development.\textsuperscript{1} Weiss et al reported similar findings in their study based on samples from a US military serum repository.\textsuperscript{9} Thus, as pointed out in our prospective study,\textsuperscript{1} important tasks for the future are to develop individualized follow-up and intervention approaches for MGUS patients. We are undertaking further clinical and laboratory studies to provide insights on the pathogenesis of MGUS, racial disparities in the incidence of MGUS and MM, and to understand the cellular and microenvironmental events that are result in the transformation of MGUS to MM or related malignant diseases. Clinical trials investigating preventive therapy for high-risk patients with MGUS are also under way.

\textbf{References}


\textbf{To the editor:}

\textbf{Does HUMARA assay for assessment of clonal hematopoiesis have shortcomings?}

Determination of clonality based on assays of inactivation of genes encoded on the X chromosome has provided important insights into the origins of neoplastic diseases. The following characteristics make a clonality assay informative: (1) the gene being assayed must undergo X inactivation such that only one allele is expressed in a somatic cell in females; (2) the gene of interest should be sufficiently polymorphic so as to be informative in a reasonably high proportion of the population; (3) the assay should be quantitative because skewing of X inactivation is a normal biologic process determined by the proportion of cells with an active paternal or maternal X chromosome that occurs randomly during embryogenesis; and (4) the assay should be sufficiently robust so that accurate determinations applicable to a variety of tissues can be made at various X chromosome loci.\textsuperscript{1} The human androgen receptor gene (HUMARA) assay has been widely used to establish clonality; however, we and others had concerns that the assay might produce spurious results.\textsuperscript{1,2} Several groups have previously reported that the HUMARA assay is not suitable for elderly females since monoallelic X-chromosome methylation or extreme skewing is detected in a significant proportion of them, suggesting clonal or oligoclonal hematopoiesis.\textsuperscript{3-6} The conclusion that hematopoiesis is monoclonal in approximately one-third of healthy elderly women seems biologically untenable. The HUMARA assay is based on indirect determination of the methylated state of 2 CCGG sites neighboring a polymorphic CAG repeat, by the \textit{HpaII} methylation sensitive restriction endonuclease, followed by amplification of the undigested methylated DNA by PCR and size fractionation on a capillary electrophoresis system. Inactive X-chromosome allelic ratios are estimated using the area under the peak of PCR amplified size fractionated alleles, which vary, based on the number of CAG repeats. Because of the baroque nature of the HUMARA assay that might result in artifactual findings, we have developed an unambiguous direct assay to measure allele-specific expression of 5 genes, subject to X-inactivation, based on quantization of transcribed single nucleotide exonic polymorphisms.\textsuperscript{1,2,7,8}

In our recent paper in the October 15, 2008 issue of \textit{Blood},\textsuperscript{9} we studied 40 healthy elderly female volunteers (range, 65-92 years; mean, 81.3 years; median, 82 years). These women were carefully screened for a lack of any acute or chronic disease and had normal blood counts. We analyzed their peripheral blood cells using a novel quantitative transcriptional assay (qTCA) based on determination of allele-specific ratios of the 5 X-chromosome genes. As reported,\textsuperscript{9} these assays distinguish each polymorphic SNP allele by more than 13 PCR cycles.\textsuperscript{9,10} We did not find any indication of clonal or oligoclonal hematopoiesis, and transcribed allelic ratios were comparable with those of younger women. However, when we analyzed granulocyte DNA from the same subjects using the HUMARA assay, we found a significant proportion with monoallelic locus and X-chromosome allelic usage. Notably, the mean age of their elderly cohort was only 71 years. We believe these contradictory results may be explained as follows:

(1) The “suppressive PCR” assay described in Busque’s paper does not appear to be the same, since they did not perform the qTCA as described in our paper.\textsuperscript{9} The PCR reagents and conditions as described by Busque et al (platinum-Taq-SYBR-super-mix and a low annealing temperature 45°C, primer Tm = 60°C) result in increased amplification of the wrong allele and thus poor allelic discrimination, and decreased specificity.\textsuperscript{12} The number of PCR cycles required to discriminate both IDS alleles using their
The general population when quantitative TCA methods are used. (2) Busque and colleagues used only one X-chromosome gene subject to inactivation, not 5 unlinked X-chromosome genes showing concordant allele-specific transcribed ratios within, and between cell lineages for any one subject as reported by us. Our assay targets 5 genes that are subject to X-inactivation, and we assay multiple tissues (ie, reticulocytes, platelets, and granulocytes). (3) Quantitation of allele-specific X-chromosome inactivation as measured by the HUMARA assay methylation PCR assay for polymorphic repeats cannot be quantitative, since alleles of different sizes are amplified with different efficiencies. Results from this assay are subject to incomplete HpaII restriction endonuclease digestion and uneven PCR amplification efficiency due to different sized alleles, as well as the presence of shadow banding on the chromatogram, which further obscures data analysis. (4) In the study by Busque and colleagues, in all 100 women from elderly and young cohorts combined (mean age, 64 years) similar skewing incidences (40% ± 2%) were noted by all methods: HUMARA, TaqMan-SNP assay, and TCA. (Only 67 samples, not 100, were tested by TCA). Given the fact that skewing rates in the subgroup of elderly women were similar to the entire cohort, it is likely that the nonelderly cohort had similar skewing (data not described). This is contrary to our prior report of nonskewed allele usage ratios in more than 200 healthy women, indicating skewing is a rare phenomenon in the general population when quantitative TCA methods are used. (5) Most importantly, Busque and colleagues did not perform a crucial experiment. One has to demonstrate that skewed methylation of the HUMARA locus is concordant with its monoallelic expression. We are currently analyzing the intriguing phenomenon regarding monoallelic methylation of the X-chromosome HUMARA exon 1 locus.

Response

To be skewed or not to be? This is no longer the question

In their letter to the editor, Dr Prchal’s group raises several issues. We agree with some of these issues and respectfully disagree with others.

First, the Tm for the 3 IDS primers used by both our groups varies between 51.6°C and 52.9°C (not 60°C). Using annealing temperatures between 45°C and 55°C, we observed identical Ct values and excellent discrimination between IDS alleles, whereas an annealing temperature of 60°C resulted in less efficient amplification. Furthermore, we obtained identical results using the carboxyfluorescein-labeled IDS-specific probe (used by Swierczek et al) and SYBR green. Thus, as reported, the quantitative allele-specific polymerase chain reaction transcription assay (TCA) that we used was not identical, but very similar, to that used by Swierczek et al.

The incidence of skewing doubles from birth to age 30, and reaches 40% in the blood cells of 60-year-old women. Our cohort was composed of relatively old subjects (mean age of subjects < 65 years was 59 years), hence our high incidence of skewing. Had we used the 80:20 criteria of skewing used by Swierczek et al, skewing incidences would have been 29%, 31%, and 31% using HUMARA, quantitative TaqMan single nucleotide polymorphism TCA, and allele-specific polymerase chain reaction TCA, respectively. The skewing incidence observed by Swierczek et al using HUMARA was similar, suggesting that groups in this age range are comparable.

The fundamental issue is whether the skewing observed in blood cells of elderly women is an artifact caused by acquired methylation...
Does HUMARA assay for assessment of clonal hematopoiesis have shortcomings?

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