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NOTCH1 fusions in pediatric T-cell lymphoblastic lymphoma: A high-risk subgroup with CCL17 (TARC) levels as diagnostic biomarker

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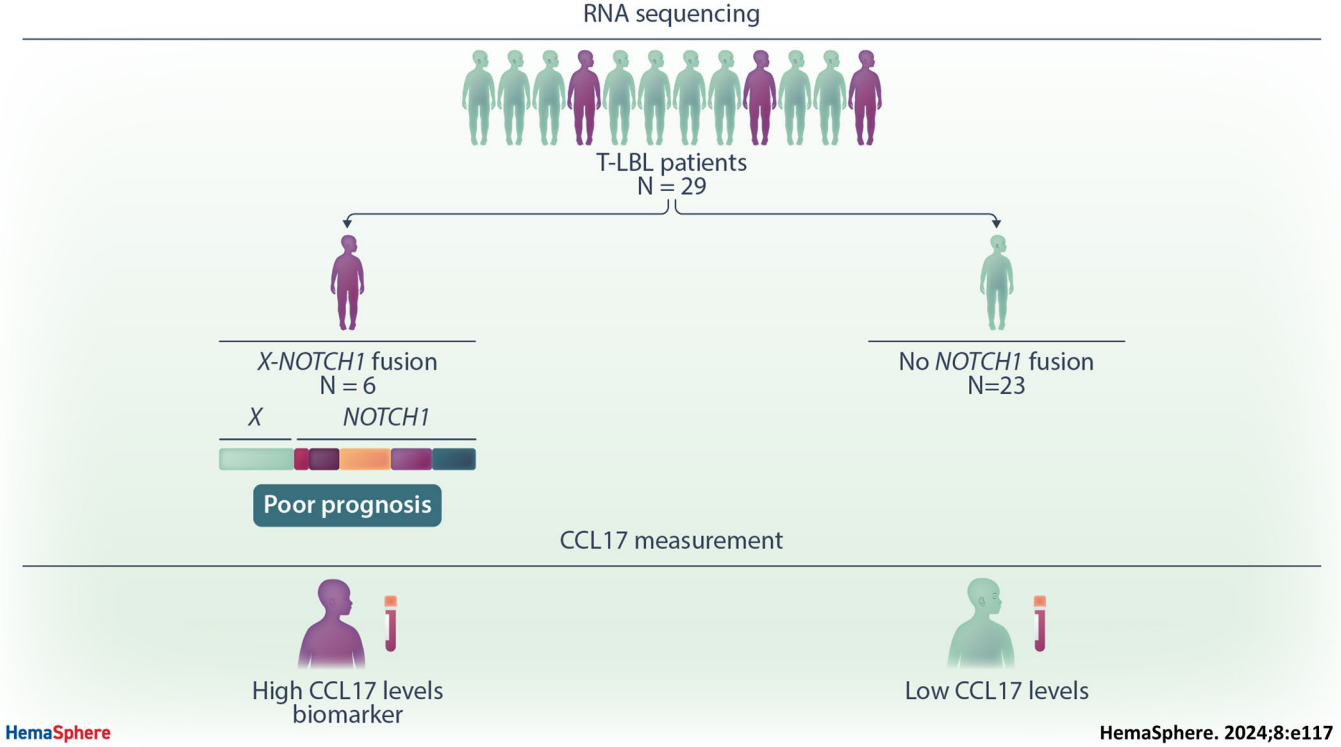
Graphical Abstract



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NOTCH1 fusions in pediatric T-cell lymphoblastic lymphoma: A high-risk subgroup with CCL17 (TARC) levels as diagnostic biomarker

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Abstract

Twenty percent of children with T-cell lymphoblastic lymphoma (T-LBL) will relapse and have an extremely poor outcome. Currently, we can identify a genetically low-risk subgroup in pediatric T-LBL, yet these high-risk patients who need intensified or alternative treatment options remain undetected. Therefore, there is an urgent need to recognize these high-risk T-LBL patients through identification of molecular characteristics and biomarkers. By using RNA sequencing which was performed in 29/49 T-LBL patients who were diagnosed in the Princess Maxima Center for Pediatric Oncology between 2018 and 2023, we discovered a previously unknown high-risk biological subgroup of children with T-LBL. This subgroup is characterized by *NOTCH1* gene fusions, found in 21% of our T-LBL cohort (6/29). All patients presented with a large mediastinal mass, pleural/pericardial effusions, and absence of blasts in the bone marrow, blood, and central nervous system. Blood CCL17 (C-C Motif Chemokine Ligand 17, TARC) levels were measured at diagnosis in 26/29 patients, and all six patients with *NOTCH1* gene fusions patients exclusively expressed highly elevated blood CCL17 levels, defining a novel and previously not known clinically relevant biomarker for T-cell lymphoblastic lymphoma. Four out of these six patients relapsed during therapy, a fifth developed a therapy-related acute myeloid leukemia during maintenance therapy. These data indicate that T-LBL patients with a *NOTCH1* fusion have a high risk of relapse which can be easily identified using a blood CCL17 screening at diagnosis. Further molecular characterization through *NOTCH1* gene fusion analysis offers these patients the opportunity for treatment intensification or new treatment strategies.

INTRODUCTION

T-cell lymphoblastic lymphoma (T-LBL) is a common pediatric malignancy accounting for approximately 20% of the non-Hodgkin lymphomas during childhood.[1] Survival rates of T-LBL are ~80%, but outcome after relapse is dismal, with salvage rates reaching only ~15%.[2,3] Considering the extremely poor prognosis after relapse and absence of clinically relevant high-risk genetics, there is an urgent need for the identification of molecular risk factors and new prognostic biomarkers in T-LBL, as well as identification of new therapeutic strategies.

