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Cytogenetic characteristics of patients with signs and symptoms of myelodysplastic syndromes in the State of Pará, Brazil

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ABSTRACT. The myelodysplastic syndromes (MDS) are clonal hematopoietic diseases characterized by medullary dysplasia, cytopenias, and frequent evolution to acute myeloid leukemia. In 1982, the French-American-British (FAB) group proposed a classification for the MDS, based on morphological characteristics of peripheral blood and of the bone marrow. Later, cytogenetics proved to be a useful tool for the refinement of prognosis, through the use of the International Prognosis Score System (IPSS), as well as through evidence of clonality. Recently, the World Health Organization (WHO) proposed a new classification for the MDS, based on significant modifications of the FAB proposal, with the inclusion of chromosome analysis. A cytogenetic analysis was made of 17 patients with symptoms of MDS in the State of Pará, based

on WHO recommendations, and application of the IPSS. Good metaphases were obtained for 13 patients; 12 had a normal karyotype and only one had a clonal abnormality, del(3)(p25). The genes related to neoplastic processes that have been mapped to 3p are: XPC in 3p25.1 and FANCD2 and VHL in 3p25-26. Four patients had classic symptoms of MDS; in the rest the possibility of MDS was excluded or several months of observation before diagnosis were recommended. Among those with MDS, it was not possible to apply IPSS and WHO recommendations, because fundamental data were lacking, specifically the medullary blast and ring sideroblast counts. We advocate the implementation of routine cytogenetic analyses for the study of MDS, especially in patients with moderate hematopoietic dysplasia.

Key words: Myelodysplastic syndromes, Cytogenetics, Classification, del(3)(p25)

INTRODUCTION

The myelodysplastic syndromes (MDS) are clonal diseases of hematopoietic stem cells, initially characterized by inefficient hematopoiesis, of one or more cell lines, and peripheral cytopenias, as well as a high risk of progression to acute myeloid leukemia (AML) (Dunbar and Saunthararajah, 2000).

In 1982, the French-American-British group (FAB; Bennett et al., 1982) proposed a classification for the MDS, based on morphological characteristics in the peripheral blood (PB) and the bone marrow (BM), in which they defined five distinct subtypes with different prognostic values (Table 1).

Table 1. French-American-British group classification of the myelodysplastic syndromes (Bennett et al., 1982).

	MB (%)	PB (%)	Others	Evolution to AML (%)
RA	<5	≤1	-	10-20
RARS	<5	≤1	>15% ring Sid.	10-35
RAEB	5-20	<5	-	50 +
CMML	5-20	<5	Monocytosis >1,000/µl	40 +
RAEB-T	21-29	≥5	Auer rods ±	60-100

MB, medullary blasts; PB, peripheral blasts; AML, acute myeloid leukemia; Sid., sideroblasts; RA, refractory anemia; RARS, RA with ring sideroblasts; RAEB, RA with excess blasts; CMML, chronic myelomonocytic leukemia; RAEB-T, RAEB in transformation.

This classification was widely accepted by pathologists and clinicians and was combined with the International Prognostic Scoring System (IPSS) (Greenberg et al., 1997), which utilizes the percentage medullary blasts, the number of cytopenic lines and cytogenetic abnormalities as prognostic factors, signifying an advance in the diagnosis and treatment of patients with MDS. Nevertheless, with time, some difficulties appeared. The heterogeneity of refractory anemias, the inclusion and definition of chronic myelomonocytic leukemia (CMML) in the

FAB system, the definition of prognosis of patients with prognosis of del(5q), and the fact that patients with medullary blast counts above 20% generally have a prognosis similar to one with AML, are some examples (Germing et al., 2000; Steensma and Tefferi, 2003).

For these reasons, the FAB proposal was recently revised by World Health Organization (WHO) specialists, resulting in a new classification for the MDS, with more homogeneous categories (Table 2) (Bennett, 2000; Germing et al., 2000; Harris et al., 2000; Jaffe et al., 2001; Vardiman et al., 2002).

