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**Brief report**

# Presence of the *P2RY8-CRLF2* rearrangement is associated with a poor prognosis in non–high-risk precursor B-cell acute lymphoblastic leukemia in children treated according to the ALL-BFM 2000 protocol

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**High-level expression of the cytokine receptor-like factor 2 gene, *CRLF2*, in precursor B-cell acute lymphoblastic leukemia (pB-ALL) was shown to be caused by a translocation involving the *IGH@* locus or a deletion juxtaposing *CRLF2* with the *P2RY8* promoter. To assess its possible prognostic value, *CRLF2* expression was analyzed in 555 childhood pB-ALL patients treated according to the Acute Lymphoblastic Leukemia Berlin-Frankfurt-Münster 2000 (ALL-BFM 2000) protocol. Besides *CRLF2* rearrangements, high-level *CRLF2* expression was seen in cases with supernumerary copies of the *CRLF2* locus. On the basis of the detection of *CRLF2* rearrangements, a *CRLF2* high-expression group (n = 49) was defined. This group had a 6-year relapse incidence of 31% plus or minus 8% compared with 11% plus or minus 1% in the *CRLF2* low-expression group (*P* = .006). This difference was mainly attributable to an extremely high incidence of relapse (71% ± 19%) in non–high-risk patients with *P2RY8-CRLF2* rearrangement. The assessment of *CRLF2* aberrations may therefore serve as new stratification tool in Berlin-Frankfurt-Münster–based protocols by identifying additional high-risk patients who may benefit from an intensified and/or targeted treatment. (*Blood*. 2010;115(26):5393-5397)**

## Introduction

Despite major improvements, for approximately 20% of children with acute lymphoblastic leukemia (ALL) therapy still fails and surviving patients often experience significant toxicities.[1-3] Therefore, an improved assessment of a patient's risk of relapse is necessary to adapt treatment accordingly and enhance the chance of cure.

In the international Berlin-Frankfurt-Münster (BFM) study group trial ALL-BFM 2000, risk-adapted treatment stratification was mainly determined by the measurement of the in vivo treatment response.[4-8] Response was assessed cytomorphologically (blast reduction in peripheral blood after 7 days of treatment [prednisone response, PR], blast clearance from bone marrow [BM] after induction therapy at week 5 [response on treatment day 33]), and molecularly by the measurement of minimal residual disease (MRD) at week 5 and after induction consolidation at week 12. Besides positivity for *BCR-ABL* or *MLL-AF4* rearrangements, patients were stratified into the high-risk group (HR) by a poor PR, nonresponse by treatment day 33 ($> 5\%$ BM blasts), and a high MRD ($> 10^{-3}$) load after induction consolidation at week 12. Whereas the relative number of relapses is greatest in the HR group, more than one half of relapses still occur in patients not classified as HR (ie, intermediate risk, standard risk).[4] If identified early, these patients may benefit from an intensified HR treatment (ie, by application of a more intensive conventional chemotherapy, by addition of stem cell transplantation, or, ideally, by addition of a specific targeted treatment). However, current strategies fail to identify these patients and indicate the need for new prognostic markers.

Recently, we and other groups identified a novel subgroup of childhood precursor B-cell ALL (pB-ALL) characterized by high-level expression of the cytokine receptor-like factor 2 gene (*CRLF2*) caused by a translocation involving the immunoglobulin heavy chain locus (*IGH@*) locus on chromosome 14q32.3 and/or an interstitial deletion centromeric to *CRLF2* juxtaposing *CRLF2* with the *P2RY8* promoter.[9-11] The incidence of these abnormalities in pB-ALL was estimated at approximately 7%.[9,10] We hypothesized that this subgroup of pB-ALL has distinct properties and that a high *CRLF2* expression might be associated with treatment outcome. To test this hypothesis, we analyzed *CRLF2* gene expression in an unselected population of 555 pB-ALL patients treated according to the ALL-BFM 2000 protocol.

## Methods

### Patients

In accordance with institutional review board regulations, clinical samples were obtained from children with ALL before treatment. The study was

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4approved by the institutional review board of the Hannover Medical School and informed consent was obtained from patients and/or their legal guardians in accordance with the Declaration of Helsinki. Diagnostics, risk group assignment, and treatment were performed according to the ALL-BFM 2000 protocol.[5,12] Between July 1999 and December 2004, 1933 patients with pB-ALL (aged ≤ 18 years) were enrolled into the ALL-BFM 2000 trial. In the present study, patients were included when spare diagnostic specimens containing more than 80% blasts were available from the German ALL-BFM biological specimen bank.

