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Disease Characteristics and Prognostic Implications of Cell Surface FLT3 Receptor (CD135) Expression in Pediatric Acute Myeloid Leukemia: A Report from the Children's Oncology Group

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Abstract

Purpose—The FLT3 cell-surface receptor tyrosine kinase (CD135) is expressed in a majority of both acute lymphoid leukemia (ALL) and myeloid leukemia (AML). However, the prognostic significance of CD135 expression in AML remains unclear. We therefore evaluated the association between FLT3 surface expression and disease characteristics and outcomes in pediatric patients with AML.

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Experimental Design—We analyzed FLT3 receptor expression on AML blasts by multi-dimensional flow cytometry and its association with disease characteristics, clinical outcomes, and FLT3 transcript level in 367 children with AML treated on the Children's Oncology Group trial AAML0531.

Results—There was high variability in blast CD135 cell-surface expression across specimens. CD135 expression measure by flow cytometry was not correlated with FLT3 transcript expression determined by quantitative RT-PCR. Overall, CD135 expression was not significantly different for patients with *FLT3*/WT and those with *FLT3*/ITD and *FLT3*/ALM ($p=0.25$). High cell-surface CD135 expression was associated with FAB M5 subtype ($p<0.001$), *KMT2A* rearrangements ($p=0.009$) and inversely associated with *inv(16)/t(16;16)* ($p<0.001$). Complete remission rate, overall survival, disease-free survival, and relapse rates were not significantly different between patients with low and high CD135 expression.

Conclusions—FLT3 cell-surface expression did not vary by *FLT3* mutational status, but high FLT3 expression was strongly associated with *KMT2A* rearrangements. Our study found that there was no prognostic significance of FLT3 expression in pediatric AML.

Keywords

CD135; AML; FLT3 expression

Introduction

The FMS-like tyrosine kinase 3 (FLT3) is a class III receptor tyrosine kinase (RTK) that regulates several key components of early hematopoietic development and cellular proliferation, and has near universal expression in B-lineage acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML).(1–4) The activation of the FLT3 RTK results in downstream phosphorylation of secondary mediators that are key regulators of cell differentiation, proliferation and survival. Mutations in *FLT3*, including both internal tandem duplications (ITD) of the juxtamembrane domain (*FLT3*/ITD) and point mutations of the activating loop (*FLT3*/ALM), are among the most common somatic mutations in AML and result in aberrant kinase activation.(5) In addition to the constitutive phosphorylation driven by the ITD, enhanced FLT3 ligand-mediated signaling can also result in increased phosphorylation and leukemic blast proliferation in AML.(6,7) Patients with a high allelic ratio (HAR) of mutant *FLT3*/ITD to wild-type *FLT3* (*FLT3*/WT) have especially poor outcomes with chemotherapy alone.(8–10) The benefit of allogeneic hematopoietic stem cell transplant (HCT) in improving the poor outcomes of HAR *FLT3*/ITD patients has been shown in pediatric and adult AML.(11–13) In contrast, the *FLT3*/ALM does not affect outcome in AML.(8, 14, 15)

Gene expression analyses in ALL have demonstrated that elevated levels of FLT3 expression are associated with translocations involving the mixed lineage leukemia (*MLL*; renamed *KMT2A*) gene at the 11q23 locus.(16) In infant ALL with *KMT2A* rearrangements, low levels of FLT3 expression have been found to identify a group of patients with more favorable outcome.(17) Lymphoblasts from patients with *KMT2A* rearrangements and elevated FLT3 expression have been shown to have enhanced *in vitro* sensitivity to FLT3

inhibitor-induced apoptosis.(18) Further, primary pediatric ALL samples with elevated FLT3 expression, but without *KMT2A* rearrangements, have also been shown to be sensitive to the apoptotic effects of FLT3 inhibitors.(19)

However, the significance of cell-surface expression of FLT3 in AML and its potential prognostic significance are less clear. In this study, we determined the cell-surface expression of the FLT3 receptor (CD135) on the blast population and retrospectively analyzed whether expression was correlated with disease characteristics and clinical outcomes in pediatric patients with *de novo* AML. We also evaluated the difference in FLT3 expression levels between patients with *FLT3*/WT and those harboring a *FLT3*/ITD or *FLT3*/ALM mutation.

