

LETTER TO THE EDITOR

Interference with pre-B-cell receptor signaling offers a therapeutic option for *TCF3*-rearranged childhood acute lymphoblastic leukemia

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Rearrangements of *TCF3* (*E2A*) occur in <5% of childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cases.¹ In 90–95% of these rearranged cases, *TCF3* (chromosome 19p13) is fused to *PBX1* (chromosome 1q23).² This *TCF3*-rearranged subtype is characterized by the expression of cytoplasmic immunoglobulin heavy chain (Cylgμ) in more than 80% of pediatric patients.^{3,4} This Cylgμ positivity is a consequence of an in-frame VDJ rearrangement of the immunoglobulin heavy chain locus (*IGH*).³ Igμ and the surrogate light chain together constitute the pre-B-cell receptor (pre-BCR).⁵ Activation of this pre-BCR triggers the clonal expansion of pre-B cells in the bone marrow, after which these cells further mature by initiating the rearrangement of the light chain locus genes *IGK* and *IGL*.⁶ The transcription factor *TCF3* is essential in the differentiation process of common lymphoid progenitors into B-lineage cells and is a key regulator of further B-cell development. *TCF3*-deficient cells are impaired in rearranging both the immunoglobulin heavy and light chain genes, and wild-type *TCF3* regulates the expression of genes important in B-cell differentiation such as *EBF1* and *PAX5*.^{7,8} These findings imply that *TCF3*-rearranged cases might be affected in pre-BCR-mediated signaling, which in turn could point to new therapeutic targets for this subtype of BCP-ALL. To address this hypothesis, we investigated components of the pre-BCR pathway in leukemic cells obtained from newly diagnosed children with *TCF3*-rearranged BCP-ALL and non-*TCF3*-rearranged BCP-ALL.

In a representative childhood BCP-ALL cohort, 11/13 (84.6%) of *TCF3*-rearranged cases were positive for Cylgμ compared with 68/325 (20.9%) of non-*TCF3*-rearranged cases ($P < 0.001$; Figure 1a), thereby confirming previous studies.^{3,4} We used a previously validated PCR heteroduplex analysis⁹ of V(D)J rearrangement patterns of the *IGH*, *IGK* and *IGL* genes to determine a difference in the heavy and light chain rearrangement patterns of *TCF3* and non-*TCF3*-rearranged cases. Four stages at which the rearrangement had been arrested were defined: samples with only rearrangements of *IGH* (*IGH*), samples with V_{κ} - J_{κ} rearrangements but no *IGK*-Kde or V_{λ} - J_{λ} rearrangements (*IGK*), samples with *IGK*-Kde and no V_{λ} - J_{λ} rearrangements (*IGK*-Kde), and samples with V_{λ} - J_{λ} rearrangements (*IGL*). Strikingly, the immunoglobulin rearrangement pattern of *TCF3*-rearranged cases was arrested at the *IGH* stage in 86.4% (19/22) of the cases. Only one case was assigned to the *IGK* group and two cases to the *IGL* group. In contrast, only 17.9% (34/190) of the non-*TCF3*-rearranged cases was arrested at the *IGH* stage and the frequency of cases with light chain rearrangements was significantly increased to 18.4% (35/190) for samples arrested at *IGK*, 38.4% (73/190) for *IGK*-Kde and 24.7% (47/190) for *IGL* ($P < 0.001$). In one sample, that was representing a *BCR-ABL1*-like patient, rearrangements of the heavy chain (*IGH*) and light chain genes (*IGK*, *IGL*) did not occur (Figure 1b). These data demonstrate that the majority of *TCF3*-rearranged BCP-ALL cases express the

immunoglobulin heavy chain without further initiating the rearrangement and expression of the light chain needed to mature further and form a functional B-cell receptor. This suggests a divergent role for the pre-BCR and its downstream signaling in *TCF3*-rearranged BCP-ALL.

