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Low NUDT15 expression levels due to bi-allelic *NUDT15* variants and 6-mercaptopurine intolerance

Masanori Yoshida[1], **Scott A Brown**[2], **Takaya Moriyama**[3], **Rina Nishii**[3], **Shin-ichi Tsujimoto**[1], **Yuji Yamada**[4], **Kaoru Yoshida**[1], **Ryota Shirai**[1], **Tomoo Osumi**[1,4], **Tomoyuki Utano**[5], **Reiji Fukano**[6], **Ko Kudo**[7], **Kimiyoshi Sakaguchi**[8], **Yuki Arakawa**[9], **Katsuyoshi Koh**[9], **Masahiro Sekiguchi**[10], **Masahiro Sekimizu**[11], **Takako Miyamura**[12], **Hisashi Ishida**[13], **Takeshi Inukai**[14], **Daisuke Tomizawa**[4], **Nobutaka Kiyokawa**[1], **Motohiro Kato**[1,4,10], **Jun J Yang**[3,15]

[1]Department of Pediatric Hematology and Oncology Research, Research Institute, National Center for Child Health and Development, Tokyo, Japan

[2]Department of Immunology, St. Jude Children's Research Hospital, Memphis, USA

[3]Department of Pharmacy and Pharmaceutical Sciences, St. Jude Children's Research Hospital, Memphis, USA

[4]Children's Cancer Center, National Center for Child Health and Development, Tokyo, Japan

[5]Department of Pharmaceuticals, National Center for Child Health and Development, Tokyo, Japan

[6]Department of Pediatrics, Yamaguchi University Graduate School of Medicine, Ube, Japan

[7]Department of Pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki, Japan

[8]Department of Pediatrics, Hamamatsu University School of Medicine, Hamamatsu, Japan

[9]Department of Hematology/Oncology, Saitama Children's Medical Center, Saitama, Japan

[10]Department of Pediatrics, Graduate School of Medicine, the University of Tokyo, Tokyo, Japan

[11]Department of Pediatrics, National Hospital Organization Nagoya Medical Center, Nagoya, Japan

[12]Department of Pediatrics, Osaka University Graduate School of Medicine, Suita, Japan

[13]Department of Pediatrics, Okayama University Hospital, Okayama, Japan

[14]Department of Pediatrics, School of Medicine, University of Yamanashi, Yamanashi, Japan

Corresponding authors: Motohiro Kato, Department of Pediatrics, the University of Tokyo 7-3-1, Hongo, Bunkyo-ku, Japan, katom-tky@umin.ac.jp. Jun J. Yang, Department of Pharmacy and Pharmaceutical Sciences, St. Jude Children's Research Hospital, 262 Danny Thomas Pl, Memphis, TN 38105, USA. jun.yang@stjude.org.

Authorship Contributions

MY, MK, and JJY planned, designed, and coordinated the research and drafted the manuscript; MY, TM, RN, SAB performed the experiments and analyzed and interpreted the data. ST, YY, KY, RS, TO, and TU performed the experiments and analyzed the data. RF, KK, KS, YA, KK, MSekiguchi, MSekimizu, TM, HI, DT, KM, and MK evaluated the patients and collected data. TI and NK discussed the results and provided comments and feedback. All the authors discussed the results and critically reviewed the manuscript.

Conflict of Interest

The authors declare no potential conflicts of interest

[15]Department of Oncology, St. Jude Children's Research Hospital, Memphis, USA

Summary

6-Mercaptopurine (6-MP) is widely used for the treatment of pediatric leukemia and lymphoma. Recently, germline variants in the *NUDT15* gene have been identified as one of the major genetic causes for 6-MP–associated adverse effects such as myelosuppression. Patients with hypomorphic *NUDT15* variants accumulate excessive levels of DNA-incorporated thioguanine in white blood cells, resulting in severe myelosuppression. Although preclinical studies suggest that these variants may influence the protein stability of NUDT15, this has not been directly characterized in patients. In this study, we report the development of a series of novel monoclonal antibodies against NUDT15, using which we quantitatively assessed NUDT15 protein levels in 37 patients with acute lymphoblastic leukemia treated with 6-MP, using Sandwich ELISA. The *NUDT15* genotype was highly correlated with its protein levels ($p < 0.0001$), with homozygous and compound heterozygous patients showing exceedingly low NUDT15 expression. There was a positive correlation between NUDT15 protein level and 6-MP tolerance ($r = 0.631$, $p < 0.0001$). In conclusion, our results point to low NUDT15 protein abundance as the biochemical basis for NUDT15-mediated 6-MP intolerance, thus providing a phenotypic readout of inherited NUDT15 deficiency.