The main changes to the WHO classification refined the definitions of low-grade subtypes (refractory anemia (RA) and RA with ring sideroblasts (RARS)) as being strictly erythrocytic, and added a new category, refractory cytopenia with multiline dysplasia (RCMD). The lower limit of percentage blasts for the diagnosis of AML, in PB or BM, was reduced from 30 to 20%, which resulted in the elimination of subtype RA with excess blasts in transformation (RAEB-T) from the WHO classification. Other changes included the recognition of new subtypes: two subtypes of RAEB; RAEB-I, with 5-9% medullary blasts and RAEB-II with 10-19%; MDS without classification, and the specific genetic subtype, 5q- syndrome (Vardiman et al., 2002).

This new classification scheme is not universally accepted; while it is criticized by some researchers (Greenberg et al., 2000; Nosslinger et al., 2001), it is supported by others (Germing et al., 2000; Bennett et al., 2002; Dunkley et al., 2002; Howe et al., 2003; Strupp et al., 2003; Giagounidis et al., 2004).

Different from the FAB proposal, the WHO classification includes a genetic subcategory specific for MDS. Consequently, cytogenetic information is indispensable, and the unavailability on short notice of these data is an obstacle for this classification (Arber, 2001).

The most common cytogenetic abnormalities in the MDS are chromosome 5, 7, 11, 12, and 20 deletions, and/or chromosome 8 trisomy (Table 3). The frequency of chromosome abnormalities in primary MDS is 30-50%, while it is 80% in secondary MDS. The latter generally has complex karyotypes (Fenaux et al., 1996; Mecucci and La Starza, 1999).

The MDS are known to have recurrent chromosome deletions. This loss of genetic material raises the hypothesis that tumor suppression genes could be involved in the pathogenic process (Hofmann et al., 2004). Table 4 lists abnormalities that are frequently found in MDS, and also lists the genes that are possibly involved in pathogenesis.

Our objective was the cytogenetic characterization of patients with signs and symptoms of MDS in the State of Pará, for confirmatory diagnosis of the clinical-morphological diagnosis, based on WHO criteria. Our specific objectives were to i) describe the recurrent break points and associate them with tumor suppressor genes, DNA repair genes, oncogenes, and other genes involved in these malignant processes; ii) relate the alterations that are observed to the evolution of the neoplasia, to determine their effect on diagnosis and prognosis, and iii) reclassify the patients that were referred based on WHO criteria and define prognosis groups based on IPSS, to develop a basis for the choice of therapy.

MATERIAL AND METHODS

BM and PB samples

Samples of BM of 17 patients suspected or diagnosed as having MDS without previous cytotoxic therapy and samples of PB of 10 control subjects were analyzed. All the study sub-

Table 2. WHO classification for the myelodysplastic syndromes (Vardiman et al., 2002).

Category	Peripheral blood	Bone marrow
RA	Anemia Blasts <1% Monocytes <1,000/µl	Erythroid dysplasia Blasts <5% Ring sideroblasts <15%
RARS	Anemia Blasts <1% Monocytes <1,000/µl	Dysplasia only in the erythroid line Ring sideroblasts ≥15% Blasts <5%
RCMD	Cytopenias (bi- or pancytopenia) Blasts <1% Monocytes <1,000/µl	Dysplasia ≥10% of the cells Blasts <5% Ring sideroblasts <15%
RCMD-RS	Cytopenias (bi- ou pancytopenia) Blasts <1% Monocytes <1,000/µl	Dysplasia ≥10% of the cells Blasts <5% Ring sideroblasts ≥15%
RAEB-I	Cytopenias Blasts <5% Monocytes <1,000/µl	Dysplasia uni- or multilines Blasts 5-9%
RAEB-II	Cytopenias Blasts 5-19% Monocytes <1,000/µl Auer rods ±	Dysplasia uni- or multilines Blasts 10-19% Auer rods ±
5q- syndrome	Anemia Blasts <5% Normal or increased platelet counts	Megakaryocytes hypolobulate normal or increased Blasts <5% del(5q) alone
MDS without classification	Cytopenias Blasts <1%	Single line dysplasia of the granulocytes or megakaryocytes Blasts <5%

RA, refractory anemia; RARS, RA with ring sideroblasts; RCMD, refractory cytopenia with multiline dysplasia; RCMD-RS, RCMD with ring sideroblasts; RAEB, RA with excess blasts; MDS, myelodysplastic syndrome.

