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High *GATA2* expression is a poor prognostic marker in pediatric acute myeloid leukemia

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In acute myeloid leukemia (AML), aberrant expression and mutations of transcription factors have been correlated with disease outcome. In the present study, we performed expression and mutation screening of *GATA2*, which is an essential transcription factor for regulation of myeloid lineage determination, in de novo pediatric AML patients. *GATA2* mutations were detected in 5 of 230 patients, representing a frequency of 2.2% overall and 9.8% in cytogenetically normal AML. *GATA2* expression analysis demonstrated that in 155 of 237 diagnostic samples (65%), *GATA2* expression was higher than in normal BM. In complete remission, normalization of *GATA2* expression was observed, whereas *GATA2* expression levels stayed high in patients with resistant disease. High *GATA2* expression at diagnosis was an independent poor prognostic factor for overall survival (hazard ratio [HR] = 1.7, *P* = .045), event-free survival (HR = 2.1, *P* = .002), and disease-free survival (HR = 2.3, *P* = .004). The prognostic impact of *GATA2* was particularly evident in specific AML subgroups. In patients with French-American-British M5 morphology, inv(16), or high *WT1* expression, significant differences in survival were observed between patients with high versus normal *GATA2* expression. We conclude that high *GATA2* expression is a novel poor prognostic marker in pediatric AML, which may contribute to better risk-group stratification and risk-adapted therapy in the future. (*Blood*. 2012;120(10): 2064-2075)

Introduction

Acute myeloid leukemia (AML) is a clinically and genetically heterogeneous disease that accounts for 15%-20% of childhood leukemia. Over the past decades, the prognosis for children with AML has improved considerably as a result of better (risk-adapted) therapeutic strategies. Currently, approximately 60%-70% of pediatric AML patients experience long-term survival.[1,2] Current risk-group classification is based mainly on cytogenetic aberrations and response to induction chemotherapy.[1] In addition, several molecular aberrations (acquired gene mutations and altered gene expression) that are correlated with clinical outcome have been identified in pediatric and adult AML. For various molecular aberrations, differences have been observed in the prevalence and prognostic significance between pediatric and adult AML.[1,3,4] For the assessment of treatment response and early detection of relapse, monitoring of minimal residual disease (MRD) is important.[5,6] Specific gene rearrangements and mutations can be used for MRD monitoring, but suitable MRD markers are lacking in the majority of AML patients.[6]

Genes that have been implicated in leukemogenesis are usually involved in the regulation of cellular survival, proliferation, and hematopoietic differentiation.[7] These processes are tightly regulated by various transcription factors. The GATA-binding protein 2 (*GATA2*) gene, located at chromosome 3q21, encodes for a zinc-finger transcription factor that plays an essential role during the development and differentiation of hematopoietic cells. *GATA2* knockout mice have profound defects in definitive hematopoiesis, resulting in embryonic lethality at day 10-11 of gestation due to a pan-hematopoietic deficit.[8] The expression and function of *GATA2* in hematopoietic cells depends largely on their differentiation status and is tightly regulated. *GATA2* is expressed broadly in hematopoietic cells, with particularly high expression in hematopoietic stem and progenitor cells and early erythroid cells, megakaryocytic cells, and mast cells.[9-14] In hematopoietic stem and progenitor cells, *GATA2* functions as an important regulator of proliferation, differentiation, and survival and acts as a regulator of stem cell quiescence.[15-19] During myeloid differentiation, *GATA2* plays an important role in the regulation of lineage determination. *GATA2* blocks terminal erythroid, monocytic, and granulocytic differentiation, and also promotes erythroblast proliferation, megakaryopoiesis, and mast cell formation.[19-22] Tight regulation of *GATA2* expression is essential in the process of myeloid lineage determination. Enforced expression of *GATA2* in myeloid progenitors induces down-regulation of the crucial myeloid transcription factors *PU.1* and CCAAT/enhancer binding protein-α (*CEBPA*), which are implicated in terminal granulocytic and monocytic differentiation.[17,21,23,24] Moreover, *GATA2* inhibits the ability of *PU.1* to transactivate critical myeloid target genes and thereby prevents the induction of a myeloid gene expression program.[25] Conversely, knockdown of *GATA2* in myeloid progenitors results in significant up-regulation of the expression of *PU.1* and *CEBPA*,[20,21]

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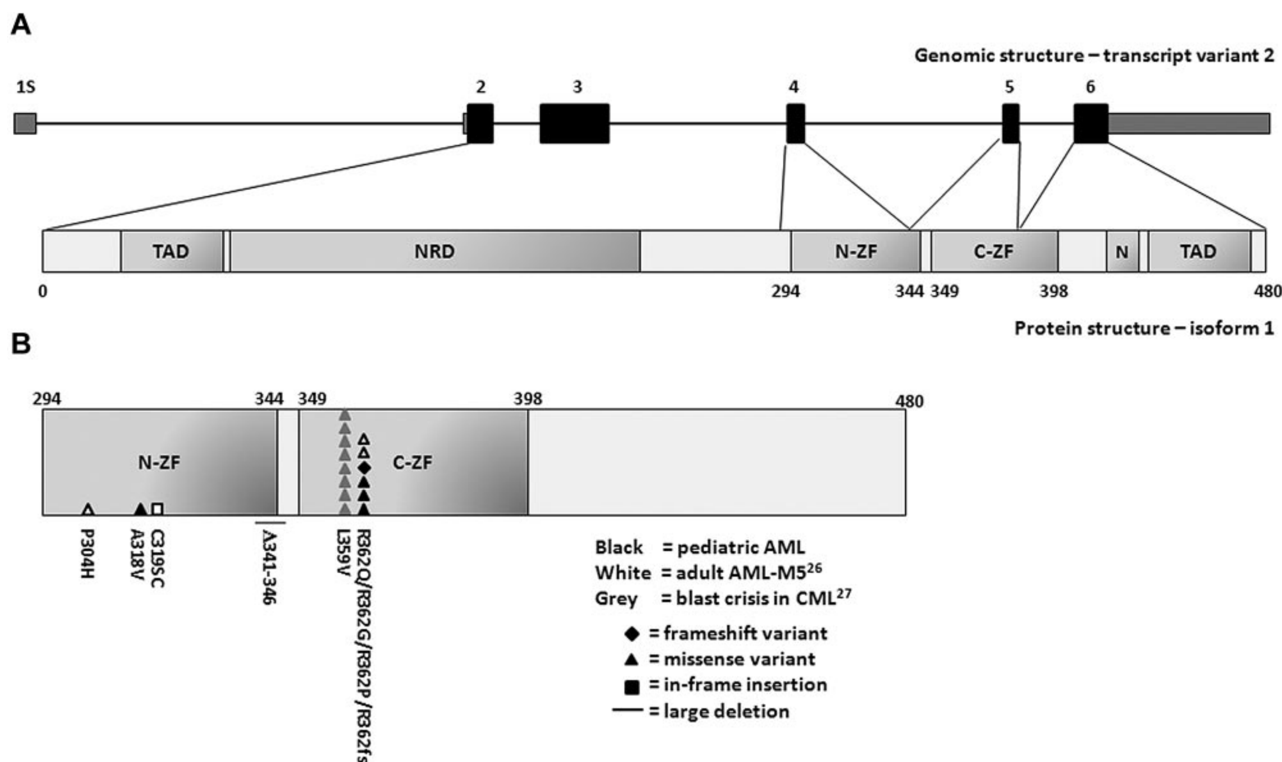