Pediatric T-LBL is typically characterized by infiltration of blasts in the mediastinum (thymus) and lymph nodes. Approximately half of the cases present with pleural effusion at diagnosis and, by definition, T-LBL patients have less than 25% blasts in the bone marrow (BM).[4,5] Based on morphology and immunophenotype, T-LBL is indistinguishable from its leukemic counterpart, T-cell acute lymphoblastic leukemia (T-ALL). T-ALL presents as leukemic disease with ≥25%

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blasts in the BM and presence of blasts in the peripheral blood (PB), but usually with mild mediastinal enlargement. Even though the clinical presentation of T-LBL and T-ALL largely differs, there has been no evidence so far that there are major differences in molecular genetics of T-LBL and T-ALL.[6,7] Therefore, T-LBL and T-ALL are regarded as different manifestations of the same disease.[8] Pediatric T-ALL has been extensively studied, and great advances in treatment protocols have been made, including minimal residual disease (MRD) measurements as a useable stratification strategy. In contrast, molecular genetics underlying T-LBL are still poorly understood and T-LBL patients are currently mainly treated according to ALL-based protocols. Thus far, MRD assessment has not proven to be usable in T-LBL and diagnostic biomarkers to identify high-risk patients are lacking.

Diagnostic biomarkers have been successfully introduced in other types of lymphomas. For example, CCL17, also known as blood *thymus and activation-regulated chemokine* (TARC) has proven to be a useful diagnostic biomarker in children with classical Hodgkin lymphoma.[9,10] It has never been studied whether CCL17 can serve as a biomarker in non-Hodgkin lymphoma and T-LBL in particular. CCL17 is constitutively produced in the thymus, acting as a powerful T-cell chemoattractant. In classical Hodgkin lymphoma patients, CCL17 production can be induced by NOTCH1 and CCL17 is highly expressed by the Reed-Sternberg cells, thereby creating a specific supporting tumor microenvironment that recruits T-cells.[10] Considering the importance of NOTCH1 and strong preference of malignant T-cells in T-LBL for the thymus, we hypothesize that CCL17 is of importance in the pathophysiology of T-LBL and creating a thymic microenvironment that favors the T-LBL cells.

Recent studies show that both T-LBL and T-ALL patients with *NOTCH1* and/or *FBXW7* DNA mutations have a better outcome compared to *NOTCH1* and *FBXW7* wildtype patients.[11,12] The pathogenic molecular mechanisms of T-LBL patients without *NOTCH1* and/or *FBXW7* mutations remain largely unknown, and this group probably contains both high-risk and low-risk patients. Considering the extremely poor prognosis after relapse, it is essential to identify these high-risk patients. Additionally, there is an urgent need for the identification of new prognostic biomarkers in T-LBL. In this study, we present a novel entity of pediatric T-LBL patients characterized by previously unknown *NOTCH1* gene fusions, high risk of relapse, and highly elevated blood CCL17 (TARC) levels.

METHODS

Patients

We included a complete cohort of all pediatric T-LBL patients ($n = 49$) that were diagnosed in the Princess Máxima Center for Pediatric Oncology between 2018 and 2024. RNA sequencing data at diagnosis were available for 29/49 patients and at relapse for two additional patients. Clinical information and hematological values at diagnosis were retrieved from patient files. All patients were treated according to the EURO-LB02 protocol[3] or its successor, the LBL2018 protocol (NCT04043494). *NOTCH1*/*FBXW7* mutational status was determined for most patients and retrieved from patient files. Pediatric T-ALL patients ($n = 39$) diagnosed between 2019 and 2022 at the Princess Máxima Center for Pediatric Oncology were included as reference cohort. All sample IDs are completely anonymized. Written informed consent was obtained from 45/49 patients and/or their legal guardians, including all patients that were used for further study. The study was performed in accordance with the Declaration of Helsinki. The Medical Research Ethical Committee Utrecht declared that the Medical Research Involving Human Subjects Act (WMO) does not apply and has approved the study (19-140/C).

RNA sequencing analysis

RNA sequencing data was obtained from the in-house diagnostics department in the Princess Máxima Center. The source of biological material is given in Supporting Information S1: Table 1. Preprocessing of the data was done with standardized and in-house pipelines and guidelines.[13] Fusion detection was performed using STAR fusion.[14] In addition, we analyzed the entire T-LBL and T-ALL cohort for exon-specific *NOTCH1* coverage as an indication for fusions, by using DepthOfCoverage of GATK v3.8.0. Whole genome sequencing data (WGS) were also obtained from the in-house diagnostics department and used for validation of genomic breakpoints.

Gene expression analyses

For expression analyses, samples with less than 30 million unique reads were excluded due to lower quality (TLBL042, TLBL046, and TLBL059). For TLBL042, expression data from the relapse were used (TLBL042_R). Gene expression alterations were assessed with log2 transformed gene length normalized read counts (transcript per million mapped reads, TPM) using R v4.4.0. Gene expression variance was determined by calculating standard deviations and the 200 most variable genes were taken in unsupervised clustering using Euclidean distance as a measure of similarity. The R package pheatmap v1.0.12 was implemented for visualizations.

Differential expression analyses were performed with DESeq2 v1.36.0[15] between NOTCH1-rearranged- and WT cases and between NOTCH1-mutated- and WT cases. Differentially expressed genes were identified after adjustment for false discovery rate (FDR-adjusted $p \leq 0.05$). Visualizations were generated using R packages EnhancedVolcano v1.12.0 and pheatmap v1.0.12. Subsequently, gene set enrichment analysis (GSEA) was conducted for biological interpretations with clusterProfiler v4.12.0.[16,17] Genes were ranked according to direction-signed log10-transformed *p*-values, as determined by DESeq2 v1.36.0, and annotations were provided by implementing the R package AnnotationHub v3.2.2. The ranked gene list was used for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Additionally, *z*-scores of log2-transformed TPM values were calculated within the complete T-LBL cohort for genes associated with the NOTCH signaling pathway. ClusterProfiler v4.12.0 and ComplexHeatmap v2.10.0 were implemented for GSEA-result visualizations.