### Real-time quantitative PCR

RNA isolation and real-time quantitative polymerase chain reaction (PCR) were performed as previously described.[13] The *succinate dehydrogenase complex subunit A* (*SDHA*) gene was chosen for normalization. QuantiTect Primer Assays were used (*CRLF2* [QT00210987], *SDHA* [QT00059486]; QIAGEN). Each sample was tested in duplicate. The expression ratio was calculated as $2^n$, where $n$ was the $C_T$ value difference normalized by the $C_T$ difference of a calibrator sample.

### Statistical analysis

Event-free survival (EFS) was calculated from date of diagnosis to last follow-up or to the first event (no complete remission [CR] as event on day 0, relapse, secondary malignancy, or death of any cause). Rates were calculated according to Kaplan-Meier and compared by log-rank test.[14,15] Cumulative incidence of relapse functions were constructed by the method of Kalbfleish and Prentice and compared with the Gray test.[16,17] Cox regression analysis was used for multivariate analysis.[18] Proportional differences between patient groups were analyzed by $\chi^2$ or Fisher exact tests. Depending on the distribution of variables, correlation analyses were performed by computing contingency tables, Pearson, or Spearman correlation coefficients.

### Genetic analysis

Fluorescence in situ hybridization (FISH) was performed on cells left from cytogenetic analysis according to routine methods. For detection of breakpoints in the *IGH@* locus, the LSI IGH BAP probe was applied (Abbott/Vysis). Detection of breakpoints affecting the *CRLF2* locus for a microdeletion upstream to *CRLF2* was performed as previously described.[9] In addition, reverse transcription PCR to detect the *CRLF2-P2RY8* fusion and sequencing of *CRLF2* to detect the *CRLF2F*232C mutation, *JAK1* (exons 13 and 14), and *JAK2* (exons 16, 20, 21) were performed as previously described.[11,19]

## Results and discussion

*CRLF2* gene expression was measured in diagnostic specimens of 555 patients (Figure 1A). Comparing characteristics of samples included in the present study and of those not analyzed, more patients older than 10 years of age (25.2% vs 20.8%, $P = .03$), with a greater white blood cell (WBC) count at diagnosis (>10 000/μL: 66.8% vs 39.9%, $P < .001$), and with an *MLL-AF4* rearrangement (0.2% vs 0.9%, $P = .05$) were included. No significant differences were observed with respect to sex, presence of *TEL-AML1* or *BCR-ABL* rearrangements, PR, MRD, and final risk stratification (supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

To define the best cutoff to distinguish a *CRLF2* high- from a *CRLF2* low-expression group, samples were screened for known *CRLF2* involving genomic aberrations beginning with those having the greatest *CRLF2* expression. The cutoff was set between positivity and negativity for *P2RY8-CRLF2* and *IGH@-CRLF2* rearrangements (Figure 1A). Screening for a *P2RY8-CRLF2* rearrangement was performed in 70 samples; additional information on the *IGH@-CRLF2* rearrangement by FISH was available in 32 of 49 (65%) samples negative for the *P2RY8-CRLF2* rearrangement. *P2RY8-CRLF2* rearrangements were detected in 21 and *IGH@-CRLF2* rearrangements in 4 samples. Remarkably, 24 of the 28 samples showed supernumerary copies of the *CRLF2* locus in the absence of a *CRLF2*-fusion, with 16 of them also having gains of the *IGH@* locus. This finding could be explained at least in part by hyperdiploidy (as determined by a DNA index > 1.16 or by cytogenetics), which was observed in 9 of 12 cases with information on either DNA index (n = 10) or cytogenetics (n = 2) available. In none of the patients was a hereditary syndrome with constitutional gain of either chromosome X or chromosome Y described. In 17 of 70 samples a *P2RY8-CRLF2* rearrangement could be excluded by reverse transcription PCR, but no cells were available for additional FISH analyses.

