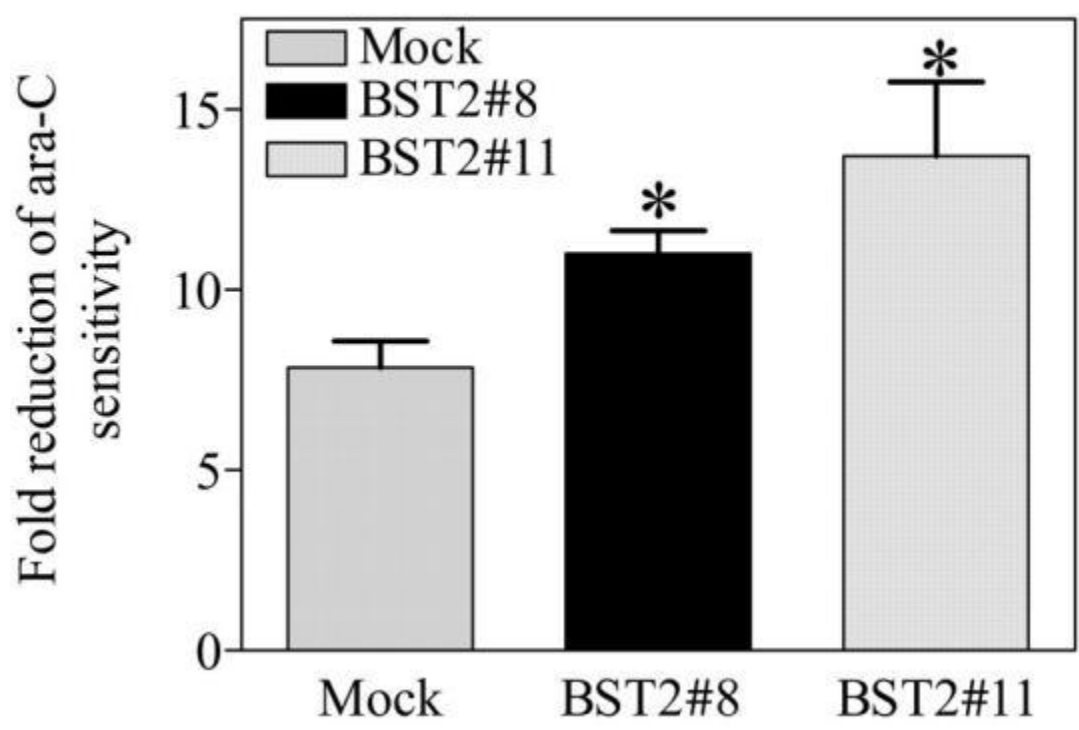
mean (\pm SD) fold increases in luciferase activity relative to a control for which pGL3B-BST2pro was cotransfected with 500 ng pPacO vector.

Figure 6.



Correlation between BST2 and GATA1 transcripts in non-DS blast cells. Correlation between BST2 and GATA1 transcripts determined by real-time RT-PCR in 17 non-DS AML and 14 non-DS AMkL patient samples. The nonparametric Spearman rank correlation coefficient was used to analyze the relationship between BST2 and GATA1 transcripts.

Figure 7.

A**B**

Functional activity of the *BST2* gene in transfected CMK sublines. (A) The coding cDNA of *BST2* gene was amplified by PCR and subcloned into a mammalian expression vector, pcDNA3. The *BST2* construct was stably transfected into the DS AMkL cell line, CMK. Overexpression of *BST2* in 2 of the stable clones was confirmed by real-time RT-PCR. (B) Protection of *BST2* stable clones from ara-C-induced apoptosis by bone marrow stromal cells, HS-5, determined with annexin-FITC on an Epics-XL MCL flow cytometer, as described in “Materials and methods.” Error bars indicate standard error of 3 independent experiments. *Statistically significant difference ($P < .005$).

Table 1.