Keywords

NUDT15 expression level; ELISA; NUDT15; 6-mercaptopurine; Pediatrics

Introduction

6-Mercaptopurine (6-MP) is one of the key agents for the treatment of acute lymphoblastic leukemia (ALL) and lymphoblastic lymphoma in children and adults.(1–4) Although prolonged exposure to 6-MP is crucial for the cure of this cancer, this class of drugs can cause severe side effects, particularly myelosuppression.(5–7), which are partly attributable to genetic variants in Thiopurine S-methyltransferase (*TPMT*) and Nudix Hydrolase 15 (*NUDT15*).(8–10)

Nine alleles of *NUDT15* polymorphisms are included in the Clinical Pharmacogenetics Implementation Consortium (CPIC) guideline.(6) In particular, alleles containing the p.R139C variant (i.e.,*2 and *3) are classified as no function, and individuals carrying two non-functional alleles show extremely low tolerance to 6-MP. In a recent international retrospective study on *NUDT15*, patients with homozygous and bi-allelic no-function alleles showed prolonged intolerance to 6-MP and excessive disruption of treatment.(11)

Previous data showed that high levels of DNA-incorporated thioguanine (DNA-TG) in white blood cells (WBCs) in patients with loss-of-function *NUDT15* variants was the pharmacological basis of their 6-MP intolerance.(8, 12, 13) Studies in laboratory model systems suggest that *NUD15* variants, particularly p.R139C, result in unstable proteins and thus reduced enzymatic activity. However, this has not been directly characterized in

patients. Thus, there is an unmet need to develop a phenotypic assay to quantify NUDT15 protein abundance and to directly examine its correlation with 6-MP toxicity clinically.

In the present study, we describe the development of NUDT15 monoclonal antibodies and a Sandwich ELISA assay for NUDT15 protein quantification. Using these assays, we examined the relationship between NUDT15 protein abundance and 6-MP tolerance in patients with ALL.

Materials and methods

Enrollment of patients and Ethical Considerations

This study enrolled patients diagnosed with ALL treated with 6-MP. Based on the hypothesis that NUDT15 protein levels in patients with the *NUDT15* variant are more variable than those in patients with the *NUDT15* wild-type (WT), we mainly collected samples from patients with the variant. These variant samples were taken from the National Center for Child Health and Development (NCCHD) in Japan and the other participating institutions that pertained to the results of *NUDT15* genotyping obtained in our previous studies.(14) Samples of patients with *NUDT15* WT were also included from NCCHD for comparison. The present study was approved by the institutional Ethics Board of the NCCHD (#2020–092) and required informed consent for participating in this study was obtained from the patients or guardians.

NUDT15 *Ab screening*

We first generated B-cell hybridomas from NUDT15 protein vaccinated *Nudt15* knock-out mice.(8, 15, 16) Then, monoclonal antibodies (mAb) were extracted from the hybridoma culture medium using HiTrap Protein G Sepharose 4 FF (Cytiva Life Sciences). Initial mAb screening using the standard ELISA method enabled the selection of 40 out of 384 mAbs. Subsequently, we selected 26 out of 40 Abs by immunoprecipitation and Western blotting, using the overexpressed NUDT15 protein in 293T cells, protein G magnetic beads, and NUDT15 Polyclonal Ab for detection (Abcam). Finally, optimal mAb pairs (clones #1, 7 and clones #4, 10) were selected based on their ability to bind strongly to two different epitopes of the NUDT15 protein, simultaneously using the analysis software, Epitope Binning on the ForteBio Octet® Platform (Sartorius).