On the basis of the aforementioned results, 49 of 555 samples (9%) were included in the *CRLF2* high-expression group (Figure 1A; supplemental Table 2): 21 cases characterized by the *P2RY8-CRLF2* fusion, 4 cases by an *IGH@-CRLF2* rearrangement, and 9 samples by additional *CRLF2* copies. Two samples (both positive for *BCR-ABL*) did not show any *CRLF2*-involving abnormality, and in 13 samples an *IGH@-CRLF2* rearrangement could not be excluded because no cells were available for additional FISH analyses. Interestingly, none of the 25 samples with the greatest *CRLF2* expression was characterized by additional copies of the *CRLF2* locus (supplemental Table 2). *JAK2* mutations were observed in 5 *P2RY8-CRLF2*–positive cases and 1 case with an *IGH@-CRLF2* rearrangement; the *CRLF2*F232C mutation was detected in 2 cases with a *P2RY8-CRLF2* rearrangement. Neither *CRLF2* nor *JAK* mutations were found in cases with a gain of the *CRLF2* locus (supplemental Table 2).

When we compared the *CRLF2* high- and low-expression groups, we observed no significant differences for sex, age and WBC at diagnosis, NCI risk groups, or the different measures of treatment response (Table 1). As expected, the number of Down syndrome–ALL (DS-ALL) patients was greater in the *CRLF2* high- compared with the *CRLF2* low-expression group (14.2% vs 1.4%, $P < .001$). There were no cases with *TEL-AML1* or *MLL-AF4* rearrangement in the *CRLF2* high-expression group in contrast to 146 (29.7%) and 5 (1.0%) cases, respectively, in the *CRLF2* low-expression group. Two *BCR-ABL*–positive cases showed a high *CRLF2* expression but were not characterized by any of the known *CRLF2* involving genomic aberrations. Within the *CRLF2* high-expression group, patients with a *P2RY8-CRLF2* rearrangement had a greater WBC count at diagnosis (> 50 000/μL, 48% vs 13%, $P = .02$) and a greater prevalence of NCI-HR status (62% vs 21%, $P = .01$) compared with patients with additional copies of the *CRLF2* gene.

### Association of *CRLF2* expression and treatment outcome

First, we analyzed the association of *CRLF2* expression and treatment outcome in the entire set of patients. Patients with a high *CRLF2* expression had a worse 6-year EFS probability compared with patients with a low *CRLF2* expression (61% ± 8% vs 83% ± 2%, $P = .003$). This effect was mainly related to a greater cumulative relapse incidence (CRI; 31% ± 8% vs 11% ± 1%, $P = .006$; Figure 1B). No differences between *CRLF2* high- and low-expression cases were seen with respect to time to relapse (< 30 months after initial diagnosis, 41.7% vs 47.3%; > 30 months, 58.3% vs 52.7%, $P = .76$) or site of relapse (isolated BM relapses: 75.0% vs 63.6%, central nervous system relapses, 25.0% vs 18.2%; combined relapses, 0% vs 18.2%; $P = .28$).

