

Karyotypic and fluorescent *in situ* hybridization study of the centromere of chromosome 7 in secondary myeloid neoplasms

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Background: Secondary myeloid neoplasms comprise a group of secondary diseases following exposure to myelotoxic agents or due to congenital diseases. The improvement of anticancer agents and immunosuppressive drugs seem to be associated with an increased incidence of secondary myeloid neoplasms. Karyotyping of bone marrow is essential for diagnosis and prognosis. Previous use of alkylating agents and radiation are associated with clonal abnormalities such as recurrent unbalanced -5/5q-, -7/7q- and complex karyotypes, whereas topoisomerase-II inhibitors lead to changes such as the balanced 11q23 rearrangement, t(8;21), t(15;17) and inv(16).

Objective: To study the clinical and cytogenetic data of patients with secondary myeloid neoplasms who took antineoplastic and/or immunosuppressive drugs or progressed from aplastic anemia.

Methods: The clinical and cytogenetic characteristics of 42 patients diagnosed with secondary myeloid neoplasms in one institution were retrospectively evaluated. Of these, 25, 11 and 6 patients had had oncological diseases, aplastic anemia and other diseases, respectively. Conventional cytogenetic and FISH analyses were performed for monosomy 7.

Results: The cytogenetic study was conclusive in 32 cases with 84.4% of clonal abnormalities. Monosomy 7 and complex karyotypes were present in 44.4% and 37%, respectively. A high prevalence of unbalanced abnormalities (96.3%) was observed. Monosomy 7 was more prevalent in patients with myelodysplastic syndromes/myeloid neoplasms after aplastic anemia (66.6%). The median survival after diagnosis of myeloid neoplasms was only 5.7 months. Normal cytogenetics was associated to better survival (p -value = 0.03). There was a slightly worse trend of survival for patients with complex karyotypes (p -value = 0.057). Abnormal karyotype was an independent risk factor for poor survival (p -value = 0.012).

Conclusion: This study enhances the importance of cytogenetic analysis of patients at the time of diagnosis of secondary myeloid neoplasms.

Keywords: Cytogenetic analysis; Myelodysplastic syndromes; Leukemia, myeloid

Introduction

The myelodysplastic syndromes (MDS), acute myeloid leukemias (AML) and secondary myeloproliferative syndromes (MPS) constitute a group of myeloid neoplasias (MNs) which result from exposure to risk factors such as chemotherapy and radiotherapy, acquired aplastic anemia associated with immunosuppression and congenital diseases.⁽¹⁻⁵⁾ A cytogenetic study of the bone marrow, fundamental for the characterization of the secondary MNs, provides essential information for the diagnosis and prognosis of the disease. Between 80 and 95% of patients with MDS/MN present abnormal and frequently complex karyotypes (an average of 5.3 abnormalities per case). Chromosome 7 monosomy is the commonest alteration in patients with secondary MN after aplastic anemia (AA).⁽⁶⁾ It is estimated that 10-20% of myeloid neoplasia cases are secondary to the use of chemotherapeutic agents and/or radiotherapy.⁽⁵⁾ The progress of therapeutic regimens used to treat primary neoplasias, as well as the new conditions of autologous hematopoietic stem cell transplantations (HSCT), have led to an increase in the rate of therapy-related myeloid neoplasia (MN-t) cases.⁽⁷⁾ Some patterns of chromosomal abnormalities are recurrent after exposure to cytotoxic agents. The previous use of alkylating agents/radiation can favor the development of MN-t, which is usually associated with cytopenia and dysplasia and often involves unbalanced clonal genetic alterations including monosomies or deletions of chromosomes 5 and/or 7, and complex karyotypes with numerous marker chromosomes that may progress to point alterations in the *P53* and *AML1* genes. Previous use of topoisomerase II inhibitors (etoposide, anthracyclines) is usually associated with the development of AML, frequently with

Conflict-of-interest disclosure:
The authors declare no competing financial interest