Methods

Patients and treatment

Pediatric patients with *de novo* AML enrolled on the Children's Oncology Group (COG) phase III trial AAML0531 were eligible for this study. Details of the study and treatment protocol have been previously described.(20, 21) In brief, COG AAML0531 tested the efficacy of the addition of the calicheamicin-linked anti-CD33 monoclonal antibody gemtuzumab ozogamicin (GO) to a 5-cycle multi-agent chemotherapy backbone. Patients with acute promyelocytic leukemia, constitutional trisomy 21, or a previous diagnosis of myelodysplastic syndrome were excluded. Morphologic, cytogenetic, and molecular analyses were performed according to study guidelines. All patients enrolled between September, 2008 and June, 2010 were eligible to undergo central FACS for staining with CD135 antibody on diagnostic bone marrow or peripheral blood to evaluate CD135 expression level.

Assessment of CD135 expression

CD135 mean fluorescent intensity (MFI) of the myeloid blast population, as defined by CD45 low and side scatter, was determined by multi-dimensional flow cytometry (MDF). Specimens were processed as previously described.(22) Bone marrow cells were incubated with CD34-APC and CD45-PerCP (BD Biosciences, San Jose, CA), CD135- PE) (BD Pharmingen, San Diego, CA), and C36-flourescein (FITC) (Beckman Coulter, Brea, CA) at room temperature in the dark. Red blood cells were then lysed with NH₄CL (0.83%) at 37°C for 5 minutes before undergoing centrifugation. Cells were then washed in buffered saline and re-suspended in 1% paraformaldehyde analyses on a FACSCalibur (Becton Dickinson Biosciences) and 200,000 events were collected for each tube. Linear MFI was determined as previously described.(23)

FLT3 mutational analysis

Mutational analysis of *FLT3*/ITD was performed using DNA from diagnostic bone marrow or peripheral blood specimens when bone marrow was not available. PCR amplification to determine the presence of an ITD and its corresponding allelic ratio was performed as described previously.(8) Mutational analysis of *FLT3*/ALM was conducted as described previously.(24)

mRNA expression by paired-end sequencing

Total RNA samples were analyzed using an Agilent Bioanalyzer RNA nanochip and 2ug arrayed into a 96-well plate. PolyA+ RNA was purified using the 96-well MultiMACS mRNA isolation kit (Miltenyi Biotec, Germany). The eluted PolyA+ RNA was ethanol precipitated and re-suspended in 10µL of DEPC treated water with 1:20 SupraseIN (Life Technologies, USA). First-stranded cDNA was synthesized from the purified polyA + RNA using the Superscript cDNA Synthesis kit (Life Technologies, USA). The second strand cDNA was synthesized following the Superscript cDNA Synthesis protocol by replacing the dTTP with dUTP in dNTP mix, allowing second strand to be digested using UNG (Uracil-N-Glycosylase, Life Technologies, USA) in the post-adaptor ligation reaction and thus achieving strand specificity. The cDNA was quantified using PicoGreen (Life Technologies, USA) and fragmented by Covaris E210 sonication. The paired-end sequencing library was prepared following the BC Cancer Agency Genome Sciences Centre strand-specific, plate-based and paired-end library construction protocol on a Biomek FX robot (Beckman-Coulter, USA). The 75 base PE libraries were sequenced on Illumina HiSeq2000 instruments. Analysis of mRNA expression was determined as previously described.(25)