CCL17 (TARC) measurements and immunohistochemistry

CCL17 measurements at diagnosis were performed in serum or plasma of 26 out of the 29 patients for whom RNAseq was available. Measurements were performed in triplo by standard enzyme-linked immunosorbent assay (ELISA) using the DuoSet ELISA kit (cat. no. DY364; R&D Systems, Inc.). Immunohistochemistry staining for CCL17 was performed on the BOND-III fully automated staining system (Leica) using CCL17 rabbit polyclonal antibody (ProteinTech Group). Significance of *CCL17* expression between *NOTCH1*-rearranged samples, and the rest of the T-LBL RNAseq cohort was determined using DESeq2 v1.36.0.

RESULTS

Patient characteristics

The pediatric T-LBL patients included in this study ($n = 49$) represented an unselected complete cohort diagnosed in the Princess

Máxima Center for Pediatric Oncology between 2018 and 2023. T-LBL diagnoses were based on histopathological classification according to the revised World Health Organization for hematological malignancies and/or flow cytometry according to the criteria of the European Group for Immunophenotyping of Leukemias.[8,18] Six out of 49 patients (12%) had a relapse. Informed consent was obtained for 45/49 T-LBL patients and these were therefore used for further analysis. The median age at diagnosis for these 45 patients was 10 years, and our cohort contained more males than females (59%), in line with previously described large T-LBL cohorts.[3] Eighty-eight percent of the patients presented with a large mediastinal mass, which was in 56% of these cases accompanied by pleural effusion. RNAseq was performed for 29/43 patients and their clinical characteristics are described in Supporting Information S1: Table 1.

Detection of *NOTCH1* rearrangements

Transcriptome sequencing was performed at diagnosis from 2019 onward for all samples with sufficient good-quality material available ($n = 29$). This technique allows for the identification and quantification of fusion transcripts and gene expression levels. We identified 12 fusions transcripts in total (12/29, 41%), of which six were *NOTCH1* gene fusions. Fusion partner of *NOTCH1* were the microRNA gene *miR142HG* on chromosome 17q22 (TLBL042 and TLBL058), *IKZF2* on chromosome 2q34 (TLBL050), and *TRBJ* on chromosome 7q34 (TLBL033, TLBL049, TLBL050) (Supporting Information S1: Table 2). The *TRBJ*::*NOTCH1* fusions were missed by fusion detection algorithms, but were detected because of pronounced expression differences between exons in the 5′ and 3′ part of the gene (see Methods section). The fusion transcripts with *miR142HG* and *IKZF2* demonstrated correct splicing to exon 27 or 28 of *NOTCH1* and genomic breakpoints were identified using available whole genome sequencing (WGS) data (Supporting Information S1: Table 2). *TRBJ*::*NOTCH1* fusion genes were previously shown to express a truncated, membrane-bound form of NOTCH1[19] (Figure 1A). None of the six samples with a *NOTCH1* gene fusion exhibited mutations in *NOTCH1*/*FBXW7*, demonstrating the mutual exclusivity of *NOTCH1* mutations and *NOTCH1*-rearrangements. Furthermore, apart from homozygous loss of the *CDKN2A/2B* locus in two cases (TLBL042 and TLBL050), no other driver events were found in these *NOTCH1*-rearranged T-LBLs. *NOTCH1* gene fusions are almost never found in T-ALL, but considering the difficulties in detecting TR-rearranged fusions with conventional pipelines, we also reanalyzed exon-specific *NOTCH1* expression in 39 T-ALL samples that were diagnosed in the Princess Máxima Center for Pediatric Oncology. In line with previous studies,[20,21] none of the T-ALL samples carried gene fusions involving *NOTCH1*, thus suggesting that the frequent occurrence of these fusions represents an important molecular genetic discriminator between T-LBL and T-ALL.

All *NOTCH1* gene fusions result in expression of intracellular NOTCH1

NOTCH1 is a transmembrane protein that is activated through ligand–receptor interaction, which induces a conformational change that results in dissociation of the heterodimerization (HD) subunits. This is followed by exposure of a cleavage site in the C-terminal part of the HD domain, resulting in the release of the intracellular domain of NOTCH1 (NICD). NICD subsequently translocates to the nucleus where it acts as a transcriptional regulator.[22]

To determine whether a truncated C-terminal version of NOTCH1 was indeed expressed in the T-LBL samples with a *NOTCH1*-rearrangement, we performed Western blotting using protein lysates of four *NOTCH1*-rearranged cases and two *NOTCH1* wildtype

