Clone-specific secondary aberrations are not detected in neonatal blood spots of children with *ETV6-RUNX1*-positive leukemia

With an incidence of approximately 25%, the *ETV6-RUNX1* (ER) gene fusion is the most common primary acquired genetic aberration in childhood B-cell precursor acute lymphoblastic leukemia (ALL). Although its leukemia-initiating role is amply documented by its occurrence in fetal life and its presence in all leukemic cells, this gene fusion is not by itself sufficient to generate clinically overt leukemia.[1-3] It is, however, the essential component that eventually promotes the acquisition of co-operating genomic lesions which then equip the affected cells with their inevitable survival advantage.[4] Investigation of the absolute and relative timing of the appearance of such secondary changes is challenging, and is at present largely deduced from the distribution of affected cells at diagnosis, and occasionally also at relapse.[5] The thorough quantification of particular abnormality patterns with fluorescence *in situ* hybridization (FISH), together with a genome-wide assessment of the mutational spectrum with sensitive sequencing techniques, have thus uncovered a hitherto unanticipated clonal diversity in virtually all types of leukemias investigated so far.[5-8] The results provide a valuable insight into their composition and architecture, as well as non-linear branching models of clonal evolution. The most common secondary aberrations are also viewed as being the most relevant ones. In ER-positive leukemia, they comprise deletions of the non-translocated *ETV6* allele and genes that regulate B-cell development and differentiation (*PAX5*, *VpreB1*), proliferation (*BTG1*) and apoptosis (*BMF*).[4,5,8] The extensive clonal heterogeneity, together with the common occurrence of partially unrelated abnormality patterns in matched diagnosis and relapse samples, stimulate an interest in exploring how, when and in which order they occur, since the answers to these questions are expected to help explain their interaction and the subsequent consequences on the evolutionary behavior and fitness of affected cell populations.

Childhood leukemias in general, and ER-positive leukemias in particular, are exceptionally suited to address these questions because they are initiated early in life and harbor well-defined changes that can be quantified and comparatively analyzed not only in diagnostic and relapse samples, but also in Guthrie card preserved neonatal blood spots and, in rare instances, also in pre-leukemic phases, cord blood and twins. Many studies have thus confirmed that the vast majority of primary changes (including ER) which define particular genetic subtypes of childhood ALL occur already *in utero*.[5,7,9,10]

Based on these prerequisites, we aimed to explore whether secondary lesions might perhaps already be identifiable at birth as well. To investigate this, we used a representative cohort of 10 ER-positive patients whose Guthrie card was available and who had at least one secondary abnormality in their leukemia that could be analyzed. Informed consent for tissue banking and research studies was obtained from the patients' parents in accordance with the Declaration of Helsinki and approved by the local ethics committees.

Breakpoint regions of leukemia-derived secondary lesions were deduced from SNP arrays and identified by multiplex long-range polymerase chain reactions (PCR). The ensuing highly sensitive PCR assays were specifically designed to backtrack 15 distinct lesions in 10 children (Table 1). According to oncogenetic tree modeling, the selected lesions occur early and, as evidenced in relapse cases, also remain stable during the course of the disease.[5] All but one of our assays sufficed for the detection of one mutated in 100,000 wild-type molecules. We also established PCR assays with an analogous sensitivity for the genomic breakpoints of ER in all cases. Despite the fact that we even evaluated two co-existing lesions in half of the patients, we were only able to recover the ER gene fusions in all instances but none of the secondary lesions (Figure 1).

To the best of our knowledge, this is the first systematic effort to backtrack different types of secondary lesions to the neonatal blood of children with ER-positive ALL. We are aware of only one previous similar attempt, which also failed to identify two leukemia-associated *ETV6* deletions in the presence of the fusion gene.[11] Although deletions of the non-translocated *ETV6* allele are the most frequent secondary lesions in *ER*-positive leukemias, we did not consider them for our analysis because of their overall intra-individual heterogeneity and instability at relapse.[5,6]

The apparent lack of secondary aberrations in Guthrie cards at similar levels to the estimated 10-100 *ER* copies contained on one such spot agrees with the prevailing view that they evolve only after birth. This notion was backed-up by observations in leukemias from twins, who share an identical *ER* fusion but harbored different secondary changes.[9,12,13] This is, however, primarily based on circumstantial evidence and, in general, neglects the fact that clonal diversification is a continuous process, which starts supposedly soon after transformation and is, therefore, already instigated during the early expansion phase of *ER*-transformed cell populations. Although we are convinced that our negative results are technically valid, we doubt that they are sufficient to definitely exclude a prenatal origin of secondary lesions. For a variety of reasons they might simply escape their detection at birth, not only because the affected cell populations might still be much too small but also perhaps too diverse to be present in the minute amounts of DNA that can be extracted from Guthrie card spots.[14] The limitations that have to be dealt with, especially in archived blood spot analyses, is further illustrated by an instructive hyperdiploid case in which the clonotypic immunoglobulin gene rearrangement was not identified in Guthrie card-derived DNA despite its presence in cord blood.[10] Moreover, the distinction between

Table 1. Genomic aberrations in ALL cases used for backtracking to birth.