Statistical analyses

Data for patients enrolled on COG AAML0531 were analyzed as of June 30, 2014. The Kaplan-Meier method was used to estimate overall survival (OS), event-free survival (EFS), and disease-free survival (DFS).(26) OS was defined as the time from study entry to death from any cause or date of last follow-up in surviving patients. EFS was defined as the time from study entry until induction failure, relapse or death. DFS was defined as the time from end of course 1 for patients in complete remission (CR) until relapse or death or date of last follow-up for those without an event. CR was defined as a bone marrow aspirate containing <5% blasts by morphology and without evidence of extramedullary disease. Relapse risk (RR) was defined as the time from end of course 1 for patients in CR to relapse where deaths without a relapse were considered competing events. Estimates of RR were obtained by the method of cumulative incidence accounting for competing events.(27) The significance of predictor variables was tested with the log-rank statistic for OS, EFS, and DFS and with Gray's statistic for RR. The Cox proportionality test was used to determine whether proportional hazards (PH) were violated. For analyses that violated the PH ($p < 0.1$), a standard Z-test that compared survival point estimates at 3 years was used. P-values determined by point estimate analysis are designated by as p^* . Patients without an event were censored at their date of last known contact. The significance of observed differences in proportions was tested using the Pearson's 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. Linear regression with determination of the coefficient of determination, R^2 , was used to assess correlation.

Results

Study population

Of the 485 patients with *de novo* AML enrolled on AAML0531 from September, 2008 to June, 2010, diagnostic specimens (bone marrow $n=301$, peripheral blood $n=65$,

unknown=1) available for 367 (75%) underwent CD135 expression analysis by MDF. The clinical and disease characteristics, as well as outcomes, of patients included in this study and those without adequate diagnostic specimens or who were enrolled prior to CD135 analysis did not differ by gender, age, race, ethnicity, white blood cell (WBC) count at diagnosis, mutation or cytogenetic makeup (Supplementary Table 1). However, there were a higher proportion of patients with standard risk disease as determined by cytogenetics and mutational status in the patients in whom CD135 expression was not evaluated. Conversely, there were a higher proportion of high-risk patients in our study cohort. Importantly, there were no significant differences in 3-year OS and DFS and RR between the patients included in the study and those for whom diagnostic specimens were not analyzed (Supplementary Table 1).

CD135 expression levels and disease characteristics

The quantitative surface expression of CD135 varied greatly among the study population, with the CD135 MFI ranging from 3-242. Patients were divided into quartiles on the basis of CD135 expression. Median MFIs for each quartile were as follows: Q1 (n=90): 7 (range 3–11); Q2 (n=85): 15 (range 12–17); Q3 (n=97): 22 (range 18–30); and Q4 (n=95): 43 (range 31–232) (Supplementary Figure 1). CD135 expression levels were then correlated with demographics and disease characteristics across the 4 quartiles to determine whether there were any significant interquartile differences. For purposes of analyses, patients in the highest CD135 expression quartile (Q4) were compared to the lower expression quartiles (Q1–3). We found that CD135 expression levels were not associated with sex, age, race, and ethnicity, diagnostic WBC count, hematocrit, platelets, or extramedullary involvement (Table 1). However, the median diagnostic bone marrow blast percentage was significantly higher in those patients with high vs. low CD135 expression (77.5% vs. 65%, $p=0.003$; Table 1).

Of the total 367 patients analyzed, 57 (16%) were positive for *FLT3*/ITD, 20 (5%) for *FLT3*/ALM and the remaining 289 (79%) were *FLT3*/WT, and 1 patient was dual-positive for an ITD and ALM. The median CD135 MFI was 17 (range 3–232) for the *FLT3*/WT patients, 22 (range 4–61) for patients with *FLT3*/ITD, and 20 (range 7–56) for the patients with *FLT3*/ALM. In comparing the *FLT3*/WT patients to the *FLT3*/ITD and *FLT3*/ALM patients, there was no significant difference between the medians of the 3 groups ($p=0.25$; Figure 1), the patient with the dual mutation was excluded from this analysis.