Figure 1. Sequence variations in the zinc-finger domains of *GATA2* in myeloid malignancies. (A) Schematic representation of the human *GATA2* gene and protein. The *GATA2* gene consists of 6 exons. The coding exons (exon 2-6) are depicted in black and the 5′ and 3′ untranslated regions are depicted in gray. The *GATA2* gene has 3 transcript variants that encode for 2 different protein isoforms with the same translation start site in exon 2. Isoform 1, encoded by transcript variant 1 or 2, contains 480 amino acids (50.5 kDa). Isoform 2, encoded by transcript variant 3, contains a smaller fifth exon resulting in a protein of 466 amino acids (49.1 kDa). Transcript variant 2, in which transcription is initiated from a distal first exon (1S), is specific for hematopoietic and neuronal cells. The encoded protein has 2 transactivation domains (TAD), a negative regulatory domain (NRD), a nuclear localization signal (N), and 2 zinc-finger domains (ZF). (B) Acquired mutations and sequence variations in the N-terminal (N-ZF) and C-terminal (C-ZF) zinc-finger domain of *GATA2* in myeloid malignancies. Sequence variations in pediatric AML from the present study are depicted in black. Previously reported mutations in adult AML with FAB-M5 morphology or in blast crisis of CML are depicted in white and gray, respectively.[26,27] Each symbol represents an independent *GATA2* mutation or variation. Numbers indicate the amino acid affected by each mutation or variation. The type of mutation is indicated by the symbol.

which allows terminal granulocytic and monocytic differentiation to proceed.[19]

In sporadic and familial myeloid malignancies, acquired and inherited mutations in the highly conserved zinc-finger domains of the *GATA2* gene have been described recently[14,26-35] (Figure 1B and supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). These 2 zinc fingers play an essential role in DNA-binding and protein-protein interactions of *GATA2* with target genes. During blast crisis in chronic myeloid leukemia (CML), 2 acquired *GATA2* mutations were found in 9 of 85 (10%) patients.[27] In adult monocytic AML (French-American-British M5 [FAB-M5]), 3 acquired *GATA2* mutations were detected in 4 of 112 (3.6%) patients.[26] In 3 other adult AML cohorts (N = 50, N = 225, and N = 268), no acquired mutations in the *GATA2* coding sequence were found.[14,28,29] So far, *GATA2* mutations have not been reported in pediatric AML. In the present study, we investigated the relevance and prognostic value of *GATA2* expression and mutations in a large cohort of pediatric AML patients.

Methods

Patient samples

Viable frozen bone marrow (BM) or peripheral blood samples taken at diagnosis from 261 children with de novo AML were provided by the Dutch Childhood Oncology Group (DCOG; The Hague, The Netherlands), the AML-Berlin-Frankfurt-Münster (BFM) study group (Hannover, Germany, and Prague, Czech Republic), and the Saint-Louis Hôpital (Paris, France). As controls, 18 BM samples form healthy donors and 62 BM samples from patients with AML (n = 42) or acute lymphoblastic leukemia (n = 20) in long-term complete remission (CR) were included. For a subgroup of 38 patients, follow up BM samples were provided by DCOG on the basis of available material for MRD analysis, including 10 paired diagnostic-relapse samples. The majority of the processed diagnosis samples (248 of 261) contained more than 80% leukemic cells as assessed morphologically on May-Grünwald-Giemsa–stained cytospin slides.

Mononuclear cells were isolated from BM or peripheral blood and contaminating nonleukemic cells were eliminated as described previously.[36] Genomic DNA and total cellular RNA were extracted from at least 5×10^6 leukemic cells using TRIzol reagent (Invitrogen), RNA-Bee (Iso-Tex Diagnostics), or the GeneElute Mammalian Total RNA isolation kit (Sigma-Aldrich). Informed consent was obtained after institutional review board approval according to local laws and regulations and in accordance with the Declaration of Helsinki. Each national study group performed central review of the morphologic classification according to the FAB classification system, cytogenetic classification, and clinical follow-up.

Survival analysis was restricted to a subset of 205 pediatric AML patients who were treated according to the BFM and Dutch pediatric AML protocols (studies AML-BFM 98, AML-BFM 04, DCOG-BFM 87, DCOG 92/94, and DCOG 97). The median follow-up time for survivors was 4.6 years (range, 0.4-22.7). In the total cohort, 4-year probability for overall survival (pOS; time between diagnosis and death from any cause) was 60% ± 4%; probability for event-free survival (pEFS; time between diagnosis and first event) was 41% ± 4%; and probability for disease-free survival (pDFS; time between achieving CR and relapse) was 53% ± 4%.

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Table 1. Nonsynonymous variations in the zinc-finger domains of *GATA2*

Exon	Variation	Nucleotide variant	Amino acid change	Age, y	Sex	WBC-count, × 10⁹/L	FAB	Karyotype	Other mutations	Follow-up	Reported previously
Exon 4	Missense	c.953C > T	A318V	18.5	M	43.1	M2	CN	*CEBPA*-DM	Relapse at 3.2 y, alive but lost to follow-up 3 mos after relapse	No
Exon 5	Missense	c.1084C > G	R362G	15.3	M	30.8	M4	CN	*NPM1* *NRAS*	CCR1, 3.4 y	No
	Missense	c.1085G > C	R362P	14.8	F	16.0	M4	CN	*NPM1* *NRAS*	CCR1, 1.8 y	No
	Missense	c.1085G > A	R362Q	4.0	M	354.0	M1	CN	*CEBPA*-DM *WT1* *NRAS*	CCR1, 3.7 y	Yes [26]
	Deletion	c.1085delG	R362fsX24	14.1	F	NA	M4	-7	*NRAS*	NA	No

CCR1 indicates continuous first CR; and NA, not available.

Cytogenetic and molecular analysis

Leukemic samples were routinely investigated for cytogenetic aberrations using standard chromosome banding analysis by the national study groups. Leukemic samples were further analyzed for recurrent nonrandom genetic aberrations characteristic of AML as described by the World Health Organization (2008) classification of myeloid neoplasms and acute leukemia,[37] including t(15;17)(q21;q22), inv(16)(p13q22), t(8;21)(q22;q22), and 11q23 translocations, using FISH and/or RT-PCR. 11q23 translocations were confirmed by RT-PCR or long-distance inverse PCR.[38]

Detection of molecular aberrations included mutation analysis of Wilms tumor 1 (*WT1*), nucleophosmin 1 (*NMP1*), *CEBPA*, FMS-related tyrosine kinase 3 internal tandem duplication (*FLT3*-ITD), *NRAS*, *KRAS*, *PTPN11*, *KIT*, and *MLL*-PTD, as described previously.[4] If positive for *MLL*-PTD, this was confirmed by a multiplex ligation–dependent probe amplification analysis.[39] High expression of *EVI1* was established previously by gene-expression profiling and real-time quantitative PCR.[40]

Sequence analysis of *GATA2*

Sequence analysis of *GATA2* was performed in 230 de novo pediatric AML patients with more than 80% blasts in their BM and for whom genomic DNA was available. Because all acquired *GATA2* mutations in myeloid malignancies so far were present within the DNA-binding zinc-finger domains encoded by exon 4, 5, and 6 (Figure 1), we performed Sanger sequencing of the coding region of these exons and their associated splice sites. Genomic DNA was PCR amplified and direct sequencing of the purified PCR products was performed unidirectionally using the described primers (supplemental Table 1). The sequence data were analyzed using Variant Reporter (Applied Biosystems). Mutations were confirmed by an independent amplification of the fragment and direct sequencing of both strands.

Microarray-based gene-expression profiling

Gene-expression profiling data (Affymetrix HGU133 Plus 2.0 microarray) were available from 237 patients.[41] The data files are available in the Gene Expression Omnibus repository (accession number GSE17855). Probe set intensities were normalized using the variance stabilization normalization (VSN) procedure (Bioconductor Package VSN). Details regarding RNA quality, microarray processing, data acquisition, and data normalization have been described previously.[41]

Three probe sets on the Affymetrix Human Genome U133 Plus 2.0 Array were annotated to the *GATA2* gene (209710_at, 210358_ × _at, and 207954_at). All probe sets are located within the 3′ untranslated region of exon 6 in regions common for all isoforms of *GATA2*. VSN-normalized expression values of *GATA2* obtained by microarray were validated with real-time quantitative PCR in a subset of patients (n = 44). VSN-normalized expression values of probe set 209710_at showed a good correlation with *GATA2* expression values obtained by real-time quantitative PCR (Spearman correlation coefficient = 0.73, $P < .00001$), which enabled us to define a conversion factor (VSN-normalized expression = 1222.3*Q-PCR expression$^{0.2955}$). *GATA2* expression was considered to be high when the expression was 2 SDs above the median *GATA2* expression obtained in normal BM as determined by microarray (n = 6) or real-time quantitative PCR (n = 74). Real-time quantitative *GATA2* expression values obtained in normal BM (n = 74) were converted into VSN-normalized expression values using the conversion factor described in this section. The cutoff value for high *GATA2* expression was determined to be 320 arbitrary units (AU).

