NIH Public Access
Author Manuscript
Br J Haematol. Author manuscript; available in PMC 2014 September 01.

Published in final edited form as:
Br J Haematol. 2013 September ; 162(5): 670–677. doi:10.1111/bjh.12444.

High *EVI1* Expression Is Associated with *MLL* Rearrangements and Predicts Decreased Survival in Paediatric AML: A Report From the Children's Oncology Group

Phoenix A. Ho[1,2,3], **Todd A. Alonzo**[3,4], **Robert B Gerbing**[3], **Jessica A. Pollard**[1,2,3], **Betsy Hirsch**[3,5], **Susana C. Raimondi**[3,6], **Todd Cooper**[3,7], **Alan S. Gamis**[3,8], and **Soheil Meshinchi**[1,2,3]

[1]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA

[2]Division of Pediatric Hematology / Oncology, Seattle Children's Hospital, University of Washington School of Medicine, Arcadia, CA

[3]Children's Oncology Group, Arcadia, CA

[4]Department of Biostatistics, University of Southern California, Los Angeles

[5]Department of Laboratory Medicine and Pathology, University of Minnesota Cancer Center, Minneapolis

[6]Department of Pathology, St. Jude Children's Research Hospital, Memphis, TN

[7]Emory University / Children's Healthcare of Atlanta, GA

[8]Division of Hematology-Oncology, Children's Mercy Hospitals and Clinics, Kansas City, MO

Abstract

EVI1 is highly expressed in certain cytogenetic subsets of adult acute myeloid leukaemia (AML), and has been associated with inferior survival. We sought to examine the clinical and biological associations of *EVI1*^{high}, defined as expression in excess of normal controls, in paediatric AML. *EVI1* mRNA expression was measured via quantitative real-time polymerase chain reaction in diagnostic specimens obtained from 206 patients. Expression levels were correlated with clinical features and outcome. *EVI1*^{high} was present in 58/206 (28%) patients. *MLL* rearrangements occurred in 40% of *EVI1*^{high} patients as opposed to 12% of the *EVI1*$^{low/absent}$ patients (p<0.001). No abnormalities of 3q26 were found in *EVI1*^{high} patients by conventional cytogenetic analysis, nor were cryptic 3q26 abnormalities detected in a subset of patients screened by next-generation sequencing. French-American-British class M7 was enriched in the *EVI1*^{high} group, accounting for 24% of these patients. *EVI1*^{high} patients had significantly lower 5-year overall survival from study entry (51% vs. 68%, p=0.015). However, in multivariate analysis including other established prognostic markers, *EVI1* expression did not retain independent prognostic significance. *EVI1* expression is currently being studied in a larger cohort of patients enrolled on subsequent

Please forward correspondence to: Phoenix Ho, MD, Fred Hutchinson Cancer Research Center, Clinical Research Division, D2-373, 1100 Fairview Ave. N, Seattle, WA 98103, USA, Phone # 206-667-7640, Fax # 206-667-6084, pho@fhcrc.org.

AUTHOR CONTRIBUTIONS

P. A. H. performed research, analysed data, and wrote the manuscript. T. A. A. and R. B. G performed statistical analysis and edited the manuscript. B. H. and S. C. R. performed cytogenetic analysis and edited the manuscript. J. A. P., T. C., and A. S. G. analysed data and edited the manuscript. S. M. designed research, analysed data, and wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no relevant conflicts of interest.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Children's Oncology Group trials, to determine if *EVI1*^{high} has prognostic value in *MLL*-rearranged or intermediate-risk subsets.

Keywords

cute myeloid leukaemia; paediatric cancer; *MLL*; *EVI1*

INTRODUCTION

Aberrant overexpression of specific genes is a common finding in acute myeloid leukaemia (AML), and may define clinically relevant biological subsets that lack other cytogenetic or molecular prognostic markers (Mawad & Estey, 2012). The ectopic viral integration site-1 *(EVI1)* gene is a proto-oncogene subject to alternative splicing, and encodes a zinc finger protein that functions as a transcriptional regulator in early development (Hoyt *et al*, 1997). The gene was first identified as a common site of viral integration in retrovirus-induced murine leukaemia, suggesting a role for *EVI1* in the transformation of haematopoietic cells (Morishita *et al*, 1988). Forced over-expression of *EVI1* in haematopoietic progenitors was later shown to induce a myeloid differentiation block, also resulting in increased self-renewal and survival of these transformed progenitors (Laricchia-Robbio & Nucifora, 2008).

Although high *EVI1* expression in adult AML is commonly found in association with rearrangements of 3q26 (Lugthart *et al*, 2008), the chromosomal location of the *EVI1* gene, cytogenetic rearrangements involving this locus are rare in paediatric AML (Harrison *et al*, 2010). However, *MLL* translocations, a cytogenetic subgroup that accounts for approximately 16% of paediatric AML patients (Harrison *et al*, 2010), have also been reported to occur at high frequencies in patients with *EVI1* overexpression (Lugthart *et al*, 2008; Balgobind *et al*, 2010). Chromosomal rearrangements involving *MLL*, a histone methyltransferase gene, frequently lead to deregulation of *HOX* genes and result in distinct aberrant methylation signatures (Bernt *et al*, 2011). Likewise, in addition to its role in transcriptional control, *EVI1* has recently been implicated in epigenetic processes due to its interaction with both the histone methyltransferase SUV39H1 (Cattaneo *et al*, 2008) and the DNA methyltransferases DNMT3A and DNMT3B (Senyuk *et al*, 2011). Though the molecular mechanism for the association between *MLL* translocations and *EVI1* expression has yet to be elucidated, it is possible that both of these events cooperate in epigenetic dysregulation, leading to myeloid leukaemia.