High CD135 expression was associated with the French-American-British (FAB) M5 subtype ($p<0.001$, Figure 2A) and inversely associated with the FAB M4 subtype ($p=0.04$, Figure 2A). Patients with the FAB M7 subtype had low CD135 expression ($p=0.004$, Figure 2A). We observed the known associations between the FAB M4 subtype and *inv(16)/t(16;16)* as well as the FAB M5 subtype and *KMT2A* rearrangements. Overall, 90% of *inv(16)/t(16;16)* cases were classified as M4 morphology and 63% of *KMT2A* rearrangements as M5 morphology.

We further evaluated the disease characteristics of patients according to CD135 MFI. Complete cytogenetic and molecular data were available for 358 of 367 (98%) of patients. Patients with normal karyotypes were evenly distributed across the expression quartiles

($p=0.48$). There was a significant correlation with high CD135 expression and *KMT2A* rearrangements ($p=0.009$), which was driven by $t(9;11)(p22;q23)$ ($p<0.001$; Figure 2B). Overall, 41% and 56% of *KMT2A* rearrangements and $t(9;11)$ respectively, occurred in Q4. CD135 expression was inversely correlated with $inv(16)/t(16;16)$ with a prevalence of 15% in Q1–Q3 compared with 2% in the Q4 ($p<0.001$, Figure 2B). There was a trend toward a lower prevalence of $t(8;21)$ in the high expressers ($p=0.06$). There were no differences in CD135 expression among patients with the high-risk cytogenetic features monosomy 5/7 or deletion 5q, although the overall numbers of patients with these features were low (Figure 2B). There were no differences in CD135 expression among patients with *NPM1*, or *WT1* mutations; however, there was a trend towards a higher prevalence of *CEBPA* mutations in patients with low CD135 expression (Table 2). We did observe a significantly higher number of patients classified as low-risk based on cytogenetic and mutational analysis in Q1–3 as compared to Q4 ($p<0.001$), as well as a higher number of patients classified as intermediate risk in Q4 vs. Q1–3 ($p<0.001$; Table 2).

CD135 expression levels and clinical outcome

Among the cohort, there were no differences in CR rates between those with low or high CD135 expression (73% vs. 75% respectively; $p=0.74$). Patients with low and high CD135 expression had similar 3-year OS ($67 \pm 6\%$ vs. $70 \pm 9\%$ respectively; $p^*=0.59$; Figure 3A), EFS ($51 \pm 6\%$ vs. $48 \pm 10\%$ respectively; $p=0.74$), and RR (36 ± 7 vs. $42 \pm 12\%$ respectively; $p=0.33$; Figure 3B). In a multivariate analysis adjusting for risk group, we observed similar results with no differences in the low vs. high expressers (Supplementary Table 2).

We analyzed the effect of CD135 expression levels on outcomes for the *FLT3*/ITD-negative cohort ($n=309$). Patients with low and high CD135 had similar rates in CR (76% vs. 77% respectively; $p=0.80$), OS ($68 \pm 6\%$ vs. $71 \pm 11\%$, respectively; $p^*=0.70$), and EFS ($52 \pm 7\%$ vs. $55 \pm 12\%$, respectively; $p=0.86$) (Kaplan-Meier curves not shown).

We further evaluated the prognostic impact of CD135 expression levels in *FLT3*/ITD-positive patients ($n=56$). There were no significant differences between low and high expressers in 3-year OS and EFS from study entry with $60\% \pm 16\%$ vs. $68\% \pm 22\%$ ($p=0.77$) and $39\% \pm 16\%$ vs. $25\% \pm 19\%$ ($p^*=0.25$), respectively. Among patients who achieved CR ($n=32$), patients with low CD135 expression as compared to those with high expression had a RR of $37\% \pm 25\%$ vs. $62\% \pm 29\%$, respectively ($p=0.20$) (Kaplan-Meier curves not shown).

