

ras* Gene Mutations and Clonal Analysis Using RFLPs of X-chromosome Genes in Myelodysplastic Syndromes

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A. Introduction

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal disorders characterized by quantitative and qualitative abnormalities of hemato-poiesis [1, 2].

Up to 30% of cases eventually develop into acute nonlymphocytic leukemia (ANLL). Previous observations by us and others have demonstrated a frequent (30%) activation of the N-*ras* oncogene in ANLL [3–6].

The involvement of the different cell lineages in MDS has been rather controversial. Various approaches such as isoenzyme studies (G-6-PD polymorphisms), cytogenetic analysis (clonal chromosome abnormalities), and autosomal DNA polymorphism as markers of clonality have been employed to study the stem cell origin of MDS. However, these analyses produced conflicting data and have not clearly identified the stem cell origin of MDS [7–12].

Although no accepted effective treatment exists for MDS, some studies have reported response rates of up to 30% to the administration of low-dose cytarabine (LD-AraC).

However, the mechanism of LD-AraC treatment is not clear. Some data suggest that LD-AraC induces differentiation

[13, 14], whereas other studies demonstrate a cytotoxic effect of LD-AraC [15, 16].

In this report we address the possible role of *ras* in MDS, the identification of its stem cell origin, and the biological action of LD-AraC treatment in MDS.

B. Materials and Methods

I. Patients

Eighty-one cases of primary MDS were screened for *ras* point mutations. The patients were classified according to FAB criteria: there were 19 cases of refractory anemia (RA), nine cases of refractory anemia with ring sideroblasts (RARS), 22 cases of refractory anemia with excess blasts (RAEB), seven cases of refractory anemia with excess blasts in transformation (RAEB-T), and 24 cases of chronic myelomonocytic leukemia (CMML). Peripheral blood or bone marrow samples, as well as a skin biopsy from one patient, were obtained with informed consent.

Moreover, seven of these patients were investigated for clonality, including three patients with RA, two with RARS, one patient with RAEB, and one with CMML.

Lymphocyte counts of the samples were between 43% and 52%. Peripheral blood did not contain any blasts except for that of the RAEB patient, who had 1% blasts.

II. Southern Blot Analysis

High-molecular-weight DNA was prepared from bone marrow or peripheral

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blood cells by standard techniques. Clonal analyses with the X-linked phosphoglycerate kinase (PGK) probe were performed as described by Vogelstein et al. [17].

III. Detection of *ras* Gene Mutations

About 150 ng of genomic DNA were amplified for *ras* sequences by means of the polymerase chain reaction (PCR) [18].

Amplified DNA was spotted onto nylon filters, fixed by UV illumination, and hybridized with an oligomer panel that is able to detect all possible amino acid substitutions at codons 12, 13, and 61 of *N-ras*, *Ki-ras*, and *H-ras* [19].

C. Results

By means of a rapid dot-blot screening procedure based on a combination of *in vitro* amplification of *ras*-specific sequences and hybridization to specific oligonucleotide probes, we analyzed 81 cases of MDS for point mutations at codons 12, 13, and 61 in *N-ras*, *Ki-ras*, and *H-ras*. Mutations of *Ki-ras* and *N-ras* were detected in four cases. One RAEB showed a *Ki-ras* mutation, two CMMoLs exhibited *N-ras* mutations, and one other CMMoL scored positive for a mutation of *Ki-ras* at codon 12. In all four *ras*-positive cases, the normal *ras* allele was also present.

As the positive MDS samples contained less than 5% blasts and our dot-blot technique is able to detect a *ras* point mutation only if more than 10% of the cells are positive, we conclude that hematopoietic cells characterized by a *ras* gene mutation have maintained the potential to differentiate *in vivo*.

In order to determine which cell lineages are involved in MDS we performed cell separation studies of two cases of *ras* mutation-positive CMMoL. Mononuclear cells and granulocytes were separated by standard Ficoll-Hypaque density gradient centrifugation. T- and B-lymphocytes and monocytes were frac-

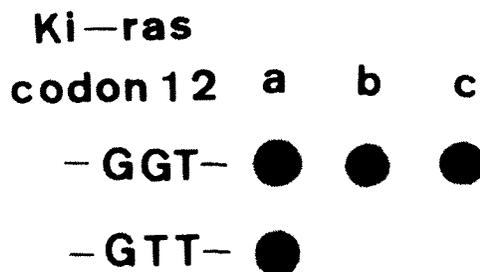


Fig. 1. Disappearance of mutated *Ki-ras* sequence after treatment with low-dose AraC. Five nanograms of amplified DNA from a patient with RAEB was spotted onto a nylon filter and hybridized to oligomers representing the *Ki-ras* wild-type allele (*top row*) and the *Ki-ras* mutated allele (*bottom row*). DNA analysis had shown a mutation of one allele at codon 12, *Ki-ras* substituting valine (*GTT*) for glycine (*GGT*) in peripheral blood cells (*a*) at presentation. Loss of *ras* mutation was observed in peripheral blood (*b*) and bone marrow (*c*) after a second course of LD-AraC

tionated by positive selection with immunomagnetic beads [20]. Thus we could show that the *ras* mutation was present in all four cell fractions, including granulocyte, monocyte, T- and B-lymphocyte lineages ([6]; unpublished results).

Figure 1 shows the analysis of a *ras*-positive case of MDS, a patient suffering from RAEB who was treated with LD-AraC. The wild-type codon 12 *Ki-ras* allele (glycine) was present before as well as after treatment. However, the mutated *ras* allele had disappeared completely after low-dose AraC treatment.

In seven female MDS patients belonging to different FAB types we investigated clonality by X-chromosome inactivation analyses. For this purpose we employed a recently published technique that utilizes the occurrence of DNA polymorphisms at X-linked loci [17]. The active and inactive alleles can be distinguished from each other by a methylation-sensitive endonuclease, as the 5' cytosine methylation pattern of

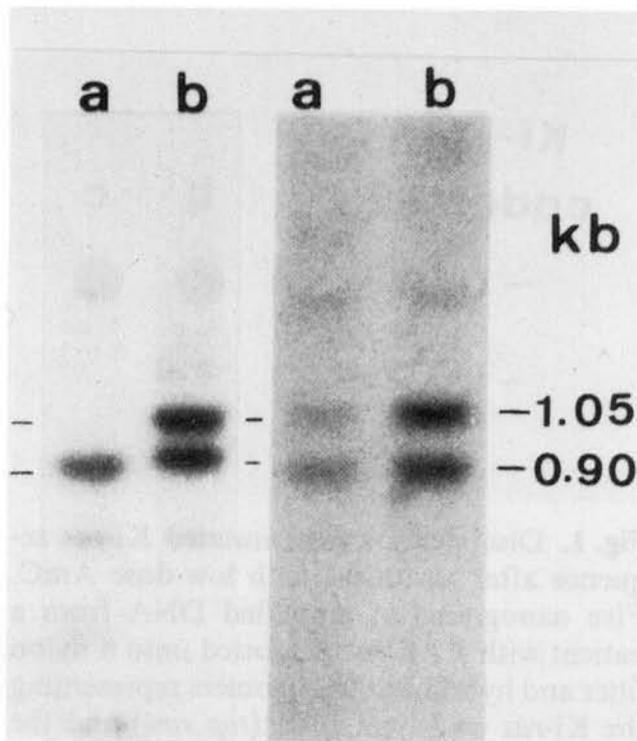


Fig. 2. Clonal analysis using a PGK probe of peripheral blood (*left panel*) and skin fibroblasts (*right panel*) from an RA patient. DNAs were digested with *Bst*XI and *Pst*I to distinguish the maternal and paternal alleles. Subsequently, the DNAs were divided into two equal aliquots; one was not digested further (*lanes b*) and the other was digested with *Hpa*II (*lanes a*) to distinguish between active and inactive X-chromosomes

housekeeping genes is changed after X-chromosome inactivation. All seven MDS patients analyzed showed a monoclonal pattern. Figure 2 shows an example of such an analysis. The right panel represents normal fibroblasts from an MDS patient before and after digestion with the methylation-sensitive enzyme *Hpa*II. Reduction in the intensity of both bands, corresponding to the two different alleles, can be observed after restriction endonuclease *Hpa*II incubation. This is expected in a polyclonal population of fibroblasts in which the X-chromosome is randomly inactivated. In contrast, peripheral blood of an MDS patient analyzed in the same way (*left panel*), shows the complete disappearance of one band (allele) after digestion with *Hpa*II, characteristic for a clonal population of cells.

D. Discussion

In this study we investigated the frequency of *ras* gene mutations in myelodysplastic syndromes. Point mutations in the Ki-*ras* and N-*ras* gene could be detected in about 5% of the cases tested. Recently, Hirai et al. [21] and Liu et al. [22] identified *ras* mutations in three of eight and two of four patients with MDS, respectively. Both articles suggest that *ras* activation might indicate the imminent conversion of an MDS into a frank leukemia. Analysis of our data has not revealed a correlation between the presence of mutated *ras* genes and the development into AML.

The small numbers of cells required for PCR analysis made it possible to study the cell lineage involvement in two MDS patients. We detected involvement of the granulocytic, monocytic, and B- and T-lymphoid lineages in both CMMoL patients tested. Identical results were obtained in our clonality studies.

This investigation revealed a clear clonal pattern in all seven MDS patients tested, and, as the samples comprised between 40% and 50% lymphocytes, it can be indirectly concluded that in these different MDS cases at least, the lymphocytic lineage was involved. The clinical heterogeneity of different MDS cases may question whether this is a common phenomenon. More cases should be studied in order to clarify this point.

Both methods, the clonality analyses based on X-linked RFLPs and the detection method for activated *ras* sequences, were used by us to investigate the action of LD-AraC in the treatment of MDS. The loss of clonality or mutated *ras* sequence in two cases supports the view that even at low doses cytarabine has a cytotoxic effect.

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