Elevated expression of the *AF1q* gene, an *MLL* fusion partner, is an independent adverse prognostic factor in pediatric acute myeloid leukemia

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The *AF1q* gene, a mixed-lineage leukemia fusion partner, is highly expressed in hematopoietic progenitor cells but has low expression in differentiated cells. We determined the expression of the *AF1q* gene by reverse transcriptase–polymerase chain reaction in 64 pediatric acute myeloid leukemia (AML) patients treated on Children's Cancer Group clinical trial CCG-2891 and correlated its expression level to clinical characteristics and outcome. *AF1q* expression in patients varied from 0- to 154-fold compared with normal marrow, and increasing expression level was associated with worsening survival, with a hazard ratio of 1.02 per fold increase in *AF1q* expression ($P = .032$). We divided patients into tertile groups based on *AF1q* expression level. Patients with high *AF1q* expression (top tertile) had a higher predominance of French-American-British M1 compared to patients with lower 2 tertiles of *AF1q* expression (43% vs 9%, $P = .003$). High AF1q expression was associated with poor survival in univariate and multivariate models. Overall survival at 8 years for patients with the high *AF1q* expression was 19% versus 50% in patients with low *AF1q* expression, ($P = .01$). *AF1q* expression may correlate with clinical outcome in pediatric AML, although it is not clear if *AF1q* is simply a marker of a more primitive phenotype or contributes directly to leukemogenesis. (Blood. 2004;104: 3058-3063)

Introduction

The normal function of the hematopoietic system depends on precise control of a series of key regulatory genes that govern self renewal, lineage commitment, and differentiation of hematopoietic stem cells and their progeny. In leukemia and lymphoma, these genes are frequently altered by chromosomal translocations, inversions, methylations, or mutations.[1-3] Hence, alterations of gene expression levels may result in gain or loss of function.[4] Many of these genetic alterations have proven to be useful molecular tools for diagnosis, risk stratification, and minimal residual disease (MRD) monitoring.[5-8] In addition, such alterations may also provide potential targets for therapy.[9,10]

The *AF1q* gene, located in chromosome 1, band 21, was initially identified as a mixed-lineage leukemia *(MLL)* fusion partner from an infant acute myelomonocytic leukemia carrying the t(1;11)(q21;q23) translocation.[11] There are more than 50 different types of chromosomal translocations involving the *MLL* gene.[12] To date, more than 20 *MLL* translocation partners have been cloned and characterized.[12] In general, the structure of the *MLL* fusion gene is diverse, and the genes seldom share functional domains, which has made it difficult to develop a unifying theory for how rearranged forms of *MLL* may contribute to leukemogenesis.[13] The *MLL* partner proteins appear to fall into 2 functional categories: signaling molecules that normally localize to the cytoplasm/cell junctions, or nuclear transcription factors implicated in transcriptional regulation.[14] The biologic function of many *MLL* fusion genes and their impact on leukemogenesis are poorly understood.

The *AF1q* gene has several unique structural features that differentiate it from other *MLL* fusion partners. *AF1q* fuses to the *MLL* gene in an in-frame fashion with its entire open reading frame (ORF), rather than with a truncated ORF portion as seen in other fusion partners.[11,15] There are no well-defined functional domains in the *AF1q* ORF. The 3′ untranslated region of *AF1q* contains a series of ATTA-rich sequences, which may contribute to the short half-life of *AF1q* protein in the intracellular environment.[11] *AF1q* is highly regulated in the human hematopoietic system, and it has been shown that *AF1q* is highly expressed in CD34-enriched stem cells, and this expression declines by differentiation and is nearly undetectable in peripheral blood.[11,16] In addition, *AF1q* is highly expressed in leukemic cell line NB4, and all-*trans* retinoic acid (ATRA)–induced differentiation leads to concomitant decline in *AF1q* expression.[17] In CD34-enriched human cord blood stem cells *AF1q* is coexpressed with other stem cell–related genes such as *GATA-2* and *STAT5*.[18]

As *AF1q* has higher expression in immature CD 34^+ cells, higher expression of *AF1q* in myeloid leukemias might identify more "primitive" leukemias with perhaps a worse prognosis. We evaluated the available diagnostic RNA from 64 pediatric AML

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cases treated on Children's Cancer Group clinical trial CCG-2891 for the expression of *AF1q* using a quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) assay and studied the association of *AF1q* expression with clinical characteristics and treatment outcome.