Given the strong association of high *FLT3* expression with *KMT2A* rearrangements, we evaluated the effect of CD135 MFI levels on the outcome in patients with *KMT2A* rearrangements ($n=73$). There were no significant differences between patients with low and high CD135 expression in 3-year OS ($55\% \pm 16\%$ vs. $71\% \pm 15\%$, respectively; $p=0.13$; Figure 4A), EFS ($33\% \pm 15\%$ vs. $49\% \pm 17\%$, respectively; $p=0.16$), and RR ($62\% \pm 19\%$ vs. $50\% \pm 19\%$, respectively; $p=0.37$; Figure 4B).

CD135 expression and FLT3 transcript expression

We analyzed the association between CD135 MFI and *FLT3* receptor transcript levels as determined by mRNA expression by paired-end sequencing. Of the 367 specimens with known CD135 MFI, 211 (57%) of specimens had mRNA transcript expression available. Similar to the variability seen in the CD135 MFI, *FLT3* transcript expression also varied greatly among the samples (range 0.0288–207.752). There was no correlation between CD135 MFI and mRNA transcript expression ($R^2=0.28$; Supplementary Figure 2A). Correlation of *FLT3* mRNA transcript expression with clinical and biologic characteristics revealed similar associations as with *FLT3* cell surface expression (Supplementary Table 3). However, *FLT3* transcript expression was significantly higher in patients with higher diagnostic WBC ($p=0.03$) and higher bone marrow blast % ($p<0.001$; Supplementary Table 3). We evaluated the effect of diagnostic WBC count on *FLT3* mRNA transcript expression and detected no correlation with transcript expression and WBC ($R^2=0.05$; Supplementary Figure 2B). Outcomes were analyzed according to *FLT3* transcript expression and in contrast to CD135 expression we found that among *FLT3*/ITD negative patients ($n=171$), low transcript expression was associated with superior 3 year EFS of $63 \pm 9\%$ vs. $34 \pm 15\%$ ($p=0.001$), although this did not lead to a difference in OS ($77 \pm 7\%$ vs. $66 \pm 15\%$ respectively, $p=0.16$; Supplementary Table 4)

Discussion

In this large prospective study, we found that CD135 expression varied greatly among the cohort and the level of CD135 MFI did not have an impact on prognosis. Further, there were no significant differences in CD135 MFI in patients with *FLT3*/WT compared to those with *FLT3*/ITD or *FLT3*/ALM. This is in line with what previous groups using qRT-PCR to examine *FLT3* transcript level have found in that significant differences in clinical outcomes were not seen among expression groups.(28) Although Ozeki *et al.* reported a higher *FLT3* transcript expression in patients with *FLT3*/ITD and *FLT3*/ALM compared to those with *FLT3*/WT, this study was performed in an adult cohort that was approximately half this size of our cohort.(29) Our study used flow cytometry to examine the CD135 surface expression on the blast population alone. The *FLT3* receptor is present on the surface of normal hematopoietic progenitors and may lead to increased RNA transcript levels that are not truly representative of only the leukemic blast population.

The association between *KMT2A* rearrangements and *FLT3* expression was first described in infant ALL, and follow-up studies have demonstrated that among this group of patients, patients with high *FLT3* transcript expression have a significantly worse prognosis.(30) In our study, high CD135 expression was strongly associated with *KMT2A* rearrangements. Our findings support what Kuchenbauer *et al.* and Ozeki *et al.* reported in adult AML, with *KMT2A* rearrangements being associated with higher *FLT3* expression, although this was measured by RNA transcript level(28,29). We found a strong association between CD135 expression and *KMT2A* rearrangements, with almost half of all *KMT2A* rearrangements occurring in patients with high expression, and this was primarily driven by t(9;11). There was no prognostic impact of CD135 expression in patients with *KMT2A* rearrangements, this in contrast to infant ALL. We observed a significantly higher number of patients with

FAB M5 morphology among those with high CD135 MFI as compared to low expression, and this finding is consistent with previous studies showing a higher incidence of *KMT2A* rearrangements in patients classified as FAB M5 subtype.(31,32) Thus it is likely that association we identified between high CD135 MFI and FAB M5 morphology is in fact driven by *KMT2A* rearrangements.

