Interpretation of X-Chromosome Inactivation Patterns

To the Editor:

We wish to comment on the recent paper by Fey et al using the M27β probe to detect clonality and X-chromosome inactivation patterns in hematopoietic cell populations. They point out that the interpretation of the results in individual cases can be highly problematic, and we are in full agreement with this view. However, we would take issue with three points raised by these authors. Firstly, they say that ‘M27β discriminates less reliably between active and inactive alleles than phosphoglycerate kinase (PGK) and hypoxanthine phosphoribosyl transferase (HPRT)’ and suggest that ‘Partial methylation of inactive X chromosomes... probably contributes to the apparent higher ascertainment of skewing in blood leukocytes [at the DXS255 locus].’ However, the failure to completely digest either allele with HpaII in hematologically normal or leukemic cell populations can be largely overcome by the use of HhaI, and the results correlate well with those obtained using HpaII. The frequent skewing of X-chromosome inactivation patterns with M27β in normal blood cells is also seen as frequently with PGK and HPRT. Fey et al refer to a major lack of concordance between M27β and either PGK or HPRT in 5 of 48 samples, but in 3 of 5 samples, the excess skewing was not observed in the M27β analysis, but was observed when using PGK. In our studies, we observed good concordance between M27β and PGK or HPRT in 46 hematologically normal leukocyte samples (provided that all digested M27β bands were included). Thus, we believe that the skewing of allele digestion with methylation-sensitive enzymes in normal leukocytes is not a methodologic artefact with M27β, but a true reflection of X-chromosome inactivation.

Secondly, they imply that their data is not compatible with the Lyon hypothesis and propose an alternative model. We do not believe this is necessary or helpful. From the Lyon hypothesis, one would predict that if random X-chromosome inactivation occurred at a time when the hematopoietic stem cell pool was small, then skewing would be seen in a significant number of individuals (Fig 1, Table 1). The lesser degree of skewed X-chromosome inactivation patterns in other tissues found by Fey et al and ourselves may then reflect a larger stem cell pool size for those tissues at the time of X-chromosome inactivation. This may mean that commitment to hematopoiesis has a restricted cell distribution in the embryo at the time of inactivation; however, it is worth noting that in the mouse, X-chromosome inactivation is not complete in all tissues at the same time, which will similarly influence the patterns obtained.

Thirdly, Fey et al refer to skewed patterns as nonrandom, but we believe that it is precisely because X-chromosome inactivation is random and occurs in a small stem cell pool that skewing occurs. If, in an individual with randomly skewed X-chromosome inactivation, a monoclonal tumor arises from a cell with the minor allele active, and if the tumor population is less than 90% pure, then X-chromosome inactivation ratios well below 10 can be seen. It can be particularly difficult to ensure such tumor purity in lymphoma samples where reactive lymphocytes are frequent. Consequently, suitable controls of normal tissue homologous to the tumor tissue of interest are not only a way around this problem, as they themselves point out, but are necessary to interpret X-chromosome inactivation patterns in individual cases. Fey et al have observed a greater degree of extreme skewing in leukocytes from normal individuals over the age of 75 years. In our studies, 4 of 12 (33%) females over the age of 75 had allelic cleavage ratios (ACR) >10 in comparison with 4 of 73 (6%) between 20 and 58 years of age. However, it should be noted that in all four elderly individuals there was extreme skewing of the X-chromosome inactivation pattern in T cells as well as granu-
lococytes, and nonhematopoietic tissues from 2 of these 4, either muscle or skin and muscle, were also found to have imbalanced X-chromosome inactivation patterns. We believe that further studies in the elderly are warranted. Even if a higher degree of skewing is confirmed, this might represent selection of only a few clones and does not mitigate against the Lyon hypothesis.

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Table 1.

<table>
<thead>
<tr>
<th>No. of Stem Cells at Time of X-Chromosome Inactivation</th>
<th>Expected Proportions of Patients With Skewed Patterns</th>
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<tbody>
<tr>
<td></td>
<td>≥75% or ≤25% of Digested Alleles (ACR &gt;3)</td>
</tr>
<tr>
<td></td>
<td>≥91% or ≤9% of Digested Alleles (ACR &lt;10)</td>
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<tr>
<td>1</td>
<td>100</td>
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<tr>
<td>4</td>
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<td>5</td>
</tr>
<tr>
<td>32</td>
<td>0.412</td>
</tr>
</tbody>
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REFERENCES