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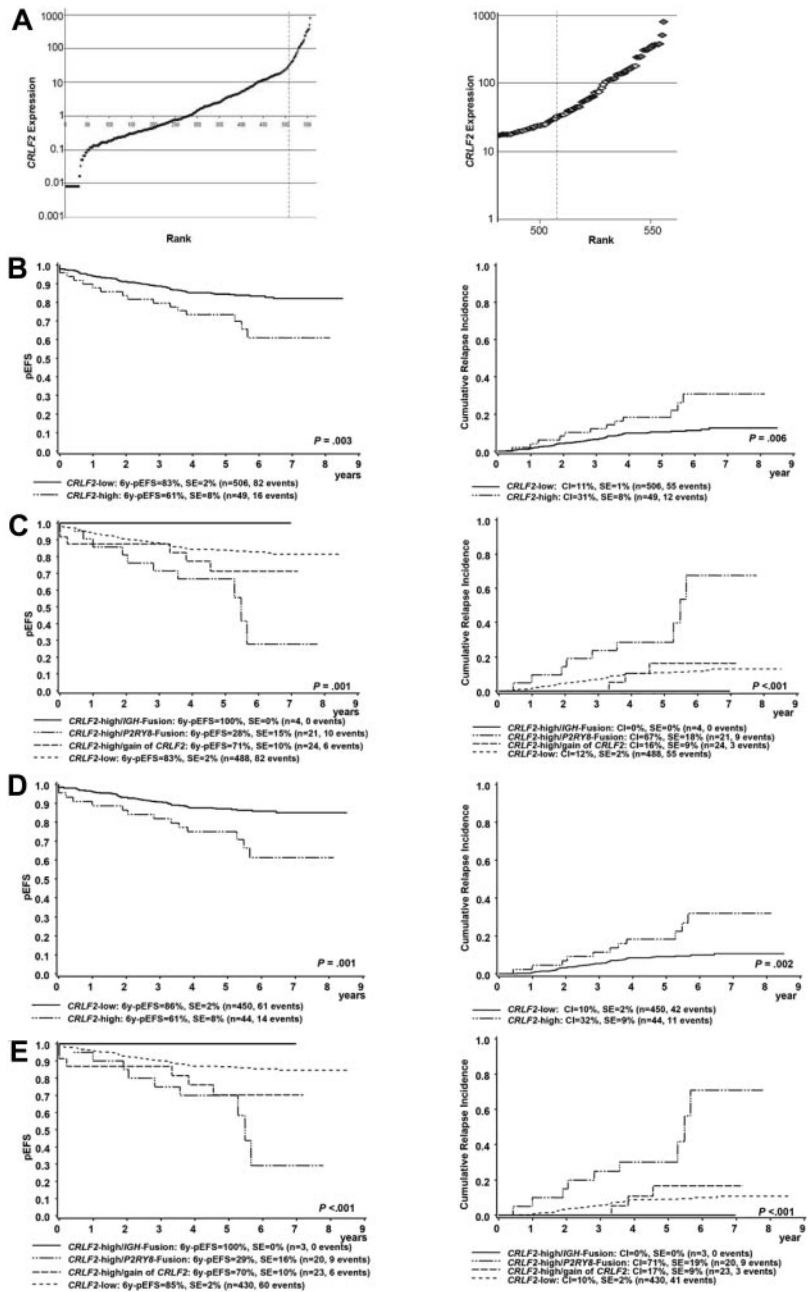


**Figure 1. CRLF2 gene expression, underlying genomic alterations, and their association with treatment outcome.** (A) Expression of *CRLF2* in 555 patients with precursor B-cell ALL (left) is shown relative to the median expression of all samples. The dashed line indicates the cutoff between a *CRLF2* high- and *CRLF2* low-expression group. (Right) Zoom on the 70 cases with greatest *CRLF2* expression analyzed for underlying genomic *CRLF2* aberrations. Cases with *P2RY8-CRLF2* or *IGH-CRLF2* rearrangement (red), additional copies of the *CRLF2* gene locus (yellow), negative for the *P2RY8-CRLF2* rearrangement but without FISH analysis (blue), and without *CRLF2* aberrations (green) are shown. (B) Kaplan-Meier estimate of EFS (left) and CRI (right) at 6 years according to *CRLF2* expression in all patients analyzed. (C) Kaplan-Meier estimate of EFS (left) and CRI (right) at 6 years according to *CRLF2* expression and detected underlying genomic *CRLF2* aberrations in all patients analyzed. For EFS, the *P* value comparing the *CRLF2* high-/*P2RY8* fusion-positive and the *CRLF2* low-expression group is shown. (D) Kaplan-Meier estimate of EFS (left) and CRI (right) at 6 years according to *CRLF2* expression in non-HR patients only. (E) Kaplan-Meier estimate of EFS (left) and CRI (right) at 6 years according to *CRLF2* expression and detected underlying genomic *CRLF2* aberrations in non-HR patients only. For EFS, the *P* value comparing the *CRLF2* high/*P2RY8* fusion-positive and the *CRLF2* low-expression group is shown.