Submitted: 3/13/2011
Accepted: 9/5/2011

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DOI: 10.5581/1516-8484.20110117

balanced chromosomal alterations involving 11q23 rearrangements, t(8;21), t(15;17), t(8;16) or inv(16).^(8,9) The high frequency of complex karyotypes in MN-t indicates a fast-emerging resistance to conventional treatments used in de novo leukemia cases.^(10,11) Typically, the clinical course progresses with high mortality and morbidity rates. Allogeneic HSCT is the only therapeutic modality with the potential to cure patients with MN-t.^(7,12) Cytogenetic aberrations which are present pre-transplantation are associated with low disease-free survival.⁽¹³⁾ A study conducted by the European Group for Blood and Marrow Transplantation (EBMT) on patients with MN-t who underwent allogeneic HSCT between 1981 and 2006 found, using multivariate analysis, that the progress of the disease was more favorable in patients with normal karyotypes.⁽⁷⁾

The development of secondary MDS/MN associated with treatment by immunosuppressants has been documented in AA. However, this subgroup also includes patients with autoimmune diseases and patients submitted to solid organ transplants.^(2,14,15) Immunosuppressants such as cyclosporine A (CyA), anti-lymphocyte or anti-thymocyte globulin (ALG/ATG), cyclophosphamide (CTX), azathioprine and the use of granulocyte colony-stimulating factor (G-CSF) appear to be involved in the etiology of MN.⁽¹⁶⁾

The cytogenetic abnormalities most frequently found in MDS/MN after AA involve chromosomes 6, 7 and 8.⁽¹⁷⁾ In some cases these alterations may progress with transient cytogenetic abnormalities, such as chromosome 8 trisomy, which is responsive to immunosuppression treatment, and trisomy of chromosome 6. However, monosomy 7, the most frequent cytogenetic abnormality, occurs in patients with minimal clinical response, in patients who relapsed with severe pancytopenia⁽¹⁸⁾ or in those who took G-CSF for a long time.⁽¹⁴⁾ Some authors admit the possibility of a pre-existent clone with chromosome 7 monosomy being present at the diagnosis of AA.⁽¹⁸⁾

Cytogenetic abnormalities involving chromosomes 5 and 7 were found in patients with rheumatic diseases that progressed to secondary MN after being treated with cumulative doses of alkylating agents for long periods.⁽¹⁵⁾ Myeloid neoplasms are rare in patients who received solid organ grafts, such as in heart, liver and kidney transplantations. However, the occurrence of these neoplasms seems to be related to maintenance immunosuppression that is basically composed of prednisone, cyclosporine and azathioprine. Thus, abnormalities involving chromosome 7 have been described in patients who received solid organ transplants, particularly those who were treated with azathioprine, a potent immunosuppressant that leads to chromosome instability.⁽²⁾

The aim of the present work was to study the clinical and cytogenetic data of patients who were exposed to antineoplastic and/or immunosuppressive drugs and those that progressed from aplastic anemia to secondary myeloid neoplasia.

Methods

A retrospective study was carried out involving 42 patients with secondary MN diagnosed between September 1987 and December 2008 at the *Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo*. Conventional cytogenetics was performed using bone marrow samples and in one case using peripheral blood, with supplemented RPMI 1640 culture medium without any mitogenic agent incubated for 24 and 48 hours in a CO₂ incubator at 37°C, followed by G-banding.⁽¹⁹⁾ The fluorescence *in situ* hybridization (FISH) technique was used to investigate possible chromosome 7 monosomies in cases in which the karyotype showed no abnormalities or in which no metaphases could be obtained. The CEP 7® Chromosome Enumeration DNA FISH Probe (Vysis, Inc) was employed according to the manufacturer's recommendations. The cut-off point for the probe had previously been established in the laboratory following international guidelines.⁽²⁰⁾ For this purpose, an inverted β function statistical test was used (Microsoft Excel), after analyzing 200 interphase nuclei from five negative control samples. For each batch of probes, a normal control sample was tested in parallel. Cases presenting more than 10% of interphase nuclei with a single signal for the chromosome 7 centromere, analyzed by two independent observers (RSST and CAK), were considered positive for monosomy 7. The molecular cytogenetic analysis was performed in samples previously fixed in Carnoy solution (methanol/acetic acid). The karyotype and FISH results were described according to the international nomenclature (ISCN 2009).⁽²¹⁾ The karyotypes were classified as normal or abnormal (chromosome 7 monosomy, complex karyotypes with 3 or more clonal alterations and other patterns with < 3 clonal alterations). Whenever possible, 20 metaphases were analyzed. To be considered a clone, the same structural alteration or the gain of the same chromosome in two metaphases or the loss of the same chromosome in three metaphases had to be observed.⁽²¹⁾