Summary of primers used for real-time RT-PCR in this study

Gene	Primer sequences		Size, bp	Annealing temperature, °C
	Forward	Reverse		
<i>18S</i>	5'-GATGCGGGGCGTTATT-3'	5'-TGAGGTTTCCCGTGTGTCA-3'	167	59
<i>GAT</i>	5'-	5'-	17	63
<i>A1</i>	TGGAGACTTTGAAGACAGAGC GGCTGAG-3'	GAAGCTTGGGAGAGGAATAGG CTGCTGA-3'	4	
<i>BST2</i>	5'-	5'-	19	62
	CCTGCTCGGCTTTTCGCTTGAA CAT-3'	CGGAGGGAGGCTCTGGAGGGA GAC-3'	9	
<i>KRT</i>	5'-	5'-	15	62
<i>18</i>	GAGTATGAGGCCCTGCTGAAC ATCA-3'	GCGGGTGGTGGTCTTTTGGAT- 3'	0	
<i>DUS</i>	5'-	5'-	17	62
<i>P6</i>	GCTGTGCCAAAGACTCCACCA ACTT-3'	GGCCTCAGGGAAAAACTGGGA CA-3'	6	
<i>BCL</i>	5'-	5'-	11	61
<i>2</i>	CTGCACCTGACGCCCTTCACC- 3'	CACATGACCCCAACCGAACTCA AAGA-3'	9	
<i>CD3</i>	5'-	5'-	19	59
<i>6</i>	ATTGGTGCTGTCCTGGCTGTGT TT-3'	GTTCATCATCACTTCCTGTGGA TTTTC-3'	5	
<i>RHA</i>	5'-	5'-	18	61
<i>G</i>	GGCATGAAAATGAAGAGTCCG CATA-3'	CTGGAGAAGGCAAAGGCTGTG A-3'	7	
<i>MDR</i>	5'-	5'-	18	59
<i>1</i>	CAGGAACCTGTATTGTTTGCCA	TGCTTCTGCCCACTCAACT	8	

Primer sequences

Gene	Forward	Reverse	Size, bp	Annealing temperature, °C
	CCAC-3'	G-3'		

Table 2.

Top 105 probe sets selected for genes underexpressed in DS AMkL compared with non-DS AmkL

Gene	Fold change	Gene symbol	GenBank no.	Chromosomal location	Description
211796_s_at	28.12	<i>TCRBV13S1-TCRBJ2S1</i>	AF043179	—	<i>Homo sapiens</i> T-cell receptor β chain (TCRBV13S1-TCRBJ2S1) mRNA, complete cds
217022_s_at	27.97	<i>MGC27165</i>	S55735	14q32.33	Hypothetical protein MGC27165
204959_at	19.34	<i>MNDA</i>	NM_002432	1q22	Myeloid cell nuclear differentiation antigen
1405_i_at	17.93	<i>CCL5</i>	M21121	17q11.2–q12	Chemokine (C-C motif) ligand 5
218847_at	12.72	<i>IMP-2</i>	NM_006548	3q28	IGF-II mRNA-binding protein 2
219279_at	12.58	—	NM_017718	2q36.3	—
201743_at	12.53	<i>CD14</i>	NM_000591	5q22–q32	CD14 antigen
34210_at	12.48	<i>CDW52</i>	N90866	1p36	CDW52 antigen (CAMPATH-1 antigen)
216035_x_at	12.48	<i>TCF7L2</i>	AV721430	10q25.3	Transcription factor 7-like 2 (T-cell specific, HMG-box)
211991_s_at	12.38	<i>HLA-DPA1</i>	M27487	6p21.3	Major histocompatibility complex, class II, DP α 1
213539_at	12.18	<i>CD3D</i>	NM_000732	11q23	CD3D antigen, δ polypeptide (TiT3 complex)
212651_at	11.96	<i>RHOBTB1</i>	AB018283	10q21.3	Rho-related BTB domain containing 1
218223_s_at	11.36	<i>CKIP-1</i>	NM_016274	1q21.3	CK2 interacting protein 1; HQ0024c protein
200800_s_at	10.97	<i>HSPA1A</i>	NM_005345	6p21.3	Heat shock 70-kDa protein