Overexpression of the *EVI1* transcription factor, as determined by calibration against normal samples taken from healthy volunteers, has been reported in 7–10% of adult AML patient samples; further, high expression of any of the common *EVI1* isoforms was found to predict significantly decreased survival in these adult AML studies (Lugthart *et al*, 2008; Groschel *et al*, 2010). In the single previous study of *EVI1* expression in paediatric AML, investigators from several European cooperative groups reported that *EVI1* overexpression was prognostic in univariate, but not multivariate, analysis (Balgobind *et al*, 2010). This study included paediatric patients enrolled on five different clinical trials, and defined *EVI1* overexpression on the basis of gene expression profiling. In the present study, we examined the clinical and biological significance of *EVI1* overexpression, as measured by quantitative real-time polymerase chain reaction (qRT-PCR), in uniformly-treated paediatric AML patients enrolled on the Children's Oncology Group (COG) pilot trial AAML03P1.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

PATIENTS, MATERIALS, AND METHODS

Patient Samples

The COG pilot trial AAML03P1 tested the safety and efficacy of the addition of the calicheamicin-linked anti-CD33 monoclonal antibody gemtuzumab ozogamicin (GO) to a five-cycle multi-agent chemotherapy backbone (Cooper *et al*, 2012). Newly diagnosed paediatric *de novo* AML patients enrolled in the COG-AAML03P1 trial were eligible for the present study. Patients with acute promyelocytic leukaemia, constitutional trisomy 21, or antecedent myelodysplastic syndrome (MDS) were excluded. Morphological, flow cytometric, cytogenetic, and molecular analyses were performed according to study guidelines (Cooper *et al*, 2012). Analysis for cytogenetic abnormalities by an AML fluorescent *in situ* hybridization (FISH) panel and G-banding of metaphase chromosomes was performed on all patients at diagnosis, and results were available for all patients included in this study. Of the 340 eligible patients enrolled in AAML03P1 between December 2003 and November 2005, 206 patients (61%) had diagnostic specimens with adequate RNA quality available for expression analysis. Demographic, laboratory, and clinical characteristics of patients with vs. without specimens adequate for analysis were compared. Median diagnostic white blood cell (WBC) count (p=0.002) and median diagnostic marrow blast percentage (p=0.039) were both significantly higher in patients with samples analysed, as is common in retrospective studies utilizing cryopreserved specimens. *FLT3* internal tandem duplication (ITD) was also more common in patients with samples available for analysis (14% vs. 4%, p=0.035). There were no significant differences in age, race, or cytogenetic distribution between the two groups. Outcome measures were not significantly different between patients with and without specimens available for analysis.

This study was approved by the COG Myeloid Disease Biology Committee, and Institutional Review Board approval was obtained from the Fred Hutchinson Cancer Research Center. In accordance with the Declaration of Helsinki, informed consent for study protocol treatment and tissue sample evaluation was obtained from patients or their legal guardians.

Molecular Genotyping and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The AllPrep DNA/RNA Mini Kit and the QIAcube automated system (Qiagen, Valencia, CA) were used to extract genetic material from cryopreserved diagnostic bone marrow specimens. Molecular genotyping for mutations in *FLT3, NPM1* and *CEBPA*, was performed as previously described (Ho *et al*, 2011a).

Reverse transcription was performed on 1 μg total diagnostic RNA per standard protocol (Invitrogen Corporation, Carlsbad, CA). *EVI1* mRNA expression was measured by performing qRT-PCR on cDNA transcripts on a StepOne Plus real-time PCR instrument, using TaqMan Universal PCR Master Mix and TaqMan *EVI1* Gene Expression Assay (Applied Biosystems, Foster City, CA) with primer / probe set designed to hybridize within a region spanning exons 2 and 3 (Figure 1). These C-terminal exons are common to all of the known major splice isoforms of *EVI1*, including the four isoforms resulting from the alternate 5' un-translated exons 1A, 1B, 1C, and 1D, as well as the *MDS1* and *EVI1* complex (*MECOM*) fusion transcript resulting from intergenic splicing; thus this assay detects "total" *EVI1* expression. Patient samples were tested in duplicate and the beta glucuronidase (*GUSB*) housekeeping gene was quantitated as an internal control. Samples with *GUSB* cycle time (Ct) >25 were excluded from further analysis. The comparative Ct method (Schmittgen & Livak, 2008) was used to determine *EVI1* relative expression levels, normalized against pooled donor normal peripheral blood (PB) controls. *EVI1* expression was reported as fold change PB.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Additionally, next-generation sequencing data from whole transcriptome sequencing (RNA-Seq; n=68) and whole genome sequencing (WGS; n=134) performed on COG paediatric AML patients as part of the National Cancer Institute's (NCI) Therapeutically Applicable Research to Generate Effective Treatments (TARGET) Initiative (www.target.cancer.gov) was examined for the presence of 3q26 alterations. RNA-Seq data was analysed by four different bioinformatic algorithms for the detection of cryptic fusion transcripts (deFuse [McPherson *et al*, 2011], TopHat-Fusion [Kim & Salzberg, 2011], FusionMap [Ge *et al*, 2011], and SnowShoes-FTD [Asmann *et al*, 2011]); WGS was performed by Complete Genomics, Inc. (CGI; Mountain View, CA) and cryptic fusions were determined by CGI proprietary algorithms.

Statistical Methods

The Kaplan-Meier method was used to estimate overall survival (OS), event-free survival (EFS) and disease-free survival (DFS). OS was defined as time from study entry to death from any cause. EFS was defined as the time from study entry to relapse or death. DFS was defined as time from course 1 for patients in complete remission (CR) to relapse or death. CR was defined as bone marrow aspirate containing <5% blasts by morphology and no evidence of extramedullary disease. The significance of predictor variables was tested with the log-rank statistic for OS and DFS. The significance of observed differences in proportions was tested by the Chi-square test and Fisher's exact test when data were sparse. The Mann-Whitney test was used to determine the significance between differences in medians. Cox proportional hazard models were used to estimate hazard ratios (HR) for univariate and multivariate analyses for OS and DFS. Statistical significance was defined as p-value less than 0.05.

RESULTS

EVI1 Expression and Correlation with Disease Characteristics

Diagnostic *EVI1* expression levels varied widely across our study cohort of paediatric AML patients, (Figure 2). The majority of patients (148/206 patients, 72%) had either undetectable *EVI1* expression, or *EVI1* expression levels lower than normal PB controls. The remaining subset of patients (58/206 patients, 28%), with *EVI1* >1.0-fold normal, were considered to have overexpression of *EVI1* ($EVI1^{high}$). Median *EVI1* expression in the $EVI1^{high}$ group was 174.67-fold normal (range 1.13- to 6660.88-fold normal).