Table 3. Most frequent chromosome alterations found in myelodysplastic syndrome patients (Hofmann et al., 2004).

Numerical	Translocations	Deletions
+8 (19%)	inv 3 (7%)	del 5q (27%)
-7 (15%)	t(1;7) (2%)	del 11q (7%)
+21 (7%)	t(1;3) (1%)	del 12q (5%)
-5 (7%)	t(3;3) (1%)	del 20q (5%)
	t(6;9) (<1%)	del 7q (4%)
	t(5;12) (<1%)	del 13q (2%)

The frequency of chromosome alterations is given in parentheses. –, chromosome loss; +, additional chromosome; inv, inversion; t, translocation; del, deletion.

Table 4. Summary of the target chromosome segments and their tumoral suppression gene candidates possibly involved in the pathogenesis of the myelodysplastic syndromes (Mhawech and Saleem, 2001).

Chromosome abnormality	Chromosome segment	Tumoral suppression gene candidate and its function
Deletion5q	5q31	IRF-1, transcriptional activator of interferon 1; EGR-1, differentiation of the monocyte-macrophage lineage and of myeloid blasts, positive regulation of cell growth; CSF-1R, regulation of growth of myeloic cells
Deletion7q	7q21-22	ASNS, control of the cell cycle
	7q22.1	Unknown gene, significant role in the malignant myeloid cells
Deletion20q	20q12-13.1	PLC1, role in signal transduction
	20q12-13.2	TOP1, transcription regulator
Syndrome 17p	17p13.1	p53, regulation of replication of DNA, cell proliferation and cell death
Deletion12p13	12p13	KIP1/p27, inhibitor of cell proliferation; TEL, negative regulation of transcription
t(5;12)(q33;p13)	5q33	PDGFRB, activation of tyrosine kinase
	12p13	TEL, negative regulation of transcription

jects were seen from February 4, 2002 to January 21, 2003. The patients, or their legal representatives, were informed about the research and they all signed a permission form, allowing us to investigate tissue samples that would already normally be collected during routine exams, so that there was no additional discomfort for these volunteers.

Methodology

The BM samples of patients were cultivated in MARROWMAX™ Bone Marrow Medium (Gibco). Three cultures were made for each BM sample: direct, 24 h and 48 h.

Lymphocytes from PB of control subjects, prepared from 0.5 ml heparinized blood obtained by venipuncture, were cultivated for 72 h in Ham's F10 medium (Gibco) supplemented with 20% fetal bovine serum and antibiotics. The cells were stimulated with 2% PHA (Gibco).

A 0.1-ml aliquot of colchicine (0.0016%) was added to all cell cultures, 2 h before each harvest. Posteriorly, these cells were centrifuged at 1000 rpm for 10 min, the supernatant was

removed and a hypotonic KCl treatment (0.075 M) was initiated at 37°C for 20 min. The cells were then centrifuged and fixed three times with methanol/acetic acid (3:1).

Cell suspensions were placed on histological slides. After drying at room temperature, the slides were stained for conventional analysis with 4% Giemsa solution, pH 6.8, for 10 min and washed with distilled water. The GTG banding technique (Scheres, 1972) was used, with some modifications. Dried slides were artificially aged overnight in a drying oven at 60°C or for 1 h at 90°C. They were then treated with 0.01% trypsin solution, and diluted in phosphate buffer, pH 6.8, for 3-5 min. The trypsin activity was halted by bathing the slides in ice-cold distilled water. After drying at room temperature, the slides were stained with 4% Giemsa solution, pH 6.8, for 10 min. The identification and classification of the chromosomes were made based on recommendations of the International System for Cytogenetics Nomenclature (ISCN, 1995), as were the criteria for the determination of cytogenetic clones. The cytogenetic data of each patient were combined with the results of their respective histopathological (BM biopsy), myelogram and hemogram, along with the clinical evaluation, for posterior analysis.