Preparation of samples

Mononuclear cells were isolated from fresh peripheral blood samples using Ficoll-Paque® PLUS (GE Healthcare) and SepMate™−15 (STEMCELL Technologies) according to the manufacture's instructions. Extraction of total protein was performed using M-PER Protein Extraction Reagent (Thermo Fisher SCIENTIFIC). The total protein level was measured by the BCA assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher SCIENTIFIC). Duplicated 50 μg of each sample (1 μg/μl, 50 μl) were measured using Sandwich ELISA.

Sandwich ELISA

We established a Sandwich ELISA system to identify NUDT15 protein with high specificity. In brief, 50 μl of the capture Ab (clone #1, 7, 10 μg/ml) in ELISA Coating Buffer (Abcam)

was added to each well of a 96-well ELISA plate (Abcam) and the plate was allowed to coat for 1 h at RT. The plate was washed three times with washing buffer (WB; 1 × PBS containing 0.05% Tween-20) and then blocked with 300 μl of 1 × Blocking Buffer (Abcam) for 1 h at RT. Then, plates were washed three times with WB and 50 μl samples or standard NUDT15 protein was purified as previously described[8]. Then, they were added and incubated for 1 h at RT. Next, plates were washed three times with WB and 50 μl of biotinylated detection Ab solution (clone #4, 10, 1 μg/ml in 1 × Blocking Buffer, biotinylated using EZ-Link™ Sulfo-NHS-LC-Biotinylation) was added and plates were incubated at RT for 15 min. Then plates were washed three times with WB, and 50 μl of Streptavidin-Horseradish Peroxidase (HRP) Conjugate (Thermo Fisher Scientific) diluted 1 : 8000 in blocking buffer was added and incubated at RT for 15 min. The plates were washed three times with WB, and 100 μl of a two-component TMB substrate system (KPL TMB Microwell Peroxidase Substrate System, SeraCare) was added to each well. After 10 min of incubation at RT, 100 μl of Stop Solution (Abcam) was added and absorbance was recorded at 450 nm.

Statistical analysis

First, Kruskal–Wallis test was applied to analyze the difference of expression levels in each *NUDT15* genotype, then multiple comparisons (Dan-Bonferroni method) were performed as post hoc comparisons. For correlation analysis between the NUDT15 expression level and the 6-MP dose, Pearson correlation was performed. The analysis was performed with the R including ggplot2 package.(17)

Results

Patient characteristics

Thirty-seven patients (21 males and 16 females) with ALL, including three relapses, were enrolled in this study (Table 1). The median age at diagnosis was five years (range, 0–17 years). All patients were treated with maintenance therapy with daily 6-MP at a standard dosage of 40–60 mg/m^2/day. These dosages were adjusted to maintain the target leukocyte counts at 2,000–3,000 /mL. Information on the *NUDT15* genotype is shown in Table 1. Twelve patients were WT, 19 were heterozygous, and six were homozygous or compound heterozygous variants, on the basis of variants *2, *3, and *5.

Comparison of 6-MP tolerances among genetic polymorphisms

As previously described,(8, 11, 14) 6-MP tolerance was lowest in patients who were homozygous /compound heterozygous for one of the *NUDT15* variant (*2, *3, and *5), followed by heterozygous and WT with a significant difference ($p < 0.0001$, Fig. 1).

NUDT15 protein level and NUDT15 genotype

Compared with patients with WT *NUDT15*, those with the heterozygous or homozygous/compound heterozygous genotype showed significantly lower NUDT15 protein levels ($p < 0.0001$, Fig. 2A). In particular, homozygous/compound heterozygous patients showed exceedingly low NUDT15 protein. In the subgroup analysis focusing on heterozygous patients, there were no significant differences among carriers of either of the three variants

(*1/*2, *1/*3, and *1/*5, Fig. 2B). Also, NUDT15 protein level did not differ by gender ($p = 0.59$) or age ($p = 0.45$) (Fig 2C and D).

NUDT15 expression levels and 6-MP tolerance

The association between the NUDT15 protein levels and 6-MP tolerance is shown in Fig. 3. There was a significant correlation between the NUDT15 protein abundance and 6-MP tolerance in the entire cohort ($r = 0.632$, $p < 0.0001$, Fig. 3A) and also within patients with at least one allele of the NUDT15 variants ($r = 0.627$, $p = 0.0008$, Fig. 3B). In particular, for patients with biallelic variants (i.e., homozygous or compound heterozygous variants), we observed a positive correlation between the NUDT15 protein level and 6-MP tolerance ($r = 0.865$, $p < 0.0001$, Fig. 3C).

