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**PAX5-ESRRB** is a recurrent fusion gene in B-cell precursor pediatric acute lymphoblastic leukemia

Genome-wide screening using genotyping microarrays and next-generation sequencing has discovered genomic rearrangements associated with the clinical outcome of pediatric patients with acute lymphoblastic leukemia (ALL). These findings have increased our understanding of pediatric ALL and have contributed to the identification of new molecular markers for diagnosis and risk stratification. Recently, we discovered a novel PAX5-ESRRB fusion transcript by RNA-sequencing in a single patient with B-cell precursor acute lymphoblastic leukemia (BCP ALL) (GEOID: ALL_619) cytogenetically karyotyped with non-recurrent chromosomal aberrations, denoted “other.”

The 5’-partner in this fusion gene is PAX5 [chr9p13], which encodes an essential B-cell transcription factor with a conserved DNA-binding motif known as the paired box. Paired box transcription factors are crucial regulators of tissue development and early cellular differentiation, and alteration of their expression is thought to promote tumorigenesis. Deletions, amplifications and rearrangements involving PAX5 occur in ∼40% of pediatric BCP ALL cases.

The Estrogen-Related Receptor Beta gene (ESRRB) [chr14q24] is a previously unknown 3’-fusion partner of PAX5, which encodes a protein similar to the estrogen receptor. ESRRB is an orphan nuclear receptor with an unknown endogenous ligand. It mediates self-renewal and pluripotency in embryonic stem cells, and is able to reprogram somatic cells to a pluripotent state by upregulating embryonic stem cell-specific genes. Alterations of ESRRB have been implicated in impaired placental development and aberrant metabolic function in mice, however, little is known regarding its role in cancer and it has not been previously associated with ALL.

Using 450k DNA methylation array data (GEO:GSE49031) available for ALL_619, copy number analysis (calculation of LogR from the probe signal intensities) revealed a deletion of chromosomal region 9p (chr9p) and a duplication of 14q (chr14q) corresponding to the PAX5-ESRRB fusion gene breakpoints. This suggested that the fusion resulted from an unbalanced t(9;14) rearrangement. To ascertain whether the PAX5-ESRRB fusion is recurrent in ALL, we screened 664 Nordic pediatric BCP ALL cases from the same dataset for concordant copy number changes on chr9p and chr14q.

### Table 1. Clinical and cytogenetic information for four BCP ALL patients with 9p13 and 14q24 breakpoints.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Karyotype*</th>
<th>WBC*</th>
<th>Age</th>
<th>NOPHO protocol*</th>
<th>Event (time in months)</th>
<th>PAX5-ESRRB breakpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL_619</td>
<td>45,XY,del(7;12)(p11p11)</td>
<td>1.4</td>
<td>16</td>
<td>NOPHO-2000, II</td>
<td>CRI (69.2)</td>
<td>Breakpoint 1</td>
</tr>
<tr>
<td>ALL_51</td>
<td>46,XY</td>
<td>3.0</td>
<td>11</td>
<td>NOPHO-82, IR</td>
<td>CRI (173.9)</td>
<td>Breakpoint 2</td>
</tr>
<tr>
<td>ALL_401</td>
<td>46,XX</td>
<td>16.0</td>
<td>7</td>
<td>NOPHO-2000, II</td>
<td>CRI (97.4)</td>
<td>Breakpoint 2</td>
</tr>
<tr>
<td>ALL_757</td>
<td>47,XY,+5,add(9)(p27),del(r(2)(t(2;20)(p21q12))</td>
<td>1.1</td>
<td>16</td>
<td>NOPHO-2008, SR</td>
<td>CRI (30.9)</td>
<td>Breakpoint 2</td>
</tr>
</tbody>
</table>

CRI: continuous first remission. *Chromosomal aberrations determined at diagnosis by karyotyping. White blood cell (WBC) count at diagnosis (x10^9/L). †Treatment group according to the Nordic Society for Pediatric Hematology and Oncology (NOPHO) 1992-2008 protocols. ‡Treatment group, intermediate; ††Treatment group, intermediate-risk; SR: standard risk.

All four cases harbored homozygous deletions of CDKN2A, including loss of a small region on the non-rearranged chromosome 9 homolog. No other alterations known to be associated with ALL including CRLF2, ETV6, ERG and BCR were identified (Online Supplementary Table S4).