Statistical analysis

The data were subjected to statistical analysis, using the chi-square test and the Friedman test to compare the variation of chromosome number in the patients in relation to the respective controls.

RESULTS AND DISCUSSION

Cytogenetic analysis was performed in all control samples but only in 13 of the 17 patients' samples. In the remainder we were not able to obtain cellular proliferation *in vitro*. The modal chromosome number was 46 in all cases (Tables 5 and 6) and no statistical differences were observed between group of patients and their respective controls ($P > 0.05$). Even with chromosome number variation ranging between 44 and 47 chromosomes (accept values), no numeric abnormalities were found in all cells analyzed and only one clonal abnormality was found in a patient sample, del(3)(p25) (Figure 1 and Table 6).

Table 5. Cytogenetic results of control subjects.

Control No.	Age/ Sex	Metaphases counted	Modal number (%)	Chromosome No. variation (%)	Cells analyzed	Karyotypes
1	25/F	100	46 (96)	45 (4)	11	46, XX [11]
2	22/M	100	46 (94)	44 (1), 45 (5)	11	46, XY [11]
3	28/F	100	46 (93)	44 (2), 45 (5)	11	46, XX [11]
4	32/F	100	46 (97)	45 (3)	11	46, XX [11]
5	35/M	100	46 (98)	45 (2)	11	46, XY [11]
6	40/M	100	46 (95)	45 (4), 47 (1)	11	46, XY [11]
7	20/M	100	46 (98)	45 (2)	11	46, XY [11]
8	31/F	100	46 (99)	45 (1)	11	46, XX [11]
9	43/M	100	46 (94)	45 (5), 47 (1)	11	46, XY [11]
10	26/M	100	46 (95)	45 (5)	11	46, XY [11]

Table 6. Cytogenetic results of patients.

Case No.	Age/ Sex	Metaphases counted	Modal number (%)	Chromosome No. variation (%)	Cells analyzed	Karyotypes
1	20/M	83	46 (96.39)	45 (3.61)	20	46, XY [20]
2	69/M	No proliferation *in vitro*				
3	24/F	No proliferation *in vitro*				
4	18/M	No proliferation *in vitro*				
5	45/M	63	46 (95.24)	45 (4.76)	15	46, XY [15]
6	8/M	88	46 (100)	-	20	46, XY [20]
7	55/F	69	46 (94.2)	44 (1.45), 45 (4.35)	20	46, XX [20]
8	22/M	No proliferation *in vitro*				
9	57/F	90	46 (94.45)	44 (1.11), 45 (4.44)	16	46, XX [16]
10	25/F	91	46 (96.7)	45 (3.3)	15	46, XX [15]
11	27/F	36	46 (97.2)	45 (2.8)	8	46, XX [8]
12	55/F	100	46 (92)	44 (2), 45 (5), 47 (1)	20	46, XX [20]
13	62/M	79	46 (97.47)	45 (2.53)	17	46, XY [17]
14	68/M	23	46 (100)	-	8	46, XY [8]
15	55/M	57	46 (98.25)	45 (1.75)	10	46, XY [10]
16	8/M	43	46 (97.67)	45 (2.33)	10	46, XY [10]
17	27/M	23	46 (95.65)	45 (4.35)	8	46, XY, del(3)(p25) [2]

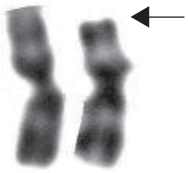

Figure 1. Case No. 17, partial karyotype with GTG banding. The arrow indicates the chromosome with del(3)(p25).

Six of the 10 control subjects and 11 of the 17 patients were males, giving a M/F proportion of 1.5 and 1.8, respectively. While the control group varied from 20-43 years old (mean 30.2) the patient group varied from 8-69 years old (mean 37.9). At the time the samples were sent for cytogenetic analysis, only three patients were over 60, four were between 50 and 59, and most of the patients (N = 10) were less than 50 years old. Nevertheless, MDS are considered geriatric diseases (Aul et al., 1995), with more than 80% of the patients being 60 years and older, and only 8-10% with less than 50 years at diagnosis. Based on these age data, it appears that most of our patients did not actually have MDS.