Multivariate regression analysis

In a multivariate model including both the NUDT15 genotype and protein levels, the *NUDT15* genotype remain associated with 6-MP doses ($p = 0.028$), whereas NUDT15 was no longer significant in terms of correlation with 6-MP doses ($p = 0.410$).

Discussion

The present study reveals NUDT15 expression levels in patient samples with different *NUDT15* variants. The NUDT15 expression levels were lower in patients with variants than with patients with the WT. Notably, patients with homozygous/compound heterozygous variants showed extremely low expressions of NUDT15. These findings indicate that low NUDT15 expression is one of the contributing factors for the low tolerance of 6-MP in patients with *NUDT15* variants.

In our study, three patients with homozygous variants (*3/*3) and three patients with compound heterozygous variants (*2/*3, [n=1] and *3/*5, [n=2]) were analyzed. In the CPIC Guidelines for thiopurines and *NUDT15*, *2 and *3 are categorized as no functional alleles, whereas *5 is as categorized as an uncertain functional allele.(6) Generally, patients with heterozygous *5 (*1/*5) show almost normal or a little lower tolerance for 6-MP.(14) In one case of homozygous *5 (*5/*5) in the recent study, the patient did not show severe intolerance.(11) However, patients with compound heterozygous variants (*2/*5 or *3/*5) showed strong intolerance to 6-MP.(11, 14) Correspondingly, in the analysis, patients with *1/*5 showed a slightly lower NUDT15 expression level than WT, and patients with compound heterozygous variants (*2/*5 or *3/*5) showed extremely low expression levels of NUDT15. These findings indicate that the effect of *5 by itself is limited to a small decrease in expression. However, when combined with *2 and *3, the effect may become apparent.

Previous data indicated that deficiency of the NUDT15 protein induces an increase in the DNA-TG level that results in excess cytotoxicity and severe side effects.(12, 13, 18) In addition, the laboratory model in those studies showed that *NUD15* variants, particularly p.R139C, result in unstable proteins. These findings are consistent with the results of the present study. However, it is still unclear how the NUDT15 expression levels decrease in

patients with variants. Further analysis is required to clarify the dynamics of the NUDT15 protein in *vivo*.

In the present study, we identified a moderate correlation between the 6-MP dose and NUDT15 expression levels for all patients. However, multiple regression analysis showed that NUDT15 genotyping was a more potent determining factor of 6-MP intolerance than were NUDT15 expression levels. In addition to *NUDT15*, many other genes are known to be involved in the metabolism of 6-MP, including *TPMT*, although patients in our cohort had no *TPMT* variants.(6) To predict the 6-MP tolerance for personalized medicine, it is necessary to develop a model that covers these genes and their protein activities.

The study has some limitations. First, we could not measure 6-MP metabolites of the same samples such as DNA-TG. Investigation of the correlation between 6-MP metabolites and NUDT15 expression levels are likely to provide further insight. In addition, the treatment protocol for the patients was not identical. Different treatments could have influenced the 6-MP tolerance. Ideally, samples from the patients using the same treatment protocol should be collected and revalidated in larger numbers in future studies.

Conclusion

NUDT15 bi-allelic variants lead to low NUDT15 protein expression levels, which contributes to intolerance of 6-MP.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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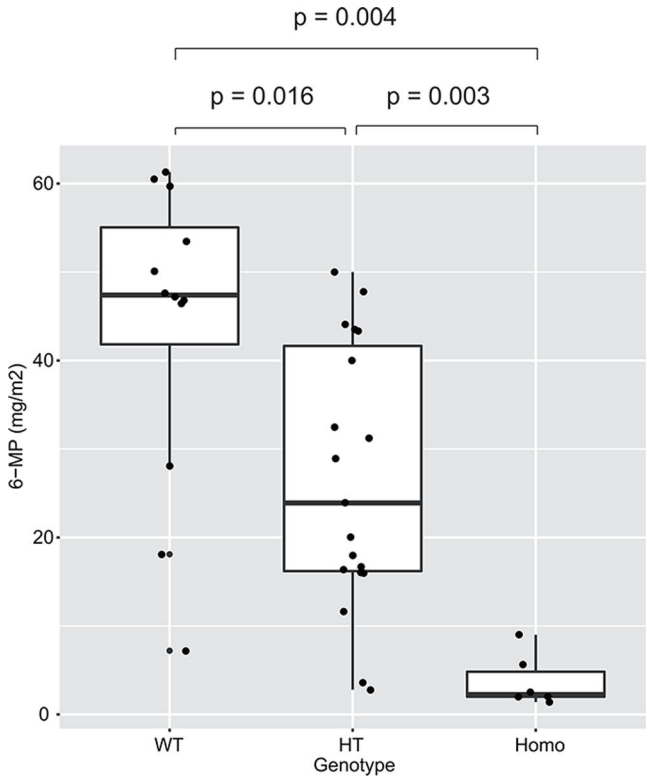