Pt ID	Age at dx, years	Genomic aberration				
		PAX5	*BTG1*	*VpreB1*	*BMF*	*ETV6-RUNX1*
1	1.7	+		+		+
2	2.9	+				+
3	3.3	+				+
4	3.4	+				+
5	3.4	+		+		+
6	4.1		+			+
7	4.6	+			+	+
8	5.3	+		+		+
9	6.1	+	+			+
10	7.3		+			+

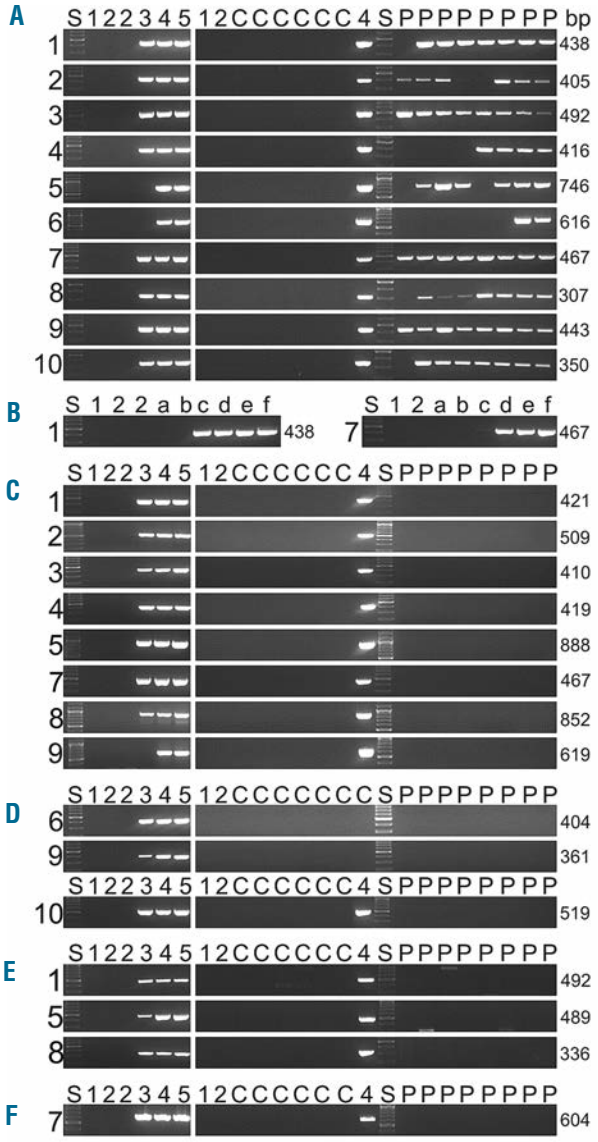

Figure 1. Detection of genomic aberrations in neonatal blood spots. Patient-specific PCR assays for genomic breakpoints of (A, B) *ETV6-RUNX1*, (C) *PAX5*, (D) *BTG1*, (E) *VpreB1* and (F) *BMF* deletions. (All patient-specific breakpoint sequences have been submitted to dbVar Accession Number nstd83). Left part of the figure shows the sensitivity of the assay of the respective patient-specific lesions after a two-round nested PCR in the diagnostic leukemia sample (patients' ID indicated left of the gels). S, size marker (100 bp ladder); lane 1, samples containing PCR mix without DNA; lane 2, PB MNC DNA; lanes 3-5, 10-log dilutions of leukemic DNA in peripheral blood MNC from healthy individuals at 10^{-5}-10^{-3} (representing 0.005 ng - 0.5 ng leukemic DNA in a total amount of 500 ng DNA). Right part of the figure shows the results of the respective specific PCRs in neonatal blood spot DNA, which was extracted from one-third of a Guthrie card blood spot with the QIAamp DNA Blood Mini Kit (Qiagen) and amplified with the REPLI-g whole genome amplification mini kit (Qiagen), as reported previously.[15] Lane 1, sample containing PCR mix without DNA; lane 2, PB MNC DNA; C, neonatal blood spot DNA from healthy newborns; lane 4, 10^{-4} dilution of leukemic DNA for the respective aberration; P, eight aliquots of patients' whole genome amplified neonatal blood spot DNA; bp, size of the PCR products in base pairs. (B) Patient-specific PCR for *ETV6-RUNX1* in 1:2 dilutions of genomic DNA from 1/10 of a neonatal blood spot. Shown are 2 representative examples (numbers left of the gels refer to patients' ID) indicating the presence of estimated 8 and 4 *ETV6-RUNX1*-positive cells in the respective samples. Lanes S, 1 and 2, as in (A); lanes a-e, dilutions from 1:32 (a), 1:16 (b), 1:8 (c), 1:4 (d) and 1:2 (e); lane f, undiluted Guthrie card DNA sample.

primary and secondary events might not always be as clear-cut as in ER-positive leukemias. For instance, we recently detected the *P2RY8-CRLF2* fusion, which must be viewed as a secondary event in the vast majority of affected children, in the Guthrie cards of cases that harbored a major clone at diagnosis but not always at relapse.[15]

Taken together, all these data underline how difficult it is to provide definite answers to the questions as to when clonal diversification starts and becomes a critical component of leukemia development and, therefore, also to which extent various leukemic subclones are already present in neonatal blood, and under which circumstances they can be clearly and reliably detected.

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