Patient No. 9, for example, had hematological data typical of a myeloproliferative disease (neutrophils: 17,025/µl; monocytes: 1,362/µl; platelets: 1,544,000/µl), incompatible with what is expected for a patient with MDS.

Patient No. 10 had alterations in the BM that were suspected to be idiopathic thrombocytopenic purpura (IPT). IPT and MDS appear to be completely distinct diseases; IPT is a typical autoimmune disease, while MDS is a clonal neoplastic disease; however, they may initially have indistinguishable symptoms. Myelodysplastic syndromes can initially present an isolated thrombocytopenia (Menke et al., 1992) that is indistinguishable from IPT on routine exams, which include full counts of the blood cells and examination of PB smears. For this reason, BM aspirates and biopsies, which are not routinely requested for the diagnosis of IPT, could be appropriate to exclude MDS in elderly patients (George et al., 1996). However, patient No. 10 was 25 years old, and had a normal karyotype, which precludes a neoplastic disease; therefore, the hypothesis of MDS can be excluded.

Not all the conditions that have pathological characteristics similar to the MDS are clonal neoplastic diseases. Even though many proposals have been made (Culligan and Jacobs, 1992; Ost and Reizenstein, 1992; Tricot, 1992; George et al., 1996; Ramos et al., 1999; Gardais, 2000), the minimum criteria for the diagnosis of MDS are not clear. Some pathologists are uncomfortable with making this diagnosis, in the absence of dysplasia in at least two cell lines, since erythroid dysplasia has many potential etiologies. However, when non-clonal conditions are eliminated, the diagnosis still cannot be assured.

The distinction between MDS and similar clonal hematopoietic diseases can also be a serious challenge, since the divisions between some chronic myeloid diseases are not clear, and sometimes they can superimpose (Neuwirtova et al., 1996; Bain, 1999).

Bain (1996) and Ramos et al. (1999), based on their findings of moderate dysplastic hematopoiesis in a large proportion of normal individuals, argued in favor of a higher threshold for the morphologic diagnosis of the MDS whenever a cytogenetic exam for clones and the associated abnormalities is not available. For this reason, we followed the recommendations of Greenberg et al. (1998), members of the NCCN (National Comprehensive Cancer Network), that "when classic characteristics are lacking, the patient needs to be examined during several

months in order to diagnose MDS". Consequently, the patients that presented light to moderate degrees of dysplasia and normal karyotypes (Nos. 1, 7, 11, and 14) were excluded from further analysis.

In about 90% of the cases, the patients with MDS had a normocellular or hypercellular BM. However, about 10% of the patients with MDS present medullary hypoplasia at diagnosis (Nand and Godwin, 1988; Rosati et al., 1996), indicating a cellularity of less than 30%, in general. Another characteristic that indicates that our sample is not typical is that 9 of the 17 patients (Nos. 1, 3, 4, 5, 8, 11, 15, 16, and 17) presented hypocellularity or medullary aplasia. The mean age of these patients was 27.3 years, compared with 62.1 years in the group studied by Goyal et al. (1999), composed of patients with hypoblastic MDS. Among these, the BM samples from patients 3, 4, 8 and, especially patient No. 2, which presented a hypercellular medulla, did not proliferate *in vitro*. This failure could be attributed to the myelofibrosis observed in patient 2, the medullary aplasia observed in patients 3 and 4, and the accentuated hypoplasia in patient No. 8.

Patients 5 and 15 presented medullary hypoplasias, with no dysplasia and a normal karyotype. Therefore, the NCCN recommendation was also followed for these cases.

Patient No. 16 is an eight-year-old child. For this reason, and because the child had medullary hypoplasia, the hypothesis of Fanconi anemia (FA) should be discarded. The cultivated lymphocytes of FA patients have a high prevalence of chromosome breaks, which are amplified by treatment with diepoxybutane or mitomicin C. Even though the karyotypic analysis of BM cells of patient 16 was normal, if there is suspicion of FA, a test for chromosome breaks induced by diepoxybutane or mitomicin C should be made, and not only conventional CTG banding analysis.

