Clonal Studies in the Myelodysplastic Syndrome Using X-Linked Restriction Fragment Length Polymorphisms

By A. Tefferi, Stephen N. Thibodeau, and Lawrence A. Solberg, Jr

We used the X-linked restriction fragment length polymorphism (RFLP)-methylation strategy to study the clonal basis of the myelodysplastic syndrome (MDS) in seven patients. RFLP-methylation analysis was performed on cell populations from bone marrow (BM) aspirates and peripheral blood using probes specific for the hypoxanthine phosphoribosyltransferase (HPRT) or phosphoglycerate kinase (PGK) gene regions. Density gradient centrifugation methods were used to separate granulocytes and monocytes, and T lymphocytes were positively selected by CD2 (a pan-T marker) immunoconjugated magnetic beads. Cell populations from BM aspirates in 6 of the 7 patients with MDS showed a monoclonal pattern of X-inactivation. The neutrophilic and T-lymphocytic cell fractions were analyzed in 4 of the 6 patients, and the monocytic cell fraction in one of these, and all fractions analyzed showed a similar monoclonal pattern. In 2 of the latter 4 patients, both of whom had normal karyotypes, DNA from a skin biopsy showed a polyclonal pattern. Our data suggest that MDS is a clonal disorder, even in the absence of detectable cytogenetic abnormalities, and that the abnormal clone is capable of myeloid, monocytic, and lymphoid differentiation.

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For hematopoietic malignancies involving the myeloid lineage, the only way of assessing clonality has been through cytogenetics or the study of glucose-6-phosphate dehydrogenase (G-6-PD) isoenzyme patterns. Restriction fragment length polymorphism (RFLP)-methylation analysis, a clonal assay based on the same principle as that of G-6-PD isoenzyme analysis, has recently been shown to be of use in clonal studies of several hematologic and nonhematologic disorders.

The assay is based on differences in X chromosome inactivation patterns between normal and neoplastic tissues of female patients. Normal cell populations contain both maternal and paternal X chromosomes randomly inactivated, as they are derived from a normal pool of precursor cells. A neoplastic cell population, on the other hand, is the progeny of a single cell that has suffered a genetic insult, and by virtue of fixed inactivation, all the cells contain either the paternal or the maternal X chromosome in the active or the inactive state. In a polyclonal cell population, the maternal X chromosome is active in half the cells and inactive in the other half, while in a monoclonal cell population, the maternal X chromosome is either active or inactive in all the cells. This difference in the pattern of X chromosome inactivation between normal and neoplastic cell population can be demonstrated, in females heterozygous for a particular RFLP, by a Southern blot analysis using X-linked DNA probes with restriction enzymes capable of differentially cleaving the maternal from the paternal X chromosome on one hand, and the active from the inactive X chromosome on the other. We used such an assay in identifying clonal populations from the bone marrow (BM), peripheral blood, and lymph nodes of patients with hematologic neoplasms, and report on results of clonal studies in the myelodysplastic syndrome (MDS).

Materials and Methods

Patients. After approval by the Mayo Clinic Institutional Review Board, tissue samples from 133 female patients were screened for heterozygosity to an RFLP within both the hypoxanthine phosphoribosyltransferase (HPRT) and the phosphoglycerate kinase (PGK) gene regions. These tissues include bone marrow buffy coats, peripheral blood, and lymph node tissue specimens. All tissues were obtained during clinically indicated procedures or from waste specimens. Seven patients who had the diagnosis of MDS and were also heterozygous to an RFLP within either the HPRT or PGK gene regions.

Peripheral blood cell separation techniques. Thirty to fifty milliliters of whole blood was collected in EDTA-anticoagulated tubes. Density gradient centrifugation methods were used to separate granulocytes and monocytes (Histopaque-1119 and Histopaque-1077; Sigma Diagnostics, St Louis, MO). T lymphocytes were positively selected by CD2 (a pan-T marker) immunoconjugated magnetic beads (Dynabeads M-450, Dynal Inc, Great Neck, NY). The procedures were performed according to the manufacturer’s recommendations. The degree of purity of each cell fraction was greater than 80%, as evaluated by a Wright-stained smear examination.

RFLP-methylation analysis. DNA was extracted from patient specimens using a nucleic acid extractor (Applied Biosystems, Model 340A, Foster City, CA). Screening for an RFLP within the HPRT or the PGK gene regions was performed by digesting 2 to 5 μg of DNA with the restriction enzymes BamHI or BglII, respectively. In patients showing heterozygosity for the corresponding restriction sites, two alleles (24 and 12 kilobases [kb] or 1.7 and 1.3 kb, respectively) carrying the gene regions complementary to the particular DNA probes described are observed.