Patients, materials, and methods

Patients and treatment

Newly diagnosed patients with de novo AML registered in Children's Cancer Group (CCG) pediatric AML protocol CCG-2891 were included in this study. Archived RNA from 64 de novo pediatric AML diagnostic marrow samples was available for analysis. The diagnosis of AML was made according to French-American-British (FAB) classification and was confirmed by the CCG-Central Review Committee. All patients had consented to use of diagnostic marrow specimens for biology studies at the time of study entry. This study was approved by the Fred Hutchinson Cancer Research Center Institutional Review Board.

CCG-2891 has been described in detail in other publications.[19,20] In brief, CCG-2891 was a prospective randomized trial that accrued 887 de novo AML patients from 1989 to 1995 (excluding Down syndrome AML, myelodysplastic syndrome, secondary AML, or granulocytic sarcoma without marrow involvement). This study randomized pediatric patients with AML at diagnosis to receive 1 of 2 induction regimens that involved 4-day cycles of 5 chemotherapeutic agents (daunomycin, cytarabine, etoposide, 6-thioguannine, and dexamethasone). The following cycles were administered either after 10 days, despite low counts (intensive timing), or after 14 days, depending on the marrow status (standard timing). Induction regimen consisted of a total of 4 cycles in both groups. At the end of induction, patients who achieved remission and had an HLA-matched related donor were eligible to receive an allogeneic bone marrow transplant (allo-BMT). Patients without related donors were randomized to receive either nonmyeloablative chemotherapy or an autologous BMT (auto-BMT).

RNA isolation and cDNA synthesis

Bone marrow samples from patients in the CCG2891 trial were obtained prior to therapy. Total RNA were prepared by using the TRIzol extraction kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The cDNAs were synthesized from 0.5 μg total RNA by using Superscript reverse transcriptase system in 20 μL volume (Invitrogen). The cDNA synthesis was performed under the following conditions recommended by Invitrogen: 42°C for 50 minutes, then 72°C for 15 minutes.

Quantitative PCR primers and fluorescent probe design

The structure of the *AF1q* gene is shown in Figure 1. The *AF1q* gene contains 2 exons, and the forward and reverse primers were designed to cross the exon/intron borders in order to avoid genomic DNA amplification. The primer sequences are as follows: *AF1q*F 5′-GCA CTC CCT CCA TCT TTG GA-3′; *AF1q*R 5′-CAG CTC CGA CAG ATC CAG TTC-3′. The size of the PCR amplicon is 133 base pair (bp). The fluorescent probe sequence was: 5′-ACC CTG TGA GTA GCC AGT ACA GTT CCT TTC TTT TC-3′ (Synthegen, LLC; Houston, TX).

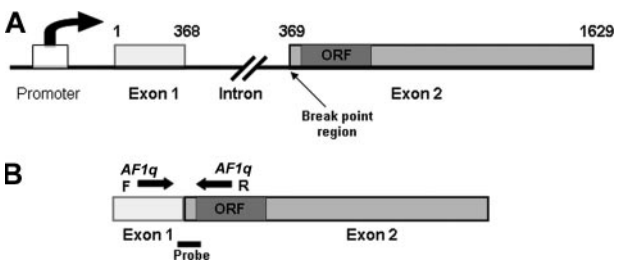

Figure 1. *AF1q* gene structure. (A) *AF1q* gene contains 2 exons separated by a 9-kb intron. Numbers above the gene represent the nucleotide sequence of the *AF1q* cDNA. Open reading frame (ORF) is located near the N-terminus of exon 2, proximal to the break-point region. (B) Primers (arrows) and TaqMan probe (solid bar) for the quantitative PCR are represented.