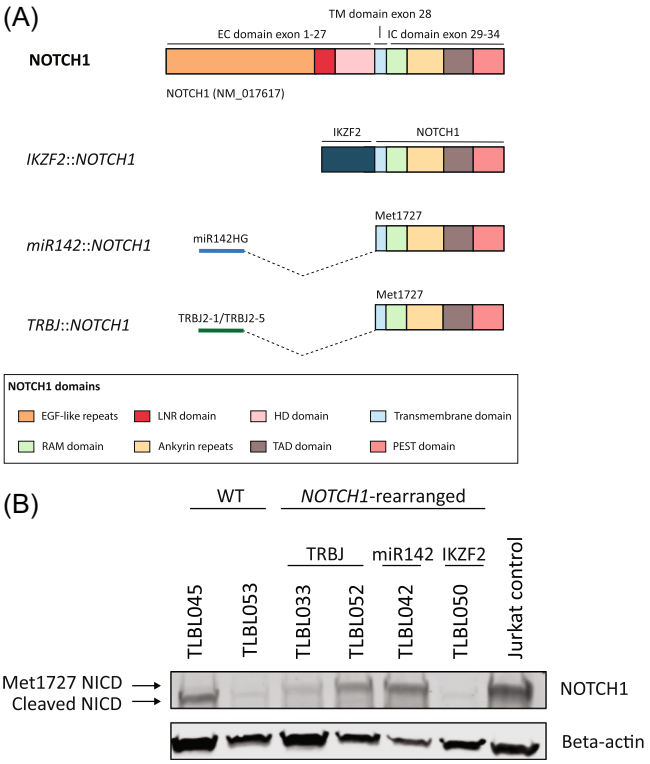


FIGURE 1 *NOTCH1*-rearrangements in T-cell lymphoblastic lymphoma. (A) Schematic representation of three different *NOTCH1* fusions with different fusion partners. The in-frame *IKZF2*::*NOTCH1* fusion generates a chimeric protein in which the N-terminal DNA binding domain of IKZF2 is fused to the C-terminal intracellular domains of NOTCH1. Fusions transcripts with *miR142HG* and *TRBJ* use an alternative translation start site in exon 28 (Met1727). (B) Western blot analysis using Val1744 antibody (Cell Signaling Inc.) shows that *miR142HG*::*NOTCH1* and the *TRBJ*::*NOTCH1* fusions lead to a larger NICD protein, likely representing uncleaved NICD with translation initiation at Met1727. Simultaneous beta-actin staining was performed for loading comparisons.

(*NOTCH1*-WT) cases. All *NOTCH1*-rearrangements lead to expression of NICD. The *IKZF2*::*NOTCH1*-positive T-LBL (TLBL050) showed expression of an NICD protein co-migrating with the ~130-kDa wild-type NICD, suggesting cleavage of the chimeric protein at the γ-secretase cleavage side (Val1744). In contrast, the *TRBJ*::*NOTCH1*-positive (TLBL033 and TLBL052) and *miR142HG*::*NOTCH1*-positive (TLBL042) cases expressed a slightly larger NICD protein, which is in line with translation initiation at methionine residue 1727 (Met1727) encoded in exon 28 of *NOTCH1*, as previously reported,[19,23] and lack of γ-secretase cleavage (Figure 1B).

To explore the downstream consequences of T-LBL cases with a *NOTCH1* fusion in our T-LBL cohort, we performed unsupervised clustering analysis with the 200 most variable genes in the dataset (Supporting Information S1: Table 3), which showed that *NOTCH1*-rearranged samples mostly clustered together (Supporting Information S1: Figure 1). These data indicate that *NOTCH1*-rearranged samples display similar expression profiles among each other. Next, we performed differential expression analysis between *NOTCH1*-rearranged and *NOTCH1*-WT T-LBLs, to determine expression differences. A total of 1288 genes were found to be significantly, differentially expressed, compared to only 101 genes in a comparison between *NOTCH1*-mutated and *NOTCH1*-WT T-LBLs (Figure 2A,B and Supporting Information S1: Tables 4 and 5). These