Patients with *KMT2A* rearrangements were classified as intermediate risk on AAML0531 and this is likely responsible in part for the strong association we observed between intermediate risk cytogenetics and high CD135 expression. There is increasing evidence that certain *KMT2A* translocation partners can predict clinical outcomes in pediatric AML.(33–36) In infant ALL, the most commonly observed *KMT2A* rearrangement, t(4;11), has been shown to cooperate with FLT3 activation, resulting in impaired differentiation of hematopoietic stem cells.(37) Our analysis supports the strong association between high FLT3 expression and *KMT2A* rearrangements and suggests their possible cooperation in leukemogenesis. The significance of the association between *KMT2A* rearrangements and FLT3 expression is of undetermined biologic and clinical significance in AML, although similar to ALL there are likely additional multiple cooperating events, including at the epigenetic level.(38) In contrast to infant ALL, we did not find any prognostic significance of CD135 expression in patients with AML and *KMT2A* rearrangements.

We found a strong inverse correlation with inv(16)/t(16;16) and CD135 expression. The significance of the association between low CD135 expression and inv(16)/t(16;16) has not been determined, but it is interesting to note that this correlation was not seen across all core binding factor AML, as there was not as strong an association with CD135 expression and t(8;21). The inverse correlation observed in our study between FAB M4 morphology and CD135 expression was likely the byproduct of the strong association with FAB M4 and inv(16)/t(16;16) (90% of patients with inv(16) in our cohort were classified as FAB M4). This finding also contributes to predominance of the low-risk cytogenetic group in patients with low CD135 expression.

The level of FLT3 cell surface expression had no impact on clinical outcomes of patients in our cohort. This finding is contrary to that of Ozeki *et al.*, who reported that among patients with *FLT3*/WT, those with high FLT3 expression experienced inferior OS compared to patients with low expression, although this analysis was based on a smaller adult cohort and expression was determined by RNA transcript level.(29) Kang *et al.* reported high FLT3 transcript level to be a poor prognostic factor in pediatric patients, but this study included only 5 patients in the highest FLT3 transcript expression level group.(39) Our results with CD135 MFI are in agreement with those from Kuchenbauer *et al.*, which showed that FLT3 transcript levels do not significantly affect OS or EFS, however they did observe a trend towards lower survival in the subset of *FLT3*/WT patients with high *FLT3* expression, which we did not observe with CD135 expression as determined by MFI.(28) It is important to note that in a smaller subset of patients with mRNA *FLT3* transcript expression we did observe among *FLT3*/ITD-negative patients a significantly lower EFS and corresponding increased RR, although there was no difference in OS. These findings align with Kuchenbauer *et al.*, although are in contrast to some of the outcomes analyzed according to FLT3 receptor surface expression by MDF.

Although our results with CD135 expression as determined by MDF are in contrast to those from some previous studies, our cohort is much larger than those in previous pediatric and adult AML studies. It is interesting to note that FLT3 expression evaluated by either method resulted in similar biologic and disease characteristics, however there were differences in the correlation with outcomes with high *FLT3* mRNA expression as demonstrating some prognostic significance with improved EFS and a lower RR, but no difference in OS. High transcript expression was associated with significantly higher diagnostic WBC and blast %, therefore it is likely that the transcript measurements reflect the overall FLT3 expression in the entire sample, and would also include normal hematopoietic cells which express the *FLT3* transcript. Significantly high WBCs could result in somewhat artificially elevated transcript levels as it is measuring all the *FLT3* transcript in the sample, and not necessarily the level on the leukemic cells. In our opinion, surface expression of FLT3, specifically as measured on the blast population, provides a more accurate representation of FLT3 among the leukemic population and its potential biologic role in AML. Kuchenbauer *et al.* reported a correlation between FLT3 expression measured by flow cytometry and transcript level determined by qRT-PCR, however we found no correlation between flow cytometry and mRNA transcript.