Quantitative PCR standard curve

The full-length *AF1q* and β_2 microglobulin cDNAs were cloned into the Bluescript plasmid at *Eco*RI and *Xba*I sites, respectively. The plasmid DNA that contains both *AF1q* and β_2 microglobulin genes was purified by standard methods (Qiagen, Valencia, CA). A standard curve stock was made that contains 10^7 molecules/μL of *AF1q*/β_2 plasmid. The standard curves were generated by amplification of a 133-bp *AF1q* sequence and 80-bp β_2 microglobulin sequence from dilution series ranging from 10^7 to 1 of *AF1q*/β_2 plasmid.

PCR quantitation

The real-time quantitative PCR analysis was performed using the standard TaqMan PCR Core Reagent Kit (Applied Biosystems, Foster City, CA) in a 50-μL reaction on patient's synthesized cDNA. Each quantitative PCR reaction mix contains the cDNA equivalence to 100 ng of total RNA. We performed the quantitative PCR reaction in ABI PRISM 7700 Sequence Detector under the following conditions: stage 1, 50° C for 2 minutes, 95°C for 10 minutes for one cycle; and stage 2, 95°C for 15 seconds, 60°C for 1 minute for a total of 40 cycles. β_2 microglobulin gene was used as an internal control to normalize the difference of amount of cDNA in each reaction. *AF1q* expression level was calculated as the ratio of *AF1q* to β_2 microglobulin amplification and then normalized to normal control bone marrow. Five marrow samples from volunteer donors were used to determine the median control *AF1q* expression.

Mutational analysis

Analyses of diagnostic marrow specimens of this population for activating mutations of the *FLT3* and *ras* genes were performed and reported previously.[21]

Statistical methods

Data obtained in CCG-2891 through July 31, 2001, were used to compare patients with various levels of *AF1q* expression. The significance of observed differences in proportions was tested using the Chi-squared test and Fisher exact test when data were sparse. For continuous data, the Mann-Whitney test was used to compare the medians of distributions.[22] Patients lost to follow-up were censored at their last known point of study, with a cutoff of January 31, 2001. Patients who received a hematopoietic cell transplant were censored at the time of transplantation. Actuarial estimates of overall survival (OS) and event-free survival (EFS) from diagnosis, defined as the time from diagnosis to either death (OS) or marrow relapse or death (EFS), were calculated using the Kaplan-Meier method.[23] The Kaplan-Meier method also was used to calculate estimates of OS and disease-free survival (DFS) from the end of induction for patients in remission at the end of induction. DFS was defined as the time from the end of induction to relapse or death by any cause. Confidence intervals were calculated using Greenwood estimate of the standard error.[24] Differences in OS, EFS, and DFS were tested for significance using the log-rank statistic.[25]

Factors significant in univariate analysis at P less than .05 were considered for inclusion in multivariate Cox regression models.[26] The likelihood ratio test was used to determine whether variables should be added or dropped from the multivariate model. Multivariate analyses included patients with complete covariate data. Statistical corrections for multiple comparisons were not employed.

The positive predictive value (PPV) of high *AF1q* expression, that is, the likelihood of a person with high *AF1q* expression dying, was estimated as 1 minus the Kaplan-Meier estimate of 8-year OS for patients with high *AF1q* expression. The negative predictive value (NPV) of low *AF1q* expression, that is, the likelihood of a person with low *AF1q* expression surviving, was estimated as the Kaplan-Meier estimate of 8-year OS for patients with low *AF1q* expression. PPV and NPV were estimated in a similar manner for nonwhite race, *FLT/ITD*, and high-risk cytogenetics.

Table 1. Patient characteristics in the study population versus CCG 2891 population

	AF1q study population	Rest of CCG2891	*P*
No.	64	823	NA
Median age, y	8.5	7.7	.532
Median WBC count, per mm^3	45 900	20 400	.0004
Male, no. (%)	34 (53.1)	421 (51.2)	.862
Intensive induction, no. (%)	40 (62.5)	499 (60.6)	.871
Race, overall			.289
White, no. (%)	47 (73)	550 (67)	.343
Nonwhite, no. (%)	17 (27)	273 (33)	
CR rate, %	68.3	77.7	.132
8-year OS from diagnosis, %	40	40	.636
8-year OS from remission, %	48	52	.445

NA indicates not applicable.

