Impact of IKZF1 deletions on IKZF1 expression and outcome in Philadelphia chromosome negative childhood BCP-ALL. *Reply to* "Incidence and biological significance of IKZF1/Ikaros gene deletions in pediatric Philadelphia chromosome negative and Philadelphia chromosome positive B-cell precursor acute lymphoblastic leukemia"

We thank Dr. Qazi and Dr. Uckun for their letter that focused on the biological relevance of *IKZF1* deletions in pediatric acute lymphoblastic leukemia (ALL), and in particular on the lack of correlation between *IKZF1* deletions and the expected deregulation of *IKZF1* expression. This information clearly supports the findings of our study[1] and our suggestion that the unfavorable prognostic role reported for Ikaros deletion may be due to general genetic instability rather than to Ikaros deletion *per se*, and justifies the hypothesis put forward by Qazi and Uckun that no specific biological effect is associated to Ikaros deletion. This issue is extremely relevant in this field, since a precise definition of the pathogenic mechanisms associated to *IKZF1* deletions is crucial for the ongoing efforts to define new targeted therapies for ALL.

Whilst the authors did infer *IKZF1* gene expression data without knowing the exact correspondence of *IKZF1* deleted and wild-type (wt) cases, to experimentally test the hypothesis, we have analyzed the gene expression data of *IKZF1* wild-type *versus* deleted cases in our own gene expression data set of pediatric ALL patients. From the MILE study[2] and further proprietary unpublished data, array-based gene expression was analyzed for 60 *IKZF1* wild-type and 10 deleted cases belonging to the cohort of 410 patients reported in our recent paper on *IKZF1*-related outcome.[1] Through analysis of the expression levels of two probe sets located in exon 4, no changes in *IKZF1* expression were observed that could be expected from heterozygous deletions of *IKZF1* (Figure 1). It has to be considered, however, that analysis of gene expression data of single probe sets is not the most appropriate method to detect gene expression variances of single genes or isoforms. In particular, probe sets 1565816_at and 1565818_s_at both show overall a very low expression, which largely reduces the probability to detect expression variance, if there is any. Therefore, we performed the analysis of *IKZF1* gene expression by real-time quantitative-PCR (Figure 2). Although there was a tendency for a lower *IKZF1* expression in *IKZF1* deleted cases compared to wt, this difference did not reach statistical significance, because of several outliers in both groups. This supports the hypothesis by Qazi and Uckun that *IKZF1* expression deregulation is very likely not a driving biological player in BCP-ALL cases with intragenic or entire deletions of *IKZF1*, thus raising the question on what is the biological mechanism related to *IKZF1* deletions and prognosis in ALL.

Our data do not support, however, the suggestion of Qazi and Uckun that in pediatric BCP-ALL *IKZF1* deletions occur in a minority of leukemic cells in an oligoclonal heterogeneous population of leukemic B-cell precursors. In our study,[1] having applied the low-sensitivity multiplex ligation-dependent probe amplification (MLPA) technique (which is not able to detect aberrations in minor cell populations), we should in fact have detected only *IKZF1* deletions in major cell subpopulations.[1] Although alternative sensitive tests could detect *IKZF1* subclonal deletions,[3] their prognostic significance should be evaluated.

On the other hand, whether *IKZF1* deletions occur in 'inactive' alleles, although fascinating, must be formally demonstrated in a context different from the canonical one for IG/TR loci,[4] as a new mechanism of escape from major damage, that, to our knowledge, has yet to be described in literature.

Certainly, the hypothesis that *IKZF1* deletion-associated adverse outcome would be a reflection of underlying genomic instability in aggressive leukemic clones, rather than lost or diminished *IKZF1* function caused by *IKZF1* haploinsufficiency, as originally proposed,[5,6] merits further investigation.