Patient No. 6 is also an eight-year-old child; however, the hypothesis of FA can be discarded, as this patient had a hypercellular BM.

Based on the available data, we consider only patients 2, 12, 13, and 17, with 69, 55, 62, and 27 years old, respectively, to have MDS. Patients 2, 12 and 13 were so-classified based on strong BM dysplasia, and patient 17, even with medullary hypoplasia and moderate erythrocytic dysplasia, based on clonal cytogenetic abnormality, del(3)(p25).

Cytogenetic abnormalities that result in deletion 3p are common in solid tumors, which indicates the presence of a tumor suppression gene in this chromosome arm.

Johansson et al. (1997) indicated that in hematological neoplasias, including the MDS, the breakpoints in chromosome 3 are more distal than those found in solid tumors, suggesting that different tumor suppression genes are involved in these processes.

There are three genes related to neoplastic processes mapped to region del(3)(p25). i) XPC, located at 3p25.1, codes for a nuclear protein involved in the premature recognition of DNA damage in the chromatin, and which affects predisposition to xeroderma pigmentosum (Stary and Sarasin, 2001). ii) FANCD2, located at 3p25-26, near the XPC gene, codes for a nuclear protein that is part of the D complement of FA (Huret, 2002). iii) VHL, located at 3p25-26, is a multifunctional tumor suppressor, which among other functions forces the cells out of the cell cycle into quiescence (Richard, 2002). These genes are contributions of this work for further molecular studies, which are needed to demonstrate its possible biological roles in MDS pathogenesis.

Six months after cytogenetic analysis of the BM of patient No. 17, there was a recommendation for an allogenic transplant, due to marked pancytopenia in the PB and intense medullary aplasia, which impeded a new karyotyping. This fact corroborates the hypothesis that del(3)(p25) is an indicator of a bad prognosis.

The FAB cooperative group (Bennett et al., 1982) proposed a classification for MDS, based on morphological characteristics of the PB cells and of the BM cells, which defined five subtypes with significant differences for prognosis. Nevertheless, it was observed that even those patients with marked dysplastic characteristics (Nos. 2, 12 and 13) were diagnosed generically as having MDS, and the FAB subtypes were not distinguished. Part of this inability to classify the patients is due to failures in laboratory procedures. Except for case No. 2, the percentage blasts in the BM was not informed. This information is of fundamental importance for classification schemes (both for FAB and WHO) and to determine a prognosis for these patients, through the use of the IPSS. Another important observation is that in none of the cases was the percentage ring sideroblasts informed. This information defines the specific MDS subtypes, RARS (in the FAB and WHO classifications) and RCMD-RS (in the WHO classification).

This study had as an objective, given cytogenetic data, to reclassify patients based on the WHO proposal and the IPSS. Nevertheless, this procedure would be arbitrary without information on the percentage medullary blasts and ring sideroblasts.

Though its prognostic value has already been proven, the IPSS is more efficient when it is combined with a classification scheme (Greenberg et al., 1997). In fact, some studies have concluded that the IPSS is limited in its ability to make a prognosis in patients with high survival rates, as well as in cases where only the erythrocytic cell line is involved (Balduini et al., 1997; Greenberg et al., 1997; Matsuda et al., 1999). Also, many other biological characteristics that can be used for prognosis are currently known. For example, the presence of abnormal location of immature precursors observed in the BM biopsy of patient No. 13 (Bellamy et al., 2001; Verburgh et al., 2003), the methylated state of the genes CDKN2B and DAPK (Tien et al., 2001; Voso et al., 2004), the length of the telomere (Ohyashiki et al., 1999), the degree of medullary apoptosis (Shimazaki et al., 2000), the mutation state of the genes, such as RAS, FMS and TP53 (Paquette et al., 1993; Padua et al., 1998), and the degree of expression of gene WT1 (Cilloni et al., 2003) can have diagnostic value.

The comparison of cytogenetic data with clinical and hematological information in patients suspected of having MDS, who were evaluated by Pará State health professionals, allows us to conclude that these patients are likely being diagnosed and treated as if they had leukemia. It is imperative that a cytogenetic analysis be made routinely of patients suspected to have MDS.

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