Results

Patient characteristics—study population versus CCG-2891

We initially examined the clinical characteristics of the study population to determine if it was representative of the CCG-2891 de novo AML patients. A comparison of median age, sex, induction regimen, race, and remission induction rate showed no statistically significant difference between our study patients and the rest of the CCG-2891 patients (Table 1). As expected, the diagnostic white blood cell count (WBC) of the archived samples was higher in our study population (45 900/mm^3) than the rest of the CCG-2891 population (20 400/mm^3) ($P = .0004$). The study population had a complete remission (CR) rate of 68%, which was lower than the CR rate of 78% for the CCG-2891 study; however, this difference was not statistically significant ($P = .21$). Actuarial overall survival (OS) at 8 years from on-study and from remission was similar between our study population and the rest of the CCG-2891 patients ($P = .636$ and .45, respectively).

AF1q expression in normal marrow and in CD34 selected progenitor cells

AF1q expression was determined in 5 marrow specimens obtained from healthy donors by real-time quantitative RT-PCR and corrected for the β_2 microglobulin expression levels. *AF1q* expression ranged from 1.54×10^5 to 2.36×10^5 *AF1q* copies per microgram of total RNA, with a median of 2×10^5 copies per microgram RNA in the normal marrow. We further evaluated the expression level in CD34 selected peripheral blood stem cells. *AF1q* expression level was 1.8×10^6 copies per microgram of total RNA, approximately 9-fold higher than that in normal marrow.

AF1q expression level varies in pediatric AML

AF1q expression level was determined for the 64 patients. *AF1q* expression ranged from 0 to3×10^7 copies per microgram RNA and normalized to the median *AF1q* expression level in the normal control bone marrows. After normalization for control marrow, *AF1q* expression levels ranged from 0- to 154.2-fold of the expression in the normal bone marrow (median, 4.7-fold). Compared with the normal marrow control, 54 of the 64 (84%) patients in our study population had elevated *AF1q* expression, and the remaining 10 (16%) either lacked *AF1q* expression (n = 1) or had expression equal to or lower than healthy volunteer marrow (n = 9). To test whether variation in *AF1q* expression might be due to the variation in blast percentage, we compared the marrow blast percentage in the lowest tertile of *AF1q* expression to that of the middle and highest tertile of *AF1q* expression of the population. Blast percentage of the one third of patients with the lowest *AF1q* expression (first tertile) was 81.5%, compared with 80% and 86% in patients with the mid-range (second tertile) or high (third tertile) *AF1q* expression ($P = .36$).

High *AF1q* expression is associated with poor outcome in pediatric AML

We studied whether *AF1q* expression level correlates with clinical outcome in pediatric patients with AML. For survival determination, patients who had received allogeneic stem cell transplantation were censored at the time of transplant. Patients who had *AF1q* expression at or below the normal range had an overall survival of 60%, versus 40% for those with elevated *AF1q* expression ($P = .41$). We subsequently evaluated the *AF1q* expression as a continuous variable, showing that increasing *AF1q* expression level was associated with worsening overall survival with a hazard ratio (HR) of 1.02 per each fold increase in *AF1q* expression ($P = .031$; Table 2). For example, a 10-fold increase in *AF1q* expression would increase risk of death by 1.20-fold (or 20%). To further delineate the clinical significance of *AF1q* expression, we divided the study population into 3 equal groups (tertiles) based on *AF1q* expression level and asked if clinical outcome differed based on *AF1q* expression. The first tertile included patients with the lowest *AF1q* expression (0- to 2.8-fold the normal control, n = 22), the second tertile included patients with mid-range *AF1q* expression (3- to 8.5-fold the normal control, n = 21), and the third tertile included patients with highest *AF1q* expression (≥ 9-fold normal control, n = 21). Cox regression analysis revealed that increasing the *AF1q* expression by tertiles was associated with worse survival, as patients in the second and third tertile had increasing risk of death compared with the patients in the first tertile. Actuarial OS at 8 years from diagnosis for the patients in the first tertile was 59% compared with 37% and 19% (5-year OS) for the patients in the second and third tertiles, respectively, (Table 2, $P = .035$).