Next, we were interested whether there were differences detectable in clinical outcome between those *CRLF2* high-expression cases with presence of a *CRLF2* rearrangement and those with additional copies of the *CRLF2* locus. Whereas all 4 patients with an *IGH@-CRLF2* rearrangement remained in long-term CR, the 6-year EFS in patients with a *P2RY8-CRLF2* rearrangement was 28% plus or minus 15% only, compared with 71% plus or minus 10% in cases with *CRLF2* high-expression and additional copies of the *CRLF2* locus and 83% plus or minus 2% in the *CRLF2* low-expression group ($P = .001$). This association was again mainly attributable to a different CRI (67% ± 18% vs 16% ± 09% vs 12% ± 2%, $P < .001$; Figure 1C). Notably, all 13 *CRLF2* high-expression patients without *P2RY8-CRLF2* rearrangement and unavailable FISH data are in long-term CR. The exclusion of cases with *MLL-AF4*, *BCR-ABL*, or *TEL-AML1* rearrangements and/or DS-ALL did not significantly change these results (supplemental Figures 1-3). Moreover, although the number of DS-ALL with high *CRLF2* expression is limited (n = 7) and does not allow any statistical analysis, those with a *P2RY8-CRLF2* rearrangement also appear to have a worse prognosis compared with those without it and low *CRLF2* expression (3 of 6 relapses vs 0 of 7 relapses).

Because *CRLF2* expression was associated with outcome but not with measures of treatment response, we next tested for potential effect modification by stratifying the analysis by risk groups (non-HR vs HR). We observed that the prognostic effect of a high *CRLF2* expression was mainly attributable to the non-HR group. Non-HR patients with a high *CRLF2* expression had an EFS probability of 61% plus or minus 9% compared with 86% plus or minus 2% for those in the low-expression group ($P = .001$). This effect was again mainly related to a greater CRI (32% ± 9% vs 10% ± 2%, $P = .002$; Figure 1D). By analyzing clinical outcome in non-HR *CRLF2* high-expression cases with presence of *CRLF2* rearrangements and those with supernumerary copies of the *CRLF2*

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**Table 1. Patient characteristics and response to treatment according to *CRLF2* expression in 555 patients with childhood precursor B-cell acute lymphoblastic leukemia**

| | *CRLF2* low, n (%) | *CRLF2* high, n (%) | *P** |
|---|---|---|---|
| Number of patients | 506 (100) | 49 (100) | |
| **Down syndrome** | | | < .001 |
| Yes | 7 (1.4) | 7 (14.2) | |
| No | 499 (98.6) | 42 (85.8) | |
| **Sex** | | | .65 |
| Male | 267 (52.8) | 24 (49.0) | |
| Female | 239 (47.2) | 25 (51.0) | |
| **Age at diagnosis, y** | | | .30 |
| 1 to less than 10 | 375 (74.1) | 40 (81.6) | |
| 10 or older | 131 (25.9) | 9 (18.4) | |
| **Presenting WBC count, cells/μL** | | | .23 |
| Less than 10 000 | 168 (33.2) | 16 (32.7) | |
| 10 000 to less than 50 000 | 223 (44.1) | 19 (38.8) | |
| 50 000 to less than 100 000 | 70 (13.8) | 5 (10.2) | |
| More than 100 000 | 45 (8.9) | 9 (18.4) | |
| ***BCR/ABL*** | | | .19 |
| Positive | 7 (1.4) | 2 (4.1) | |
| Negative | 499 (98.6) | 47 (95.9) | |
| ***MLL/AF4*** | | | > .999 |
| Positive | 5 (1.0) | 0 (0.0) | |
| Negative | 501 (99.0) | 49 (100.0) | |
| ***TEL/AML1*** | | | < .001 |
| Positive | 146 (28.9) | 0 (0.0) | |
| Negative | 345 (68.2) | 45 (90.5) | |
| Unknown | 15 (2.9) | 4 (9.5) | |
| **NCI risk group** | | | .76 |
| Standard | 295 (58.3) | 30 (61.2) | |
| High | 211 (41.7) | 19 (38.8) | |
| **Prednisone response†** | | | > .999 |
| Good | 466 (92.1) | 46 (93.9) | |
| Poor | 38 (7.5) | 3 (6.1) | |
| No result | 2 (0.4) | 0 (0.0) | |
| **MRD‡** | | | .42 |
| Less than $10^{-3}$ | 462 (91.3) | 42 (85.7) | |
| More than $10^{-3}$ | 19 (3.8) | 3 (6.1) | |
| No result | 25 (4.9) | 4 (8.2) | |

MRD indicates minimal residual disease; NCI, National Cancer Institute; and WBC, white blood cell.

*Fisher exact test comparing the *CRLF*2 high and *CRLF2* low groups.

†Good: < 1000 leukemic blood blasts/μL on treatment day 8; poor: > 1000/μL.