Diagnostic clinical and laboratory parameters were compared between $EVI1^{high}$ patients and the remainder of the study cohort (Table I). There was no difference in gender or racial distribution between the two groups. Infant patients (less than 1 year of age) accounted for 40% of the $EVI1^{high}$ group compared to 14% of remaining patients (p<0.001). Median diagnostic bone marrow blast percentage was similar among the 2 groups, but $EVI1^{high}$ patients had significantly lower median WBC counts at diagnosis (15.4×10^9/l vs. 35.0×10^9/l, p=0.021). French-American-British (FAB) class was non-randomly distributed between the two groups defined by *EVI1* expression. FAB class M7 (acute megakaryoblastic leukaemia) was significantly more common in $EVI1^{high}$ patients, accounting for 24% of this group as opposed to 1% of the remaining patients (Figure 3, p<0.001), while FAB class M4 accounted for 18% of $EVI1^{high}$ patients vs. 33% of remaining patients (p=0.038). In terms of cytogenetic subgroups, $EVI1^{high}$ patients were significantly less likely to harbour either of the favourable-risk core binding factor (CBF) translocations (4% vs. 15% prevalence of t(8;21), p=0.030, and 0% vs. 21% prevalence of inv(16), p<0.001). Conversely, all cases of the high-risk monosomy 7 abnormality occurred in $EVI1^{high}$ patients, accounting for 8% of this group (p=0.006). Translocations involving the *MLL* gene on 11q23 were also enriched in the $EVI1^{high}$ cohort, occurring in 40% of

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

patients vs. 12% of the remaining patients (p<0.001). Although *EVI1* expression has been linked to 3q26 rearrangements in adult AML, no chromosome 3 abnormalities at the level of conventional cytogenetics were detected in *EVI1*$^{\text{high}}$ patients in our study.

We next examined the relationship between diagnostic *EVI1* expression and the presence of prognostic mutations (Table I; Meshinchi *et al*, 2006; Brown *et al*, 2007; Ho *et al*, 2009). *FLT3*-ITD mutations were present at similar frequencies in both the *EVI1*^{high} and low / undetectable *EVI1* groups. No *EVI1*^{high} patients harboured the favourable-risk *NPM1* mutation, and only a single *EVI1*^{high} patient harboured a *CEBPA* mutation (biallelic). Complete cytogenetic and molecular profiles were available for 195 of the 206 patients included in this study. In recent COG AML trials, cytogenetic and molecular prognostic markers are combined to define the following risk groups: a) favourable-risk: patients without *FLT3*-ITD who presented with either a CBF translocation, *NPM1* mutation, or *CEBPA* mutation; b) high-risk: patients with either *FLT3*-ITD with high mutant to wild-type allelic ratio (>0.4) or adverse cytogenetics (either monosomy 5, deletion of 5q, or monosomy 7); and c) intermediate-risk patients: all remaining patients not classified as either favourable-risk or high-risk. The prevalence of cytogenetic / molecular risk groups was non-randomly (p<0.001) distributed among the *EVI1* expression groups. The majority (81%) of *EVI1*^{high} patients belonged to the intermediate-risk group, lacking other cytogenetic or molecular prognostic markers. Favourable-risk patients accounted for only 4% of the *EVI1*^{high} patients as compared to 48% of remaining patients.

Absence of 3q26 Rearrangements in Paediatric AML

In adult AML, overexpression of *EVI1* is often associated with chromosomal abnormalities involving the 3q26 locus itself, most commonly inv(3)(q21q26) and t(3;3)(q21;q26) (Lugthart *et al*, 2008). These rearrangements are rare in paediatric AML. A recent study of cytogenetic abnormalities in childhood AML from the British Medical Research Council (MRC) reported 3q26 abnormalities in only 2 patients, out of 729 children with AML treated on MRC trials AML 10 and AML 12 (Harrison *et al*, 2010), and no 3q26 abnormalities were found in *EVI1*^{high} patients in the single previous paediatric study of *EVI1* expression (Balgobind *et al*, 2010). None of the *EVI1*^{high} patients in our study harboured a 3q26 rearrangement at the level of conventional cytogenetics. However, novel cytogenetically cryptic 3q26 rearrangements have recently been described in adult AML in association with *EVI1* overexpression (Lugthart *et al*, 2008; Haferlach *et al*, 2012).

As part of the NCI TARGET Initiative, a cohort of COG paediatric AML patient samples were subjected to either whole transcriptome (n=68) and/or whole genome sequencing (n=134); a proportionate subset of *EVI*^{high} patients were represented in each group. Given the paucity of chromosome 3 abnormalities detected in paediatric AML detected by conventional methodologies, next-generation sequencing data was bioinformatically examined for fusion transcripts involving 3q26. No cryptic rearrangements involving the *EVI1* locus at 3q26 were detectable in this childhood AML population by any of the 4 algorithms performed on RNA-Seq data (McPherson *et al*, 2011; Kim & Salzberg, 2011; Ge *et al*, 2011; Asmann *et al*, 2011), or by CGI proprietary algorithms performed on WGS data. In our study, 3q26 cytogenetic abnormalities were absent in paediatric AML.

MLL Translocation Partners

Rearrangements of the *MLL* gene on 11q23, with a variety of translocation partners, were detected in 40% of *EVI1*^{high} patients (Table II). The most common *MLL* translocation in this group was t(9;11)(p22;q23), as is the case in unselected paediatric AML patients. The *EVI1*^{high} group of patients also included all cases of t(11;19)(q23;p13) (n=3), t(2;11) (q35;q23) (n=1), and t(6;11)(q27;q23) (n=1). A large international retrospective study of

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

MLL translocations in paediatric AML recently identified an association between t(6;11) and inferior survival outcome (Balgobind *et al*, 2009). No patient with t(1;11)(q21;q23), t(2;11)(q33;q23), t(10;11)(p11.2;q23), or t(X;11)(q13;q23) translocations had high expression of *EVI1*.

Diagnostic *EVI1* Expression and Clinical Outcome

Response to therapy and survival outcomes were compared between *EVI1*^{high} patients and patients with low / undetectable *EVI1* (Figure 4). *EVI1*^{high} patients had a CR rate of 73% after the first course of induction therapy, as compared to 82% for the remaining patients (p=0.151). Patients in the *EVI1*^{high} cohort had significantly lower rates of 5-year OS (51 ± 14% vs. 68 ± 8%, p=0.015) and EFS (40 ± 13% vs. 52 ± 8%, p=0.042). For patients who achieved CR, 5-year DFS was 50 ± 16% for the *EVI1*^{high} cohort vs. 59 ± 9% for the remaining patients, p=0.140. For the 99 intermediate-risk patients included in this study, 5-year OS was 50 ± 16% for *EVI1*^{high} patients (n=44) vs. 63 ± 13% for the remaining patients (n=55, p=0.263).