We also compared the clinical outcome of the patients with the highest *AF1q* expression (ie, third tertile) to the remaining two thirds of the patients with lower *AF1q* expression. Remission induction rate was similar between the patients with low and high *AF1q* expression (70% vs 66%, $P = .922$). Patients with lower *AF1q* expression (n = 43) had an actuarial OS at 8 years from diagnosis of 50% compared to 19% for the patients with high *AF1q* expression (n = 21, $P = .01$) (Figure 2). Actuarial DFS at 8 years from remission in patients with lower *AF1q* expression (n = 28) was 50% compared with 0% (n = 12) in patients with high *AF1q* expression ($P < .001$), as patients with high *AF1q* expression had a relapse rate of 100% at 8 years compared with 50% in patients with lower *AF1q* expression (Table 3, $P = .002$). Thus, increasing *AF1q*

Table 2. Survival of patients with elevated *AF1q* expression

AF1q expression by tertiles	HR versus first tertile	*P*	Overall survival, %*	*P*
First tertile	NA	NA	59	.035
Second tertile	1.4	.483	37	NA
Third tertile	2.9	.019	19	NA

AF1q expression as a continuous variable: HR per fold increase in *AF1q* expression = 1.02, and $P = .32$. First tertile versus second tertile, $P = .476$; second tertile versus third tertile, $P = .063$; first tertile versus third tertile, $P = .026$. For the first tertile, N = 22; for the second and third, N = 21.

NA indicates not applicable.

*Pairwise comparisons for overall survival.

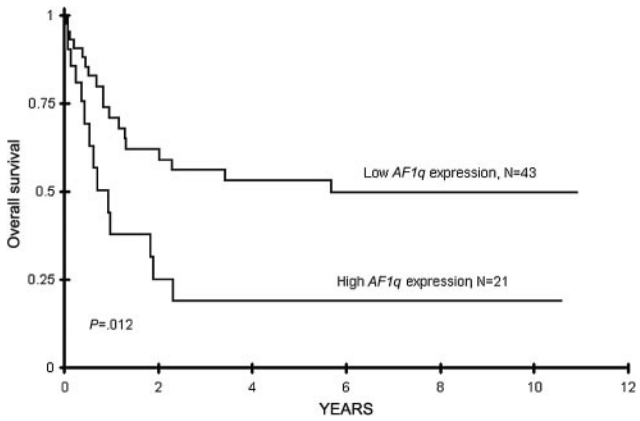

Figure 2. The Kaplan-Meier estimates for overall survival from diagnosis in AML patients with high or low *AF1q* expression. High *AF1q* expression is defined as highest tertile and low expression is defined as the lower 2 tertiles of *AF1q* expression.

appeared to correspond with poor clinical outcome, and high *AF1q* expression level distinguished patients with significantly higher relapse rate and worse outcome. In contrast, of the 14 high-*AF1q* patients who achieved a CR, 4 went on to receive a matched family donor stem cell transplant in first CR. Of these 4 patients, 3 are long-term survivors, with 1 patient dying of nonleukemic causes more than 6 years after transplantation. The remaining 10 patients with high *AF1q* who were in CR and did not have matched family donors were randomized to autologous stem cell transplantation or continued chemotherapy. All of these patients relapsed and died (6 with early relapse, 2 after autologous stem cell transplantation, and 2 from relapse after completing chemotherapy).

Characteristics of the patients with elevated *AF1q* expression

We compared the laboratory and clinical characteristics of patients with high (top tertile) and low (lower 2 tertiles) *AF1q* expressions. The parameters of sex, age, diagnostic WBC, and induction regimen in patients with high *AF1q* expression were compared to that of patients with lower *AF1q* expression. Patients with high or low *AF1q* expression had similar demographics and clinical characteristics (Table 1). Diagnostic WBC was lower in patients with high *AF1q* expression, but this difference did not achieve statistical significance (43 700 vs 63 700, $P = .4$, Table 3). Of the 21 patients with high *AF1q* expression, 9 (43%) had an M1 FAB morphology compared with 4 (9%) of the 43 of the patients with low *AF1q* expression ($P = .003$).