Gene	Fold change	Gene symbol	GenBank no.	Chromosomal location	Description
					1A
214677_x_at	10.96	<i>IGLJ3</i>	X57812	22q11.1–q11.2	Immunoglobulin λ joining 3
211675_s_at	10.62	—	AF054589	7q31.2//7q31.2	—
210982_s_at	10.19	<i>HLA-DRA</i>	M60333	6p21.3	Major histocompatibility complex, class II, DR α
209138_x_at	10.17	—	M87790	22q11.1–q11.2	—
215379_x_at	9.819	<i>IGLJ3</i>	AV698647	22q11.1–q11.2	Immunoglobulin λ joining 3
211368_s_at	9.609	<i>CASP1</i>	U13700	11q23	Caspase-1, apoptosis-related cysteine protease (interleukin 1, β , convertase)
205081_at	9.594	<i>CRIP2</i>	NM_001311	7q11.23	Cysteine-rich protein 2
218559_s_at	9.557	<i>MAFB</i>	NM_005461	20q11.2–q13.1	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)
211571_s_at	9.541	<i>CSPG2</i>	D32039	5q14.3	Chondroitin sulfate proteoglycan 2 (versican)
208891_at	9.516	<i>DUSP6</i>	BC003143	12q22–q23	Dual specificity phosphatase 6
219229_at	9.488	<i>SLCO3A1</i>	NM_013272	15q26	Solute carrier organic anion transporter family, member 3A1
209040_s_at	9.468	<i>PSMB8</i>	U17496	6p21.3	Proteasome (prosome, macropain) subunit, β type, 8 (large multifunctional protease 7)
215646_s_at	9.385	<i>CSPG2</i>	R94644	5q14.3	Chondroitin sulfate proteoglycan 2 (versican)
219505_at	9.344	<i>CECR1</i>	NM_017424	22q11.2	Cat eye syndrome chromosome region, candidate 1
204891_s_at	9.093	<i>LCK</i>	NM_005356	1p34.3	Lymphocyte-specific protein tyrosine kinase
204661_at	8.961	<i>CDW52</i>	NM_001803	1p36	CDW52 antigen (CAMPATH-1 antigen)
204619_s_at	8.71	<i>CSPG2</i>	BF590263	5q14.3	Chondroitin sulfate proteoglycan 2 (versican)
217523_at	8.62	—	AV700298	11p13	AV700298 GKC <i>Homo sapiens</i> cDNA clone GKCBVGO5 3', mRNA sequence

Gene	Fold change	Gene symbol	GenBank no.	Chromosomal location	Description
201506_at	8.592	<i>TGFBI</i>	NM_000358	5q31	Transforming growth factor, β -induced, 68 kDa
208791_at	8.552	<i>CLU</i>	M25915	8p21-p12	Clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)
201641_at	8.506	<i>BST2</i>	NM_004335	19p13.2	Bone marrow stromal cell antigen 2
203949_at	8.477	<i>MPO</i>	NM_000250	17q23.1	Myeloperoxidase
204646_at	8.461	<i>DPYD</i>	NM_000110	1p22	Dihydropyrimidine dehydrogenase
218927_s_at	8.401	<i>CHST12</i>	NM_018641	7p22	Carbohydrate (chondroitin 4) sulfotransferase 12
208450_at	8.353	<i>LGALS2</i>	NM_006498	22q12-q13	Lectin, galactoside-binding, soluble, 2 (galectin 2)
221731_x_at	8.257	<i>CSPG2</i>	BF218922	5q14.3	Chondroitin sulfate proteoglycan 2 (versican)
214500_at	8.033	<i>H2AFY</i>	AF044286	5q31.3-q32	H2A histone family, member Y
208892_s_at	7.959	<i>DUSP6</i>	BC003143	12q22-q23	Dual specificity phosphatase 6
209374_s_at	7.528	<i>IGHM; MU</i>	BC001872	14q32.33	Synonym: MU; <i>Homo sapiens</i> immunoglobulin heavy constant μ , mRNA (cDNA clone MGC:1228 IMAGE:3544448), complete cds
211429_s_at	7.463	—	AF119873	—	PRO2275 mRNA, complete cds
204202_at	7.244	<i>KIAA1023</i>	NM_017604	7p22.3	<i>Homo sapiens</i> KIAA1023 protein (KIAA1023), mRNA
212063_at	7.063	<i>CD44</i>	BE903880	11p13	CD44 antigen (homing function and Indian blood group system)
201301_s_at	7.045	<i>ANXA4</i>	BC000182	2p13	Annexin A4
209304_x_at	6.957	<i>GADD45B</i>	AF087853	19p13.3	Growth arrest and DNA-damage-inducible, β
210915_x_at	6.881	<i>TCRB</i>	M15564	—	T-cell receptor precursor; human T-cell receptor