Expression of *WT1* was analyzed using VSN-normalized expression values of probe set 206067_s_at on the Affymetrix Human Genome U133 Plus 2.0 Array, which is annotated to the *WT1* gene. Previously, we showed a good correlation with *WT1* expression values obtained by real-time quantitative PCR.[42] In the present study, we could define a conversion factor (VSN-normalized expression = 523.86*Q-PCR expression$^{0.1613}$). *WT1* expression was considered to be high when the expression was 2 SDs above the median *WT1* expression obtained in normal BM as determined by microarray (n = 6) or real-time quantitative PCR (n = 69). Real-time quantitative *WT1* expression values obtained in normal BM (n = 69) were converted into VSN-normalized expression values using the conversion factor described in this section. The cutoff value for high *WT1* expression was determined to be 143 AU.

Real-time quantitative PCR

For quantification of *GATA2* expression, a TaqMan Gene Expression Assay (Hs_00231119_m1; Applied Biosystems) was used. For quantification of *WT1* expression, we used the European LeukemiaNet *WT1* assay that was developed previously in our laboratory[43] and was recently evaluated in normal control and AML patient samples by the European LeukemiaNet.[44] For quantification of *EVI1* expression, we used a forward primer (5′-GGTCAACAAACCAATTTAGACAGACA-3′), reverse primer (5′-TTCAGAATGAGGCGACGATGT-3′), and probe (5′-ATGGGAACATG-TCCGGTAC-3′). For quantification of the *AML1-ETO* fusion-gene expression, we used a forward primer (5′-CCACAGAGCCATCAAAATCACA-3′), reverse primer (5′CAGCCTAGATTGCGTCTTCACAT-3′), and probe (5′-TGAGAAGCACTCCACAAT-3′). As a reference gene, we used *GAPDH* (premixed assay 4326317E; Applied Biosystems) or *PBGD* (forward primer: 5′-GCGGAGCCATGTCTGGTAA-3′; reverse primer: 5′-GGG-TACCCAACGCGAATCAC-3′; probe: 5′-CTCATCTTTGGGCTGTT-TTCTTCCGCC-3′). Samples were considered eligible for testing only when the threshold cycle value (Ct) of the reference gene *GAPDH* was lower than 26. Quantitative PCR reactions contained 2.5-5.0 μL of cDNA (10 ng/μL), 1.25 μL of premixed assay, or 0.75 μL of forward primer (10 pmol/μL), 0.75 μL of reverse primer (10 pmol/μL), and 0.35-0.5 μL of probe (10 pmol/μL). The 25-μL reactions were incubated in a 96-well plate for 2 minutes at 50°C, 10 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C, and 1 minute at 60°C. Quantitative PCR reactions were performed on the ABI/PRISM 7900 Sequence Detection system.

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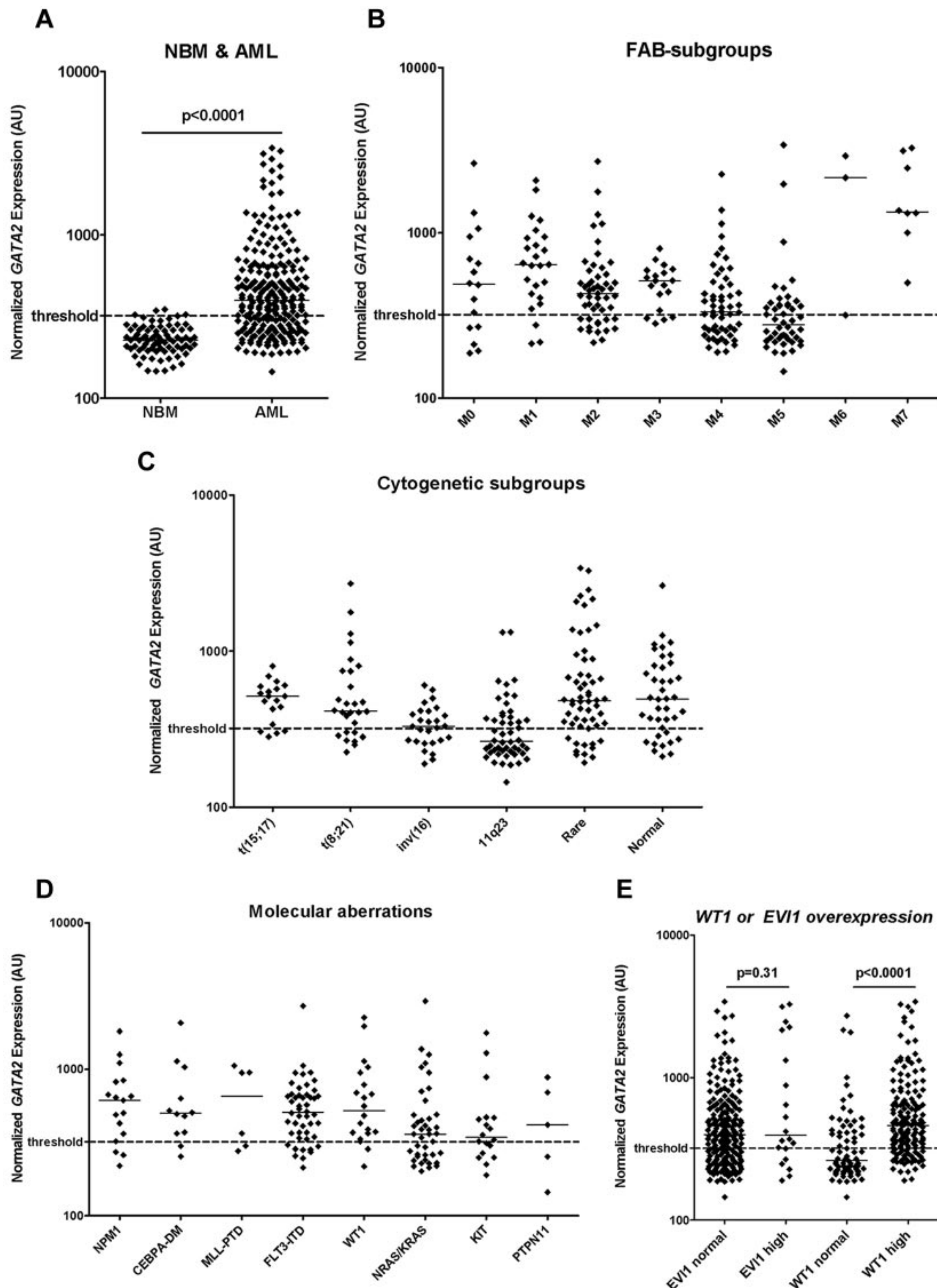

Figure 2. *GATA2* expression in normal BM and different morphologic, cytogenetic, and molecular AML subgroups. (A) Normalized expression levels of *GATA2* in pretreatment samples of 237 children with de novo AML and 80 normal BM samples. Expression was significantly higher in AML compared with normal BM. Normalized expression levels of *GATA2* in different morphological FAB subgroups (B), in cytogenetic subgroups (C), and in molecular subgroups including molecular aberrations (D) and AML with *WT1* or *EVI1* overexpression (E). *GATA2* expression was determined using gene-expression arrays (Affymetrix HGU133 Plus 2.0 microarray, probe set 209710_at) or real-time quantitative PCR. Median values are depicted by the horizontal lines. The Mann-Whitney *U* test was used to compare expression levels between groups. The threshold for high versus normal *GATA2* expression is based on median *GATA2* in normal BM + 2 SD and is depicted as a dashed line.

Statistical methods and analysis

To compare categorical variables, χ^2 analysis and the Fisher exact test for small patient numbers were used. The nonparametric Mann-Whitney *U* test was applied for continuous variables. Correlations between continuous variables were calculated using the Spearman correlation coefficient.

