

### The activating STAT5B N642H mutation is a common abnormality in pediatric T-cell acute lymphoblastic leukemia and confers a higher risk of relapse

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of thymocytes that accounts for approximately 15% of pediatric acute lymphoblastic leukemias. A variety of genetic events affecting cellular processes such as the cell cycle, differentiation and survival have been identified in T-ALL and result in uncontrolled cell growth, developmental arrest and clonal expansion of T cells.

Aberrant interleukin 7 receptor (IL7R)/Janus activated kinase (JAK)-signaling by either activating somatic mutations of the IL7R gene<sup>1,2</sup> or by activating mutations in JAK3<sup>3</sup> have been described in approximately 20% of pediatric T-ALL. Deletions or inactivating mutations of the protein phosphatase PTPN2 gene result in increased STAT5 phosphorylation in 6% of adults with T-ALL.<sup>4</sup> Less commonly, activating mutations of JAK1 have also been found in T-cell leukemias.<sup>5</sup>

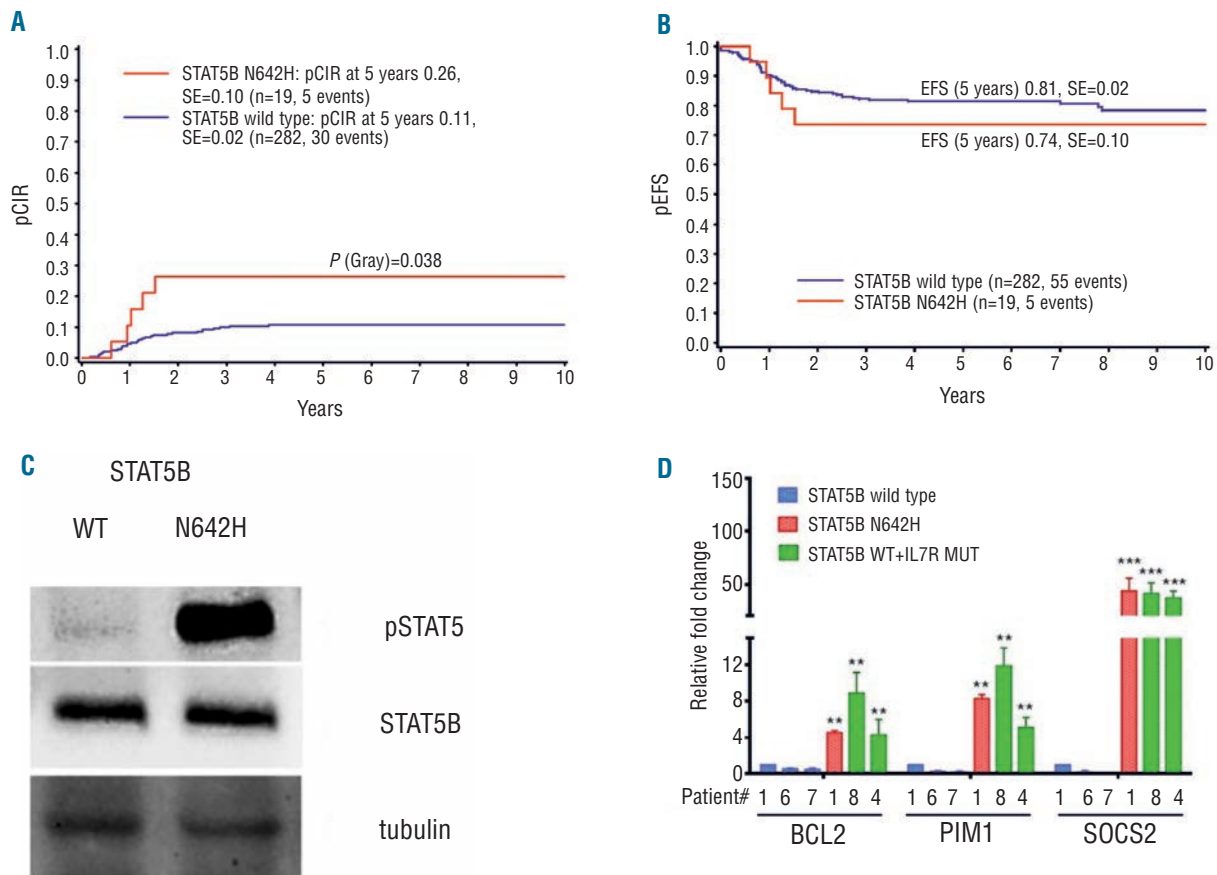
STAT5 represents one of the downstream effectors of IL7R/JAK signaling and activating STAT5B mutations have only recently been suggested to be leukemogenic in 2% of