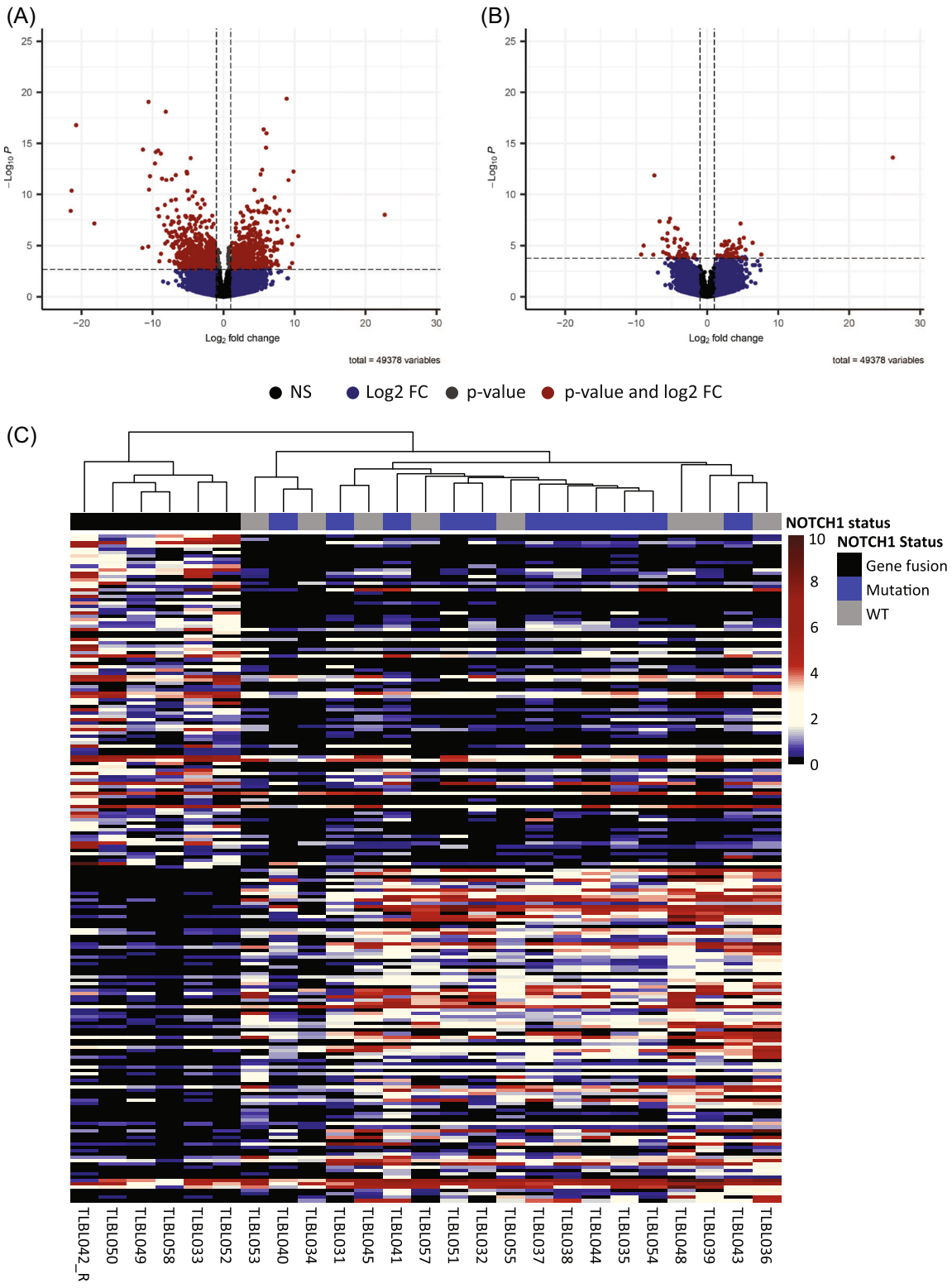


FIGURE 2 Gene expression differences in *NOTCH1*-rearranged, mutated and wildtype T-cell lymphoblastic lymphoma. (A, B) Volcano plots showing differentially expressed genes between *NOTCH1*-rearranged and *NOTCH1*-WT samples ($n = 1288$; A) and between *NOTCH1*-mutated and *NOTCH1* WT samples ($n = 101$; B) (C) Expression analysis of the 200 most significantly upregulated and downregulated genes (from a total of 1288 genes) in *NOTCH1*-rearranged compared to *NOTCH1*-WT samples, revealed that *NOTCH1*-rearranged samples cluster separately from *NOTCH1* WT and *NOTCH1*-mutated samples using Euclidean distance as a measure of similarity. The relapse sample of TLBL042 was used because of better quality. Range of 0–10 showing the log2-transformed TPM values. Significance was determined using false discovery rate (FDR)-adjusted *p*-values.

data indicate that *NOTCH1*-mutated and *NOTCH1*-WT cases exhibit comparable expression profiles, whereas *NOTCH1*-rearranged cases differ substantially from the rest of the cohort. Subsequently, we selected the 200 most significantly, differentially expressed genes between *NOTCH1*-rearranged and *NOTCH1*-WT T-LBLs for supervised clustering. This analysis revealed that *NOTCH1*-rearranged samples formed a separate cluster, whereas the NOTCH1 mutated and *NOTCH1*-WT cases are mixed in a second cluster (Figure 2C), confirming the unique characteristics of the *NOTCH1* fusion samples.

To explore downstream characteristics of *NOTCH1*-rearranged cases, we performed GSEA, to determine enriched KEGG pathways (Supporting Information S1: Figure 2A). Among others, the NOTCH signaling pathway was significantly activated in *NOTCH1*-rearranged samples compared to *NOTCH1*-WT samples, with an enrichment score of 0.695 (adj. $p = 0.0003$; Figure 3A,B). Enrichment of KEGG pathways was to a lesser extent observed in *NOTCH1*-mutated cases compared to *NOTCH1*-WT samples, including no significant enrichment of the NOTCH signaling pathway (Supporting Information S1: Figure 2B). These findings suggest that the downstream characteristics and mechanisms of action of the *NOTCH1*-rearranged samples are different from both wildtype and mutant T-LBL samples.

Clinical presentation of patients with *NOTCH1* gene fusions

All six patients with *NOTCH1* gene fusions presented with a massively enlarged mediastinum, combined with pleural/pericardial effusion. Moreover, all *NOTCH1* gene fusion-positive patients were bone marrow negative, peripheral blood negative, and cerebral spinal fluid negative. These patients therefore had a uniform clinical presentation of disease, although not differentiating between this group and the rest of the T-LBL cohort. Flow cytometry performed at diagnosis revealed positivity for cytoplasmatic CD3 (cyCD3) in all cases, as well as positivity for other T-cell markers. Precursor-marker Terminal Deoxynucleotidyl Transferase (TdT) was expressed in 50% of the *NOTCH1*-rearranged cases, which was lower than expected (~90%)[24] (Supporting Information S1: Table 6). Next, we analyzed blood CCL17 levels, which were highly elevated in all patients with a *NOTCH1* gene fusion (range from 2345 to >10,000 pg/mL), compared to 31–638 pg/mL in 16 patients who did not have a *NOTCH1* gene fusion ($p < 0.0001$, *t*-test) (Figure 4A). Follow-up CCL17 levels during first remission were available for 3/6 patients and revealed normalized values (range 57–152 pg/mL) (Figure 4B). Three of the patients with a clinical relapse (TLBL042, TLBL050, and TLBL058) also showed substantially elevated CCL17 levels at relapse (TLBL042:4613 pg/mL, TLBL050:8654 pg/mL, TLBL058:1662 pg/mL), which could be an indication that CCL17 levels in blood might also increase upon progression of relapse. One patient, whose relapse was discovered with routine imaging, presented with relatively little tumor load and low LDH levels, and did not have increased CCL17 levels (TLBL033) at time of establishing the relapse. CCL17 levels decreased again in second remission (range 69–1331 pg/mL) (Figure 4C). Although immunohistochemistry did not reveal positivity of the T-LBL cells for CCL17 (Figure 4D), based on gene expression, *CCL17* was significantly upregulated in the *NOTCH1*-rearranged cases compared to the rest of the T-LBL cohort