Analysis of the FAB subgroup AML-M3 was restricted to patients harboring a translocation t(15;17)(q21;q22). Patients with rare recurrent cytogenetic aberrations such as t(7;12)(q36;p16), t(6;9)(p23;q34), t(16; 21)(p11;q22), t(8;16)(p11;p13), monosomy 7, trisomy 8, and complex karyotype were classified as 1 cytogenetic subgroup entitled "rare cytogenetics."

To assess outcome, the following parameters were used: CR rate, resistant disease (RD; defined as failure to achieve CR after ≤ 2 cycles of chemotherapy, excluding patients with early death), pOS (defined as

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Table 2. Clinical and biological characteristics of the de novo pediatric AML cohort

	Overall population (N = 237)	Normal *GATA2* expression (n = 82)	High *GATA2* expression (n = 155)	*P*
Sex, n (%; n = 237)				
Male	137	51 (37.2%)	86 (62.8%)	.320*
Female	100	31 (31.0%)	69 (69.0%)	
Median age, y (n = 237)	9.6	8.9	9.8	.059†
< 2 y	38	18 (47.4%)	20 (52.6%)	.071*
2-10 y	87	31 (35.6%)	56 (64.4%)	.799*
> 10 y	112	33 (29.5%)	79 (70.5%)	.116*
Median WBC count, × 10^9/L (n = 216)	41.8	34.5	42.7	.276†
< 50 × 10^9/L	123	44 (35.8%)	79 (64.2%)	.905*
> 50 × 10^9/L	93	34 (36.6%)	59 (63.4%)	
FAB, n (%; n = 226)				**< .001***
M0	16	5 (31.3%)	11 (68.8%)	.747*
M1	25	3 (12.0%)	22 (88.0%)	**.011***
M2	51	14 (27.5%)	37 (72.5%)	.201*
M3	19	4 (21.1%)	15 (78.9%)	.184*
M4	56	24 (42.9%)	32 (57.1%)	.153*
M5	48	28 (58.3%)	20 (41.7%)	**< .001***
M6	3	1 (33.3%)	2 (66.7%)	.953‡
M7	8	0 (0%)	8 (100%)	.053‡
Cytogenetic abnormalities, n (%; n = 222)				**.001***
t(8;21)(q22;q22)	28	8 (28.6%)	20 (71.4%)	.499*
inv(16)(p13q22)	27	12 (44.4%)	15 (55.6%)	.233*
t(15;17)(q22;q21)	19	4 (21.1%)	15 (78.9%)	.205*
11q23 translocations	50	30 (60.0%)	20 (40.0%)	**< .001***
Rare cytogenetics	58	12 (20.7%)	46 (79.3%)	**.044***
Normal cytogenetics (CN)	40	10 (25.0%)	30 (75.0%)	.174*
Other genetic abnormalities, n (%)				
NPM1 mutations (n = 237)	17	3 (17.6%)	14 (82.4%)	.127*
CEBPA mutations (n = 227)	16	2 (12.5%)	14 (87.5%)	.056*
CEBPA single mutations (n = 227)	4	0 (0%)	4 (100%)	.301‡
CEBPA double mutations (n = 227)	12	2 (16.7%)	10 (83.3%)	.227‡
MLL-PTD (n = 237)	6	2 (33.3%)	4 (66.7%)	1.000‡
FLT3-ITD (n = 237)	48	9 (18.7%)	39 (81.3%)	**.010***
WT1 mutations (n = 230)	20	2 (10.0%)	18 (90.0%)	**.015***
NRAS/KRAS mutations (n = 237)	41	17 (41.5%)	24 (58.5%)	.310*
KIT mutations (n = 237)	18	7 (38.9%)	11 (61.1%)	.691*
PTPN11 mutations (n = 237)	5	2 (40.0%)	3 (60.0%)	1.000‡
High *EVI1* expression (n = 237)	20	6 (30.0%)	14 (70.0%)	.651*
High *WT1* expression (n = 237)	176	42 (23.9%)	134 (76.1%)	**< .001***

Patients with high *GATA2* expression are compared with patients with normal *GATA2* expression. *P* values in bold indicate whether differences are significant at the level of .05.
*χ^2 test.
†Mann-Whitney *U* test.
‡Fisher exact test.

time between diagnosis and death from any cause), pEFS (defined as time between diagnosis and first event, including relapse, death by any cause, second malignancy, or failure to achieve CR after ≤ 2 cycles of chemotherapy (included as an event on day 0), and pDFS (defined as time between achieving CR and relapse; only patients who achieved CR were included). pOS, pEFS, and pDFS were estimated by the Kaplan-Meier method and compared using the log-rank test. Two different analyses were performed according to the selected population: on the overall population and on the different morphological, cytogenetic, and molecular subgroups. The independence of prognostic factors was examined by multivariate Cox regression analysis, including in pediatric AML–established prognostic factors such as age, WBC count at diagnosis, favorable karyotype, *NPM1* mutation, *CEBPA* double mutation (*CEBPA*-DM), and *FLT3*-ITD.[45] Age and WBC count were included as continuous variables. Favorable karyotype was defined as patients harboring t(15;17)(q21;q22), inv(16)(p13q22), or t(8;21)(q22;q22). The results of univariate Cox regression analysis of the established prognostic factors are included in supplemental Table 4. $P < .05$ was considered statistically significant (2-tailed testing). All analyses were performed with SPSS Version 16.0 or 18.0 software.

Results

Genomic variations in the zinc-finger domains of the *GATA2* gene

Sequence analysis of *GATA2* was performed in 230 de novo pediatric AML patient samples. In 9 patients, a synonymous alteration (conversion of GCG to GCA) was observed within codon 411 of the coding sequence of exon 6, which is a known polymorphism (dbSNP release 132). A novel synonymous alteration was observed in codon 471 of exon 6 (supplemental Table 2). Nonsynonymous *GATA2* variations were found in 5 of 230 (2.2%) pediatric AML patients (Figure 1B and Table 1). No correlation was observed between the presence of these variations and the expression levels of *GATA2*. The sequence abnormalities included 4 distinct heterozygous missense variants and 1 heterozygous single nucleotide deletion. In 1 patient, a

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heterozygous missense variant was detected in exon 4, leading to a A318V substitution. Neither this variant nor the affected codon has been described previously. The other 3 heterozygous missense variants and the single nucleotide deletion were present within codon 362 of exon 5. The 3 heterozygous missense variants resulted in 3 distinct amino acid substitutions (R362Q, R362G, and R362P). The single nucleotide deletion resulted in a frameshift with premature termination. Because neither germline nor remission material was available, the origin of the variations (acquired or inherited) could not be determined in the present study.

Patients who harbored nonsynonymous *GATA2* variations were classified morphologically as FAB-M1 (n = 1), FAB-M2 (n = 1), or FAB-M4 AML (n = 3; Table 1). The nonsynonymous *GATA2* variants were mainly present in cytogenetically normal AML (CN-AML) with a frequency of 9.8% (4 of 41). In addition, we detected a nonsynonymous *GATA2* variation in a patient with monosomy 7. No *GATA2* variations were found in the favorable cytogenetic subgroup (n = 67). In all cases, we observed cooccurrence of *GATA2* variations with various other recurrent mutations, including *NRAS* mutations (n = 4), *NPM1* mutations (n = 2), *CEBPA*-DM (n = 2), and *WT1* mutations (n = 1). The outcome of patients with nonsynonymous *GATA2* variations is described in Table 1. Because of the low frequency of nonsynonymous *GATA2* variants in this pediatric AML cohort, prognostic implications of *GATA2* variations could not be determined.

GATA2 expression in morphological and genetic AML subgroups

In 237 de novo pediatric AML patients, microarrays were used to determine the expression of *GATA2* at diagnosis. In these AML samples, the expression of *GATA2* varied more than 20-fold (range, 145-3405 AU; median, 379 AU). In 80 normal BM samples, a relatively homogeneous *GATA2* expression was observed (range, 146-349 AU; median, 226 AU). The *GATA2* expression was significantly higher in AML compared with normal BM ($P < .0001$; Figure 2A). *GATA2* expression was not related to sex ($P = .22$), age ($P = .52$), or WBC count ($P = .33$; supplemental Figure 2).