adults with large granular lymphocyte leukemia<sup>6</sup> and also in T-ALL.<sup>7,8</sup> Here, we aimed to identify novel leukemogenic driver mutations and first analyzed trios of bone marrow DNA of 5 patients with T-cell acute lymphoblastic leukemia obtained at initial diagnosis, remission and relapse. Exome sequencing revealed somatic nonsynonymous mutations in NOTCH1 (3 of 10 samples), PHF6 (2 of 10 samples), genes which are involved in T-ALL leukemogenesis<sup>9,11</sup> and a missense mutation in STAT5B in 2 patients resulting in amino acid substitution N642H in the phosphotyrosine binding pocket of the SH2 domain of STAT5B. According to the allele frequency (AF), one patient was homozygous at relapse (AF=0.78, proportion of blasts 0.72). The same mutation was detected in the primary disease sample with an AF of 0.43, indicating that either it was present in a heterozygous state in approximately all leukemic blasts or that it was in a homozygous state in approximately half of the leukemic blasts. These somatic genotypes were confirmed by Sanger sequencing. Sanger sequencing of xenograft cells confirmed a homozygous STAT5B N642H mutation in one of the patients at relapse; however, in the primary disease sample from this patient, the mutation was not detected in the xenograft cells although the other mutations detected by WES were confirmed by Sanger sequencing. In the second patient, STAT5B N642H was detected only at first diagnosis (AF

**Table 1.** Clinical and genetic characteristics of 301 T-ALL patients treated in the ALL-BFM 2000 study according to STAT5B mutational status.

Characteristic	Total cohort (%) n=301	STAT5B N642H mutation		P
		Positive (%) n=19	Negative (%) n=282	
Sex				
Male	224 (74)	17 (89)	207 (73)	0.17*
Female	77 (26)	2 (11)	75 (27)	
Age at diagnosis				
< 10 years	159 (53)	7 (37)	152 (54)	0.16*
10 years ≥ older	142 (47)	12 (63)	130 (46)	
WBC count at diagnosis				
Less than 10x10 <sup>9</sup> /L	41 (14)	2 (11)	39 (14)	0.94**
10x10 <sup>9</sup> /L to < 50x10 <sup>9</sup> /L	83 (28)	6 (32)	77 (27)	
50x10 <sup>9</sup> /L to < 100x10 <sup>9</sup> /L	58 (19)	3 (16)	55 (20)	
100x10 <sup>9</sup> /L or ≥	119 (40)	8 (42)	111 (39)	
T-cell immunophenotype ***				
Pro	11 (4)	0	11 (4)	0.71**
Pre	50 (17)	2 (11)	48 (17)	
Cortical	197 (65)	15 (79)	182 (65)	
Mature	38 (13)	2 (11)	36 (13)	
T not further classified	5 (2)	0	5 (2)	
Activating IL7 R mutations				
Present	34 (11)	1 (6)	33 (12)	0.71*
Absent	267 (89)	18 (94)	249 (88)	
Activating Notch1 mutations				
Present	150 (50)	8 (42)	142 (50)	0.64*
Absent	151 (50)	11 (58)	140 (50)	
Inactivating Fbxw7 mutations				
Present	42 (14)	1 (6)	41 (15)	0.49*
Absent	259 (86)	18 (94)	241 (85)	
Inactivating PTEN mutations				
Present	50 (17)	4 (21)	46 (16)	0.53*
Absent	251 (83)	15 (79)	236 (84)	

WBC: white blood cell; \*Fisher's exact test; \*\* $\chi^2$  test; \*\*\*Pro/Pre (cyCD3<sup>+</sup>, CD7<sup>+</sup>/cyCD3<sup>+</sup>, CD2<sup>+</sup> and/or CD5<sup>+</sup> and/or CD8<sup>+</sup>), cortical (CD1 $\alpha$ ), mature (CD1 $\alpha$ , sCD3<sup>+</sup>). cyCD3<sup>+</sup>: cytoplasmic CD3<sup>+</sup>; sCD3<sup>+</sup>: surface CD3<sup>+</sup>.



