healthy women belonging to 3 age groups: neonates (umbilical cord blood), women 25 to 32 years old (young women group), and women more than 75 years old (elderly women). The frequency of skewed X inactivation in polymorphonuclear cells (PMNs) increased with age: in fact, a cleavage ratio of at least 3.0 was found in 3 of 36 cord blood samples, 5 of 30 young women, and 14 of 31 elderly women. The inactivation patterns found in T lymphocytes were significantly related to those observed in PMNs in both young ($P < .001$) and elderly women ($P < .01$). Based on the above estimates, the probability that the 4 women in our family simply had age-related skewing would be 8 divided by 10 000 ([5 of 30]²), while the probability that skewing was familial is 9992 divided by 10 000. Consequently our conclusion had a strong scientific basis.

Aivado et al suggest that a comparison of leukocyte XCIP with XCIP from other tissues is needed. To define the best control tissue for the interpretation of X chromosome inactivation patterns in hematopoietic cells, we previously analyzed X chromosome inactivation patterns in different peripheral blood cell populations and in hair bulbs from healthy women belonging to different age groups. When PMNs were compared with hair bulbs, no relationship was found with respect to the inactivation ratio ($r = .31, \ P > .05$). There was no difference between young and elderly women in this respect, a cleavage ratio of at least 3.0 in PMNs being associated with a similar value only in about 50% of hair bulb DNA from either young or elderly women.

In summary, findings of our study clearly indicate that the most likely explanation of the above findings is that the proband, despite a markedly congenitally unbalanced X chromosome inactivation in her hematopoietic cells, was able to produce normal amounts of red cells for the first 6 decades of her life, as her daughters and granddaughter do. In the seventh decade she developed acquired skewing, as do about one third of elderly women. She unfortunately further inactivated the parental X chromosome carrying the normal ALAS2 gene, and when nearly all red cell precursors expressed the mutant gene, she became severely anemic.

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References

To the editor:

Prognostic value of $p16^{INK4a}$ gene deletions in pediatric acute lymphoblastic leukemia

Recently, Carter et al\(^1\) reported the prevalence of $p16^{INK4a}$ gene deletions and their prognostic value in a cohort of 45 children with initial acute lymphoblastic leukemia (ALL). Using a real-time quantitative duplex polymerase chain reaction (PCR) assay, the prevalence of homozygous and hemizygous $p16^{INK4a}$ deletions was 25% and 13%, respectively. We performed a similar assay on 125 samples from children with first relapse of ALL and would like to comment on their data.

To date, real-time PCR is not widely used for detecting hemizygous deletions in genomic DNA because concentration differences as small as 0.5-fold have to be detected. These differences become even smaller when nonmalignant cells are present in the sample (up to 25% contaminating cells in the specimens investigated by Carter et al). To allow the unequivocal detection of hemizygous deletions, the assay must be extremely reproducible. In our hands, despite thorough optimization of a real-time PCR assay analyzing target and control genes in separate tubes, the intra- and interassay reproducibility is insufficient. Carter et al have not assessed the reliability of their assay. They defined no experimental rationale to support these values is given. Their mixing experiments were carried out on DNA from cell lines with a $p16^{INK4a}$ homozygous deletion and wild type, respectively, but did not include a cell line hemizygous for a $p16^{INK4a}$ deletion. Values between 0.25 and 0.5, as well as between 0.75 and 1, are difficult to interpret, and there is neither experimental nor theoretical background for the values the authors defined to discriminate hemizygously deleted from homozygously deleted and wild-type genotypes. Allowing for no more than 25% contaminating normal cells, ratios of samples with hemizygous deletions should range from 0.5 to 0.75, while homozygous deletions should be no more than 0.25, and wild-type genotypes should always be 1. Values interpreted as hemizygosity could theoretically represent polyclonality, aneuploidy, a combination of these, or true hemizygosity. Considering methodological limitations and the difficulties in data interpretation, we suggest using the real-time PCR technique only for the detection of homozygous deletion. For a unequivocal diagnosis of hemizygous deletion, fluorescence in situ hybridization (FISH) should be used.