Substantial biologic differences exist between pediatric and adult AML, such as the increased prevalence of *KMT2A* rearrangements in pediatric AML, and underscore the importance of evaluating the impact of CD135 expression in a pediatric cohort. It is possible that CD135 expression may have prognostic value in conjunction with other biomarkers in subsets of patients, including additional cell surface markers, as Sharawat et al found when CD135 was evaluated in conjunction with CD117 expression.(40)

In infant ALL, *in vitro* assays have shown that specimens with high *FLT3*/WT expression are more sensitive to FLT3 inhibition.(41) To date, FLT3 inhibition studies in AML have mainly evaluated response to FLT3 inhibition in *FLT3*/ITD vs *FLT3*/WT patients and early phase studies detected a higher rate of response in *FLT3*/ITD patients, however the level of FLT3 expression was not assessed in the WT patients.(42,43) *FLT3*/ITD samples were noted to be more sensitive to inhibition of phosphorylation as well as cytotoxic effects of FLT3 inhibitors when evaluated by *in vitro* cytotoxicity assays as compared to *FLT3*/WT, however level of WT FLT3 expression was not assessed.(42,43) The use of FLT3 inhibitors alone and in combination with conventional chemotherapy can be beneficial in inducing remissions in AML patients with *FLT3*/ITD mutations and has recently been shown to improve survival. (44–48) As FLT3 is expressed on the cell surface, it could also serve as a target for immunotherapeutic strategies. Although the potential use monoclonal antibodies have been investigated, newer immunotherapeutics including chimeric antigen T cell receptors and bi-specific T cell engagers may offer additional therapeutic benefit.(49) Current trials will be helpful in elucidating the best ways in which to use FLT3-targeted therapies, especially given the presence of CD135 on normal hematopoietic cells which may result in significant myelotoxicity.

Our study shows that there is no prognostic significance of cell-surface FLT3 receptor expression in pediatric AML. Our observation of increased CD135 expression among patients with *KMT2A* rearrangements confirms previous findings in infant ALL and AML,

and suggests possible cooperation in leukemogenesis. A better understanding of cooperative mutations, both at the genetic and epigenetic level, will provide further insight into optimal ways to direct therapy at dysregulated pathways and thus improve outcomes for patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance

In this study, we assessed the prognostic significance of FLT3 cell surface expression (CD135) in pediatric patients with acute myeloid leukemia (AML) and demonstrated that FLT3 expression levels do not have a prognostic impact in this group of patients. Our report is the largest study to date to examine the effect of FLT3 expression in pediatric AML. We observed no prognostic impact in this pediatric cohort, although previous reports have suggested that, similar to ALL, high expression is a poor prognostic feature in AML. We found that FLT3 expression did not differ by FLT3 mutational status. We did identify a strong correlation between elevated expression and *KMT2A* rearrangements, which has been previously reported in infant ALL, thereby confirming the biologic cooperation between these events in leukemogenesis. Our study suggests that FLT3 expression should not be used for risk stratification of pediatric patients with AML.

Figure 1.
Distribution of CD135 expression according to *FLT3* mutation status.

Figure 2. FAB classification and CD135 expression (2A); CD135 expression and association with cytogenetic features (2B). * significant p-value

Figure 3. Correlation of clinical outcome with CD135 expression quartile for the entire cohort. (A) OS for Q1–3 versus Q4. (B) RR for Q1–3 versus Q4. p^* indicates 3-year point estimate analysis.

Figure 4. Correlation of clinical outcome with CD135 expression quartile in *KMT2A* rearranged patients. (A) OS for Q1–3 vs. Q4. (B) RR for Q1–3 versus Q4.

Table 1

Disease characteristics of patients according to CD135 expression quartile.