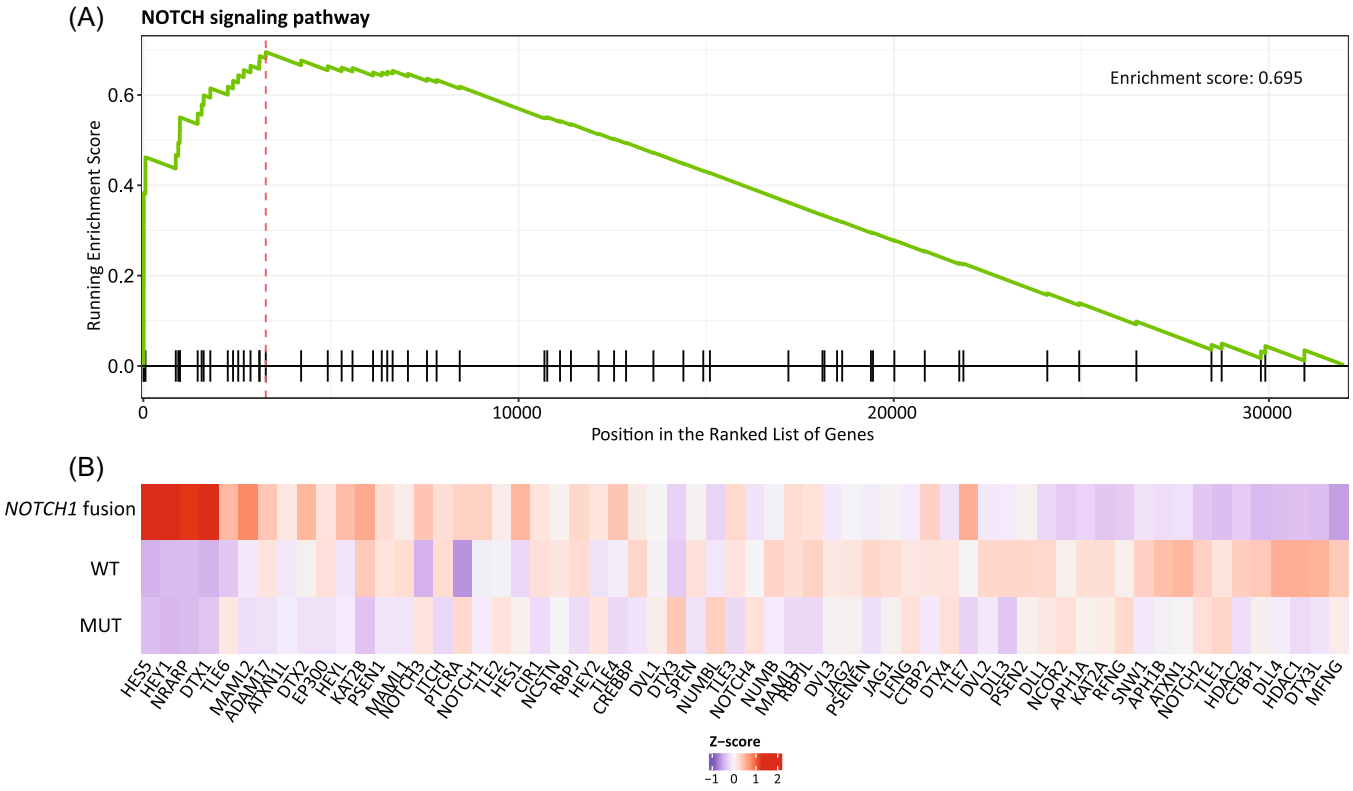


FIGURE 3 Gene set enrichment analysis (GSEA) results of the NOTCH signaling pathway. (A) Enrichment plot for the NOTCH signaling pathway in the *NOTCH1*-rearranged versus wild-type (WT) samples, showing the profile of the running enrichment score and positions of genes associated with this pathway on the rank-ordered gene list. (B) *Z*-scores of log2-transformed TPM values of genes associated with the NOTCH signaling pathway within the complete T-LBL cohort. Mean *z*-scores are depicted for *NOTCH1*-rearranged, *NOTCH1*-mutated, and WT samples. Genes are ranked based on their position in the rank-ordered gene list used for GSEA between *NOTCH1*-rearranged and WT samples.