Figure 1. The *NUDT15* genotypes and the average dose of 6-MP.

WT, wild-type; HT, heterozygous variants; Homo, homozygous/bi-allelic variants

There was a significant difference in the Kruskal–Wallis test ($p < 0.0001$). The result of post-hoc comparisons are shown.

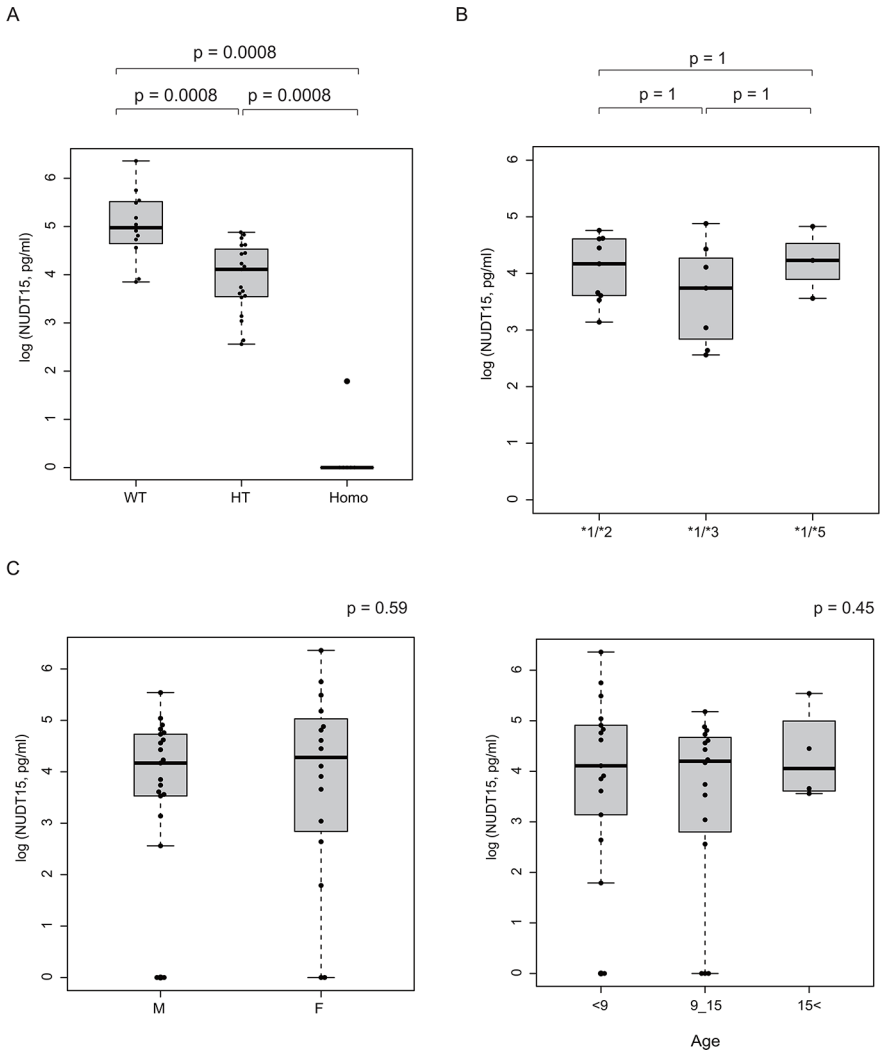

Figure 2. Difference in NUDT15 expression levels

(A) NUDT15 expression levels in patients with *NUDT15* wild-type (WT), heterozygous variants (HT), and homozygous/bi-allelic variants (Homo). (B) NUDT15 expression levels in the respective polymorphisms in heterozygous patients. In (A) and (B), there was a significant difference in the Kruskal–Wallis test ($p < 0.0001$) and the result of post-hoc comparisons are shown. (C, D) NUDT15 expression levels by gender and age. In (C) and (D), the result of the Kruskal–Wallis test are shown.