Expression of the PAX5-ESRRB fusion was verified by reverse-transcription polymerase chain reaction (RT-PCR) followed by Sanger sequencing in all cases (Figure 1B; Online Supplementary Figure S3; Online Supplementary Table S2). Fusion of PAX5 exon 8 with ESRRB exon 5 (breakpoint 1) was confirmed in ALL_619, while a fusion between PAX5 exon 5 and ESRRB exon 5 (breakpoint 2) was confirmed in ALL_51, ALL_401 and ALL_757 (Figure 1B; Table 1). Both gene fusion variants were in-frame and predicted to produce a functional PAX5-ESRRB protein. Regardless of the breakpoint, the fusion protein deletions of chr9p and duplications of chr14q, within PAX5 and ESRRB respectively, totaling <1% (4/664) (Table 1). No additional cases were identified after screening 78 patients with cytogenetically unclassified BCP ALL by RNA-sequencing (data not shown).

To validate the findings from the methylation array, DNA from the four ALL cases was subjected to copy number analysis on HumanOmni2.5M SNP arrays (Illumina, Inc). The breakpoints on chr9p and chr14q within PAX5 and ESRRB were confirmed in all four cases, with the corresponding LogR and B-allele frequency values providing evidence of their presence in the dominant clone. The deletion of chr9p included the terminal region of PAX5, while the duplication on the distal region of chr14q suggested an unbalanced translocation on derivative chr9 (Online Supplementary Figures S1 and S2). Although there were indications of an abnormal chr9p in ALL_757, the exact alteration could not be detected by G-banding. Using fluorescent in situ hybridization analysis, a der(9)t(9;14) rearrangement was verified in ALL_619 and ALL_757 (Figure 1A). Besides our earlier study in which we reported PAX5-ESRRB for the first time, a single additional case with der(9)t(9;14)(p13;q24) was found in the literature, but with no information other than the karyotype available. We identified two breakpoints in the PAX5 gene by copy number analysis. One breakpoint was found in intron 8 of PAX5 in ALL_619 (breakpoint 1) and a second in intron 5 of PAX5 in cases ALL_51, ALL_401 and ALL_757 (breakpoint 2). The breakpoint in ESRRB was located in intron 4 in all four cases (Figure 1B). Additionally, both ALL_51 and ALL_757 had a duplication of chromosome 5 and ALL_401 and ALL_757 shared a subclonal duplication of chromosome 13 (Online Supplementary Figures S1 and S2).
retains functionally important domains, such as the paired box domain, the highly conserved octapeptide motif for protein-protein interactions, and the nuclear localization signal of PAX5 (Figure 1C). Retention of the paired box domain is a key feature of all PAX5 fusion proteins, required for binding PAX5 to its target regions. The fused ESRRB protein retains the ligand binding domain of hormone receptors and a zinc finger motif in the DNA binding domain (Figure 1C). Consistent with the fluorescent in situ hybridization analysis demonstrating an unbalanced translocation der(9)t(9;14)PAX5-ESRRB, no evidence for expression of the reciprocal ESRRB-PAX5 fusion gene was found by RNA-sequencing in ALL_619. Gene expression levels of PAX5 and ESRRB in cases with the der(9)t(9;14)PAX5-ESRRB were assessed by RNA-sequencing (only for ALL_619) and by quantitative real-time PCR (qRT-PCR) in all four PAX5-ESRRB cases (Figure 2A,B; Online Supplementary Figure S4; Online Supplementary Table S2). RNA-sequencing data for ESRRB revealed a high expression in ALL_619 compared to that in 41 BCP ALL cases and five normal samples of whole blood sorted CD19⁺ B cells (Figure 2A; Online Supplementary Table S3). The observed ESRRB expression in ALL_619 derived from the retained ESRRB region in the der(9)t(9;14) translocation as seen in the RNA-sequencing data (Online Supplementary Figure S5). Similarly, qRT-PCR analysis of ESRRB in the four PAX5-ESRRB cases showed higher expression of fused ESRRB compared to that in normal CD19⁺ B cells (Figure 2B). Expression of wild-type ESRRB was not detectable by qRT-PCR in any of the PAX5-ESRRB cases or in the CD19⁺ B cells. Collectively, this suggests that expression of fused ESRRB is driven by the translocated PAX5 promoter in cases with the der(9)t(9;14) rearrangement, retaining key functional domains from both proteins.