Statistical analysis was performed using the Kaplan-Meier product limit estimator (survival curves), log rank test to compare groups and Fisher's exact test for associations between variables. In addition to the cytogenetic parameters, other clinical, hematological, bone marrow aspirate and histological factors were submitted to the binary logistic regression test to evaluate overall survival (OS). The data were analyzed using version 16.0 of the Statistical Package for the Social Sciences (SPSS) Computer program.

Results

Clinical characteristics of the patients

A total of 42 patients were evaluated, of which 23 (54.8%) were male and the median age was 53.5 (range: 4-88) years. Their previous conditions included oncohematological

neoplasias (n= 19), aplastic anemia (n= 11), solid tumors (n= 6), autoimmune diseases (n= 4) and solid organ transplantations (n=2). The treatments of the previous

diseases are listed in Table 1. The median latency between the previous disease and secondary MN was 85.4 months (range: 23.8- 221.3 months).

Table 1 - Clinical characteristics of patients with secondary MDS/MN

Patient	Previous disease	Chemotherapy or immunosuppressive agents	RT
1	Plasmocytoma L3	Melphalan + Lomustine	Y
2	Plasma cell myeloma IgG lambda	Melphalan	N
3	Plasma cell myeloma IgG lambda	Melphalan + Lomustine	Y
4	Plasma cell myeloma IgG kappa	Melphalan + Doxorubicin	Y
5	Plasma cell myeloma IgG kappa	Melphalan	Y
6	Plasma cell myeloma IgG kappa	Melphalan + Lomustine	N
7	Plasma cell myeloma IgG kappa	Melphalan + Lomustine	N
8	Plasma cell myeloma IgG kappa	Melphalan	N
9	Plasma cell myeloma IgG kappa	Melphalan	N
10	Plasma cell myeloma IgG kappa	Melphalan	N
11	Primary amyloidosis	Melphalan	N
12	LH nodular sclerosis	CTX + Procarbazine	Y
13	Follicular lymphoma	Chlorambucil + CTX + Fludarabine phosphate	Y
14	Follicular lymphoma	CTX + Fludarabine phosphate	N
15	Follicular lymphoma (1990) [Sjögren's syndrome (1988)]	Chlorambucil + CTX + Fludarabine phosphate	N
16	Diffuse large B-cell lymphoma	CTX + Doxorubicin + Ifosfamide + Etoposide	N
17	Burkitt's lymphoma	CTX + Doxorubicin + Ifosfamide + Etoposide	N
18	Burkitt's lymphoma	CTX + Vincristine + Methotrexate + Adriamycin	Y
19	AML + cardiac transplant	protocol for AML	N
20	Breast cancer	Not available	?
21	Breast cancer	CTX + Methotrexate + 5-Fluorouracil	Y
22	Breast cancer	Chemotherapy (data not available)	Y
23	Ovarian cancer (1991)	Chemotherapy (data not available)	Y
	+ Breast cancer (1998)	Tamoxifen + Chemotherapy for lung metastases	Y
24	Prostate cancer	No chemotherapy	Y
25	Rhabdomyosarcoma	CTX + Etoposide + Doxorubicin + Carboplatin	Y
26	Behcet's syndrome	CTX + Chlorambucil + Infliximab	N
27	Polyarteritis nodosa	CTX	N
28	Ulcerative colitis	Azathioprine	N
29	Systemic lupus erythematosus	Prednisone + CyA	N
30	Liver transplant	Not available	N
31	1st Renal transplant (1992)	[CyA + Azathioprine + Prednisone] + EPO + G-CSF	
	2nd Renal transplant (2001)	[ATG + Tacrolimus + Azathioprine + Prednisone] + EPO + G-CSF	N
32	SAA	CyA + G-CSF	N
33	AA	Androgen	N
34	SAA	CyA + ATG/ALG + G-CSF	N
35	SAA (PNH)	CyA + G-CSF	N
36	SAA	CyA + G-CSF	N
37	SAA (PNH)	CyA + ATG/ALG	N
38	SAA congenital dyskeratosis	Androgen	N
39	SAA	CyA + ATG/ALG + G-CSF	N
40	Bone marrow hypoplasia	No medication	N
41	AA (PNH)	CyA	N
42	AA	CyA	N