Thirty-seven patients had cytogenetic information available, of which 12 had high *AF1q* expression and the other 25 had low *AF1q* expression. Of the 12 patients with high *AF1q* expression, 6 (50%) and 8 of 25 patients (32%) with low *AF1q* expression had normal karyotype ($P = .47$). There was no predominance of a particular cytogenetic abnormality in the patients with high *AF1q* expression. Of the 7 patients with 11q23, 6 had low *AF1q* expression ($P = .389$), and there were no t(1;11) translocations. Only 1 patient with high *AF1q* expression had high-risk cytogenetics (deletion 5) (Table 3).

All 64 patients in this study were previously screened for activating mutations of the *FLT3* and *ras* genes. In the high *AF1q* expression group, 5 (24%) patients had *FLT3* internal tandem duplication *(FLT3/ITD)*, and an additional 3 (14%) patients had activation loop mutation of the *FLT3* gene *(FLT3/ALM)*. Activating mutations of the *FLT3* gene was seen in 7 of 43 (16%) patients with low *AF1q* expression (5 *FLT3/ITD* and 2 *FLT3/ALM*). Prevalence of *FLT3* activating mutations was 38% versus 16% in the high versus low *AF1q* expression group ($P = .066$, Table 3). Mutations in the *ras* gene were present in 9.5% of the patients with high *AF1q* expression, compared with 28% in the low *AF1q* expression ($P = .118$), and specifically, *N-ras* mutations were not seen in the high *AF1q* group (21% vs 0%, $P = .025$, Table 3). Thus, activating mutations of the *FLT3* or *ras* genes were seen in 10 of 21 (48%) of the patients with high *AF1q* and in 20 of 43 (47%) patients in the patients with low *AF1q* expression ($P = .93$).

Prognostic significance of high *AF1q* expression

Cox regression analysis was used to determine the prognostic significance of high *AF1q* expression and compare it with that of other known prognostic factors. Specifically, we used univariate analysis to assess the prognostic significance of *AF1q* expression level (high vs low), induction timing (intensive vs standard timing), diagnostic WBC ($<$ vs $>$ 100 000/mm^3), race (nonwhite vs white), *ras* (activated vs wild type), *FLT3/ITD* (positive vs negative), FAB (M1 vs other FAB phenotypes), and cytogenetics (high risk vs other). The HR for death for patients with high *AF1q* expression was 2.4 compared to those with low *AF1q* expression ($P = .015$). Induction timing, diagnostic WBC count higher

Table 3. Patient characteristics in the *AF1q* study population

	Low *AF1q* expression*	High *AF1q* expression†	*P*
No.	43	21	NA
Median age, y	8.6	8.3	.721
Median WBC count per mm^3	637 000	437 000	.403
No. of males (%)	21 (49)	13 (62)	.473
No. of nonwhites (%)	10 (23)	7 (33)	.578
Intensive induction, no. (%)	25 (58)	15 (71)	.45
CR rate, %	70	65	.922
FAB classification, no. (%)			.057
M0	1 (2.3)	0 (0)	.99
M1	4 (9.3)	9 (42.8)	.003
M2	12 (27.9)	4 (19.0)	.547
M3	3 (12)	1 (4.8)	.99
M4	15 (43.8)	4 (19.0)	.251
M5	6 (13.9)	2 (9.5)	.99
M6	2 (4.6)	0 (0)	.99
M7	0 (0)	1 (4.8)	.328
Cytogenetics, no. (%)			
No.	25	12	.569
Normal	8 (32)	6 (50)	.47
t(15;17)	2 (8)	1 (8.3)	.99
t(8;21)	3 (12)	1 (8.3)	.99
11q23	6 (24)‡	1 (8.3)§	.389
Del 7	0 (0)	0 (0)	NA
Del 5	0 (0)	1 (8.3)	.324
Others	5 (20)	1 (8.3)	.641
Mutation status, no. (%)			
No.	43	21	.082
No mutation	23 (53.5)	10 (47.6)	.791
K-ras	3 (7)	2 (9.5)	.99
N-ras	9 (21)	0 (0)	.025
Flt3/ITD	5 (11.6)	5 (23.8)	.275
Flt3/ALM	2 (4.6)	3 (14.3)	.320
All *FLT3* mutations	7 (16.2)	8 (38.1)	.066
Survival/relapse			
8-year OS from diagnosis, %	50%	19%	.01
8-year DFS from remission, %	50%	0%	.001
Relapse rate at 8 years, %	50%	100%	.002

NA indicates not applicable.
*Low is defined as lower 2 tertiles of *AF1q* expression.
†High is defined as highest tertile of *AF1q* expression.
‡Two t(9;11), 2 del (11)(q23), 1 t(4;11).
§t(9;11).