Next, we used DNA methylation data to establish whether cases with the der(9)t(9;14)PAX5-ESRRB were among those with specific methylation changes. Methylation data from the four PAX5-ESRRB cases was analyzed together with data from 24 additional ALL cases representing six cytogenetic subtypes of BCP ALL: dic(9;20), HeH, t(1;19)TCF3-PBX1, t(12;21)EVT6-RUNX1, t(9;22)BCR-ABL1 and 11q23/MLL (Online Supplementary Table S4). Six additional BCP ALL cases from the same DNA methylation data set known to
harbor PAX5-ELN (n=1), PAX5-JAK2 (n=1), PAX5-ETV6 (n=2) or PAX5-ZCCHC7 (n=2), were also included. Using unsupervised hierarchical clustering across the 2000 most variable CpG sites, the der(9)t(9;14) PAX5-ESRRB cases formed a distinct cluster separated from the other subtypes including the other PAX5 chimeras (Figure 2C). We then interrogated differentially methylated CpG sites (DMC) genome-wide between the PAX5-ESRRB group and the six BCP ALL subtypes. Based on a maximum false discovery rate of 10% and an absolute mean methylation difference >20%, 825 CpG sites annotated to 648 unique genes were differentially methylated (Figure 2D; Online Supplementary Table S5). The majority of the detected DMC (96.8%) were hypomethylated in the der(9)t(9;14) PAX5-ESRRB group compared to the six BCP ALL subtypes (Figure 2D). Similarly, the identified DMC were more hypomethylated in the PAX5-ESRRB group than in PAX5-ELN, PAX5-JAK2 and PAX5-ZCCHC7, which clustered with the other BCP ALL subtypes. The PAX5-ETV6 cases were more hypomethylated and clustered close to the PAX5-ESRRB cases.

Genes containing DMC were enriched to the Wnt/Beta catenin pathway (P value=2.25x10^{-5}), affecting genes associated with leukemia such as CREBBP, LEF1 and TCF7L2 (Online Supplementary Figure S6). Functional annotation of DMC to nearby genes revealed enrichment towards genes involved in hematopoietic functions; furthermore, binding motif analysis revealed an enrichment of the DMC in relation to the leukemia-associated transcription factors EGR1 (P value=3.68x10^{-5}) and EBF1 (P value=6.05x10^{-5}).
motifs (Online Supplementary Figure S7). Taken together, these analyses demonstrate that the der(9)t(9;14) PAX5-ESRRB, like other recurrent groups of BCP ALL, is associated with distinct molecular features.

This is the first study to describe the recurrence of a PAX5 fusion involving the estrogen related receptor ESRRB and its molecular characteristics in ALL. Although the cause of frequent PAX5 associated rearrangements is still unclear, it has become evident that PAX5 chimeras harbor diverse molecular features driven by the 3’ partner gene. BCP ALL with PAX5-JAK2 fusions are known to display a different expression signature compared to other PAX5 fusions, characterized by upregulation of JAK-STAT target genes, suggesting that PAX5 fusions interact not only with PAX5 target genes but also various downstream targets depending on the 3’ partner.

Previously, we have demonstrated the utility of RNA-seq to detect novel fusion genes such as PAX5-ESRRB in childhood ALL. Recently, the recurrence of a novel fusion gene, EP300-ZNF384, was first detected using RNA-seq and subsequently screened for by RT-PCR. By combining RNA-seq with copy number analysis data from high-density single nucleotide polymorphism arrays and Human Methylation 450 Bead Chips, we identified a group of rare cases harboring the same gene fusion within a large ALL cohort, in which screening by RT-PCR would not be feasible. While genotyping arrays can be useful in screening for unbalanced rearrangements, such as the der(9)t(9;14), they are not suitable for the detection of balanced rearrangements.

In conclusion, we describe the recurrent chromosomal rearrangement der(9)t(9;14)(p13.2;q24.3), an unbalanced translocation resulting in an in-frame fusion transcript involving PAX5 and ESRRB in four BCP ALL cases. We show that der(9)t(9;14)PAX5-ESRRB is associated with a distinct hypomethylated pattern compared to other BCP ALL subtypes including other PAX5 fusions analyzed herein. Whether patients with der(9)t(9;14)PAX5-ESRRB share additional biological or clinical features important for diagnosis and therapy warrants further investigation.

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