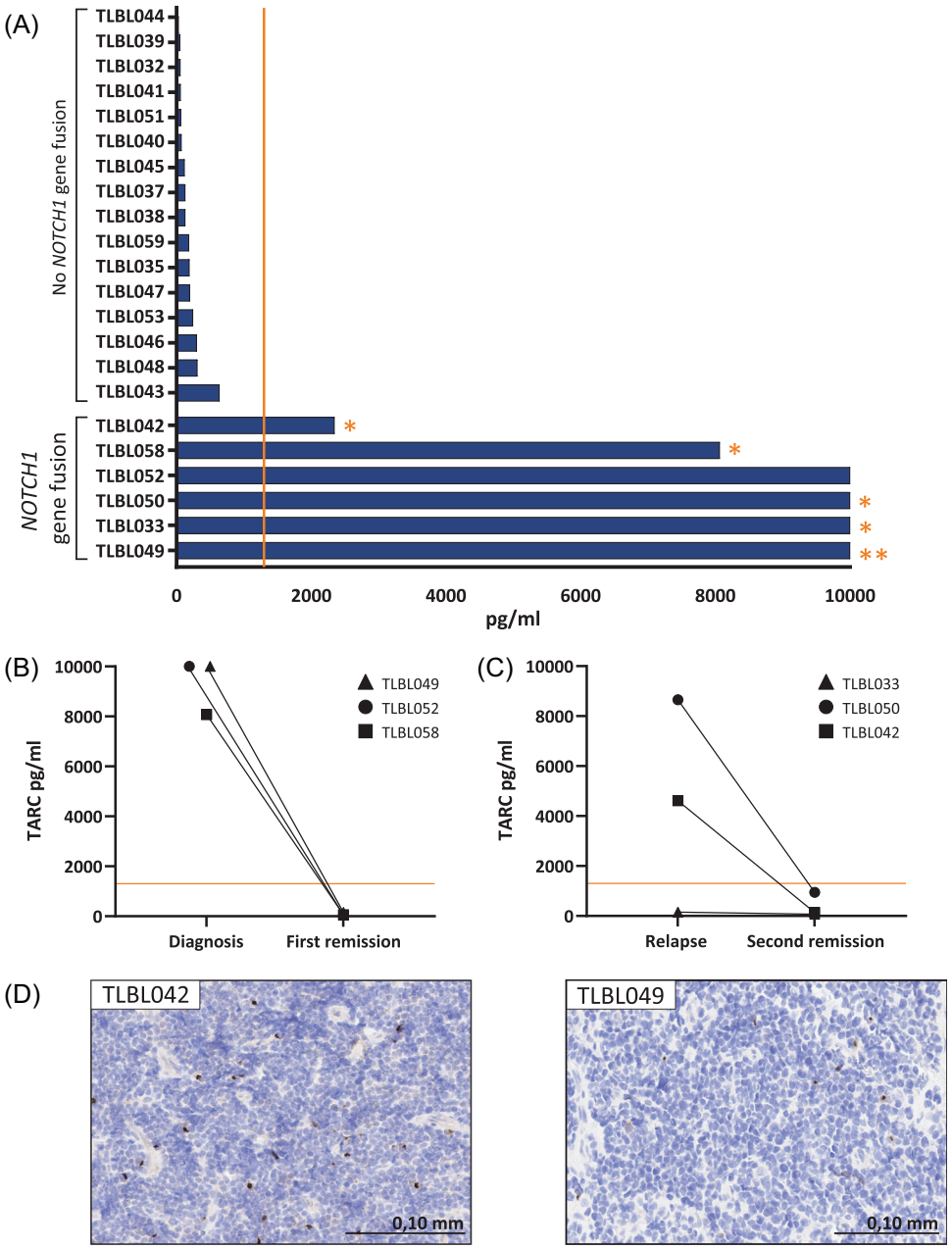


FIGURE 4 CCL17 (TARC) in *NOTCH1*-rearranged patients. (A) CCL17 levels in pg/mL per patient, showing highly elevated CCL17 in blood of *NOTCH1*-rearranged T-LBL patients but none of the other patients. 10,000 pg/mL is the maximum measurable CCL17 level with used assay. Orange line in (A–C) represents maximum normal CCL17 level (1300 pg/mL) based on what has been described in Hodgkin lymphoma.[9] Patients that had a relapse are indicated with an asterisk. Patient TLBL049 developed a therapy-related acute myeloid leukemia (double asterisk). (B) For three *NOTCH1*-rearranged patients, blood CCL17 levels could be determined for a time point of remission after diagnosis, revealing normalized CCL17 levels in all three cases. (C) For four *NOTCH1*-rearranged patients blood CCL17 levels were determined at time point of relapse and remission after relapse (second remission), revealing increased levels in three relapses that again normalized in second remission. (D) Staining for TARC using anti-CCL17 antibody for four *NOTCH1*-rearranged patients showing that T-LBL cells do not express high levels of CCL17 based on immunohistochemistry.

(FDR-adjusted $p = 0.019$). Together, our data strongly indicate that CCL17 can serve as a high-risk biomarker at diagnosis.

NOTCH1 gene fusions as poor prognostic marker in T-LBL

Finally, we aimed to determine the prognostic relevance of *NOTCH1* gene fusions in T-LBL. We found that five out of six *NOTCH1* fusion-positive patients had an event. Four patients had a relapse during therapy (TLBL033, TLBL042, TLBL050, TLBL058), one of them is still under treatment, the other three could not be rescued. Additionally, one patient had a therapy-related acute myeloid leukemia (t-AML) during maintenance therapy of T-LBL (TLBL049), leaving just one *NOTCH1*-fusion-positive patient without an event. The t-AML carried the typical *KMT2A*::*MLLT3* fusion. This patient was rescued with AML induction chemotherapy followed by allogeneic stem cell transplantation. The sixth *NOTCH1*-fusion-positive patient did not

have an event, yet this patient is still under maintenance treatment. In the rest of the cohort, one event occurred, which was death due to pancreatitis complicated by a septic shock (Figure 5). Our data therefore shows a significant difference in cumulative incidence of events between the *NOTCH1*-fusion group and the rest of the cohort ($p < 0.001$).

The unselected 5-year T-LBL cohort ($n = 49$) contained six patients who relapsed, of whom four had a *NOTCH1* gene fusion. For the other two patients, only RNAseq data at relapse was available, revealing a *DDX3X*::*MLLT10* fusion and a *JAKMIP2*::*PDGFRB* fusion at relapse, respectively, established high-risk ALL aberrations.[20,25] Thus, *NOTCH1*-rearranged T-LBL caused the majority of relapses in our cohort, suggesting that they cause an aggressive T-LBL phenotype, with a significantly higher cumulative incidence of relapse ($p < 0.001$, Gray's test) compared to the *NOTCH1*-fusion negative patients.

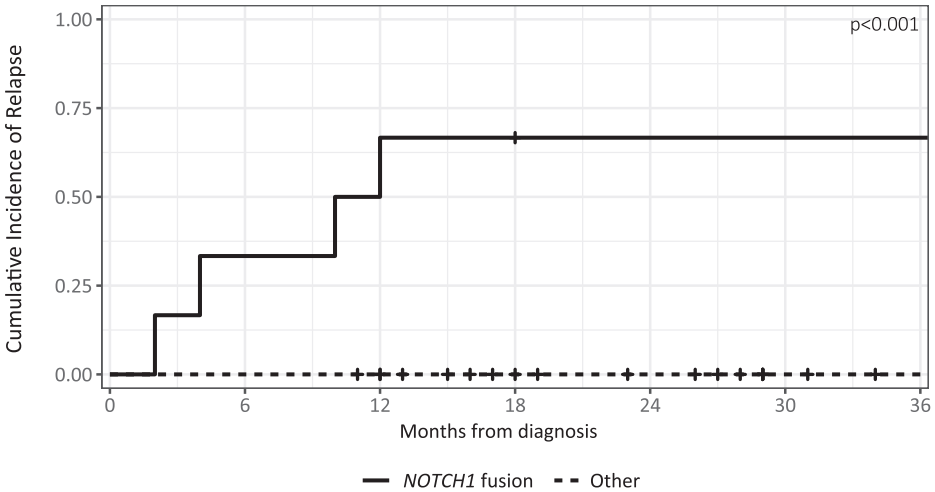
DISCUSSION

To date, mainly clinically applicable low-risk genetics have been described for T-LBL. This implicates that molecular genetic high-risk patients who need intensified or alternative treatment strategies are undetected. Moreover, patients with unknown low-risk molecular genetic profiles could also be overtreated with current treatment strategies.