In patients with FAB-M4 (n = 56) or FAB-M5 (n = 48) morphology, the median *GATA2* expression was lower than in patients with other FAB subtypes ($P = .019$ and $P < .0001$, respectively), whereas higher expression was observed in patients with FAB-M1 (n = 25) or FAB-M7 (n = 8) morphology ($P = .001$ and $P < .0001$, respectively; Figure 2B). In cytogenetic subgroups, *GATA2* expression was lower in patients with 11q23 translocations (n = 50) or inv(16) (n = 27; $P < .0001$ and $P = .012$, respectively), whereas higher *GATA2* expression levels were observed in patients with CN-AML (n = 40) or rare cytogenetic aberrations (n = 58; $P = .039$ and $P = .003$, respectively; Figure 2C). No particular rare cytogenetic aberrations were associated with high levels of *GATA2* expression. In addition, no chromosomal abnormalities involving the *GATA2* locus (3q21) or located close to the *GATA2* locus (eg, 3q26 aberrations) were present. No evident correlation between *GATA2* expression levels and specific 11q23 translocation partners was observed. In the molecular subgroups, we observed that the median *GATA2* expression was higher in patients with *FLT3*-ITD (n = 48), *NPM1* (n = 17), *CEBPA* (n = 16), or *WT1* mutations (n = 20; $P = .003$, $P = .046$, $P = .02$, and $P = .035$, respectively; Figure 2D-E) compared with the patients with wild-type *FLT3*, *NPM1*, *CEBPA*, or *WT1*, respectively. When *CEBPA* single mutants (n = 4) and double mutants (n = 12) were analyzed as 2 distinct entities, a trend toward higher *GATA2* expression was observed in both groups compared with patients with wild-type *CEBPA* ($P = .11$ and $P = .05$, respectively). The expression of *GATA2* showed a positive correlation with expression of the transcription factor *WT1* (Spearman correlation coefficient = 0.51, $P < .0001$), which is known to be highly expressed in several hematopoietic malignancies including AML and has been identified previously as a molecular marker associated with disease outcome in AML.[44,46,47]

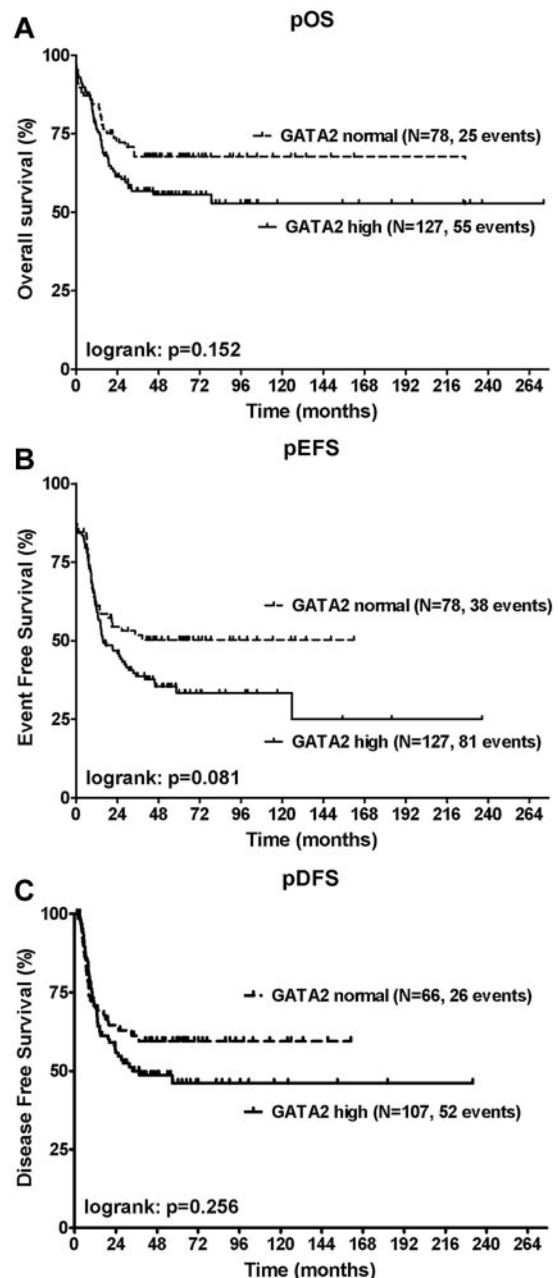

Figure 3. Correlation of *GATA2* expression with OS, EFS, and DFS. Shown are Kaplan-Meier estimates for pOS (A), pEFS (B), and pDFS (C). Patients with high *GATA2* expression were compared with patients with normal *GATA2* expression using a log-rank test. High *GATA2* expression suggested a worse pOS and pEFS, although the differences did not reach statistical significance at the $P = .05$ level in univariate analysis.

High *GATA2* expression is an independent poor prognostic factor

To determine the clinical significance of *GATA2* expression in pediatric AML, we studied the relation between *GATA2* expression at diagnosis and parameters of long-term outcome (pOS, pEFS, and

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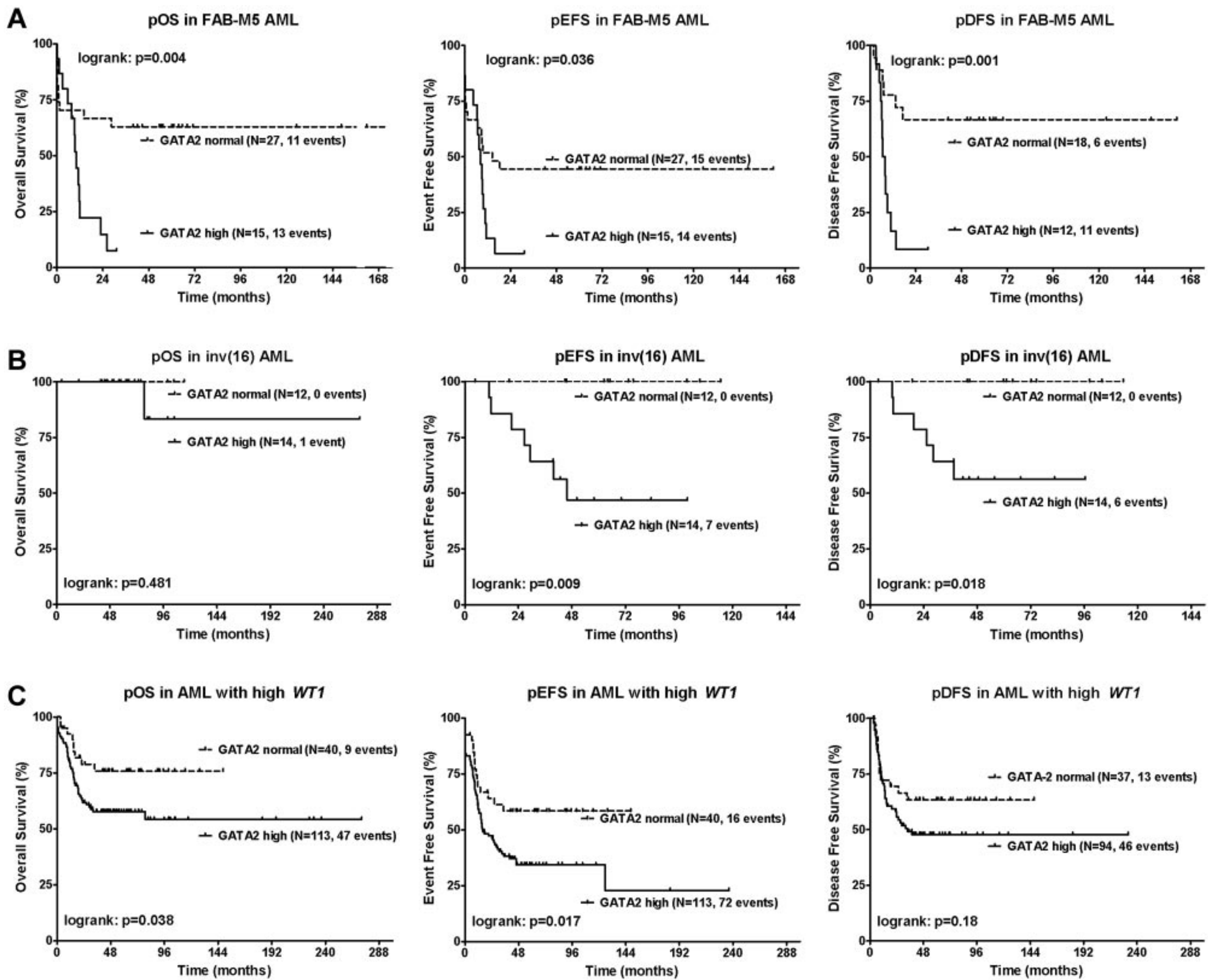