Table 4. Cox regression analysis of prognostic factors for survival from diagnosis

	Univariate			Multivariate		
	HR	95% CI	*P*	HR	95% CI	*P*
AF1q expression, high versus low	2.41	1.19-4.88	.015	3.05	1.31-7.09	.009
Timing, standard versus intensive	1.24	1.02-1.49	.03	0.88	0.39-1.98	.758
WBC, at least 100 versus fewer than 100, mm^3	1.29	1.02-1.62	.033	1.41	0.60-3.35	.430
Race, nonwhite versus white	1.30	1.07-1.59	.008	0.94	0.41-2.19	.893
ras gene, activated versus wild type	2.04	1.15-3.63	.015	1.76	0.67-4.65	.254
FLT3/ITD, positive versus negative	1.78	0.89-3.54	.101	1.13	0.39-3.30	.820
FAB, M1 versus other FAB	1.24	0.51-3.03	.636	0.74	0.27-2.03	.557
Cytogenetics, high-risk versus other	1.53	0.91-2.59	.111	—	—	—

— indicates we were unable to obtain estimate because cytogenetics had zero variance when added to the multivariate model.

than 100 000, activated *ras*, and nonwhite race also were univariately associated with adverse overall survival (Table 4). Patients with *FLT3/ITD*, FAB M1 classification, and high-risk cytogenetics had HR for death of 1.78, 1.24, and 1.53, respectively, although they did not reach statistical significance (Table 4). Multivariate analysis of the univariate predictors of poor outcome (high *AF1q*, standard timing induction regimen, high diagnostic WBC, race, activated *ras*, and *FLT3/ITD*) revealed that high *AF1q* expression was the single independent prognostic factor for adverse survival from diagnosis (Table 4).

We also used the univariate and multivariate analyses to ascertain the prognostic significance of the aforementioned parameters on death or relapse from the time of achieving remission. Patients with high *AF1q* expression had a nearly 5-fold higher risk of relapse or death from the time of achieving remission ($P = .002$). After accounting for induction regimen, diagnostic WBC, race, activated *ras*, and *FLT3/ITD* in the multivariate model, the hazard ratio for relapse or death remained elevated at more than 6 ($P = .002$). In the univariate model induction regimen, race, activated *ras*, and diagnostic WBC were associated with poor DFS; however, in the multivariate model that included high *AF1q* expression, all parameters lost clinical significance while high *AF1q* expression remained a strong predictor of outcome.

Finally, we assessed the predictive values (positive and negative) of high *AF1q* expression for survival and compared the values with those of other established prognostic factors. In this study, positive predictive value (PPV) for *AF1q* expression (likelihood of a person with high *AF1q* expression dying) was 81% compared with negative predictive value (NPV) of 50% (likelihood that a person with low *AF1q* expression would survive). The PPV and NPV for WBC higher than 100 000, nonwhite race, *FLT3/ITD*, and high-risk cytogenetics were 65% and 42%, 65% and 43%, 78% and 44%, and 73% and 42%, respectively.