Next, we analyzed the expression levels of several key components of the pre-BCR signaling complex: ZAP70, SYK, LYN, SLP65 (BLNK), BTK, PLCγ2, PI3K-p110δ, and IRF4 by reverse phase protein arrays¹⁰ of leukemic cell lysates (containing >90% leukemic blasts) obtained from 19 *TCF3*-rearranged and 113 non-*TCF3* BCP-ALL pediatric patients. As visualized in Figure 1c, most of these proteins were expressed significantly more in *TCF3*-rearranged compared with non-*TCF3*-rearranged BCP-ALL: ZAP70 1.3-fold ($P < 0.001$), SLP65 3.4-fold ($P = 0.002$), BTK 1.5-fold ($P < 0.001$), PI3K-p110δ 1.2-fold ($P = 0.02$) and IRF4 5.2-fold ($P < 0.001$). In addition, the expression of the *TCF3*-target gene *EBF1* was 2.7-fold increased in *TCF3*-rearranged BCP-ALL cases ($P < 0.001$; Figure 1c).

The activity of this divergent pre-BCR pathway was evaluated by responsiveness to the BTK inhibitor Ibrutinib (PCI-32765, Selleckchem, Houston, TX, USA). Ibrutinib was demonstrated to be of clinical benefit to patients suffering from chronic lymphoblastic leukemia,^{11,12} B-cell non-Hodgkin lymphomas¹¹ and multiple myeloma.¹³ We tested in primary BCP-ALL samples and leukemic cell lines in a 72-h *in vitro* drug cytotoxicity assay using methyl-thiazol-tetrazolium conversion as read-out.¹⁴ In correspondence with preclinical studies showing effective anti-leukemic effect of Ibrutinib in primary chronic lymphoblastic leukemia cells,¹⁵ a concentration range between 0 and 50 μM Ibrutinib was used. In our study the growth and viability of the *TCF3*-rearranged cell line MHH-CALL3 decreased upon exposure to increasing concentrations of Ibrutinib (Figure 2a), resulting in a growth inhibitory concentration (GI50) of 43.5 μM. In contrast, no GI50 value was reached for non-*TCF3*-rearranged cell lines (Nalm6 and MHH-CALL4) nor the *TCF3*-rearranged cell line 697 (Figure 2a). This latter cell line is known for its lack of functional BTK,^{16,17} suggesting relative specificity of Ibrutinib for BTK-mediated pre-BCR signaling in BCP-ALL. In correspondence, the level of phosphorylated ERK1/2, which is an important downstream effector of BTK contributing to cell survival, decreased upon a 4-h exposure to Ibrutinib in the *TCF3*-rearranged MHH-CALL3 but not in both non-*TCF3*-rearranged cell lines Nalm6 and MHH-CALL4 (as shown on western blot, Figure 2c). Figure 2b shows the differential cytotoxic effect of Ibrutinib in primary patients' leukemic cells of six *TCF3*-rearranged and six non-*TCF3*-rearranged BCP-ALL patients (>90% leukemic blasts) taken at the initial diagnosis (measured by methyl-thiazol-tetrazolium assay). All *TCF3*-rearranged samples expressed Cylgμ in contrast to 50% (3/6) non-*TCF3* samples, which represented two hyperdiploid (>50 chromosomes), two *ETV6-RUNX1*-translocated, one *BCR-ABL1*-like and one *BCR-ABL1*-positive case. Ibrutinib significantly reduced the cell viability of all *TCF3*-rearranged cases at each inhibitor concentration compared with non-*TCF3*-rearranged cases ($P < 0.001$). As leukemic cells of patients do not proliferate in *ex vivo* culture conditions, the concentration of Ibrutinib, which is