Figure 4. Relation of *GATA2* expression with survival in morphologic and genetic AML subgroups. Kaplan-Meier estimates for pOS, pEFS, and pDFS in AML patients with FAB-M5 morphology (A), inv(16) (B), or high *WT1* expression (C). Patients with high *GATA2* expression were compared with patients with normal *GATA2* expression using a log-rank test. High *GATA2* expression was associated with worse outcome. $P < .05$ was considered statistically significant (2-tailed testing). Patient characteristics are described in supplemental Tables 5 through 7.

pDFS). Because the level of expression of *GATA2* appears to be a continuum, we defined a threshold for high versus normal *GATA2* expression based on the expression of *GATA2* in normal BM (threshold: median expression in normal BM + 2 SD). In 155 patients (65%) *GATA2* levels were expressed above this threshold of 320 AU (range, 320-3405; median, 506 AU). The remaining 82 patients (35%) were classified as having normal *GATA2* levels (range, 145-319 AU; median, 250 AU; Table 2).

Survival data were available for 205 patients, including 127 patients with high *GATA2* expression (62%). High *GATA2* expression was associated with worse pOS and pEFS, although the differences did not reach statistical significance at the $P = .05$ level in univariate analysis. No effect on pDFS was observed. The 4-year pOS was 56% ± 5% for patients with high *GATA2* expression (n = 127) compared with 68% ± 6% for patients with normal *GATA2* levels (n = 78; $P = .15$; Figure 3A); the 4-year pEFS was 35% ± 5% compared with 50% ± 6% ($P = .08$; Figure 3B) and the 4-year pDFS was 49% ± 5% compared with 59% ± 6% ($P = .26$; Figure 3C), respectively.

The prognostic impact of *GATA2* was particularly evident in patients with FAB-M5 morphology (n = 42) and inv(16) (n = 26). In patients with FAB-M5 morphology, high *GATA2* expression was associated with worse pOS, pEFS, and pDFS. The 4-year pOS was 7% ± 7% in FAB-M5 patients with high *GATA2* expression (n = 15) compared with 63% ± 9% in FAB-M5 patients with normal *GATA2* levels (n = 27; $P = .004$); the 4-year pEFS was 7% ± 6% compared with 44% ± 10% ($P = .036$) and the 4-year pDFS was 8% ± 8% compared with 67% ± 11% ($P = .001$; Figure 4A), respectively. In patients with inv(16), high *GATA2* expression was associated with worse pEFS and pDFS, whereas no effect on pOS was observed. The 4-year pEFS was 47% ± 14% in inv(16) patients with high *GATA2* expression (n = 14) compared with 100% in inv(16) patients with normal *GATA2* levels (n = 12; $P = .009$); the 4-year pDFS was 56% ± 14% compared with 100% ($P = .018$; Figure 4B), respectively. Moreover, in patients with high *WT1* expression, 11q23 translocations or *FLT3*-ITD positivity, a trend toward worse outcome was observed in patients with high *GATA2* expression versus normal *GATA2* expression (Figure 4C and supplemental Figure 3). Within other morphological, cytogenetic, or molecular subgroups, we did not observe differences in outcome between patients with high versus normal *GATA2* expression (supplemental Table 3).

To assess the independent prognostic contribution of *GATA2* expression, a multiple Cox regression analysis was conducted. In

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Table 3. Multivariate analysis for pOS, pEFS, and pDFS

	HR	95% CI Lower	95% CI Upper	*P*
pOS				
High *GATA2* expression	1.724	1.012	2.938	**.045**
Age	1.031	0.982	1.083	.219
WBC count	1.003	1.001	1.006	**.008**
FLT3-ITD	0.924	0.502	1.700	.799
NPM1 mutation	0.169	0.040	0.714	**.016**
Favorable karyotype	0.145	0.069	0.306	**< .001**
CEBPA double mutation	0.128	0.017	0.949	**.044**
pEFS				
High *GATA2* expression	2.050	1.312	3.204	**.002**
Age	1.058	1.014	1.104	**.010**
WBC count	1.002	0.999	1.004	.189
FLT3-ITD	0.791	0.467	1.342	.385
Favorable karyotype	0.204	0.121	0.345	**< .001**
CEBPA double mutation	0.181	0.054	0.601	**.005**
NPM1 mutation	0.130	0.039	0.426	**.001**
pDFS				
High *GATA2* expression	2.290	1.300	4.034	**.004**
Age	1.073	1.016	1.133	**.011**
WBC count	1.000	0.997	1.004	.960
FLT3-ITD	0.874	0.456	1.677	.687
Favorable karyotype	0.160	0.083	0.307	**< .001**
NPM1 mutation	0.140	0.041	0.471	**.002**
CEBPA double mutation	0.134	0.030	0.588	**.008**

Multivariate Cox regression model for pOS, pEFS, and pDFS. Age and WBC count are analyzed as continuous variables. *GATA2* expression is dichotomized. *P* values in bold indicate whether differences are significant at the level of .05.

95% CI indicates 95% confidence interval.

pediatric AML, several prognostic factors have been identified previously: age, WBC count, favorable karyotype, *NPM1* mutation, *CEBPA*-DM, and *FLT3*-ITD.[45] When *GATA2* was entered into a multiple Cox regression analysis adjusting for these established prognostic factors, we observed a significant correlation between high *GATA2* expression with worse pOS (HR = 1.7, *P* = .045), pEFS (HR = 2.1, *P* = .002), and pDFS (HR = 2.3, *P* = .004; Table 3 and supplemental Table 4).

GATA2 expression during treatment and follow-up

To study the prognostic relevance of *GATA2* expression after induction and consolidation treatment, consecutive *GATA2* expression levels were determined using real-time quantitative PCR in a representative subgroup of 38 patients (supplemental Table 10). Thirty-three patients reached CR, with a median time to first CR (CR1) of 1.4 months (range, 0.9-4.4), 3 patients died within 1 week after diagnosis (categorized as early death), and 2 patients had RD. Twelve patients relapsed, with a median time to relapse of 12.6 months (range, 6.0-65.4).

Both at diagnosis and clinical relapse, *GATA2* expression was heterogeneous ($>$ 2-log difference between highest and lowest expression). No differences were observed in median *GATA2* expression at diagnosis versus relapse (*P* = .87). After induction and consolidation therapy, normalization of *GATA2* expression was observed in patients who reached CR (Figure 5A-B), whereas *GATA2* expression levels stayed high in patients with RD (Figure 5C). In 2 of 38 AML patients, *GATA2* was sufficiently highly expressed to allow detection of at least a 2-log reduction (ie, sensitivity < 0.01). *GATA2* expression was assessed in parallel with additional markers that have been used previously for the detection of residual disease: *WT1* expression (n = 38), *AML1-ETO* fusion gene expression [patients with t(8;21); n = 3], and *EVI1* expression (patients with high *EVI1* expression, n = 3; Figure 6).[6,44,47,48] The expression patterns of *GATA2* were comparable to the expression patterns of the *AML1-ETO* fusion gene, *EVI1* and *WT1*. However, the log reduction for *WT1* and *AML1-ETO* was higher than for *GATA2*.