Prognostic Effect of *EVI1* Expression In Cytogenetic / Molecular Risk Groups

Cox regression analysis was then performed to evaluate the significance of *EVI1* expression as a predictor of outcome in the context of established cytogenetic and molecular risk groups (favourable-risk, intermediate-risk, and high-risk, as defined above). Risk groups were used as a covariate for both univariate and multivariate models (Table III). In separate univariate models, favourable-risk group was a strong predictor of improved OS (HR for death from study enrollment compared to intermediate-risk group: 0.31, p<0.001) and improved DFS (HR for relapse or death from initial remission: 0.55, p=0.043); for high-risk group, HR was 1.72 for OS (p=0.065) and 1.72 for DFS (p=0.108). In a separate univariate model, high *EVI1* expression was also a significant predictor of decreased OS (HR=1.79, p=0.016) but not DFS (HR=1.48, p=0.142). In a multivariate model including high *EVI1* expression and the aforementioned risk groups, *EVI1*^{high} did not retain independent prognostic significance for OS (HR for death from study enrollment: 1.17, p=0.554).

DISCUSSION

This retrospective study presents an evaluation of the biological associations and clinical relevance of diagnostic *EVI1* expression in paediatric AML patients uniformly treated on the COG pilot trial AAML03P1. *EVI1* expression levels varied broadly in our study, but only 28% of patients had overexpression of this gene (in excess of normal controls). Even within the cohort of *EVI1*^{high} patients, a wide range of expression was noted, although the magnitude of this variation is probably amplified by the use of normal tissue, in which the gene is expressed at low levels, as a reference control. Nonetheless, by using overexpression above normal as a threshold for determining high *EVI1* expression, we were able to detect intriguing biological differences between *EVI1*^{high} patients and the remaining patients with low or absent *EVI1* expression.

The prevalence of *EVI1* overexpression in our study was higher than the 6–10% reported in adult AML (Lugthart *et al*, 2008; Groschel *et al*, 2010); this age-dependent discrepancy is not surprising given the preponderance of *MLL*-rearranged infant patients in the *EVI11*^{high} group. The prevalence of *EVI1* overexpression in our study was also higher than the prevalence in the single prior paediatric report (Balgobind *et a*l, 2010), although this may reflect a difference in definition. *EVI1* over-expression was reported on the basis of gene expression profiling in the Balgobind study, whereas our study defined *EVI1*^{high} as over-expression relative to normal on the basis of qRT-PCR. The majority (81%) of *EVI1*^{high} patients in our trial belonged to the intermediate-risk group based on current cytogenetic /

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

molecular risk stratification; only 2 patients with either favourable-risk CBF chromosomal abnormalities, and / or favourable-risk gene mutations, exhibited overexpression of *EVI1*. Monosomy 7, a rare high-risk cytogenetic abnormality in *de novo* pediatric AML, occurred in only 4 patients included in our study; all 4 monosomy 7 patients were found to have high *EVI1* expression. Although FAB class is not incorporated into current risk-stratification schemes, *EVI1* overexpression was also significantly associated with FAB class M7 unrelated to trisomy 21, which has been reported to confer adverse prognosis in paediatric AML (Barnard *et al*, 2007). High *EVI1* expression was a significant predictor of inferior survival outcomes in univariate, but not multivariate, analysis in our study.

As advances in genomic technology improve our molecular understanding of AML, it is becoming increasingly clear that paediatric and adult forms of the disease are biologically distinct (Ho *et al*, 2011b). Overexpression of *EVI1* in adult AML is frequently associated with, and presumed to directly result from, alterations of 3q26. We did not detect any chromosomal rearrangements of 3q26 in our paediatric AML patients, either at the level of conventional cytogenetics, or cryptically in our analysis of whole genome and transcriptome sequencing data. The mechanisms of *EVI1* overexpression in paediatric AML appear to be distinct from *EVI1* overexpression in the setting of chromosome 3 abnormalities in adult AML.

However, the deregulation of EVI1 function, as a result of *EVI1* overexpression, may explain the association between *EVI1^{high}* patients and certain clinical features common to both paediatric and adult AML. For example, *EVI1* overexpression and resultant deregulation occurs in the setting of the adult AML "3q21q26 syndrome". This syndrome of myelodysplasia and abnormal megakaryopoiesis in acute myeloblastic leukaemia with 3q26 rearrangements is highly associated with acquired monosomy 7, often in the setting of underlying or preceding MDS (Martinelli *et al*, 2003). It is possible that the paediatric *EVI1^{high}* patients with monosomy 7 in our study had underlying MDS but were not diagnosed until after the transformation to AML. Further, the association with abnormal megakaryopoiesis may hint at one of the roles of *EVI1* in haematopoiesis. *In vitro* overexpression of *EVI1* in murine emybronic stem (ES) cells has been demonstrated to result in cell proliferation, clonogenicity, and differentiation shifted to enhance megakarypoiesis (Sitailo *et al*, 1999). Thus, it is not surprising that nearly all cases of acute megakaryoblastic leukaemia (FAB class M7) had high *EVI1* expression in our study.

Rearrangements of the *MLL* gene on 11q23 are present in 15–20% of *de novo* paediatric AML patients. *MLL*-rearrangements comprise a biologically and clinically heterogeneous group, as the *MLL* gene has over 50 known translocation partners (Balgobind *et al*, 2009). Thus, this cytogenetic group as a whole is considered intermediate-risk in the present COG risk-stratification scheme, although the recent large international study of 11q23-rearranged paediatric AML identified specific translocations with prognostic associations (Balgobind *et al*, 2009). Further, in a recent report of nearly 300 11q23-rearranged adult AML patients, overexpression of *EVI1* identified a subset of high-risk patients with poor survival outcomes within the *MLL*-rearranged cytogenetic group (Groschel *et al*, 2013). Our present study is not powered to determine the prognostic relevance of *EVI1* overexpression in paediatric AML with *MLL* translocations, or robust correlation between *EVI1* expression and specific *MLL* translocation partners. A larger cohort of patients from the AAML03P1 successor Phase III trial, COG-AAML0531, is currently being evaluated for *EVI1* expression. This should allow for analysis of outcome based on *EVI1* expression in the 11q23-rearranged cohort, as well as expanded analysis of the significance of *EVI1* expression in the cytogenetic / molecular intermediate-risk group. The identification of *EVI1^{high}* patients at diagnosis may have therapeutic as well as prognostic relevance. High *EVI1* expression has been recently linked to aberrant overexpression of CD52, a surface glycoprotein normally

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

present on lymphocytes, which is the target of the monoclonal antibody alemtuzumab (Saito *et al*, 2011).

Acknowledgments

We would like to thank the patients and families who consented to the use of biological specimens in this trial, and we thank the COG AML Reference Laboratory for providing diagnostic specimens. This work was supported by the National Institutes of Health grants R21 CA10262 (S.M.), R01 CA114563 (S.M.), and COG Chair's Grant U10 CA98543, as well as the Mary Claire Satterly Foundation, Alex's Lemonade Stand Foundation, and the St. Baldrick's Foundation (P.A.H.).