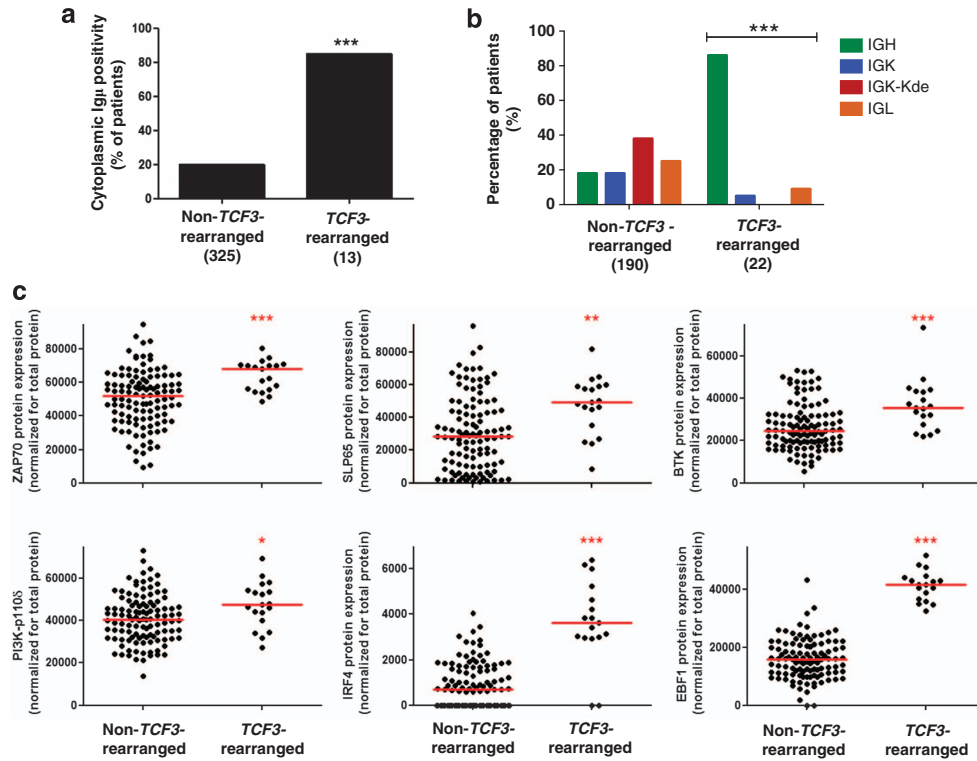


Figure 1. Cytoplasmic Ig μ expression in *TCF3* non-*TCF3*-rearranged B-cell precursor ALL (a). Patients were considered CyIg μ positive if >30% of leukemic cells were stained positively for this marker as detected by flow cytometry. Distribution of immunoglobulin rearrangement pattern of *TCF3*-rearranged and non-*TCF3*-rearranged BCP-ALL cases (b). IGH group contains samples with IGH rearrangement only. IGK group consists of cases with V κ -J κ rearrangements without IGK-Kde or V λ -J λ ; IGK-Kde group contains *IGK*-deleted cases without V λ -J λ rearrangements. IGL group contains cases with V λ -J λ -rearranged IGL locus. V(D)J rearrangement patterns were detected by genomic PCR heteroduplex analysis as previously described.⁹ Expression level of proteins involved in pre-BCR signaling in *TCF3*-rearranged and non-*TCF3*-rearranged BCP-ALL samples (c). Expression levels of indicated proteins were determined by reverse phase protein arrays using leukemic cell lysates of newly diagnosed BCP-ALL patients. The protein level was detected in six spots per patient by specific antibodies and was normalized for total protein loaded on each array. Red line indicates the median expression level per group. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ (a and b: χ^2 -test; c: Mann-Whitney *U*-test). See Supplementary Materials and methods document for more information.