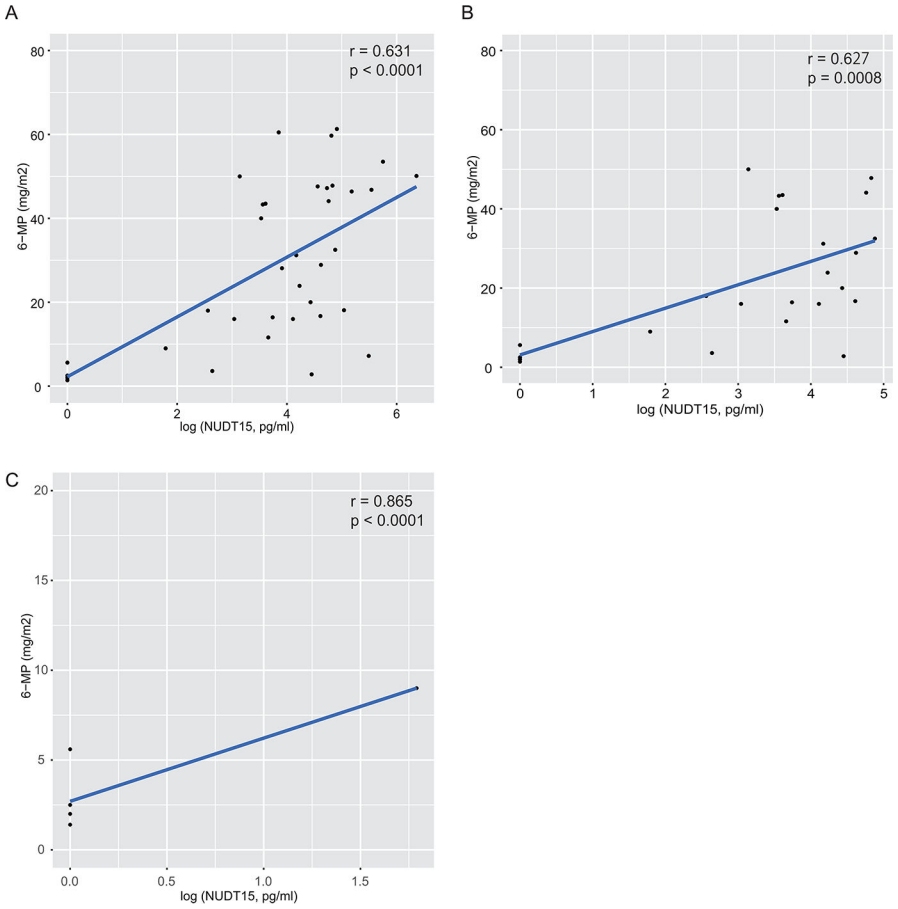

Figure 3. The correlation between NUDT15 exression levels/*NUDT15* genotypes and 6-MP tolerance

The NUDT15 expression levels (log scale) and 6-MP dose in all patients (A), the patients with *NUDT15* variants (B), and the patients with homozygous/bi-allelic variants (C) are shown.

Table 1

Patient Characteristics

Total, n	**37**
Male/Female, n	21/16
Median age at diagnosis, years (range)	5 (0–17)
Diagnosis	
B-ALL (Relapse)	33 (3)
T-ALL	4
***NUDT15* genotype, n**	
Wild-type (*1/*1)	12
Heterozygous	19
*1/*2	9
*1/*3	7
*1/*5	3
Homozygous/Compound heterozygous	6
*3/*3	3
*2/*3	1
*3/*5	2

B-ALL, B-Cell Acute Lymphoblastic Leukemia; T-ALL, T-Cell Acute Lymphoblastic Leukemia