‡After induction consolidation at week 12, MRD > $10^{-3}$ qualifies for the high-risk group.

locus separately, we observed an extremely poor outcome in *P2RY8-CRLF2*–positive cases (EFS 29% ± 16%, CRI 71% ± 19%) compared with cases with more than 2 copies of the *CRLF2* locus (EFS 70% ± 10%, CRI 17% ± 9%) or those with low *CRLF2* expression (EFS 85% ± 2%, CRI 10% ± 2%, $P < .001$ for EFS and CRI, respectively; Figure 1E). Remarkably, all 8 *P2RY8-CRLF2*–positive relapses with information on MRD were negative for MRD after induction consolidation treatment at week 12. Only 5 of the 49 patients with a high *CRLF2* expression were stratified as HR according the ALL-BFM 2000 protocol; 2 of them were positive for *BCR-ABL*, and only 1 patient (positive for *BCR-ABL*) experienced a relapse.

Altogether, patients with *CRLF2* aberrations appear to be sensitive to in vivo treatment as measured by MRD. Therefore, most of them were not identified as being HR for relapse. When we examined outcome in NCI HR and NCI low-risk patients, we found that the *CRLF2* high-expression status was associated with a poor outcome in both groups (supplemental Figures 1-3). We detected *JAK2* mutations in 6 and *CRLF2*F232C mutations in 2 cases with *CRLF2* rearrangement (supplemental Table 2): only 1 patient with *JAK2*R683S and 1 patient with *CRLF2*F232C mutation experienced a relapse. On the basis of the limited number of patients no statement can be given whether there are differences in outcome between patients with aberrant *CRLF2* expression with or without *JAK* or *CRLF2* mutations.

In a multivariate analysis considering initial WBC count, age at diagnosis, presence of *TEL-AML1* rearrangement, presence of *BCR-ABL* or *MLL-AF4* rearrangement, and MRD after induction consolidation in addition to either a high *CRLF2* expression or presence of the *P2RY8-CRLF2* rearrangement, the presence of a *P2RY8-CRLF2* rearrangement but not a high *CRLF2* expression irrespective of the underlying aberration provided independent prognostic information (risk ratio for relapse 3.11, 95% confidence interval 1.40-6.92, $P = .005$; supplemental Table 3).

In summary, high-level *CRLF2* expression was associated with a poor EFS in childhood pB-ALL treated according to the ALL-BFM 2000 protocol. Similar observations were recently made by other groups.[11,19,20] However, in contrast to published studies, we witnessed that this effect was mainly related to a greater CRI in non-HR patients with the presence of the *P2RY8-CRLF2* rearrangement. Once confirmed independently, the assessment of *CRLF2* status may, therefore, serve as a new stratification tool on BFM treatment regimens by identifying additional patients who are deemed HR for relapse. On the basis of the data presented here, however, it is not yet clear whether a high *CRLF2* expression independent of the underlying aberrations per se or specifically the detection of the *P2RY8-CRLF2* rearrangement is the decisive prognostic factor. However, with the detection of *CRLF2* aberrations, there is for the first time a prognostic marker for a relative large group of patients (5%-10% of pB-ALL) with a HR of relapse who currently remain unrecognized by the stratification regimen because 90% of them are regularly stratified and treated as standard-risk or intermediate-risk patients. Whether these cases, which in the majority are sensitive to treatment (as measured by MRD), may benefit from an intensification of conventional therapy or whether they need the addition of hematopoietic stem cell transplantation has to be evaluated in future clinical trials. Moreover, high-level CRLF2 expression and/or aberrant CRLF2/JAK signaling may serve as therapeutic targets for this important subgroup of patients.

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## Authorship

Contribution: G.C., M.Z., and M. Stanulla designed the study, analyzed the data, and wrote the manuscript; R.R. and A.S. performed gene expression and MRD analysis and contributed to the writing of the manuscript; A.M. contributed to data analysis and interpretation and to the writing of the manuscript; S.G., J.H., and

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R.S. performed FISH analysis and contributed to the writing of the manuscript; I.V. performed sequencing and contributed to the writing of the manuscript; and S.I., T.A., M.J.S.D., R.S., and M. Shrappe made initial observations, were involved in the initiation of the study, and contributed to data interpretation and the writing of the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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