We discovered a biological high-risk subgroup of T-LBL, characterized by *NOTCH1* gene fusions. This subgroup represents 21% (6/29 patients) of our T-LBL cohort. All patients had a similar although not unique presentation of disease predominantly consisting of a large mediastinal mass, pleural/pericardial effusion, and highly elevated CCL17 (TARC) levels in blood. Moreover, four out of six patients with a *NOTCH1* gene fusion had a relapse and did not survive, indicating that *NOTCH1* fusions lead to an aggressive T-LBL phenotype. Fifty percent of the *NOTCH1*-rearranged cases exhibited expression of TdT, which is lower than the expected 90%. It has been described before that the TdT-negative subset often represent diagnostically challenging cases with phenotypic features that are consistent with a late cortical subtype, coinciding with what we found in our cohort.[24]

The presence and frequency of *NOTCH1* gene fusions can currently be regarded as a major molecular genetic difference between T-LBL and T-ALL, since fusions involving *NOTCH1* are only extremely rarely described in T-ALL (<0.1%).[21,26–31] The uniform absence of BM and PB involvement in T-LBL patients with a *NOTCH1* gene fusion coincides with the fact that these rearrangements have almost never been detected in T-ALL. *NOTCH1* gene fusions have been described in T-LBL before,[28] but given the small number of samples in these studies, as well as difficulties in detecting TR-rearranged fusions with conventional pipelines, these fusions might have been missed explaining the lower contribution of *NOTCH1* gene fusions in these studies.

NOTCH1 gene fusions appear to have more impact on T-LBL cells compared to *NOTCH1* mutations, with more and larger changes in the downstream gene expression and NOTCH1 activity, independent of the type of *NOTCH1*-fusion. Furthermore, whereas patients with *NOTCH1* and/or *FBXW7* mutations are considered low risk and have a better outcome compared to *NOTCH1* and/or *FBXW7* WT patients,[11,12] we demonstrate that the outcome of these recurrent *NOTCH1*-rearranged T-LBLs is poor. It has recently been described that *NOTCH1* intronic single nucleotide variants (SNVs) and *NOTCH1* intragenic losses were also associated with an inferior event-free and overall survival,[31] further substantiating that distinct genetic aberrations in *NOTCH1* have a different impact on outcome. The T-LBL patients with high-risk *NOTCH1* aberrations will probably need intensified or alternative treatment strategies. A second consequence of our findings may be that



***NOTCH1*-fusion**							
At Risk	6	4	3	2	1	1	1
Events	0	2	4	4	4	4	4

Other							
At Risk	23	22	21	15	12	5	3
Events	0	0	0	0	0	0	0

FIGURE 5 Cumulative incidence plot of events reveals a significant higher cumulative incidence of relapse in *NOTCH1*-rearranged cases compared to the rest of the T-LBL cohort (3 years) ($p < 0.001$). The *p*-value is estimated using Gray's test. Four relapses occurred in the *NOTCH1*-rearranged cohort. No relapses occurred in the cohort. In both cohorts, one other event occurred, which was a therapy-related-AML in the *NOTCH1*-rearranged cohort and death due to pancreatitis complicated by a septic shock in the other cohort.

when *NOTCH1* gene fusions are recognized as a separate high-risk group, the survival characteristics of the remaining group of T-LBL patients with unknown molecular genetics improves and may benefit from less intensified treatment.

The highly elevated blood CCL17 levels were exclusively observed in all T-LBL patients with a *NOTCH1*-rearrangement, even though almost all T-LBL patients had an enlarged mediastinum. Elevated CCL17 levels may therefore serve as an important biomarker to assist in the diagnosis of this high-risk group at diagnosis. Moreover, there might be a possibility that CCL17 levels could be used during follow-up as well, but these findings need to be validated in larger cohorts. In classical Hodgkin lymphoma, It has been suggested that inhibiting CCL17, produced by the Reed-Sternberg cells, may have therapeutic consequences in classical Hodgkin lymphoma as inhibiting CCL17 can decrease the recruitment of T-cells, thereby affecting the supporting microenvironment.[32] Our data suggest that CCL17 protein levels in the tumor cells of *NOTCH1*-rearranged tumor cells is only slightly increased and rapidly secreted based on immunohistochemistry, while *CLL17* gene expression and blood levels are highly increased. This likely points toward active crosstalk between the tumor cells and the microenvironment.[33] It is therefore intriguing to further explore whether *NOTCH1*-rearranged T-LBLs are dependent on CCL17 expression and whether this would provide opportunities for targeted treatment in a potentially high-risk subgroup of T-LBL. Measuring blood CCL17 levels could also serve as an easily applicable technique to identify high-risk T-LBL patients in low- and middle-income countries with restricted access to next-generation sequencing techniques.

In conclusion, we discovered that, in contrast to T-ALL, *NOTCH1* gene fusions are common in T-LBL and represent a high-risk subtype with an easily applicable biomarker. The discovery of this clinically relevant high-risk T-LBL subgroup offers opportunities to develop intensified and targeted treatment strategies for this subgroup and decrease overtreatment in the remaining group of T-LBL patients.

AUTHOR CONTRIBUTIONS

Roland P. Kuiper, Jan L. C. Loeffen, and Auke Beishuizen designed the study. Emma Kroeze wrote the manuscript and analyzed the data. Michelle M. Kleisman and Lennart A. Kester performed the bioinformatic analyses. Jessica G. C. Buijs-Gladdines performed the wet lab analyses. Edwin Sonneveld provided laboratory supervision. Marijn A. Scheijde-Vermeulen performed the histological analyses. Melanie M. Hagleitner, Friederike A. G. Meyer-Wentrup, and Margreet A. Veening were involved in data curation. Jules P. P. Meijerink acquired the funding.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in EGA at https://ega-archive.org/, reference number EGAS00001007703.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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