immunosorted cells. They suggest that our positive results (and their own “first-round” results) could have been false-positives with reverse transcription–PCR (RT-PCR) contamination. We think this is very unlikely in Mori et al.2

First, we used 4 separate laboratories for extracting RNA, making cDNA, running PCR arrays, and handling our positive leukemia samples or cell lines. Second, we performed parallel quantitative PCR (Q-PCR; TaqMan) screens on all samples and the results were entirely concordant between RT-PCR and Q-PCR. Third, we confirmed positivity (by RT-PCR and Q-PCR) on a separate sample of cells retrieved from the frozen whole cord blood unit. Fourth, and critically, we validated positive samples by fluorescence in situ hybridization (FISH). This latter result cannot be attributed to contamination. Lausten-Thomsen et al also suggest that our FISH data are unconvincing. They say that the image we provided (Figure 5C in Mori et al) is compromised by overlapping signals or subjective interpretation. On this they are plainly wrong. The FISH scoring was very stringent in only including those cells in which there was 1 large and 1 small RUNX1 signal which were well separated. As further examples, we are including 5 other “preleukemic” cells from cord blood that we screened in the same study (Figure 1).2 The designation of these cells as having a ETV6-RUNX1 fusion signal plus a small truncated RUNX1 (= remnant of translocation) plus larger noninvolved RUNX1 (chromosome 21) is unambiguous. This was the case for all 35 cells scored as positive. We also draw to the attention of Lausten-Thomsen et al and readers of Blood that all ETV6-RUNX1+ cells in cord blood were exclusively CD19 and/or CD10 positive (as in ETV6-RUNX1+ ALL itself). In the one cord blood positive for AML1-ETO, the FISH-positive cells were myeloid lineage. FISH screening was also concordant with Q-PCR with levels of ETV6-RUNX1+ cells recorded in both tests as 10−3 to 10−4 in samples scored as positive.

Lausten-Thomsen et al further suggest that our control cord blood sample (ETV6-RUNX1 negative by both RT-PCR and Q-PCR) for FISH was also “positive.” In that control (Table 1 in Mori et al), one CD10-positive cell was scored as ETV6-RUNX1+ of 3199 cells screened giving a crude frequency of < 0.03, some 10-fold less than in our designated “positive” cord samples. Little can be said on the significance of a single cell, though it would have been helpful to have screened by FISH more cord blood that was negative by RT-PCR/Q-PCR. Collectively, the FISH data cannot be explained by artefacts.

If our original data2 were entirely correct, as we firmly believe, then the negative results of Lausten-Thomsen et al do require an explanation. We have none to offer except to point out that in their first round of PCR screening on freshly extracted mRNA, they did in fact find 14 of 1417 positive, that is, as in Mori et al,2 ~ 1% positives. They failed to confirm that any of these 14 samples were positive with frozen mRNA or on mRNA extracted from frozen cells. Perhaps the authors should ask whether, in their frozen material, ETV6-RUNX1 mRNA stability is robust and whether their assay with stored cord blood cells or mRNA really does have the sensitivity implied by their model experiment with ETV6-RUNX1+ leukemic cells (Figure 1 in Lausten-Thomsen et al). Another group has independently produced a similar result to ours with ~ 1%-2% ETV6-RUNX1+–positive samples.4 Nevertheless, it would be helpful if others with access to a large series of cord blood samples would carry out another, carefully controlled, independent screen for ETV6-RUNX1 and perhaps other leukemic fusion genes.

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References


Response

Challenges and pitfalls in the mapping of the natural history of t(12;21)–positive childhood ALL

We thank Zuna et al and Greaves et al for their interest in our recent report in Blood1 and for addressing the conflicting observations regarding levels of ETV6/RUNX1 (previously TEL/AML1)–positive cells in healthy neonates.2 Clarification of this issue is highly important both for the understanding and continued mapping of the natural history of ETV6/RUNX1–positive acute lymphoblastic leukemia (ALL) as well as for potential future disease preventive measures. Unfortunately, the differences are not easily reconciled from the available data and we agree with Greaves et al that further studies are needed.

All 3 groups acknowledge that the ETV6/RUNX1-translocation is a frequent prenatal hit in children who later develop this ALL subtype, and all groups also found ~ 1% ETV6-RUNX1+–positive cord blood samples from healthy neonates in their initial screening rounds. Zuna et al report that 5 of 253 cord blood samples and 1 of 1 spleen sample were positive in the qualitative endpoint ETV6/RUNX1 polymerase chain reaction (PCR) screening, but the published data do not allow estimation of the level of positive cells. However, there is a remarkable ~ 100-fold difference in the estimated frequencies of positive cells between our data (~ 10−5) and perhaps other leukemic fusion genes.
and the data of Mori et al\textsuperscript{2} \((10^{−3}−10^{−4})\). Since all studied populations were of European descent, variation in the levels of \(ETV6/RUNX1\)-positivity of such a magnitude is likely to have technical rather than biologic explanations.

Our screening was based on isolation of mRNA from fresh cord blood samples within 24 hours from birth and on linear standard curves down to a level of \(10^{−5}\). We therefore find it unlikely that we would have failed to identify \(ETV6/RUNX1\)-positive mononuclear cells at levels of \(10^{−3}−10^{−4}\). Zuna et al detected the \(ETV6/RUNX1\) transcript only in the second-run PCR in their initial screening,\textsuperscript{3} which would be compatible with low levels. Meanwhile, Mori et al\textsuperscript{2} only classified sample as positive if levels were above \(10^{−5}\) by quantitative PCR, and although no information was provided regarding samples positive at lower levels, cDNA contamination at levels of \(10^{−3}−10^{−4}\) seems highly unlikely.

All 3 groups attempted to confirm their initial PCR-based screening results by alternative methods. Because our low-level findings in the first screening round precluded confirmatory fluorescence in situ hybridization (FISH) analyses, we used cell sorting of thawed fresh-frozen cord blood samples. A relative increase in the detected level of positivity by quantitative PCR would confirm the true positivity of the first screening. We screened \(10^6\) CD19-positive cells, but without positive results. Greaves et al suggest instability of our frozen samples may have played a role. However, our previous comparison between \(ETV6/RUNX1\) and \(ABL\) housekeeping gene mRNA stability\textsuperscript{4} and the low \(ABL\) cycle threshold values in the present study do not indicate such instability.

Both Zuna et al and Greaves et al used FISH to confirm the PCR results of their initial screening, and the illustrations provided by Zuna et al and Greaves et al are convincing and support their findings. Irrespective, manual screening by FISH of almost 25 000 mononuclear cells (as done by Greaves et al) is difficult, and without appropriate adjustment, which should be based on blinded screening of a similar number of cells classified as \(ETV6/RUNX1\)-negative in their initial screening, the number of FISH-positive cells might be overestimated. Thus, in the original report by Mori et al,\textsuperscript{2} one such “false-positive” cell by FISH was found among 3199 cells, and the difference (1/3198 PCR-negative vs 35/20 901 PCR-positive) is not significant, although it strongly indicates the presence of \(ETV6/RUNX1\)-positive cells in healthy neonates.

A reliable accurate characterization of the occurrence of preleukemic cells in healthy neonates is highly relevant to both the design and execution of future investigations of the natural history of \(ETV6/RUNX1\)-positive ALL. We therefore fully support Greaves et al’s call for further studies of the issue.

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References
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