For the RFLP-methylation analysis, approximately 10 μg of DNA was initially digested with either the restriction enzymes BamHI or PvuII. This resulted in the formation of two alleles, 18 and 12 kb or 1.7 and 1.3 kb, respectively. The digested DNA was then precipitated with 2 vol of ethanol, and two equal aliquots were prepared. One aliquot was further digested with the methyl-sensitive enzyme, HpaII, while the other was left undigested. The HpaII digested and undigested DNA were then size fractionated on a 0.8% agarose gel and transferred to a nylon filter (Micron Separations Inc, Homey Falls, NY) by Southern blotting. The filters were then hybridized to 32P-labeled probes specific for the PGK (pSPF/PGK) or the HPRT (pHPRT-800) gene loci. All the

From the Division of Hematology and Internal Medicine, Division of Laboratory Medicine, Molecular Genetics Laboratory, Mayo Clinic and Mayo Foundation, Rochester, MN.

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Address reprint requests to Lawrence A. Solberg, Jr, MD, PhD, Mayo Clinic and Mayo Foundation, 200 First St, SW, Rochester, MN 55905.

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CLONAL STUDIES IN MYELODYSPLASTIC SYNDROME

Fig 1. RFLP-methylation analysis on specimens from the BM (patients 1 through 7), neutrophilic cell fraction (N) (patients 2, 4, 6, and 7), T-lymphocytic cell fraction (T) (patients 2, 4, 6, and 7), monocytic cell fraction (M) (patient 4), and skin tissue (S) (patients 6 and 7) of patients with myelodysplastic syndrome. In each column, (a) represents bands resulting from digestions with restriction enzymes detecting polymorphisms, and (b) represents the effect of additional digestion with a methyl-sensitive enzyme. The particular patterns are indicated in Table 1.

Results: Restriction enzymes were purchased from New England Biolabs (Beverly, MA), and the conditions of digestion were according to the manufacturer’s recommendations. The DNA probes pHRPT-800 and pSPT/PGK were kindly provided by Dr. B. Vogelstein (Johns Hopkins Medical School, Baltimore, MD).

Defining clonality from the pattern of X-inactivation. The radiographic bands obtained from the HpaII undigested and digested DNA aliquots were compared visually. A monoclonal pattern of X-inactivation was defined as a greater than 80% signal loss by one of the paired alleles accompanied by a less than 40% signal loss by the other. Because of occasional differences in the amount of DNA in the two aliquots containing HpaII digested and undigested DNA, we also considered a pattern monoclonal if there was a greater than 50% disproportionate signal loss between the two alleles after digestion with the methyl-sensitive restriction enzyme. A polyclonal pattern was defined as a near-equivalent degree of signal reduction between the two alleles.

Results

The results of both RFLP-methylation analysis and cytogenetic analysis performed on specimens from seven patients with MDS are shown in Fig 1 and summarized in Table 1. The results indicate the presence of a clonal hemopathy in these patients even in the absence of a detectable karyotypic abnormality. They also suggest a multipotent origin for the abnormal clone, which may involve neutrophils, monocytes, and T lymphocytes.

Discussion

Clonal studies with both G-6-PD isoenzyme analysis and the RFLP-methylation strategy rely on differences in the pattern of X chromosome inactivation between normal and neoplastic tissues. The former approach has been invaluable in defining the clonal nature of several myeloid disorders. Despite its enormous contributions to the understanding of hematopoietic stem cell diseases, the use of G-6-PD isoenzyme analysis has been limited due to the rarity within the indigenous female population of G-6-PD heterozygosity.

The RFLP-methylation strategy analyzes DNA polymorphisms rather than protein polymorphisms and has a wider applicability. In our survey of 133 patients, the incidences of heterozygosity to an RFLP within the HPRT, PGK, or either one or the other gene regions, were 20%, 32%, and 45%, respectively. The corresponding incidences from the study by Vogelstein et al. were similar to our own (29%, 33%, and 53%). In contrast, the incidence of BamHI polymorphism in another survey of 80 females was only 11%. The discrepancy may reflect differences in allele frequencies among different geographic regions. Nevertheless, the assay is potentially applicable for approximately half the North American female population using the aforementioned restriction enzymes and DNA probes.

We found the assay useful in readily identifying clonal excess in the BM, lymph node, and peripheral blood of patients with known clonal hematologic neoplasms. We have used

Table 1. Results of RFLP-Methylation Analysis and Bone Marrow Cytogenetic Studies of Patients With Myelodysplastic Syndrome

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease</th>
<th>Karyotype</th>
<th>BM</th>
<th>Neut</th>
<th>T-Lymph</th>
<th>Mono</th>
<th>Skin</th>
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<td>1</td>
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<td>NN</td>
<td>M</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>2</td>
<td>RA</td>
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<td>M</td>
<td>M</td>
<td>M</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>RA</td>
<td>AN (4/20)</td>
<td>P</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>M</td>
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<td>ND</td>
</tr>
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</tr>
<tr>
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<td>M</td>
<td>M</td>
<td>ND</td>
<td>P</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent ratios of abnormal to total number of metaphases studied.