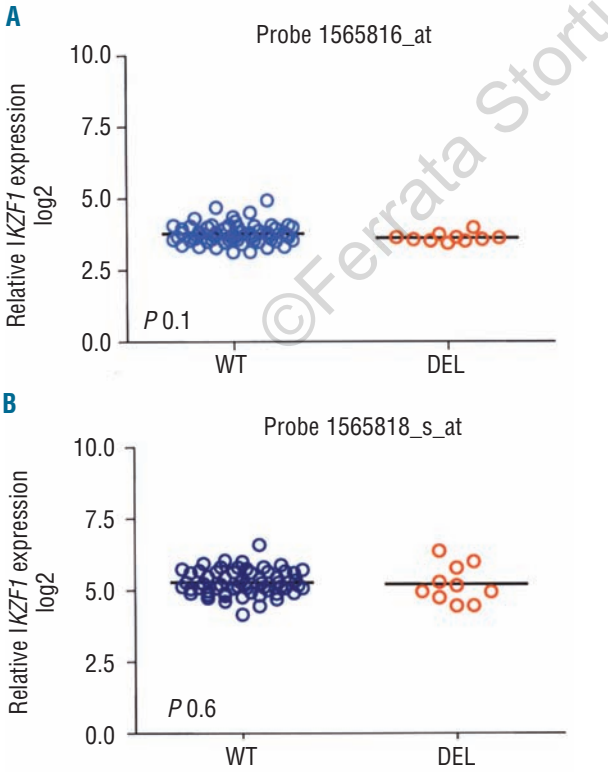

Figure 1. Gene expression values for *IKZF1* probes 1565816_at (A) and 1565818_s_at (B) in patients with (DEL) and without (WT) *IKZF1* deletions.

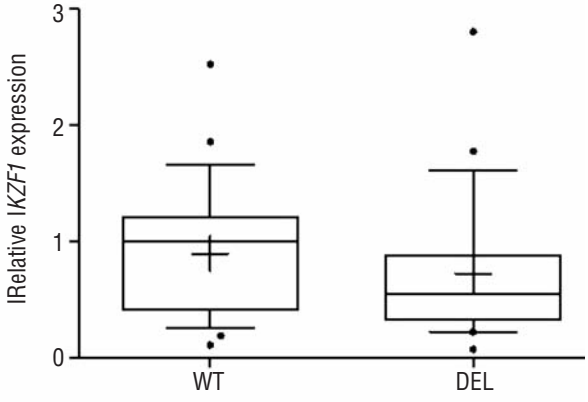

Figure 2. Gene expression values for *IKZF1* by RQ-PCR in patients with (DEL) and without (WT) *IKZF1* deletions. Boxes include 10-90 percentile, mean (internal line) and median (cross) are shown. Dots indicate outliers.

The prognostic significance of *IKZF1* deletions in our study[1] was, indeed, enhanced when additional copy number abnormalities (CNA) involving other genes were present. We specifically looked by MLPA 385 kit at *CDKN2A/B*, PAR region, *PAX5*, *ETV6*, *BTG1*, *RB1* and EBF1 deletions. In our series, out of 54 *IKZF1* deleted cases, 28 carried additional CNA. More precisely, 16 had at least one additional lesion (in 11 of 16 this was the *ETV6* deletion), 6 had 2 and 6 had 3 additional CNA. The incidence of additional CNA was the same in final Standard or Intermediate Risk groups. Out of the only 4 cases at High Risk, one had no CNA additional to *IKZF1* deletion. Nine were the relapses among the 28 *IKZF1* deleted cases with additional CNA *versus* only 3 out of 25 *IKZF1* deleted cases without additional CNA, pointing to a poor outcome when a major genetic instability was observed.[1]

Still, we are also puzzled by having observed no difference in outcome in haploinsufficient patients (with whole *IKZF1* gene deletion) *versus* cases carrying the dominant negative Δ4-7 deletion variant.[1] Interestingly, in Ph$^+$ ALL, which frequently carry *IKZF1* deletions, the haploinsufficient cases have an even worse outcome compared to cases carrying the Δ4-7 deletion variant (A van der Veer *et al*., 2013, *submitted manuscript*). This further observation indicates that either the suspected deleterious (post-transcriptional) effect of the dominant negative Δ4-7 deletion variant is not worse than losing one *IKZF1* copy, or that both aberrations are epiphenomena of genetic instability. It is also interesting to observe that *IKZF1* deletions and other CNA are over-represented in the so-called 'Ph-like' subgroup of BCP-ALL cases with gene expression signatures similar to Ph$^+$ ALL without the presence of the t(9;22) translocation.[6-8] Thus, it remains difficult to separate the contribution of single gene aberrations from a cumulative effect of several aberrations on the biology of leukemic cells, as reflected by a common gene expression signature.

In any case, if the hypothesis of *IKZF1* deletions in inactive alleles holds true, and/or assuming that the *IKZF1* deletion is only an epiphenomenon of the accumulation of CNA, it still remains to be demonstrated that other CNA occur in active alleles, and altogether (or specifically some of them) are responsible for the worse outcome as a manifestation of broader chromosomal instability. One interesting candidate to look at is the *CRLF2-P2RY8* fusion, which has been shown to have an independent worse outcome.[9] It would be very instructive to collect cases carrying both *IKZF1* deletions and *CRLF2-P2RY8* fusion and analyze the effect of their combination on outcome and evaluate whether this could be separated from additional CNA.

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