Gene	Fold change	Gene symbol	GenBank no.	Chromosomal location	Description
208894_at	6.854	<i>HLA-DRA</i>	M60334	6p21.3	rearranged β a-chain V-region (V-D-J) mRNA, complete cds Major histocompatibility complex, class II, DR α
210473_s_at	6.844	—	M37712	—	<i>Homo sapiens</i> p58/GTA protein kinase mRNA, complete cds
212068_s_at	6.822	<i>KIAA0515</i>	AB011087	9q34.3	KIAA0515
203069_at	6.8	<i>SV2A</i>	NM_014849	1q21.3	Synaptic vesicle glycoprotein 2A
202284_s_at	6.784	<i>CDKN1A</i>	NM_000389	6p21.2	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
212592_at	6.749	<i>IGJ</i>	AV733266	4q21	Immunoglobulin J polypeptide, linker protein for immunoglobulin α and μ polypeptides
217762_s_at	6.711	<i>RAB31</i>	BE789881	18p11.3	RAB31, member RAS oncogene family
203275_at	6.701	<i>IRF2</i>	NM_002199	4q34.1–q35.1	Interferon regulatory factor 2
203431_s_at	6.602	<i>RICS</i>	NM_014715	11q24–q25	Rho GTPase-activating protein
203148_s_at	6.58	<i>TRIM14</i>	NM_014788	9q31.1	Tripartite motif-containing 14
203408_s_at	6.548	<i>SATB1</i>	NM_002971	3p23	Special AT-rich sequence-binding protein 1 (binds to nuclear matrix/scaffold-associating DNAs)

Table 3.

Top 105 probe sets selected for genes overexpressed in DS AMkL compared with non-DS AMkL

Gene name	Fold change	Gene symbol	GenBank no.	Chromosomal location	Description
219870_at	0.5	<i>ATF7IP2</i>	NM_024997	16p13.2	Activating transcription factor 7 interacting protein 2
203377_s_at	0.498	<i>CDC40</i>	NM_015891	6q22.1	Cell division cycle 40 homolog (yeast)