**Figure 1.** STAT5B N642H confers a higher risk of relapse in pediatric T-ALL; (A) Cumulative incidence of relapse (pCIR) and (B) probability of event-free survival (pEFS) for T-ALL patients enrolled in ALL-BFM 2000 with STAT5B wild type (blue) or STAT5B N642H (red). (C) Xenografted cells from Patient 1 at first diagnosis (STAT5B wild type) and at relapse (STAT5B N642H) were harvested from the spleen of leukemic mice, lysed and subjected to SDS-gel electrophoresis and Western blotting. Membranes were probed with anti STAT5B and anti pSTAT5. Tubulin was assessed as a loading control. (D) Expression of downstream STAT5B targets in xenografted leukemia cells: mRNA abundance of the STAT5 downstream targets BCL2, PIM1 and SOCS2 was measured by quantitative RT-PCR in triplicate and set to 1 in STAT5B wild-type cells.

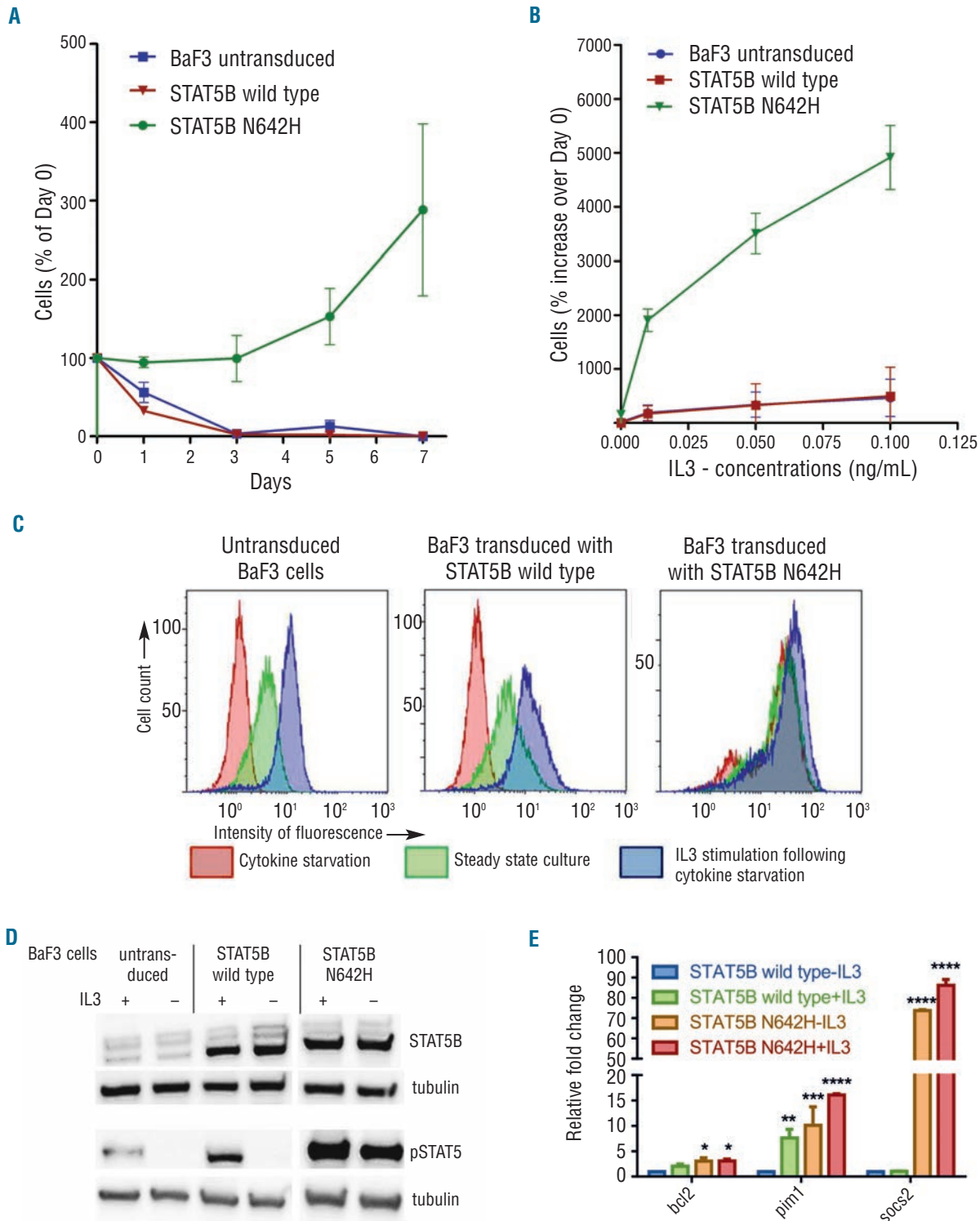
0.48), not at relapse (0 variant reads, coverage 176), indicating that treatment had reduced the leukemic clone carrying the STAT5B mutation by more than 99%. In the xenograft cells from the second patient, STAT5B N642H was detected in the sample from primary disease at a low AF (estimated 10%).