References

Asmann YW, Hossain A, Necela BM, Middha S, Kalari KR, Sun Z, Chai HS, Williamson DW, Radisky D, Schroth GP, Kocher JP, Perez EA, Thompson EA. A novel bioinformatics pipeline for identification and characterization of fusion transcripts in breast cancer and normal cell lines. Nucleic Acids Research. 2011; 39:e100. [PubMed: 21622959]

Balgobind BV, Raimondi SC, Harbott J, Zimmermann M, Alonzo TA, Auvrignon A, Beverloo HB, Chang M, Creutzig U, Dworzak MN, Forestier E, Gibson B, Hasle H, Harrison CJ, Heerema NA, Kaspers GJ, Leszl A, Litvinko N, Nigro LL, Morimoto A, Perot C, Pieters R, Reinhardt D, Rubnitz JE, Smith FO, Stary J, Stasevich I, Strehl S, Taga T, Tomizawa D, Webb D, Zemanova Z, Zwaan CM, van den Heuvel-Eibrink MM. Novel prognostic subgroups in childhood 11q23/MLL-rearranged acute myeloid leukemia: results of an international retrospective study. Blood. 2009; 114:2489–2496. [PubMed: 19528532]

Balgobind BV, Lugthart S, Hollink IH, Arentsen-Peters ST, van Wering ER, de Graaf SS, Reinhardt D, Creutzig U, Kaspers GJ, de Bont ES, Stary J, Trka J, Zimmermann M, Beverloo HB, Pieters R, Delwel R, Zwaan CM, van den Heuvel-Eibrink MM. EVI1 overexpression in distinct subtypes of pediatric acute myeloid leukemia. Leukemia. 2010; 24:942–949. [PubMed: 20357826]

Barnard DR, Alonzo TA, Gerbing RB, Lange B, Woods WG. Comparison of childhood myelodysplastic syndrome, AML FAB M6 or M7, CCG 2891: a report from the Children's Oncology Group. Pediatric Blood & Cancer. 2007; 49:17–22. [PubMed: 16856158]

Bernt KM, Armstrong SA. Targeting epigenetic programs in MLL-rearranged leukemias. Hematology / the Education Program of the American Society of Hematology. 2011; 2011:354–360.

Brown P, McIntyre E, Rau R, Meshinchi S, Lacayo N, Dahl G, Alonzo TA, Chang M, Arceci RJ, Small D. The incidence and clinical significance of nucleophosmin mutations in childhood AML. Blood. 2007; 110:979–985. [PubMed: 17440048]

Cattaneo F, Nucifora G. EVI1 recruits the histone methyltransferase SUV39H1 for transcription repression. Journal of Cellular Biochemistry. 2008; 105:344–352. [PubMed: 18655152]

Cooper TM, Franklin J, Gerbing RB, Alonzo TA, Hurwitz C, Raimondi SC, Hirsch B, Smith FO, Mathew P, Arceci RJ, Feusner J, Iannone R, Lavey RS, Meshinchi S, Gamis A. AAML03P1, a pilot study of the safety of gemtuzumab ozogamicin in combination with chemotherapy for newly diagnosed childhood acute myeloid leukemia: a report from the Children's Oncology Group. Cancer. 2012; 118:761–769. [PubMed: 21766293]

Ge H, Liu K, Juan T, Fang F, Newman M, Hoeck W. FusionMap: detecting fusion genes from next-generation sequencing data at base-pair resolution. Bioinformatics. 2011; 27:1922–1928. [PubMed: 21593131]

Gröschel S, Lugthart S, Schlenk RF, Valk PJ, Eiwen K, Goudswaard C, van Putten WJ, Kayser S, Verdonck LF, Lübbert M, Ossenkoppele GJ, Germing U, Schmidt-Wolf I, Schlegelberger B, Krauter J, Ganser A, Döhner H, Löwenberg B, Döhner K, Delwel R. High EVI1 expression predicts outcome in younger adult patients with acute myeloid leukemia and is associated with distinct cytogenetic abnormalities. Journal of Clinical Oncology. 2010; 28:2101–2107. [PubMed: 20308656]

Groschel S, Schlenk RF, Engelmann J, Rockova V, Teleanu V, Kühn MW, Eiwen K, Erpelinck C, Havermans M, Lübbert M, Germing U, Schmidt-Wolf IG, Beverloo HB, Schuurhuis GJ, Ossenkoppele GJ, Schlegelberger B, Verdonck LF, Vellenga E, Verhoef G, Vandenberghe P,

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Pabst T, Bargetzi M, Krauter J, Ganser A, Valk PJ, Löwenberg B, Döhner K, Döhner H, Delwel R. Deregulated Expression of EVI1 Defines a Poor Prognostic Subset of MLL-Rearranged Acute Myeloid Leukemias: A Study of the German-Austrian Acute Myeloid Leukemia Study Group and the Dutch-Belgian-Swiss HOVON/SAKK Cooperative Group. Journal of Clinical Oncology. 2013; 31:95–103. [PubMed: 23008312]

Haferlach C, Bacher U, Grossmann V, Schindela S, Zenger M, Kohlmann A, Kern W, Haferlach T, Schnittger S. Three novel cytogenetically cryptic EVI1 rearrangements associated with increased EVI1 expression and poor prognosis identified in 27 acute myeloid leukemia cases. Genes, Chromosomes & Cancer. 2012; 51:1079–1085. [PubMed: 22887804]

Harrison CJ, HIlls RK, Moorman AV, Grimwade DJ, Hann I, Webb DK, Wheatley K, de Graaf SS, van den Berg E, Burnett AK, Gibson BE. Cytogenetics of childhood acute myeloid leukemia: United Kingdom Medical Research Council Treatment trials AML 10 and 12. Journal of Clinical Oncology. 2010; 28:2674–2681. [PubMed: 20439644]

Ho PA, Alonzo TA, Gerbing RB, Pollard J, Stirewalt DL, Hurwitz C, Heerema NA, Hirsch B, Raimondi SC, Lange B, Franklin JL, Radich JP, Meshinchi S. Prevalence and prognostic implications of CEBPA mutations in pediatric acute myeloid leukemia (AML): A report from the children's oncology group. Blood. 2009; 113:6558–6566. [PubMed: 19304957]