Gene name	Fold change	Gene symbol	GenBank no.	Chromosomal location	Description
217793_at	0.494	<i>RAB11B</i>	AL575337	19p13.3	RAB11B, member RAS oncogene family
206875_s_at	0.491	<i>SLK</i>	NM_014720	10q25.1	SNF1 sucrose nonfermenting like kinase (yeast)
203910_at	0.491	<i>PARG1</i>	NM_004815	1p22.1	PTPL1-associated RhoGAP1
221832_s_at	0.491	<i>AOF2</i>	AV741657	1p36.11	Amine oxidase (flavin containing) domain 2
210718_s_at	0.49	<i>LOC51326</i>	AF119889	17q21.32	ARF protein
218712_at	0.489	<i>FLJ20508</i>	NM_017850	1p34.2	Hypothetical protein FLJ20508
214801_at	0.485	—	W88821	—	CDNA FLJ11392 fis, clone HEMBA1000575
212246_at	0.482	<i>MCFD2</i>	BE880828	2p21	Multiple coagulation factor deficiency 2
202008_s_at	0.48	<i>NID</i>	NM_002508	1q43	Nidogen (enactin)
202421_at	0.476	<i>IGSF3</i>	AB007935	1p13	Immunoglobulin superfamily, member 3
222329_x_at	0.463	—	AW974816	—	Transcribed sequence with weak similarity to protein sp: P39188 (<i>Homo sapiens</i>) ALU1_HUMAN Alu subfamily J sequence contamination warning entry
202557_at	0.46	<i>STCH</i>	AI718418	21q11.1	Stress 70 protein chaperone, microsome-associated, 60 kDa
209045_at	0.459	<i>XPNPEP1</i>	AF195530	10q25.3	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble
218344_s_at	0.457	<i>FLJ10876</i>	NM_018254	1q32.3	Hypothetical protein FLJ10876
202468_s_at	0.451	<i>CTNNAL1</i>	NM_003798	9q31.2	Catenin (cadherin-associated protein), α -like 1
203015_s_at	0.451	<i>SSX2IP</i>	AW136988	—	Synovial sarcoma, X breakpoint 2 interacting protein
203224_at	0.447	<i>FLJ11149</i>	BF340123	9q21.31	Riboflavin kinase
203225_s_at	0.443	<i>FLJ11149</i>	NM_018339	9q21.31	Riboflavin kinase
202932_at	0.442	<i>YES1</i>	NM_005433	18p11.31–p11.21	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
214205_x_at	0.44	<i>TXNL2</i>	AK022131	6p25.3	Thioredoxin-like 2
204417_at	0.439	<i>GALC</i>	NM_000153	14q31	Galactosylceramidase (Krabbe disease)
210818_s_at	0.437	<i>BACHI</i>	AF026199	21q22.11	BTB and CNC homology 1,

Gene name	Fold change	Gene symbol	GenBank no.	Chromosomal location	Description
					basic leucine zipper transcription factor 1
207332_s_at	0.433	<i>TFRC</i>	NM_003234	3q26.2-qter	Transferrin receptor (p90, CD71)
202949_s_at	0.432	<i>FHL2</i>	NM_001450	2q12-q14	Four and a half LIM domains 2
221580_s_at	0.425	<i>MGC5306</i>	BC001972	11q21	Hypothetical protein MGC5306
207386_at	0.42	<i>CYP7B1</i>	NM_004820	8q21.3	Cytochrome P450, family 7, subfamily B, polypeptide 1
207845_s_at	0.42	<i>APC10</i>	NM_014885	4q31	Anaphase-promoting complex subunit 10
212884_x_at	0.414	<i>APOE</i>	AI358867	19q13.2	Apolipoprotein E
212978_at	0.413	<i>TA-LRRP</i>	AU146004	1p22.2	T-cell activation leucine repeat-rich protein
211810_s_at	0.405	<i>GALC</i>	D25284	14q31	Galactosylceramidase (Krabbe disease)
202506_at	0.405	<i>SSFA2</i>	NM_006751	2q32.1	Sperm-specific antigen 2
206693_at	0.403	<i>IL7</i>	NM_000880	8q12-q13	Interleukin-7
203640_at	0.401	—	BE328496	13q32.2	hs98f09.x1 NCI_CGAP_Kid13 <i>Homo sapiens</i> cDNA clone IMAGE:3145289 3', mRNA sequence
208398_s_at	0.392	<i>TBPL1</i>	NM_004865	6q22.1-q22.3	TBP-like 1
214252_s_at	0.388	—	AV700514	13q21.1-q32	AV700514 GKC <i>Homo sapiens</i> cDNA clone GKCDQC04 3', mRNA sequence
204624_at	0.387	<i>ATP7B</i>	NM_000053	13q14.2-q21	ATPase, Cu ⁺⁺ transporting, β polypeptide (Wilson disease)
213757_at	0.386	<i>EIF5A</i>	AA393940	17p13-p12	Eukaryotic translation initiation factor 5A
203789_s_at	0.382	<i>SEMA3C</i>	NM_006379	7q21-q31	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C
209267_s_at	0.373	<i>SLC39A8</i>	AB040120	4q22-q24	Solute carrier family 39 (zinc transporter), member 8
216063_at	0.369	<i>HBBP1</i>	N55205	—	Transcribed sequence with moderate similarity to protein sp: P02096 (<i>Homo sapiens</i>) HBG_HUMAN hemoglobin γ -A and γ -G chains
208691_at	0.367	<i>TFRC</i>	BC001188	3q26.2-qter	Transferrin receptor (p90,