Discussion

In this study we demonstrate significant variation in expression of *AF1q* gene in pediatric AML and show that *AF1q* expression level correlates with clinical outcome in this population. Patients with high *AF1q* expression had a remission induction rate similar to those with lower expression, but suffered a higher relapse rate. The poor outcome associated with high *AF1q* expression remained after adjustment for other factors associated with poor outcome. As this preliminary study was conducted in a subset of patients from the CCG-2891 study, these results should be interpreted with caution. However, the study population did not significantly differ from the CCG-2891 population except for having a higher white count, which is difficult to avoid in retrospective analyses. The underlying reason for such a difference is likely the higher proportion of samples with high white counts in the reference laboratory, as higher WBC may also explain the somewhat lower (though statistically not significant) CR rate in this study group. Taken together, the data suggest that high *AF1q* expression may be a new determinant of poor outcome in pediatric AML. This study also suggested that high expression of *AF1q* was an independent prognostic factor for poor outcome. Patients with high *AF1q* had lower white counts, had higher proportion of patients who received intensive induction (which should affect the outcome favorably in this population). Despite these 2 favorable factors among patients with high *AF1q* expression, these patients had a substantially worse outcome. Interestingly, patients with high *AF1q* expression had a higher percentage of FAB M1 but a lower percentage of M4 subtype compared to patients with low *AF1q* expression (M1: 42.8% vs 9.3%, $P = .003$; M4: 43.8% vs 19%, $P = .251$). The data seemed to support that high *AF1q* expression might be related to less-differentiated FAB subtypes. However, increased HR in patients with M1 subtype did not reach statistical significance (HR 1.24, $P = .636$). This was consistent with recent observations reported by CCG2891 investigators that patients with minimally differentiated AML-M0 had no differences in demographic, cytogenetics, and clinical outcome when compared to patients with more-differentiated non-M0 subtypes.[27] There was no predominance of high-risk cytogenetics in the high *AF1q* group, and indeed, more than 90% of the patients with high *AF1q* had normal or traditionally considered "favorable risk" cytogenetics. Furthermore, in multivariate analysis, which adjusted for known prognostic factors including WBC, race, *FLT3/ITD*, and activated *ras*, high *AF1q* expression was the sole independent prognostic factor in this patient population.

To date, there have been more than 20 *MLL* fusion partner genes cloned. The functions of most of these genes are unknown, as are the gene expression levels in leukemia cases. This study demonstrates the variation in expression of this *MLL* fusion partner in leukemia and shows that higher expression level of the *AF1q* gene is associated with an adverse clinical outcome. *AF1q* expression is highly regulated in human and murine hematopoietic cell differentiation processes,[11,17] and *AF1q* expression is markedly elevated in undifferentiated progenitor cells.[16] This observation was further supported by the fact that *AF1q* expression was highest in acute promyelocytic leukemic (APL) cell line NB4 entering S phase and declined to nearly undetectable levels after ATRA-induced differentiation.[16] Gene expression profile studies have demonstrated direct correlation between *AF1q*, *WT-1*, and *c-kit* gene expression and indirect correlation with CEBPα expression (D.L.S. and W.T., unpublished data, June 2003). These observations suggest that *AF1q* gene expression may be dysregulated in leukemia and that the level of expression might reflect the differentiation and proliferative state of the leukemia. There are, however, examples of genes that are expressed in immature

hematopoietic cells yet not related to clinical significance or FAB classification. For example, *c-kit* expression predicted a higher CR rate to induction therapy in adult AML but had no effect on OS, DFS, and FAB classification.[28] In addition, the expression of *ELF-1* and *MEF* genes are linked to hematopoietic cell differentiation and proliferation, but may be differentially associated with phenotype and outcome, as a study showed that *ELF-1* expression appears not related to FAB classification and clinical outcome, whereas *MEF* expression level is associated with FAB M2/M3 and a favorable prognosis.[29]

The function of *AF1q* currently is unknown. However, given that its high expression appears clinically relevant and associated with a significantly poor outcome, studies examining the structure, function, and biologic properties of *AF1q* may clarify its clinical significance. Although it is possible that high *AF1q* may play an integral part in leukemogenesis, it may also be that high *AF1q* expression is simply a surrogate marker for more-primitive, less-differentiated leukemia with high resistance to nonmyeloablative chemotherapy. Clearly further basic research is needed to understand the biology of this gene. In addition, the clinical use of *AF1q* expression needs to be further evaluated in large clinical trials. Such large studies would have adequate power to appropriately validate its clinical significance in the context of other prognostic markers. Evaluation of the prognostic significance of the *AF1q* gene expression in large multi-institutional trials are currently under way.

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