Ho PA, Kuhn J, Gerbing RB, Pollard JA, Zeng R, Miller KL, Heerema NA, Raimondi SC, Hirsch BA, Franklin JL, Lange B, Gamis AS, Alonzo TA, Meshinchi S. The *WT1* synonymous SNP rs16754 correlates with higher mRNA expression and predicts significantly improved outcome in favorable-risk pediatric AML: a report from the Children's Oncology Group. Journal of Clinical Oncology. 2011a; 29:704–711. [PubMed: 21189390]

Ho PA, Kutny MA, Alonzo TA, Gerbing RB, Joaquin J, Raimondi SC, Gamis AS, Meshinchi S. Leukemic mutations in the methylation-associated genes DNMT3A and IDH2 are rare events in pediatric AML: a report from the Children's Oncology Group. Pediatric Blood & Cancer. 2011b; 57:204–209. [PubMed: 21504050]

Hoyt PR, Bartholomew C, Davis AJ, Yutzey K, Garner LW, Potter SS, Ihle JN, Mucenski ML. The Evi1 proto-oncogene is required at midgestation for neural, heart, and paraxial mesenchyme development. Mechanisms of Development. 1997; 65:55–70. [PubMed: 9256345]

Kim D, Salzberg SL. TopHat-Fusion: an algorithm for discovery of novel fusion transcripts. Genome Biology. 2011; 12:R72. [PubMed: 21835007]

Laricchia-Robbio L, Nucifora G. Significant increase of self-renewal in hematopoietic cells after forced expression of EVI1. Blood Cells, Molecules, & Diseases. 2008; 40:141–147.

Lugthart S, van Drunen E, van Norden Y, van Hoven A, Erpelinck CA, Valk PJ, Beverloo HB, Löwenberg B, Delwel R. High EVI1 levels predict adverse outcome in acute myeloid leukemia: prevalence of EVI1 overexpression and chromosome 3q26 abnormalities underestimated. Blood. 2008; 111:4329–4337. [PubMed: 18272813]

Martinelli G, Ottaviani E, Buonamici S, Isidori A, Borsaru G, Visani G, Piccaluga PP, Malagola M, Testoni N, Rondoni M, Nucifora G, Tura S, Baccarani M. Association of 3q21q26 syndrome with different RPN1/EVI1 fusion transcripts. Haematologica. 2003; 88:1221–1228. [PubMed: 14607750]

Mawad R, Estey EH. Acute myeloid leukemia with normal cytogenetics. Current Oncology Reports. 2012; 14:359–368. [PubMed: 22806102]

McPherson A, Hormozdiari F, Zayed A, Giuliany R, Ha G, Sun MG, Griffith M, Heravi Moussavi A, Senz J, Melnyk N, Pacheco M, Marra MA, Hirst M, Nielsen TO, Sahinalp SC, Huntsman D, Shah SP. deFuse: an algorithm for gene fusion discovery in tumor RNA-Seq data. Public Library of Science Computational Biology. 2011; 7:e1001138. [PubMed: 21625565]

Morishita K, Parker DS, Mucenski ML, Jenkins NA, Copeland NG, Ihle JN. Retroviral activation of a novel gene encoding a zinc finger protein in IL-3-dependent myeloid leukemia cell lines. Cell. 1988; 54:831–840. [PubMed: 2842066]

Meshinchi S, Alonzo TA, Stirewalt DL, Zwaan M, Zimmerman M, Reinhardt D, Kaspers GJ, Heerema NA, Gerbing R, Lange BJ, Radich JP. Clinical implications of FLT3 mutations in pediatric AML. Blood. 2006; 108:3654–3661. [PubMed: 16912228]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Saito Y, Nakahata S, Yamakawa N, Kaneda K, Ichihara E, Suekane A, Morishita K. CD52 as a molecular target for immunotherapy to treat acute myeloid leukemia with high EVI1 expression. Leukemia. 2011; 25:921–931. [PubMed: 21394097]

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nature Protocols. 2008; 3:1101–1108.

Senyuk V, Premanand K, Xu P, Qian Z, Nucifora G. The oncoprotein EVI1 and the DNA methyltransferase Dnmt3 co-operate in binding and de novo methylation of target DNA. PLoS ONE. 2011; 6:e20793. [PubMed: 21695170]

Sitailo S, Sood R, Barton K, Nucifora G. Forced expression of the leukemia-associated gene EVI1 in ES cells: a model for myeloid leukemia with 3q26 rearrangements. Leukemia. 1999; 13:1639–1645. [PubMed: 10557037]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

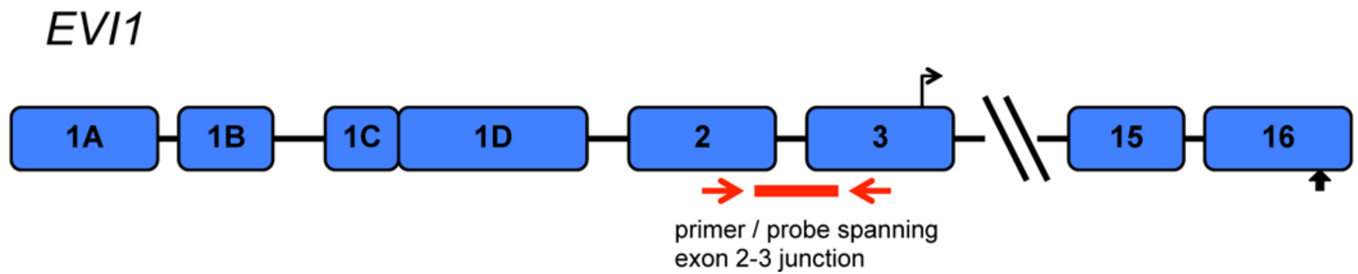

Figure 1. Location of qRT-PCR primer / probe

The primer / probe set utilized is designed to hybridize within a region spanning the exon 2–3 junction. These exons are common to all major splice isoforms resulting from alternate splicing of the first exon, as well as the *MDS1* and *EVI1* complex (*MECOM*) fusion transcript, which results from intergenic splicing.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