Gene name	Fold change	Gene symbol	GenBank no.	Chromosomal location	Description
					CD71)
212990_at	0.366	<i>SYNJI</i>	AB020717	21q22.2	Synaptojanin 1
213358_at	0.365	<i>KIAA0802</i>	AB018345	18p11.22	KIAA0802 protein
202558_s_at	0.361	<i>STCH</i>	NM_006948	21q11.1	Stress 70 protein chaperone, microsome-associated, 60 kDa
203019_x_at	0.36	<i>SSX2IP</i>	NM_014021	—	Synovial sarcoma, X breakpoint 2 interacting protein
205667_at	0.358	<i>WRN</i>	NM_000553	8p12-p11.2	Werner syndrome
219177_at	0.355	<i>BRIX</i>	NM_018321	5p13.3	BRIX
209676_at	0.354	<i>TFPI</i>	J03225	2q31-q32.1	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)
212558_at	0.351	<i>GDAP1L1</i>	BF508662	20q12	Ganglioside-induced differentiation-associated protein 1-like 1
203017_s_at	0.346	<i>SSX2IP</i>	R52678	—	Synovial sarcoma, X breakpoint 2 interacting protein
204976_s_at	0.338	<i>AMMECRI</i>	AK023637	Xq22.3	Alport syndrome, mental retardation, midface hypoplasia, and elliptocytosis chromosomal region, gene 1
216398_at	0.338	<i>GYPE</i>	U05255	4q31.1	Glycophorin E
219795_at	0.334	<i>SLC6A14</i>	NM_007231	Xq23-q24	Solute carrier family 6 (neurotransmitter transporter), member 14
203544_s_at	0.329	<i>STAM</i>	NM_003473	10p14-p13	Signal transducing adaptor molecule (SH3 domain and ITAM motif) 1
210664_s_at	0.318	<i>TFPI</i>	AF021834	2q31-q32.1	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)
202479_s_at	0.316	<i>TRB2</i>	BC002637	2p25.1	Tribbles homolog 2
202481_at	0.31	<i>DHRS3</i>	NM_004753	1p36.1	Dehydrogenase/reductase (SDR family) member 3
217388_s_at	0.31	<i>KYNU</i>	D55639	2q22.3	Kynureninase (L-kynurenine hydrolase)

Table 4.

Summary of median transcript levels in DS and non-DS megakaryoblast samples

Gene	DS AMkL	Non-DS AMkL	Fold difference by real-time PCR	<i>P</i>	Fold difference by microarray
<i>BST2</i>	0.220	1.600		7.3 .004	8.5
<i>KRT18</i>	1.430	26.020		18.2 .020	5.7
<i>DUSP6</i>	0.600	3.150		5.3 .003	7.9
<i>BCL2</i>	0.163	1.067		6.5 .070	5.1
<i>CD36</i>	1.473	0.476		3.1 .012	4.6
<i>RHAG</i>	0.185	0.114		1.6 .384	8.6
<i>MDR1</i>	0.881	0.430		2.0 .195	4.5

Data are shown for median transcript levels determined by real-time RT-PCR assays, as described in “Materials and methods.” Statistical differences between the groups for the real-time RT-PCR results were determined by the nonparametric Mann-Whitney 2-sample test.

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