The prognostic importance of $p16^{INK4a}$ deletions in childhood ALL is still controversial. Using multivariate Cox regression analysis for the probability of relapse-free survival on 45 patients, Carter et al found hemizygous and homozygous deletion of $p16^{INK4a}$ to be an independent predictor of poor outcome. These results support a previous report from their laboratory and from Heyman et al.\(^2\) In contrast, 2 studies not cited by the authors on a larger number of patients did not find a prognostic impact of $p16^{INK4a}$ deletions.\(^3,4\) The number of patients Carter et al investigated is too low for the use of multivariate Cox regression analysis with so many variables, and neither did they show any odds ratios of the confounding factors included in their analyses nor did they present data about the association that might exist between $p16^{INK4a}$ loss and relevant prognostic factors. In particular, it would be of interest whether $p16^{INK4a}$ deletion remains of independent prognostic significance within the subgroups of patients with T-cell or B-precursor ALL, respectively.

We have analyzed 125 samples from children with first relapse of ALL for $p16^{INK4a}$ deletions using a real-time quantitative PCR assay with minor differences from the method of Carter et al. The children were treated according to trials ALL-REZ BFM 90 and 96.
of the Berlin-Frankfurt-Münster Relapse Study Group. The prevalence of homozygous \textit{p16\textsuperscript{INK4a}} deletions was found to be 35% (44 of 125). Due to considerations mentioned above, hemizygous deletions were not assessed. Homozygous \textit{p16\textsuperscript{INK4a}} deletions showed a highly significant association with the most important adverse prognostic factors for relapsed ALL, T cell immunophenotype and early occurrence of relapse (less than 6 months after cessation of front-line therapy). Despite this strong correlation with adverse prognostic factors, no independent prognostic impact of \textit{p16\textsuperscript{INK4a}} deletion could be found in our cohort. The probability of event-free survival at 5 years for \textit{p16\textsuperscript{INK4a}} deletion and nondeletion was 0.46 ± 0.08 and 0.43 ± 0.07, respectively.

The prevalence of the homozygous \textit{p16\textsuperscript{INK4a}} deletion in relapsed ALL is not much higher than at first diagnosis, a fact one should expect from a prognostically nonsignificant alteration. With the absence of any prognostic impact of the \textit{p16\textsuperscript{INK4a}} deletion in relapsed ALL, it is unlikely that a prognostic influence of the \textit{p16\textsuperscript{INK4a}} deletion in initial ALL is independent from other confounding variables. To finally clarify this issue, which has generated controversy for nearly 6 years, we agree with Carter et al that prospective evaluation on a larger number of patients with appropriate methods should be carried out.

In conclusion, real-time PCR is a convenient method to rapidly analyze homozygous deletions in a large number of patient samples. Detection of hemizygous deletions with this method is questionable and should be evaluated by FISH. Our data on \textit{p16\textsuperscript{INK4a}} gene deletion analysis in 125 children with first relapse of ALL do not support the hypothesis presented by Carter et al regarding an independent negative prognostic influence in childhood ALL.

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References


Response:

\textit{p16\textsuperscript{INK4a}} gene deletion in pediatric acute lymphoblastic leukemia

Einsiedel et al comment on the prognostic value of the \textit{p16\textsuperscript{INK4a}} gene deletion in pediatric acute lymphoblastic leukemia, with particular reference to the methodology used in our recent publication. The real-time polymerase chain reaction (PCR) was developed in our laboratory to detect deletion of the \textit{p16\textsuperscript{INK4a}} exon 2 gene since the Southern blotting method used in our previous studies does not allow accurate quantitation of gene deletion. The novel method described by us is performed in a multiplex format and is similar to the Southern blotting method but employed in our laboratory to detect deletion of the \textit{p16\textsuperscript{INK4a}} gene since the Southern blotting method used in our previous studies does not allow accurate quantitation of gene deletion. The real-time polymerase chain reaction (PCR) was developed in our laboratory to detect deletion of the \textit{p16\textsuperscript{INK4a}} gene since the Southern blotting method used in our previous studies does not allow accurate quantitation of gene deletion. To finally clarify this issue, which has generated controversy for nearly 6 years, we agree with Carter et al that prospective evaluation on a larger number of patients with appropriate methods should be carried out.