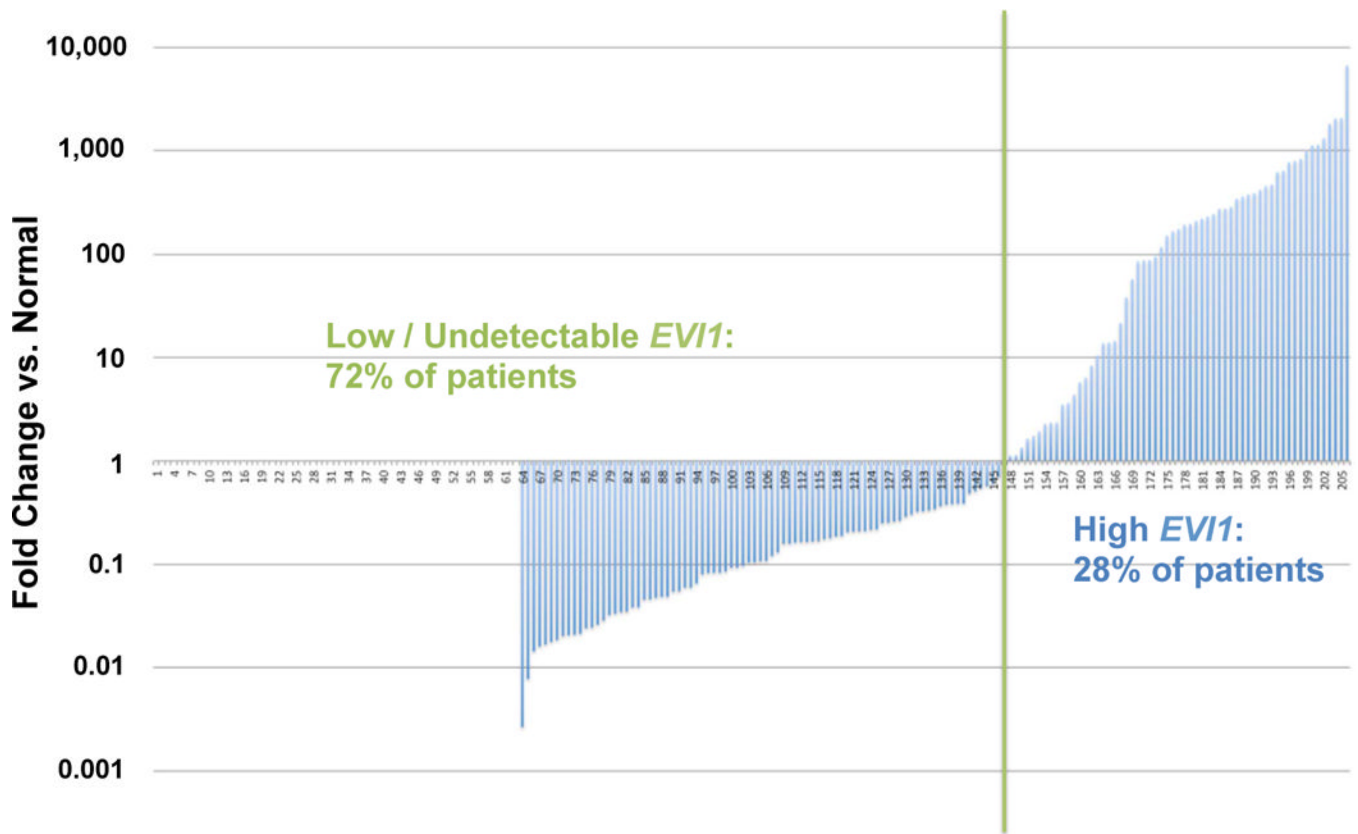

Figure 2. Distribution of *EVI1* expression in 206 diagnostic pediatric AML specimens

EVI1 expression ranged from 0 to 6660.88-fold normal. Overexpression of *EVI1* was detected in 58/206 (28%) of patients. *EVI1* expression is presented graphically on a logarithmic scale.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

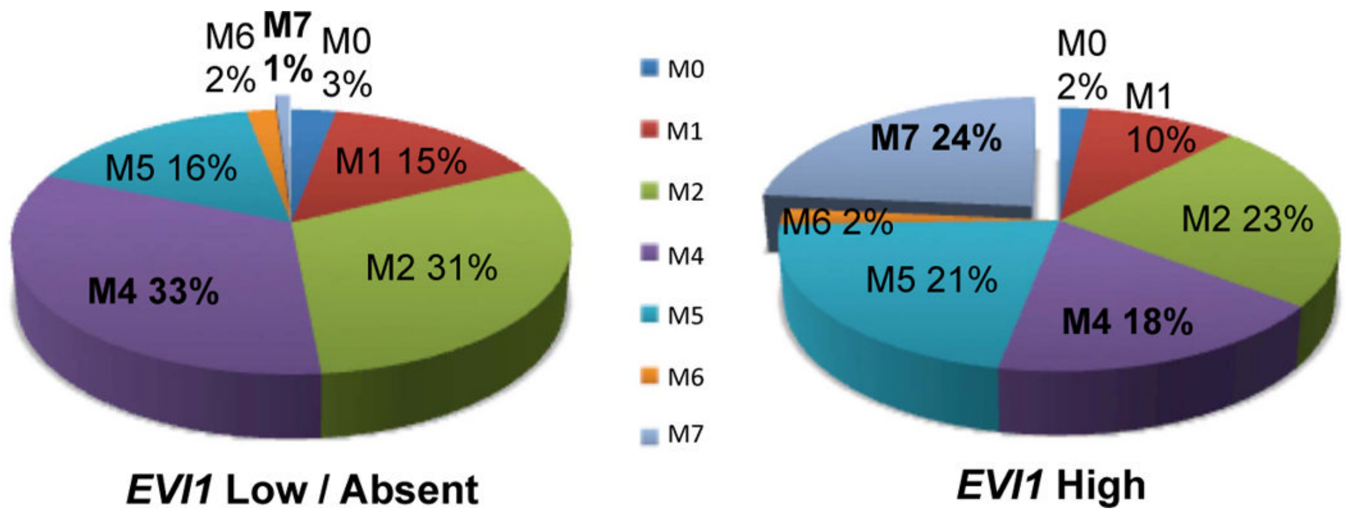

Figure 3. Distribution of FAB class in patients with low / undetectable *EVI1* compared to patients with *EVI1* overexpression

French-American-British (FAB) class M7 was significantly over-represented in the $EVI1^{high}$ group, while FAB class M4 was significantly less common.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