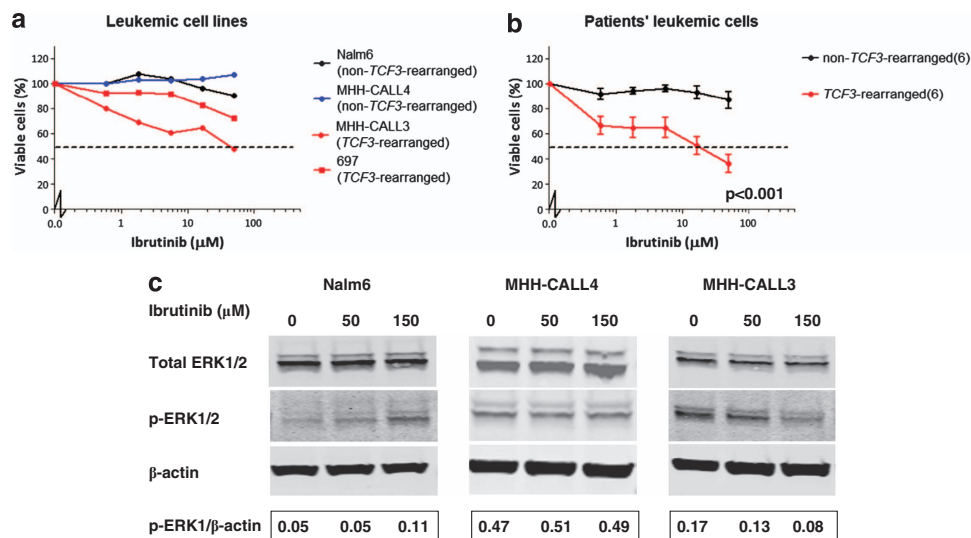


Figure 2. Sensitivity of leukemic cells to the BTK inhibitor Ibrutinib. (a) Leukemic cell lines. (b) Patients' leukemic cells. Experiments were performed in duplicate. Dashed line indicates the Ibrutinib concentration affecting growth (GI50, cell lines) or inducing cell death (LC50, patients' cells) in 50% of the cells. Bars indicate 95% confidence interval. *P*-value was calculated by one-way analysis of variance test with repeated measurements. Effect of Ibrutinib on ERK protein levels in leukemic cell lines (c). Cell lines were exposed to 0, 50 and 150 μ M Ibrutinib for 4 h. The level of phosphorylated ERK1/2 (p-ERK1/2) was reduced in *TCF3*-rearranged MHH-CALL3 compared with non-*TCF3*-rearranged Nalm6 and MHH-CALL4 cell lines. β -Actin served as a loading control. P-ERK1/ β -actin: ratio of the intensities. See Supplementary Materials and methods document for more information.

lethal to 50% (LC50) of the primary leukemic cells, was calculated. The median LC50 value was 16.7 μM for *TCF3*-rearranged cases, whereas virtually no cell death was induced up to 50.0 μM of Ibrutinib in leukemic samples of non-*TCF3*-rearranged patients ($P < 0.001$; Figure 2b). Strikingly, in our study the leukemic cell samples of patients were more sensitive to cell death induced by Ibrutinib than the growth inhibitory concentration needed for cell lines. This suggests that *TCF3*-rearranged patients' leukemic cells have an activated pre-BCR signaling pathway. The observed LC50 values for *TCF3*-rearranged cells are comparable to previously reported drug cytotoxicity data in primary chronic lymphoblastic leukemia cells.¹⁵ In addition, Ibrutinib was shown to impair the migration and adhesion of mature B cells to mesenchymal stromal cells at sub-micromolar levels.^{18,19} Taken together, these studies suggest that Ibrutinib is highly effective to target leukemic cells by interfering with (pre)BCR-driven survival/proliferation signaling and with migration/adhesion pathways.

Remarkably, we observed that *TCF3*-rearranged BCP-ALL cases intrinsically express higher protein levels of pre-BCR pathway including IRF4 (Figure 1c). In normal B-cell development, IRF4 triggers the rearrangement of the immunoglobulin light chain genes *IGK* and *IGL*, thereby contributing to the maturation of the B-cell receptor,²⁰ whereas the present study shows that these light chain genes are infrequently rearranged in *TCF3*-rearranged BCP-ALL. Inhibition of *TCF3* is essential for the successful production of high-affinity immunoglobulins, a process that is mediated via a negative-feedback loop of the mature B-cell receptor.²¹ Given the presently available data, we would like to postulate that the differentiation of *TCF3*-rearranged BCP-ALL is arrested at an early stage in B-cell development, most likely resembling the compartment of pre-B-II-large cells in normal hematopoiesis before IRF4 triggers the rearrangements of immunoglobulin light chains.²⁰ The aberrantly high levels of IRF4 together with activated pre-BCR signaling may trigger an uncontrolled expansion of *TCF3*-rearranged cells *in vivo* at the expense of differentiation into more mature B cells. In correspondence, the relative sensitivity of *TCF3*-rearranged leukemic cells for Ibrutinib—without need for external stimuli other than those provided by the culture medium—and the aberrant expression profile of pre-BCR-related proteins suggests that the pre-BCR pathway is more activated in *TCF3*-rearranged compared with non-*TCF3*-rearranged leukemic cells.

The prognosis of children with *TCF3*-rearranged leukemia has improved during consecutive trials by optimizing the regimens with traditional chemotherapeutic drugs.²² Implementation of more specific, that is, targeted, drugs may further improve prognosis and/or reduce the side effects of current chemotherapeutics by allowing a dosage reduction. Early clinical trials show that objective response rates can be obtained with Ibrutinib in 60–75% of patients with relapsed or refractory B-cell malignancies.^{11,23} A phase II study in adult patients with chronic lymphoid leukemia indicates that monotherapy with Ibrutinib might be effective even in high-risk cases with limited adverse effects.²³ These early clinical trial observations together with the findings presented in this preclinical study provide a strong rationale to design studies with agents interfering with pre-BCR signaling (for example, Ibrutinib) in children with *TCF3*-rearranged BCP-ALL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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