Sequencing of STAT5B exon 16 (which contains position 642) in the cohort of 301 children with primary T-ALL identified the N642H mutation in 19 samples (6.3%). No other mutations in this exon were detected. For patients carrying the STAT5B mutation, there was a trend towards older age (>10 years) and male sex (Table 1), but no correlation with treatment response as measured by prednisone response or PCR-based MRD kinetics. However, patients carrying the STAT5B N642H mutation were at significantly higher risk of relapse (pCIR 0.26) compared to STAT5B wild-type patients (pCIR 0.11; relative risk 2.47;  $P=0.038$ ) (Figure 1A) with a trend towards poorer event-free survival in STAT5B mutated patients (pEFS 0.74 vs. 0.81) (Figure 1B). The presence of the STAT5B N642H mutation was not significantly associated with the presence or absence of mutations in NOTCH1, PTEN, JAK1 or IL7R. By Sanger sequencing, we did not identify somatic PTPN2 mutations in our patient cohort. As reported before, NOTCH1 mutations were associated with a better early response to treat-

ment and a reduced risk of relapse, while for PTEN mutations the opposite was observed.<sup>12</sup> The additional effect of STAT5B N642H on the risk of developing a relapse could not be subjected to a meaningful statistical analysis because of the small sample sizes of the subgroups.

Consistent with the observation that T-ALL patients with STAT5B N642H carry an increased risk of relapse, of the 78 patients who had been treated according to the ALL-REZ BFM 95/96 and 2002 trials, 7 carried the STAT5B N642H mutation (9%). As in primary disease, there were no significant differences between STAT5B wild-type and STAT5B N642H patients with regard to age, sex, immunophenotype. Five year event-free survival after first relapse was 14.3% in STAT5B N642H patients and 27.6% in STAT5B wild-type patients (not significant;  $P=0.611$ ). Sequencing of the complete STAT5B gene in 96 samples from patients with primary T-ALL and in the cohort of 78 relapse patients did not detect any further mutations, indicating that activating mutations in STAT5B are restricted to the SH2 domain. Sequencing of the corresponding region of STAT5A and of hotspot regions in STAT3 (exon 21) did not detect any mutation in the cohort of 78 relapse patients or in a cohort of 96 primary disease patients, respectively.

We next analyzed the effects of STAT5B N642H in pri-



**Figure 2.** STAT5B N642H results in IL3-independent proliferation in BaF3 cells. BaF3-cells were either left untransduced or transduced with either STAT5B wild type or STAT5B N642H. (A) Cells were grown without IL3 and counted on Days 1, 3, 5 and 7. Error bars represent SE from 3 independent experiments. (B) Cells were grown for five days in the presence of increasing concentrations of IL3. Error bars represent SE from 3 independent experiments. (C) BaF3 cells were either harvested after 4 h of cytokine starvation (red), during the steady state phase of growth in medium containing IL-3 (green), or following 4 h of cytokine starvation and subsequent 20 min stimulation with IL3 (10 ng/mL; blue). Cells were fixed, stained for pSTAT5 and analyzed by FACS for STAT5p levels. (D) BaF3 cells were incubated without IL3 for 5 h before IL3 (10ng/mL) was added. After another 20 min, cells were harvested, lysed and subjected to SDS-gel electrophoresis and Western blotting. All samples were analyzed on one gel and blot for STAT5B (upper panel) and pSTAT5 (lower panel). The blots were cut at the indicated positions to excise unrelated tracks. Tubulin was assessed as a loading control. (E) BaF3 cells were transfected with either STAT5B wild type or with STAT5B N642H. Cells were harvested and RNA isolated after 4 h of cytokine deprivation (-IL3) or after a 20 min incubation with IL3 (10 ng/ml) following 4 h cytokine deprivation (+IL3). Expression of the STAT5 target genes BCL2, PIM1 and SOCS2 was quantified by RT-PCR. \* $P=0.05$ , \*\* $P=0.01$ , \*\*\* $P=0.001$ , \*\*\*\* $P=0.0001$ .