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We elected to conduct the mixing experiment using 2 cell lines, rather than a cell line showing hemizygous deletions. Apart from the fact that we do not have access to a line showing this particular feature, such a cell line may contain submicroscopic lesions in \textit{p16} not detected by cytogenetics. The mixing experiment provides much more information as it allows titration over the entire range, from 0 to 100 percent. This is of critical importance as it was necessary to focus on the range between 0% and 25% to assess the suitability of the test for patient specimens containing normal cells. The ratios to determine the \textit{p16} genotype of the patient specimens was based on the reproducibility of the assay (see above), and we opted to use conservative values of 0.4 and 0.8. Most importantly, the patient specimens showed a clear triphasic distribution, consistent with discrete populations having G/G, G/D, or D/D alleles.

Einsiedel et al were not able to establish a technique as accurate and reproducible as ours, which may be due to the instrument used or/to the fact that test and reference genes were not measured in a multiplex reaction. Our results on many standard calibration curves showed that the conditions optimized for multiplexing pass the test for the comparative efficiency test, which means that they should allow detection and comparison of the test gene and reference gene in separate tubes. Although we did not expect to achieve the required accuracy to conduct the analysis in separate tubes, we verified whether our conditions would be suitable. Indeed, the results confirmed this to be the case, but as expected, the accuracy in repeat tests is not as high as by the multiplexing method.

As stated in our paper, we intended to confirm the status of the G/D specimens by using an independent technique. Due to lack of material, it was not possible to conduct fluorescence in situ
hybridization studies. Similarly we refer to the controversy regarding p16 deletion as a prognostic factor in pediatric ALL. Rather than quoting many individual publications, we referenced the reviews by Drexler et al. and by Tsihlias et al. which contain all relevant publications. Moreover, as stated in our paper, we agree with Einsiedel et al regarding the need for a larger study to assess the significance of p16 deletion as a prognostic marker, and such a study is currently in progress in our laboratory.

In order to determine the frequency of p16 deletion at relapse, we studied patients from whom we obtained diagnosis and relapse bone marrow specimens. The data showed the rate to be much higher at the time of relapse, which is in agreement with a study by Maloney et al.

We are intrigued by the motivation underlying the statement “The number of patients Carter et al investigated is too low for the use of multivariate Cox regression analysis with so many variables, and neither did they show any odds ratios of the confounding factors included in their analyses nor did they present data about the association that might exist between p16INK4A loss and relevant prognostic factors.” The first part is not substantiated; we would strongly recommend that personal beliefs about the theoretical validity of an analysis might usefully be accompanied by an explanation of the theoretical basis of those beliefs. There are plenty of unique failure times to ensure that the number of risk sets underpinning the generation of the partial log likelihood in this case is adequate to permit the number of parameters we use in our analysis to be estimated. Obviously the data set is relatively small, but that is why we quote confidence intervals and why we state that further investigation is essential. As is almost always the case, severe space limitations prevented us from including (1) the results of formal model checking (completeness of linear predictor; analysis of Martingale residuals; checks of leverage and influence), which showed that our primary models fitted well, or (2) the associations between baseline potentially confounding covariates and outcome and between the covariates and p16INK4A loss. We happen to agree that such data ought to be provided, and they were in fact included in earlier longer versions of the paper, but the reality is that requirements for radical abridgment ultimately meant that they had to be removed. It is of relevance to note that, had these results been reported, it would not in any way have changed the conclusions of the paper. In essence, we agree with much of what Einsiedel et al write, and in particular are delighted that they support our call for further research. There is no question that the differing results from the various studies to which we and they refer are intriguing and need to be properly understood. In particular, it would be of interest whether p16INK4A deletion remains of independent prognostic significance within the subgroups of patients with T-cell or B-precursor ALL, respectively.


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