Abbreviations: RFLP, restriction fragment length polymorphism; BM, bone marrow; Neut, neutrophilic fraction of peripheral blood; T-Lym, T-lymphocyte fraction of peripheral blood; Mono, monocytic fraction of peripheral blood; RA, refractory anemia with ringed sideroblasts; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; NN, normal; AN, both normal and abnormal metaphases present; M, monoclonal; P, polyclonal; ND, not done.
in general, elected to use relatively pure tumor samples because in a separate mixing experiment performed to investigate the effect of normal tissue contamination, we found that in the presence of greater than 50% normal tissue in the test material, we could not appreciate a distinct pattern visually different from that derived from pure normal tissue (Fig 2). This limits the use of the assay to tissues with a relatively pure clonal representation. As such, the assay may not be helpful in detecting minimal residual disease or confirming complete remissions. However, the inability to detect minor clonal populations has its own benefits. For example, in studying specific cell lineages, minor contamination with other cells may not affect the resultant X-inactivation pattern, which is representative of the majority cell population. Similarly, in a tissue sample comprised of different cell types where the percentage of any one cell type does not exceed 50%, the demonstration of a clonal excess implies multilineage clonal involvement.

The assay was particularly helpful in determining the clonal basis of MDS in seven patients. In two of these patients (both with refractory anemia [RA]), a monoclonal pattern of X-inactivation was demonstrated in the BM, granulocytic, and T-lymphocytic components of the peripheral blood, while a polyclonal pattern was obtained from corresponding skin tissue. This suggests a multipotent stem cell origin of the neoplastic clone, which is capable of myeloid and lymphoid differentiation. Furthermore, clonal dominance was indicated in these patients with MDS in the absence of a detectable cytogenetic abnormality, suggesting that the acquisition of a cytogenetic abnormality may not occur with the initial clonal development. This interpretation is supported by the demonstration by G-6-PD isoenzyme analysis of clonal, Ph' negative B lymphocytes in a patient with Ph' positive CGL. However, the recognition of early clonal dominance does not necessarily indicate neoplasia and may be caused by stem cell depletion or damage.

Four of the remaining five patients with MDS showed a monoclonal pattern in their BM samples, and of these two showed a similar monoclonal pattern in the neutrophilic and T-lymphocytic cell fractions, and one also in the monocytic cell fraction. The only polyclonal pattern of X-inactivation was seen in a patient with RA (Table 1, patient 3), despite the presence of a cytogenetically abnormal marker in 4 of 20 analyzed metaphases. The discrepancy is probably due to the inability of the RFLP-methylation assay to detect minor clonal populations, as shown by our mixing experiments (Fig 2). Interestingly, another patient with RA (Table 1, patient 2) also had karyotypic abnormality in 4 of 20 analyzed metaphases, but displayed a monoclonal pattern of X-inactivation. These observations suggest heterogeneity in the clonal composition of a hematopoietic tissue among patients with MDS belonging to the same disease subgroup as defined by the French-American-British (FAB) cooperative group, independent of their karyotypic status. This may indicate different stages of clonal evolution that may have prognostic significance.

The occasional occurrence of extreme lyonization may result in a monoclonal pattern of X-inactivation in the absence of a neoplastic clone. Thus, clonal dominance should be validated, whenever possible, by the demonstration of a polyclonal pattern from a corresponding normal tissue. The use of corresponding normal tissue as an internal control presupposes positive correlations between the mosaic compositions of different tissues within an individual female. This was found to be the case by a previous study of G-6-PD isoenzyme ratio determination in different tissues from single heterozygote females. We have further confirmed this by comparing X-inactivation patterns, using RFLP-methylation analysis, in corresponding normal blood and colon tissue in heterozygous females. This consistency in X-inactivation ratios within an individual female allows corresponding

**Figure 2.** RFLP-methylation analysis on mixtures of DNA from pure monoclonal and pure polyclonal tissues at different proportions in three patients. Monoclonal to polyclonal DNA ratios in columns A, B, C, D, and E were 1:0, 3:1, 1:1, 1:3, and 0:1, respectively. In column A, which represents DNA from a pure monoclonal tissue, there is a disproportionate signal reduction between the two alleles after digestion with the methyl sensitive enzyme (a monoclonal pattern), while in column E, which represents a pure polyclonal tissue, the signal reduction between the two alleles is proportional (a polyclonal pattern). (a) and (b) are per the Fig 1 legend. Quantitative densitometric measurements of signal reduction from (a) to (b) of the two alleles in each column (expressed as percentage ratio) showed a greater than 50% difference for columns A and B, and very little difference in columns D and E.
normal tissues to be used as internal controls in the assessment of clonality in a given cell population.

In conclusion, the results of our clonal studies of patients with MDS parallel those of a recent report, and suggest a multipotent stem cell origin of the neoplastic clone, which may exist in the absence of a detectable cytogenetic abnormality, and may involve neutrophils, T lymphocytes, and monocytes. Furthermore, heterogeneity in the clonal composition of a hematopoietic tissue was demonstrated among patients with RA carrying a similar degree of karyotypic abnormality, and the neoplastic clone was not always dominant over a coexisting normal clone.

REFERENCES

9. Taylor KMeD, Shetta M, Talpaz M, Kantarjian HM, Har-
Clonal studies in the myelodysplastic syndrome using X-linked restriction fragment length polymorphisms

A Tefferi, SN Thibodeau and LA Jr Solberg