mary leukemic cells following xenotransplantation in immunodeficient mice. Cells were obtained from Patient 1, either at the time of primary disease (STAT5B wild type) or at the time of relapse (STAT5B N642H). The STAT5B N642H mutated cells showed strongly increased phosphorylation as compared to the cells not carrying STAT5B N642H (Figure 1C). Quantitative RT-PCR analyses of the RNA revealed that BCL2, PIM1 and SOCS2 were specifically up-modulated in the range of 4-fold to 45-fold in cells with STAT5B N642H and in cells with activating mutations in IL7R (Figure 1D). Control xenografts that did not carry mutations in genes related to the JAK/STAT pathway showed low expression of the analyzed target genes, indicating that phosphorylation of STAT5B and transcriptional activation of downstream targets is specific for leukemias that carry activating mutations in the JAK/STAT pathway.

Stable expression of STAT5B wild type and STAT5B N642H in BaF3 cells revealed that STAT5B wild-type cells survived only in the presence of IL3, whereas expression of STAT5B N642H resulted in IL3-independent survival and proliferation (Figure 2A). Furthermore, the analysis of IL3 dose response curves showed that both the untransduced cells and the cells transduced with STAT5B wild type were viable and proliferated at high concentrations of IL3 (Figure 2B).

Because STAT5B activation requires tyrosine phosphorylation,<sup>15</sup> we analyzed the effect of the N642H mutation on the abundance of phospho-STAT5. Staining and FACS analysis of BaF3 cells with a phospho-specific STAT5 antibody showed that untransduced cells and cells transduced with STAT5B wild type induce STAT5 phosphorylation following IL3 treatment and lose phosphorylation of STAT5 following IL3 starvation. By contrast, cells that have been transduced with STAT5B N642H show strong STAT5 phosphorylation that is completely independent of IL3 (Figure 2C). Western blotting confirmed this result (Figure 2D).

We next analyzed the expression of the known transcriptional STAT5 downstream targets. BCL2 and the serine/threonine protein kinase and oncogene PIM1 affect cell viability and proliferation by inhibiting apoptosis and facilitating homing into the bone marrow. SOCS2 was originally identified as a feedback suppressor of cytokine signaling, but has also been shown to enhance JAK/STAT signaling by degrading other members of the SOCS family. Expression of STAT5B N642H in comparison to STAT5B wild type in BaF3 cells resulted in a strongly increased expression of all tested STAT5 target genes that was independent from IL3 (Figure 2E).

Recently, the same mutation has been found in one of 15 pediatric and 5 of 84 adult T-ALL patients in addition to four other STAT5B mutations in adult T-ALL patients (S434L, T648S, Y665F, I704L).<sup>7,8</sup>

In large granular lymphocytic leukemia, the STAT5B N642H mutation has been shown to be involved in constitutive phosphorylation, nuclear localization and enhanced transcriptional activity of STAT5B in a reporter gene assay.<sup>6</sup>

Because human STAT5A and STAT5B show 92% identity and can substitute each other in many, but not all, functions,<sup>14</sup> one might hypothesize that STAT5A and STAT5B may be redundant proteins and subjected to the same mode of activation in T-ALL. However, an analysis of the corresponding area in STAT5A in our cohort of relapsed pediatric T-ALL did not reveal any mutations including position N642H. This finding is in line with the observation that activation of STAT5B, but not STAT5A, supports proliferation in squamous cell carcinoma.<sup>15</sup> Furthermore, we did not find mutations in the SH2-domain of STAT3 indicating that STAT5B is unique within

the STAT family in its ability to promote pediatric T-ALL.

The increased risk of developing a relapse in STAT5B N642H mutated patients as reported here represents an important clinical finding. As STAT5B N642H seems to be independent both from established risk factors, such as MRD response, and from other genetic markers, such as activating NOTCH1 mutations, it may contribute to risk stratification algorithms of pediatric T-ALL if this effect is confirmed in current T-ALL treatment protocols.

In conclusion, we demonstrate here that a common mutation of STAT5B results in constitutive STAT5B phosphorylation, growth factor-independent proliferation in cell-based assays, in the activation of downstream targets in leukemia cells of children with T-ALL, and in a higher risk of relapse. This report thus implicates STAT5B activation as an important molecular abnormality in pediatric T-ALL.

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