To determine whether *GATA2* expression was related to outcome, we compared the *GATA2* expression of patients who relapsed versus patients in continuous CR1 at different time points. Neither at diagnosis nor during chemotherapy were differences in *GATA2* expression levels detected between the groups. After consolidation therapy, an increase of *GATA2*, *WT1*, and *EVI1* expression was observed in 1 patient who was diagnosed with a clinical relapse 3 months later (Figure 6B bottom panel). In 2 other patients who suffered an early relapse with high *GATA2* expression, we did not observe a rise in *GATA2* expression levels after consolidation therapy (2.6-5.4 months before clinical relapse).

Discussion

In the present study, we investigated the relevance and prognostic value of *GATA2* expression and mutations in a large cohort of de novo pediatric AML patients.

Mutational analysis was performed by sequencing of the DNA-binding zinc-finger domains of *GATA2* in 230 patients. We detected nonsynonymous variations in the *GATA2* gene with a frequency of 2.2% overall and 9.8% in pediatric CN-AML. All *GATA2* variants affected highly conserved residues within the critical functional DNA-binding domain consisting of 2 zinc fingers. Functional studies have recently shown significant effects of 6 different mutations located within the N-terminal and C-terminal zinc-finger domain of *GATA2* on transactivation of target genes,

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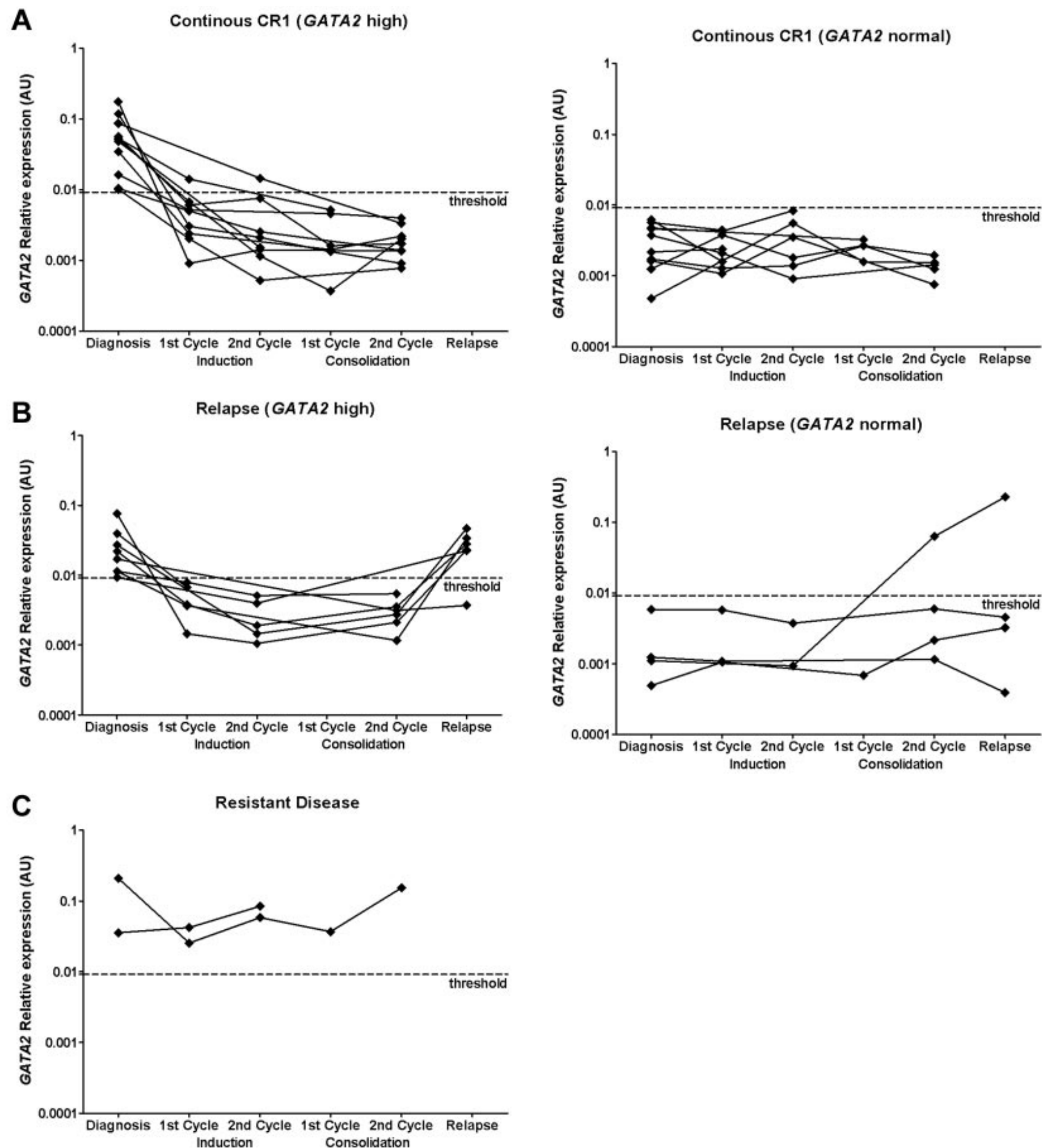

Figure 5. *GATA2* expression in continuous CR, RD, and clinical relapse. Consecutive *GATA2* expression at diagnosis and at different time points during treatment in a cohort of 38 pediatric AML patients from whom material was available for MRD analysis. *GATA2* expression was determined using real-time quantitative PCR at diagnosis (n = 38), after the first cycle of induction therapy (n = 27), after completion of induction therapy (n = 23), after the first cycle of consolidation therapy (n = 12), after completion of consolidation therapy (n = 25), and at relapse (n = 10). Expression relative to *GAPDH* is plotted. *GATA2* expression in patients with continuous CR1 (A; n = 21), relapse (B; n = 12), and RD (C; n = 2). Patients were subdivided into patients with high *GATA2* expression at diagnosis versus normal *GATA2* expression at diagnosis. *GATA2* expression was considered to be high when the expression was 2 SD above the median *GATA2* expression obtained in normal BM (n = 74), which was determined by real-time quantitative PCR. The threshold is depicted as a dashed line.

DNA-binding ability, protein-protein interactions, cellular differentiation, apoptosis, and global gene expression.[27,29,35] In 1 patient, we detected a variation in codon 318 of the N-terminal zinc-finger domain adjacent to codon 319 in which an acquired mutation was described recently in an adult AML patient with FAB-M5 morphology.[26] In codon 362 of the C-terminal zinc-finger domain, we identified 3 heterozygous missense variants and 1 heterozygous single nucleotide deletion resulting in loss of the C-terminal part of the protein. Because 4 of 5 *GATA2* variants were present within codon 362, this suggests that these variations are indeed true mutations. One of these variants (R362Q) was identified recently as an acquired mutation in 2 adult AML patients with FAB-M5 morphology.[26] In the highly conserved zinc-finger domains of the *GATA2* gene, acquired and inherited mutations have been reported in myeloid malignancies. In (familial) myelodysplastic syndrome/AML and syndromes with a predisposition to developing myelodysplastic syndrome/AML, inherited *GATA2* mutations are mainly clustered in codon 354 and codon 396-398 of the C-terminal zinc-finger domain (supplemental Figure 1).[29-35] In contrast, in sporadic AML, the majority of acquired *GATA2* variations are located within codon 362 in the C-terminal zinc-finger domain (Figure 1B),[26] suggesting that this location may be a mutational hot spot.