	Q1 (n=90)	Q2 (n=85)	Q3 (n= 97)	Q4 (n=95)	Q1-3 (n=272)	Q1-3 vs. Q4 p-value
Gender, n (%)						
Male	43 (48%)	37 (44%)	51 (53%)	49 (52%)	131 (48%)	0.57
Female	47 (52%)	48 (56%)	46 (47%)	46 (48%)	141 (52%)	
Median age, yrs (range)	7.8 (0.06–21.7)	10.2 (0.09–20.7)	10.4 (0.6–29.8)	10.6(0.01–23.5)	9.8 (0.06–29.8)	0.93
Race, n (%)						
American Indian or Alaskan Native	2 (2%)	0 (0%)	0 (0%)	0 (0%)	2 (1%)	1
Asian	4 (5%)	6 (8%)	1 (1%)	3 (3%)	11 (4%)	1
Hawaiian/Pacific Islander	0 (0%)	0 (0%)	1 (1%)	0 (0%)	1 (0%)	1
Black	7 (8%)	10 (13%)	22 (25%)	9 (10%)	39 (16%)	0.21
White	70 (84%)	59 (79%)	65 (73%)	75 (86%)	194 (79%)	0.12
Unknown	7	10	8	8	25	
Ethnicity						
Hispanic or Latino	23 (27%)	14 (17%)	17 (18%)	18 (20%)	54 (20%)	0.90
Not Hispanic or Latino	63 (73%)	70 (83%)	78 (82%)	73 (80%)	211 (80%)	
Unknown	3	2	4	4	9	
Hepatomegaly	25 (29%)	18 (22%)	23 (25%)	35 (38%)	66 (25%)	0.02
Splenomegaly	26 (30%)	17 (20%)	24 (25%)	31 (34%)	67 (25%)	0.10
CNS involvement	7 (8%)	3 (4%)	6 (6%)	5 (5%)	16 (6%)	0.82
Chloroma	12 (13%)	9 (11%)	8 (8%)	16 (17%)	29 (11%)	0.11
WBC $\times 10^3/\mu\text{L}$ median (range)	30.9 (1.1–473.1)	24.2 (0.7–340.7)	25.6 (0.7–446)	32.1 (0.2–519)	27.2 (0.7–473.1)	0.83
BM blasts % (range)	62 (0.4–96)	68 (9–96)	71 (0–100)	77.5 (29–100)	65 (0–100)	0.003
Median platelet count, 1000/ μL (range)	40 (5–1,177)	52(2–357)	48 (5–245)	47 (6–389)	48 (2–1,117)	0.71
Median hemoglobin, g/dL (range)	8.2 (2.6–14.4)	7.8 (2.4–13.2)	7.5 (3.1–13.2)	8.2 (3.1–14.3)	7.9 (2.4–14.4)	0.92

Table 2

Mutational and risk factor classification based on cytogenetic and mutation status by CD135 expression quartile.

	Q1	Q2	Q3	Q4	Q1-3	Q1-3 vs Q4
	n (%)	n (%)	n (%)	n (%)	n (%)	p-value
Mutations						
<i>FLT3/ITD</i>	10 (11%)	11 (13%)	17 (18%)	20 (21%)	38 (14%)	0.10
<i>FLT3/ALM</i>	5 (6%)	5 (6%)	6 (6%)	5 (5%)	16 (6%)	0.82
<i>CEBPA</i>	10 (11%)	7 (8%)	3 (3%)	2 (2%)	20 (7%)	0.06
<i>NPM1</i>	6 (7%)	6 (7%)	7 (7%)	7 (7%)	19 (7%)	0.91
<i>WT1</i>	5 (6%)	4 (5%)	11 (11%)	6 (6%)	19 (7%)	0.73
Risk Groups						
Low	33 (37%)	37 (44%)	46 (48%)	17 (18%)	116 (43%)	<0.001
Intermediate	43 (48%)	34 (46)	29 (38)	57 (76)	108(40%)	< 0.001
High	13 (15%)	13 (15%)	18 (19%)	14 (15%)	44 (16%)	0.73
Unknown	1	1	2	1		NA