2. Gale RE, Linch DC: Investigation of methylation at HhaI sites using the hypervariable probe M27β allows improved clonal analysis in myeloid leukaemia and demonstrates differences in methylation between leukaemic and remission samples. Leukemia 8:190, 1994

Response

We would like to thank Dr. Belmont as well as Drs. Gale and Linch for their interesting comments on our work on clonality and M27β X-inactivation patterns in hematology. Dr. Belmont suggests that our way of expressing the results of M27β X-inactivation analysis by calculating allelic cleavage ratios would lead to a distortion of allelic cleavage ratio (ACR) values towards very large numbers as allelic contribution gets increasingly skewed. For practical reasons we have set the limits of the ACRs at 1 (lower end representing random X inactivation) and at 100 (upper end representing 100% unilateral use of one X chromosome) as outlined in Fig 2 of our paper. As a corollary, an extreme distortion of the results would be avoided. However, data compiled in this way were not normally distributed, and hence, nonparametric tests were chosen for statistical analysis. We share Dr. Belmont’s concern that our observations seem to undermine the correlation between X-chromosome activation status and particular methylation patterns at the M27β locus. His find-
ings of mismatches between results of M27β analysis and clonal analysis using the highly informative androgen receptor locus (HAR) are of particular interest in this respect. We do not as yet have full results of HAR–X-inactivation analysis in our cases. However, our own preliminary data suggest that indeed correlation between clonal HAR and M27β analyses might not be consistent in all cases (unpublished results). When comparing various approaches to study clonal X inactivation, we find it difficult to judge at this stage whether data obtained with M27β X-inactivation analysis are more likely to be inaccurate than results obtained with the phosphoglycerate kinase and the hypoxanthine phosphoribosyl transferase gene polymorphisms, the HAR marker, or the glucose-6-phosphate-dehydrogenase protein variants. Unfortunately, the gold standard reflecting the true X-inactivation pattern of a particular case has not been defined. Therefore, we would certainly agree with Dr Belmont’s warning, which is also expressed in our paper, that results of M27β clonality assessment should be interpreted with great caution in hematologic or any other samples if no homologous normal tissue control is available, because extreme skewing might be constitutional rather than reflecting the clonal neoplastic nature of a cell population. Unfortunately, such control material is rarely if ever at hand in hematologic cases. We have offered a few speculative explanations in our paper as to why skewed X inactivation might be more frequent at the M27β locus than at others, and Dr Belmont has thoughtfully extended our discussion on this interesting point. Although we cannot offer any formal proof, we still favor the hypothesis of a clonal succession model in hematopoiesis to explain age-related skewing in our patient population and we believe that Dr Belmont’s discussion of our findings may be taken as favoring the same basic idea.

Drs Gale and Linch suggest that the problem of discriminating between active and inactive alleles at M27β may be overcome by using Hha I instead of Hpa II. We are grateful for this helpful comment and we are indeed currently testing our series of samples with Hha I. In a second comment, they point out that the smaller the number of hematopoietic stem cells at the time of X inactivation, the higher the chances are for observing constitutionally skewed X inactivation in blood cells. If, for example, hematopoiesis would depend on a single stem cell, severe skewing would be seen not only in a clonal leukaemia from such an individual but equally in her normal leukocyte DNA. Assessment of clonality in such patients would obviously be problematic. Similarly, the degree of constitutive skewing in hematologic cell populations would increase over time if, with advancing patient age, hematopoiesis would depend on fewer and fewer actively dividing stem cells producing mature blood cells. Our data would support this notion. However, there is no way to determine in a given normal individual whether skewing at the M27β locus is caused by small size of the original hematopoietic stem cell pool present at the time of lyonization or whether a clonal succession model might be responsible. Based on our observation of an increasing rate of skewing with advancing age of the population under investigation, we believe that both ideas must be considered. However, it was never our intention, nor is it our current view that our data should be read as overthrowing Dr Mary Lyon’s X-inactivation hypothesis, and we feel that this should appear clearly from our model shown in Fig 4. We were very interested to note that in their small series of elderly women, Gale et al. were able to confirm our finding of an increased rate of severe skewing in blood leukocytes. We absolutely agree with their point that this phenomenon would warrant further investigation and we have never pretended that this finding, if confirmed, would mitigate in any way against the Lyon hypothesis.

In summary, we believe that although the M27β assay is very useful to assess clonality in solid tumors, its limitations for this purpose in hematology are such that its use should be discouraged.

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REFERENCES


Interpretation of X-chromosome inactivation patterns [letter; comment]

RE Gale and DC Linch