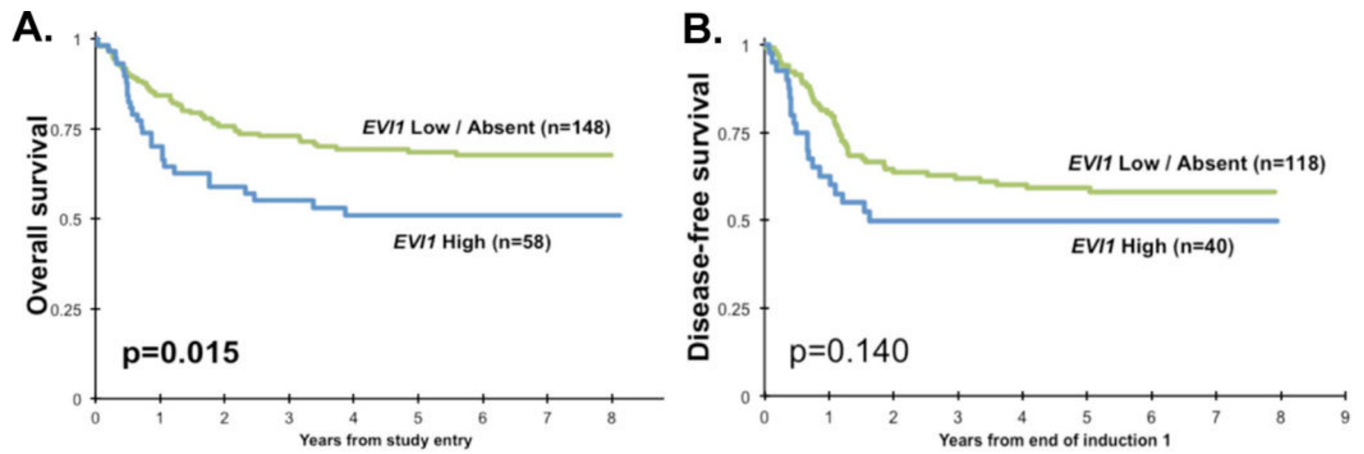

Figure 4. Survival outcomes by *EVI1* expression

EVI1^{high} patients had significantly worse (A) overall survival from study entry and (B) trended towards worse disease-free survival from complete remission.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

TABLE I

Clinical and Laboratory Characteristics by *EVI1* expression status

	EVI1 0–1 (n=148)		*EVI1* >1 (n=58)		
Characteristic	**N**	**%**	**N**	**%**	**p-value**
Gender					
Male	92	62%	29	50%	0.111
Female	56	38%	29	50%	
Age (years)					
Median (range)	10.8	(0.1 – 20.8)	4.8	(0.1 – 20.8)	**0.002**
0–1 years	21	14%	23	40%	**<0.001**
2–10 years	54	36%	15	26%	0.146
11–29 years	73	49%	20	34%	0.054
Race					
American Indian or Alaska Native	0	0%	1	2%	0.277
Asian	10	7%	2	4%	0.516
Hawaiian or other Pacific Islander	3	2%	0	0%	0.562
Black or African American	21	15%	9	17%	0.755
White	102	75%	40	77%	0.784
Unknown	12		6		
Ethnicity					
Hispanic or Latino	23	16%	8	15%	0.761
Not Hispanic or Latino	118	84%	47	85%	
Unknown	7		3		
WBC (x 10^9/l) – median (range)	35	(0.8 – 409)	15.4	(1.4 – 495)	**0.021**
Bone marrow blasts %	70	(5 – 100)	70.5	(2 – 100)	0.615
Platelet count (x 10^9/l)) – median (range)	45.5	(4 – 369)	52.5	(7 – 578)	0.403
Haemoglobin (g/l)– median (range)	83	(33 – 137)	83	(33 – 156)	0.707
Cytogenetics					
Normal	34	25%	9	17%	0.257
t(8;21)	21	15%	2	4%	**0.030**

	EVI1 0–1 (n=148)		*EVI1* >1 (n=58)		
Characteristic	**N**	**%**	**N**	**%**	**p-value**
inv(16)	29	21%	0	0%	**<0.001**
11q23	17	12%	21	40%	**<0.001**
t(6;9)(p23;q34)	6	4%	0	0%	0.189
monosomy 7	0	0%	4	8%	**0.006**
del(7q)	3	2%	0	0%	0.562
−5/5q-	1	1%	1	2%	0.479
8	11	8%	4	8%	1.000
Other	16	12%	12	23%	0.053
Unknown	10		5		
FLT3-ITD status					
ITD +	22	15%	5	9%	0.286
ITD –	124	85%	49	91%	
Missing	2		4		
CEBPA status					
CEBPA mutant	10	7%	1	2%	0.293
CEBPA wild type	129	93%	49	98%	
Missing	9		8		
NPM1 status					
NPM1 mutant	10	8%	0	0%	0.065
NPM1 wild type	111	92%	49	100%	
Missing	27		9		

WBC, white blood cell count; ITD, internal tandem duplication

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

TABLE II

MLL Translocation Partners by *EVI1* Expression Status

***MLL* Translocation Partner Distribution**		
	High *EVI1*	**Low *EVI1***
t(1;11)(q21;q23)	0	1
t(2;11)(q33;q23)	0	1
t(2;11)(q35;q23)	1	0
t(6;11)(q27;q23)	1	0
t(9;11)(p22;q23)	10	9
t(10;11)(p11.2;q23)	0	1
t(10;11)(p12;q23)	1	1
t(11;17)(q23;q21)	2	2
t(11;19)(q23;p13)	3	0
t(X;11)(q13;q23)	0	1
del(11)(q23)	1	0
add(11)(q23)	1	0
11q23 not otherwise specified	1	1

TABLE III

Univariate and multivariate analysis of high *EVI1* expression and cytogenetic / molecular risk groups

Univariate Cox Analyses								
	OS from study entry				**DFS from end of course 1**			
	N	**HR**	**95% CI**	**p**	**N**	**HR**	**95% CI**	**p**
EVI1 Expression								
Low / Absent *EVI1*	148	1			118	1		
High *EVI1*	58	1.79	1.11 – 2.89	**0.016**	40	1.48	0.88 – 2.49	0.142
Risk group								
Standard	99	1			70	1		
Favourable	70	0.31	0.16 – 0.59	**<0.001**	59	0.55	0.31 – 0.98	**0.043**
High	26	1.72	0.97 – 3.07	0.065	19	1.72	0.89 – 3.35	0.108
Multivariate Cox Analyses								
	OS from study entry				**DFS from end of course 1**			
	N	**HR**	**95% CI**	**p**	**N**	**HR**	**95% CI**	**p**
EVI1 Expression								
Low / Absent *EVI1*	141	1			111	1		
High *EVI1*	54	1.17	0.70 – 1.96	0.554	37	1.11	0.61 – 2.01	0.728
Risk group								
Standard	99	1			70	1		
Favourable	70	0.33	0.16 – 0.65	**0.002**	59	0.58	0.31 – 1.08	0.087
High	26	1.76	0.99 – 3.14	0.056	19	1.76	0.91 – 3.44	0.096

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript