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Primitive hematopoietic stem cells show a polyclonal pattern in myelodysplastic syndromes

Francesca Guidetti
Sandra Grazioli
Francesca Capelli
Claudia Marini
Margherita Gallicchio
Daniela De Micheli
Luigi Castello
Pier Paolo Sainaghi
Gian Paolo Fra
Giuseppe Saglio
Gian Carlo Avanzi

A B S T R A C T

Background and Objectives. Clonal hematopoiesis is the hallmark of myelodysplastic syndromes, but the role played by pluripotent stem cells and progenitor cells in these disorders remains unclear.

Design and Methods. Eight female patients with myelodysplastic syndrome were studied. X-chromosome inactivation patterns were analyzed in peripheral blood granulocytes, T-lymphocytes, single colonies originating from bone marrow progenitors and pluripotent stem cells, using the human androgen receptor locus polymorphism assay.

Results. Granulocytes and progenitor cells were monoclonal in 7/8 cases. Immature stem cells showed a non-clonal pattern of X-inactivation and were detectable at diagnosis in the presence of clonal hematopoiesis. T-lymphocyte clonality was heterogeneous.

Interpretation and Conclusions. In myelodysplastic syndromes, hematopoiesis may be dominated by a neoplastic clone by virtue of its biological advantage over a residual polyclonal, probably still normal, population of immature stem cells still able to grow *in vitro*.

Key words: myelodysplastic syndromes, clonality, LTC-IC, HUMARA.

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From the Department of Medical Science, University of Piemonte Orientale Amedeo Avogadro Novara, Italy (FG, SG, FC, CM, LC, PPS, RR, GPF, GCA); Department of Pharmacy and Pharmacology, University of Turin, Italy (MG); Department of Biological and Clinical Science, University of Turin, Italy (DDM, GS).

Correspondence: Gian Carlo Avanzi, MD, Dipartimento di Scienze Mediche, via Solaroli 17, 28100 Novara, Italy. E-mail: avanzi@med.unipmn.it

The myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal disorders of a hematopoietic progenitor cell, characterized by impaired proliferation and maturation of one or several cell lineages in the bone marrow and by pancytopenia in peripheral blood. According to the WHO classification there are five different MDS subgroups with variable clinical courses but, overall, an unfavorable prognosis.[1]

The hallmark of MDS is clonal hematopoiesis.[2] The clonal origin of MDS can be demonstrated by a variety of different techniques, including karyotyping,[3-5] fluorescent *in situ* hybridization (FISH) analysis,[6,7] and detection of gene mutation.[8] However, if genetic changes have occurred after the clonal evolution, cytogenetic analyses and studies of non-specific gene mutations might fail to identify the real clones; moreover, many MDS patients have a normal karyotype. X-chromosome inactivation assays, on the other hand, provide a means of assessing clonality without any requirement for tumor-specific genetic or cytogenetic markers[2,5,8-13] and are potentially applicable to all women. Recent studies have demonstrated that the human androgen-receptor (HUMARA) gene,[14] which has the polymorphic short tandem repeat (STR) CAG near the HpaII recognition site, is useful for clonality analysis[15,16] and that its heterozygosity is higher (20 alleles, 90% heterozygosity) than that of other genes.[14]

It is still a matter of debate whether the primary clonal mutation in MDS arises in the most primitive common hematopoietic stem cell[2,8,9,17] or in a more committed daughter cell,[3-7] although the weight of evidence seems to support the latter hypothesis. Furthermore, the evidence that non-

Table 1. Clinical characteristics of the patients affected by myelodysplastic syndrome enrolled in the study.

N.	Age (years)	WBC ($x10^9$/L)	Hb (g/dL)	PLTS ($x10^9$/L)	BM % Blast	Cytogenetics	Lineages involved	Disease duration (months)	WHO category
1	65	2.10	9.5	64	11	47,XX,+8	3	14	RAEB II
2	70	2.61	8.3	133	16	45,XX, -6, -7, +der(inv6?)	3	8	RAEB II
3	77	5.56	7.4	232	<5	46,XX	1	72	RA
4	80	2.10	8.0	162	<5	failure	2	38	RCMD
5	79	2.04	8.2	44	8	46,XX	3	18	RAEB I
6	81	2.67	8.7	96	6	47,XX,+8	3	16	RAEB I
7	77	3.10	8.3	18	<5	46,XX	3	32	RCMD
8	78	2.30	7.5	26	6	46,XX,del(20q)	3	20	RAEB I

clonal hematopoiesis still persists in the bone marrow of patients with MDS, despite the expansion of the neoplastic clone, is indirect.[18,19] For example, cytokine therapy,[20,21] low-dose cytarabine[8] and, more importantly, intensive chemotherapy[22,23] are all able to induce polyclonal hematopoiesis in MDS patients. Non-clonal myeloid cells can be isolated in untreated MDS patients,[12,18] though, to date, their replicative ability has not been thoroughly investigated. In order to address these questions, we evaluated the clonal nature of granulocytes, lymphocytes, very immature stem cells and progenitor cells in female MDS patients at diagnosis. Clonality studies were performed by analysis of X-chromosome inactivation patterns using the human androgen receptor (HUMARA) locus polymorphism assay.[14]

Design and Methods

Patients' characteristics

Eight female patients, heterozygous for HUMARA alleles, aged 65 to 81 years (mean, 75.9 years) were studied; all had primary MDS, according to the WHO classification.[1] Four female subjects, aged 68 to 78 years (mean, 74 years), affected by non-hematopoietic disease and heterozygous for HUMARA alleles, served as controls. Patients and controls gave informed consent to their participation in the study, which was conducted in strict accordance in the principles of the Declaration of Helsinki. The characteristics of the patients with MDS are summarized in Table 1.

Samples

DNA was extracted from bone marrow, peripheral blood and urine from each patient at diagnosis. A preliminary study on the polymorphism of the HUMARA gene was made to exclude those patients in whom the two alleles could not be separated by standard electrophoresis.

Cell isolation

Bone marrow mononuclear cells (BMMC) and peripheral blood cells were subjected to gradient centrifugation on a Ficoll Hypaque (1.077 g/mL, Pharmacia) cushion at 850 μg for 30 min at room temperature. The low density BMMC were collected in the interface, washed twice with phosphate buffered solution (PBS) and processed for $CD34^+$ and $CD34^+/CD38^-/lin^-$ isolation. The $CD34^+$ cells were purified by immunomagnetic adsorption on MiniMACS separation columns using a CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Milan, Italy). The cell fraction enriched for $CD34^+$ progenitors was subsequently resuspended in Iscove's modified Dulbecco's medium (IMDM)/20% fetal calf serum (FCS) and cells were counted. Likewise, the $CD34^+/CD38^-/lin^-$ cells were purified by immunomagnetic adsorption on MiniMACS separation columns using a $CD34^+/CD38^-/lin^-$ Progenitor Cell Isolation Kit (Miltenyi Biotec). The cell fraction enriched for $CD34^+/CD38^-/lin^-$ stem cells was subsequently resuspended in IMDM/20%FCS and cultured as described below. Polymorphonuclear cells (PMN) were isolated from citrated peripheral blood by standard gelatin sedimentation followed by Ficoll-Hypaque gradient centrifugation. Residual erythrocytes were removed by hypotonic lysis. The PMN cells were resuspended in PBS. The average purity of the final cell suspension was 98%, as assessed by direct light microscopy.

T-lymphocytes were obtained as follows: the layer of light density BMMC was subjected to a panning procedure on a plastic surface coated with CD2 monoclonal antibody (Becton-Dickinson, immunocytometry systems, San José, CA, USA). The T-lymphocytes were resuspended in PBS and tested for purity by staining

with an anti-CD3 FITC-conjugated monoclonal antibody (Becton-Dickinson). The purity was approximately 85%. Transitional epithelium cells were obtained by centrifuging a 50 mL urine sample at 1000 μg for 10 minutes. DNA was extracted from cells recovered in the pellet.

Cell cultures and progenitor cell colony assays

Cultures were set up by seeding 1×10^3 $CD34^+$ enriched cells in 35 mm Petri dishes in IMDM containing 0.9% methylcellulose (StemCell, Vancover, Canada), 20% FCS (Hyclone, Celbio, Milan, Italy), 1% bovine serum albumin (BSA) (Sigma-Aldrich-Fluka, Milan, Italy), 10^{-4}M β-mercaptoethanol (Sigma-Aldrich-Fluka) with 10 ng/mL of recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sandoz, Basel, Switzerland), 10 ng/mL of rh interleukin-3 (IL-3), (Sandoz), and 2 U/dish of rh erythropoietin (R&D System, Oxon, UK). Progenitor cell colonies (CFU-GM, BFU-E) were scored at day 14 for colony formation. Some experiments were also performed using low density BMMC. In these instances, 5×10^4 BMMC were seeded in 35 mm Petri dishes in semisolid medium, as described above, and scored at day 14 for colony formation. Colonies that had grown sufficiently were sucked up by a Pasteur pipette under inverted light microscopy control. Single colonies were separately subjected to DNA extraction.

Long-term culture-initiating cells (LTC-IC) assay

LTC-IC assays were performed using a modification of the method described by Petzer *et al.*[24] $CD34^+$/$CD38/Lin^-$ cells were seeded in IMDM supplemented with 20% FCS, 100 ng/mL stem cell factor (SCF) (R&D system, Milan, Italy), 20 ng/mL thrombopoietin (TPO) (MPL ligand) (Amgen Thousand Oaks, CA, USA), 5% human bone marrow stromal cell culture supernatant (obtained in our laboratory),[25] 100 ng/mL of Flt-3 ligand (R&D system, Milan, Italy), 10 ng/mL of G-CSF (Sandoz), and 10 ng/mL of rh IL-3 (Sandoz).

After four weeks, all cultured cells were washed twice with PBS and seeded to obtain colonies as described above.

DNA extraction and processing

DNA was extracted using standard protocols for human tissues, as described in the NucleoSpin Tissue kit (Macherey-Nagel GmdH, Duren, Germany).

High molecular weight DNA was half digested overnight at 37°C after the addition of 100 U of HhaI and 100 U of HpaII (New England BioLabs, Beverly, MA, USA) for the digestion of unmethylated (or active) alleles as previously described.[26] After overnight incubation, restriction enzymes were inactivated by heating at 95°C for 10 min. For all cell fractions, clonality at the HUMARA locus was further assessed with a nested polymerase chain reaction (PCR) using two sets of primers flanking the trimeric CAG repeats as previously described.[19] PCR conditions were as follows: for both digested and undigested samples, 10 μL of DNA obtained from single colony extraction and 3 μL of DNA obtained from PMN cells or transitional epithelium cells or T-lymphocytes were subjected to 40 cycles (first step) at 60°C and to 30 cycles (second nested step) at 65°C both in PCR Gold buffer II (AmpliTaq Gold, Applied Biosystems, Roche, Branchburgh, NJ USA) including 2.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, 0.3 U AmpliTaq Gold (Applied Biosystems) and 15 pmol/L of each primer in a Perkin Elmer Thermocycler 8500 (Perkin Elmer, Monza, Italy).

Amplification of male T-lymphocytes DNA failed after all restriction enzyme incubation procedures, indicating that restriction enzyme digestion with HpaII and HhaI was complete.

Three microliters of PCR products were loaded on an 8% non-denaturing 19:1 acrylamide/bisacrylamide gel (1×TAE) followed by electrophoresis at 15 W overnight. Gels were stained with ethidium bromide using standard methods. Stained gels were photographed and amplified bands were subjected to density analysis by Gel Doc software (Amersham/Pharmacia Biotech, Cologno Monzese, Italy).

Analysis of results

Clonality analysis involving differences in DNA methylation was performed and quantified with Gel Doc software (Amersham/Pharmacia Biotech). The allele ratio was defined as the ratio between the intensity of PCR product from the two HUMARA alleles in a given sample, before and after digestion with methylation sensitive enzymes (upper allele/lower allele). We used the corrected ratio (CR), obtained by dividing the allele ratio of the digested DNA sample (upper allele/lower allele), divided by the allele ratio of the not digested (ud) sample (ud upper allele/ud lower allele).[27] Clonality of granulocytes, lymphocytes and transitional epithelium cells was defined as CR ≥ 3.00 or ≤ 0.33, which corresponds to the finding of one allele present on the same inactive chromosome in ≥ 75% of cells.[27] The presence of clonality in transitional epithelium cells was interpreted as an excess of skewing and led to a patient being excluded from the study.

We calculated allele ratios between colonies (colony allele ratios, CAR) dividing the number of lanes containing the upper band by the lanes containing the lower band.[16,19] A CAR between 0.50 to 2.00 was interpreted as a polyclonal pattern, while a CAR ≤ 0.33 or ≥ 3.00 was interpreted as a clonal pattern. Values

between those two ranges were considered ambiguous. The minimum number of colonies analyzed was 15 for LTC-IC and 20 for progenitor cells.

Results

Among the 8 patients, 2 were affected by RAEB II, 3 by RAEB I, 2 by RCMD and 1 by RA. CFU-GM and BFU-E were obtained from all patients, and LTC-IC from all except one. The polymorphism pattern of the HUMARA gene was studied in granulocytes, T-lymphocytes, progenitor and immature stem cells colonies. Moreover, a preliminary study of the clonality of urothelium cells was performed to rule out the extreme lyonization that can be seen in the elderly. The HUMARA polymorphism of control tissue revealed a polyclonal pattern in all patients (*data not shown*). This pattern was also observed in all the control subjects (*data not shown*).

Mature cells

In all patients, except one, the corrected ratio of the PMN cells showed a predominant methylation of one allele after restriction enzyme digestion. This is consistent with the presence of a dominant myelodysplastic clone. All the control subjects showed a polyclonal pattern. T- lymphocytes were found to be polyclonal in 50% of patients and in one out of four control subjects. These results are summarized in Table 2.

Table 2. Corrected ratio of mature cells of MDS and control subjects (c). *represents a corrected ratio ≥ patients 3.00 or ≤ 0.33, which was considered indicative of a clonal pattern.

Patient No.	*Granulocytes*	*Lymphocytes*
1	3.28*	3.70*
2	0.26*	0.26*
3	0.82	1.77
4	3.27*	1.62
5	0.30*	1.17
6	3.21*	1.63
7	3.40*	3.73*
8	0.21*	4.30*
C	0.78	3.93*
C	1.00	1.93
C	0.96	0.72
C	1.63	1.22

Progenitor cells

Progenitor cell colonies (CFU-GM and BFU-E) were cultured by plating bone marrow mononuclear cells obtained by density gradient separation. The analysis of the clonality of colonies harvested singularly showed the presence of only one band for each colony indicating the lack of contamination between colonies. Progenitor cell colonies were found to be clonally derived in all but one patient, demonstrating perfect correlation with the PMN clonality pattern. All control subjects showed a polyclonal pattern. These data are shown in Table 3.

Table 3. Colony allele ratios of CFU-GM/BFU-E and LTC-IC. A ratio between 0.50 to 2.00 was interpreted as polyclonal pattern while a ratio ≤ 0.33 or ≥3.00 was interpreted as a clonal pattern (marked with an *). Values between those two ranges were considered ambiguous. The minimum number of colonies analyzed was 15 for LTC-IC and 20 for progenitor cell colonies.

Patient No.	*CFU-GM / BFU-E*	*LTC-IC*
1	4.50*	0.67
2	0.11*	0.87
3	0.82	1.50
4	9.00*	–
5	0.18*	0.50
6	5.00*	1.14
7	5.67*	1.28
8	0.18*	1.67
C	0.54	0.45
C	1.00	1.42
C	0.82	1.12
C	1.22	1.37

Immature stem cells

Bone marrow mononuclear cells were used to isolate the most immature population belonging to the $CD34^+$, $CD38^-$, Lin^-, phenotype. Given the low number of these very immature stem cells, we amplified the $CD34^+$, $CD38^-$, Lin^- cells obtained with Minimacs negative cell sorting, culturing them in a liquid culture system.[24] After four weeks of liquid culture, the cells which had grown were plated to obtain CFU-GM and BFU-E. Singles colonies were then picked up and analyzed in the same way as that used for progenitors cells. As expected, there were few colonies and they were small; nevertheless, there were sufficient cells to perform DNA extraction, enzyme digestion, and PCR amplification. At least fifteen colonies from each patient were analyzed.

As shown in Table 3, the lyonization pattern of LTC-IC at diagnosis was polymorphic in 7 patients, including in two patients with RAEB II; in one patient the LTC-IC colony assay failed. Moreover, in one case we repeated the analysis when leukemic transformation occurred. Surprisingly, we were still able to find a polyclonal pat-

Table 4. Summary of the results of HUMARA gene polymorphism studies in separated cell populations.

Patient N	Diagnosis (WHO)	Pattern of HUMARA polymorphism in			
		PMN cells	T-lymphocytes	CFU-GM/BFU-E	LTC-IC
1	RAEB II	Clonal	Clonal	Clonal	Polyclonal
2	RAEB II	Clonal	Clonal	Clonal	Polyclonal
3	RA	Polyclonal	Polyclonal	Polyclonal	Polyclonal
4	RCMD	Clonal	Polyclonal	Clonal	–
5	RAEB I	Clonal	Polyclonal	Clonal	Polyclonal
6	RAEB I	Clonal	Polyclonal	Clonal	Polyclonal
7	RCMD	Clonal	Clonal	Clonal	Polyclonal
8	RAEB I	Clonal	Clonal	Clonal	Polyclonal

tern in LTC-IC, even in the presence of 50% of blasts in the bone marrow (Figure 1). Table 4 summarizes the clonality pattern of all the cell types analyzed.

Discussion

Our data show that granulocytes and progenitor cells were monoclonal in 7/8 MDS cases, demonstrating that the hematopoiesis in these patients originated from a neoplastic clone. This is in agreement with previously published data.[8-10,12,28] Delforge *et al.* showed clonal maturation in the myeloid, erythroid and megakaryocytic lineages in MDS patients while residual polyclonal hematopoiesis could be detected after complete hematologic remission induced by chemotherapy.[19] Similar results were reported by the same authors in another paper in which they demonstrated that most hematologic remissions in chemotherapy-treated MDS are associated with restoration of polyclonal hematopoiesis.[29]

We unequivocally demonstrated that very immature stem cells not involved in the neoplastic process were present and detectable at diagnosis in MDS in the presence of clonal hematopoiesis. This was evident even in a patient with leukemic transformation (Figure 1). Patient #3 showed a polyclonal pattern in granulocytes, T-lymphocytes, progenitors and immature

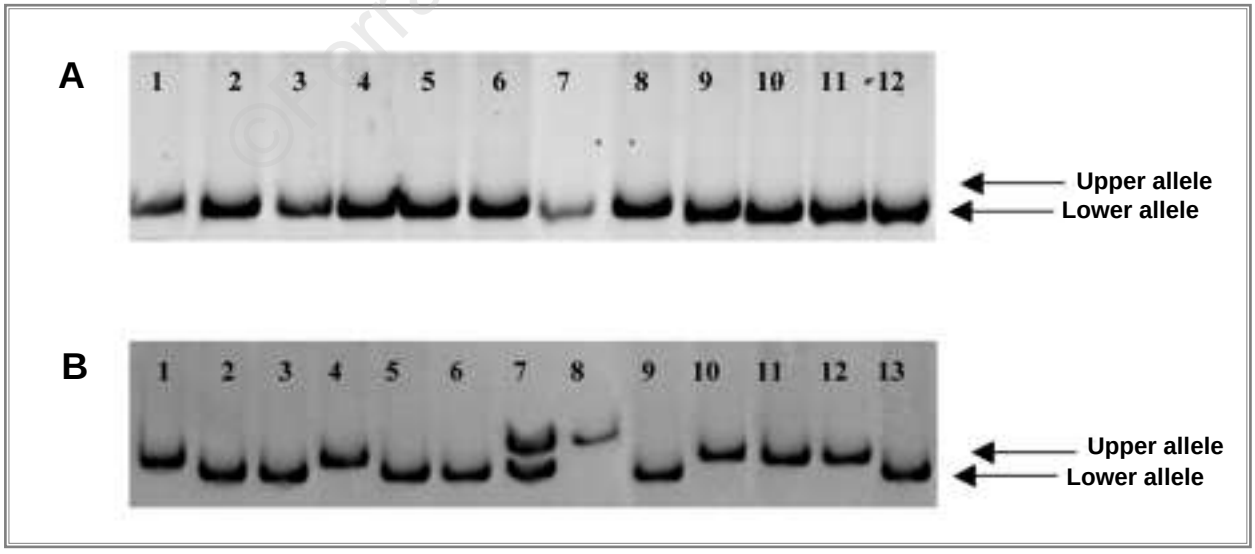

Figure 1. Acrylamide gel electrophoresis of PCR products of several colonies and control tissue from patient 2 affected by RAEB II. Panel A shows the clonal pattern of CFU-GM (lanes 1-6) and BFU-E (lanes 7-12) colonies. Panel B shows the polyclonal pattern of LTC-IC (lanes 1-6 and 9-13) of the same patient as in A. Lanes 7 and 8 represent transitional epithelium cells and a molecular weight marker, respectively.

stem cells. Although this patient fulfilled the clinical and histologic criteria for RA, several features in the course of her disease were distinctly unusual for MDS: a long survival (in excess of 6 years), a complete response to low dose erythropoietin, and the presence of chronic renal failure. Despite this case, the results of the present study confirm the hypothesis that, in the majority of MDS patients, a residual population of immature stem cells is still able to grow *in vitro* and to generate polyclonal hematopoiesis. The results also indicate that LTC-IC were probably not involved in the neoplastic process in these MDS patients, which differs from the conclusions reached by other researchers.[30] Nilsson *et al.* analyzed 13 MDS cases, all with the +8 karyotype abnormality, in whom FISH analysis demonstrated the presence of +8 in 0-70% of $CD34^+CD38^-Thy\text{-}1^+$ cells. Moreover, in a few cases those (unselected) populations were demonstrated to be unable to repopulate the bone marrow of irradiated NOD-SCID mice. The authors concluded that the LTC-IC compartment may be involved in the neoplastic process in MDS patients carrying the +8 abnormality. However, all the cases in their study population had a very complex karyotype in which trisomy 8 was only one among several abnormalities: this could have strongly influenced the capacity of this cell population to grow both *in vivo* and *in vitro*. The authors also showed that many LTC-IC obtained from $CD34^+$ $CD38^-$ $Thy\text{-}1^+$ cells were disomic for chromosome 8 and most probably normal. This is in agreement with our findings of polyclonal LTC-IC also in +8 patients.

In contrast to myelopoiesis, we found that HUMARA polymorphism of T-cells was heterogeneous, as previously reported.[2,3,9,10,12,17,28] However, some points need to be taken into consideration when MDS lymphocytes are judged to be polyclonal or monoclonal based on their X-chromosome inactivation patterns:

a) one possibility is that, due to their long life-span, polyclonal lymphocytes in MDS are part of a population that originated before the onset of a primary mutation affecting a progenitor cell common to the myeloid and lymphoid lineages. Delforge *et al.* studied four low risk MDS patients with polyclonal lymphocytes; samples taken a median of 3 years after diagnosis confirmed stable polyclonal lymphopoiesis. Longer longitudinal follow-up of these patients is, however, required to reveal the influence of disease progression on the clonal patterns of lymphocytes.[29]
b) polyclonal peripheral blood lymphocytes do not exclude the presence of a minor monoclonal lymphoid population which is selectively retained in the bone marrow.[21,31] We obtained T-lymphocytes from the bone marrow of our patients and we cannot exclude the possibility of having selected a T-lymphocyte clone.
c) autoimmune syndromes are common in MDS patients. Clinical manifestations include acute systemic vasculitis, polyneuropathy, inflammatory bowel disease and glomerulonephritis, and classic connective tissue disorders, most notably relapsing polychondritis. Asymptomatic immunologic abnormalities are also common and include hypergammaglobulinemia and a positive antinuclear antibody test.[32-36] The concept of oligoclonality in the context of cellular immune responses is based on the presence of immunodominant T-cell clones within distinct T-cell subpopulations. Under normal circumstances, a limited number of clones undergo periodic expansion in reaction to foreign antigens. Under pathologic conditions, abnormal immune system regulation allows expansion of specific and potentially pathogenic T-cell clones. For example, large granular lymphocyte leukemia represents an extreme expansion of a single T-cell clone associated with a distinct autoimmune pathology.[37] Moreover, clonal T-cell expansion has been described in immune-mediated bone marrow failure and MDS.[38,39]
d) monoclonal X-chromosome inactivation patterns can be due to constitutional or acquired skewing, mimicking monoclonality in 25-40% of all females.[33,40] It has been proven that hematopoietic cells are more prone than non-hematopoietic tissues to excessive skewing with increasing age.[27,41]

The clonal pattern observed in T-cells in cases #1, 2 and 7 could be due to involvement of the T cells in the malignant process or to one of the other aforementioned hypotheses. In patient #8, the X-chromosome inactivation pattern of T-lymphocytes was different from that observed in granulocytes, excluding the possibility that the T-cells belonged to the neoplastic clone. We cannot exclude that autoimmune phenomena were acting in this patient, since she had a mild joint effusion (in her right knee) and psoriasis. One subject in the control group showed clonality of T-lymphocytes, but this may occur in normal subjects.[40] Previous reports have described that the stem cells involved in MDS are committed to the myeloid lineage and are not able to generate clonal lymphopoiesis,[27,29,31,40,41] although this issue has not been clarified even by studies in which polyclonal hematopoiesis was obtained after intensive chemotherapy.[29] Our results indirectly indicate that MDS are a heterogeneous group of diseases in which the neoplastic process can affect different stages of stem cell development, as previously demonstrated in the 5q- syndrome.[42,43] However, even if a pluripotent stem cell is involved in MDS, this does not exclude the possibility that a normal population can

be recruited and grown *in vitro*.

Our study gives the first evidence of the presence of a consistent, detectable non-clonal immature stem cell population in MDS at diagnosis. Our results support the hypothesis that hematopoiesis in MDS is dominated by a neoplastic clone which shows a biological advantage over a residual polyclonal, probably still normal, population of immature stem cells. These results could give support to the idea of growing and generating hematopoietic progenitors *in vivo* that would be able to differentiate *in vivo* in MDS patients. Whether such a population would be substantial enough to offer complete hematologic reconstitution after chemotherapy in all MDS patients is unpredictable. It has been shown that depletion of early hematopoietic cells is an essential part of the pathophysiology of MDS, this phenomenon being particularly prominent in those patient who have hypoplastic bone marrow.[44,45] We suggest that a preliminary study on the *in vitro* growth of immature stem cells and X-chromosome inactivation patterns in female patients might allow clinicians to define a therapeutic approach.[46,47] Unfortunately, the above clonal assay cannot be employed in male patients.

Contributions. All the authors contributed substantially to the study. FG contributed to drafting and reviewing the paper, SG, FC and DDM contributed to perform experiments of DNA extraction and PCR, CM was involved in the analysis of the results and statistical methods, MG contributed to cell cultures, LC, PPS and GPF were involved in the diagnosis and recruitment of patients, GS contributed to reviewing the paper and GCA contributed to the conception and design of the study. The authors reported no conflict of interest.

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