The low frequency of *GATA2* variations found in the present study is consistent with recent studies in adult AML, which reported acquired *GATA2* mutations with a frequency of 3.6% in adult AML patients with FAB-M5 morphology.[14,26,28,29] Interestingly, no *GATA2* variations were observed in any of our pediatric patients with FAB-M5 morphology (n = 51). We detected mainly *GATA2* variations in CN-AML. In all cases, we observed

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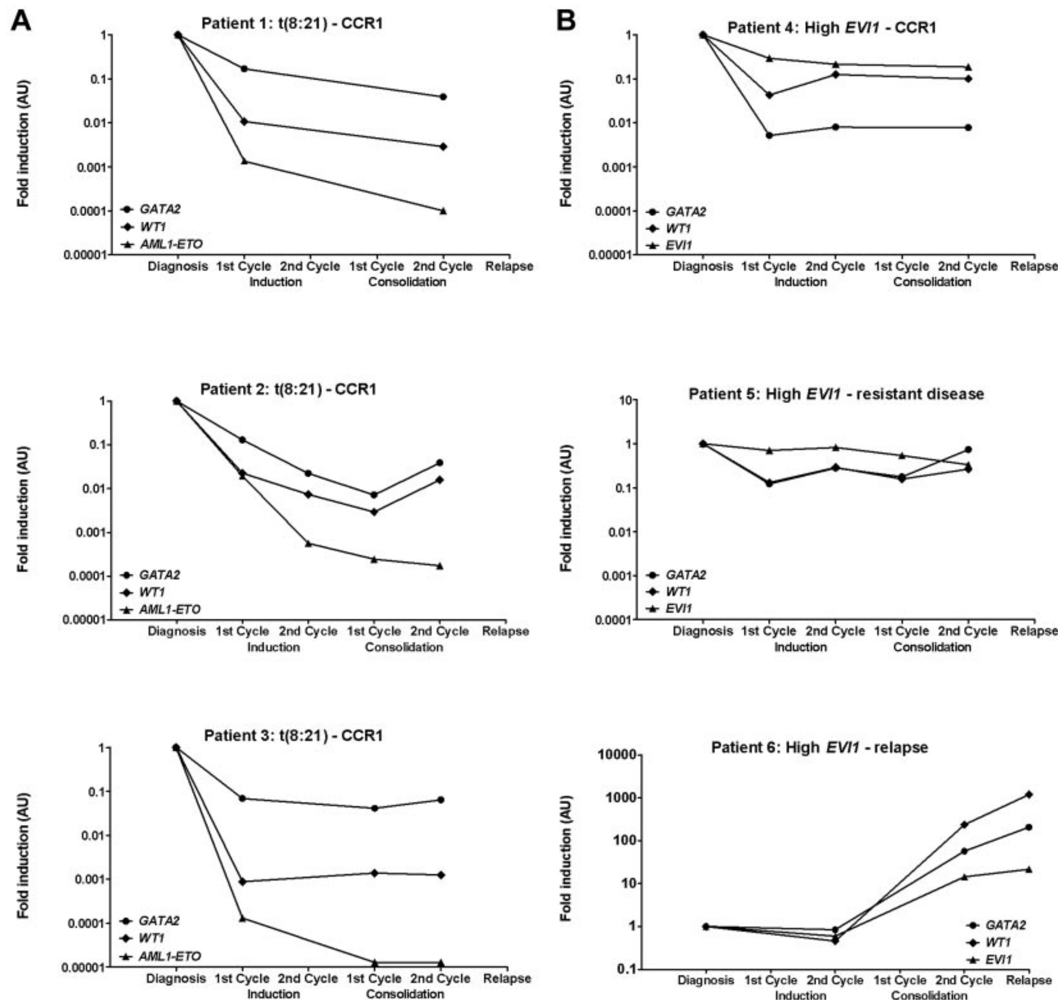

Figure 6. Relation between *GATA2* expression and other residual disease markers. Consecutive *GATA2*, *WT1*, *EVI1*, and fusion gene expression at diagnosis and at different time points during treatment in pediatric AML patients with t(8;21) (A; n = 3) or high *EVI1* expression (B; n = 3) determined by real-time quantitative PCR. Expression of *GATA2* and *WT1* relative to *GAPDH* is plotted. Expression of *EVI1* and *AML1-ETO* relative to *PBGD* is plotted. For all of the different markers, expression at different time points during treatment was normalized to expression at diagnosis. CCR1 indicates continuous first CR.

cooccurrence of nonsynonymous *GATA2* variations with various other recurrent mutations, including *NRAS* mutations (n = 4), *NPM1* mutations (n = 2), *CEBPA*-DM mutations (n = 2), and *WT1* mutations (n = 1). In adult AML, cooccurrence of *GATA2* mutations with other recurrent mutations (eg, *WT1* mutation and *CEBPA* single mutation) has also been reported.[26] Although the cooccurrence of these lesions remains a rare event in pediatric AML, this observation may suggest that these aberrations cooperate in leukemia development. Determination of the clinical significance of *GATA2* variations in a larger cohort of pediatric CN-AML is highly recommended.

In the present study, *GATA2* expression analysis demonstrated that in 155 of 237 diagnostic samples (65%), *GATA2* expression was higher than in normal BM. Normalization of *GATA2* expression was observed in patients who reached CR, whereas *GATA2* expression levels stayed high in patients with RD. Although *GATA2* expression during follow-up was studied in a limited number of patients, these data suggest that *GATA2* may be an interesting marker for residual disease detection. However, because of the heterogeneous expression of *GATA2* in AML and the relatively high background expression in normal hematopoietic cells, the sensitivity of *GATA2* as a marker for residual disease may be limited. In only 2 of 38 AML patients was *GATA2* sufficiently highly expressed to allow detection of at least a 2-log reduction (ie, sensitivity < 0.01). Assessment of quantitative fusion-gene expression was shown to be a more robust marker for residual disease. However, this strategy is limited to specific AML subgroups. In addition, MRD detection by multiparameter flow cytometry is a promising strategy that is applicable to the majority of AML patients.[5,6]

We have shown herein that high *GATA2* expression at diagnosis is an independent poor prognostic factor for pOS (HR = 1.7, $P = .045$), pEFS (HR = 2.1, $P = .002$), and pDFS (HR = 2.3, $P = .004$) in pediatric AML overall. In adult AML, studies of the prognostic value of *GATA2* have yielded conflicting results, which might be because of the relatively small patient cohorts and technical differences between studies in quantification of *GATA2* expression.[49-52] In a cohort of 112 adult AML patients, Vicente et al showed recently that high *GATA2* expression was associated with worse outcome (pOS, pEFS, and pDFS). The prognostic value of high *GATA2* expression was most evident in adult CN-AML.[51,52] In the present study of pediatric AML, we did not observe a difference in survival based on *GATA2* expression in CN-AML (n = 40), which may be because of differences in distribution of genetic aberrations underlying pediatric compared with adult CN-AML.

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Interestingly, the prognostic impact of *GATA2* was particularly high in specific morphologic and genetic AML subgroups. High *GATA2* expression identified a group of patients with a significant worse outcome among patients with high *WT1* expression, which is consistent with recent observations in adult AML.[51] Moreover, in patients with FAB-M5 morphology and inv(16), we observed pronounced differences in survival between patients with high versus normal *GATA2* expression, and a trend toward worse survival was observed in patients with 11q23 translocations or *FLT3*-ITD.

Based on the results of the present study, we conclude that *GATA2* expression is a novel independent poor prognostic factor in pediatric AML. The negative correlation between *GATA2* expression and outcome, especially in specific morphologic and genetic AML subgroups, suggests that *GATA2* may be a useful marker for the identification of patients with a dismal prognosis, which could lead to better risk-group stratification and risk-adapted therapy in the future. In addition, we identified the presence of *GATA2* mutations as a recurrent but infrequent event in childhood AML, which mainly occurs in CN-AML. The clinical significance of *GATA2* mutations should be determined in a larger cohort of pediatric CN-AML patients, and the value of *GATA2* expression as a predictor for poor OS, EFS, and DFS should be validated in prospective studies.

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Authorship

Contribution: M.L., I.H.I.M.H., V.H.J.v.d.V., B.A.v.d.R., M.M.v.d.H.-E., C.M.Z., and J.H.J. designed the study; M.L., I.H.I.M.H., and R.H.J.N.K. performed the laboratory research; M.L., I.H.I.M.H., R.H.J.N.K., J.B.M.B., and J.H.J. analyzed and interpreted the data; V.d.H., J.T., A.B., and D.R. collected the patient samples and clinical data; M.L. and J.H.J. wrote the manuscript; and all authors critically reviewed the manuscript and gave their final approval.

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