AA: aplastic anemia; SAA: severe aplastic anemia; AML: Acute myeloid leukemia; ALG: anti-lymphocyte globulin; ATG: anti-thymocyte globulin; CTX: cyclophosphamide; CyA: cyclosporine A; EPO: erythropoietin; G-CSF: granulocyte colony-stimulating factor; LH: Hodgkin disease; PNH: paroxysmal nocturnal hemoglobinuria; RT: radiotherapy; N: no; Y: yes

The median age of patients with previous aplasia was 24 years (range: 15-61), six (54%) were male and the latency period until developing secondary MN was 97 months. Of the other 31 patients, median age was 60 years (range: 4-88), 16 (51.6%) were male and the median latency period was 76 months.

Cytogenetic characteristics

This study includes cytogenetic studies of 38 patients performed when secondary MDS/MN was suspected. In six cases, no cell growth was achieved; clonal abnormalities were

Table 2 - Cytogenetic characteristics of patients with secondary MDS/MN

Patient	Conventional karyotype	Abnormalities B/NB	FISH (-7)	Cytogenetic profile
1	46,XY[13]	NB	(+)	-7
2	45,XY,del(5)(q12q33),-7[8]/45,XY,-15[4]/45,XY,-21[8]	NB	NP	C
3	45,XY,-7[23]/45,XY,-1,der(1;7)(q10;p10),add(12)(p12), +mar,inc[7]	NB	NP	C
4	AM			
5	43,X,-Y,-5,-7,add(11)(p15),add(12)(p13),add(17)(p12)[20]	NB	NP	C
6	33-40,X,-Y,-4,der(5),-8,-9,-12,-15,-17,-18,-22, +mar[cp7]/46,XY[2]	NB	NP	C
7	NP			
8	44-47,XX,-5,-17,-18, +19, +21, +2mar[cp23]/46,XX[4]	NB	NP	C
9	46,XX,t(3;21)(q26;q22)[7]/46,XX[2]	B	NP	O
10	NP			
11	45,XY,-7[17]/46,XY[3]	NB	NP	-7
12	AM		(-)	AM
13	49-52,XY, +1,-4,-5, +6, +7,-8,-9,-17,-20, +21, +21, +mar[cp18]	NB	NP	C
14	46,XX,del(5)(q?14q?31),del(13)(q?21q?31)[16]/47,XX,idem, +8[4]	NB	NP	C
15	47,XX,add(21)(q21), +mar[10]/46,XX[12]	NB	NP	O
16	46,XY[19]	NL	(-)	NL
17	45,XY,-7,add(11)(p15)[7]/45,XY,idem,add(17)(p12)[12]	NB	NP	C
18	46,XY,del(7)(q22)[16]/46,XY[4]	NB	NP	O
19	AM		(-)	AM
20	46,XX,-7, +mar[18]	NB	NP	-7
21	NP	NP	NP	NP
22	AM		NP	
23	AM		(-)	AM
24	46-49,-X,Y,-5, +2mar +r[cp3]	NB	NP	C
25	46,XY[20]	NB	(+)	-7
26	48,XX, +8, +8[20]	NB	NP	O
27	47,XX, +der(1;7)(q10;p10),-7, +21[20]	NB	NP	C
28	47,XX,inc,-6, +2mar[15]	NB	NP	C
29	44,XX,-5,-7, +8,add(17)(q10),-18,-21, +2mar[10]	NB	NP	C
30	47,XY, +der(1)add(q11)[7]	NB	NP	O
31	46,XY[21]	NL	(-)	NL
32	45,XX,-7[18]	NB	NP	-7
33	45,XY,-7[10]/46,XY[3]	NB	NP	-7
34	45,XY,-7[3]/46,XY,-7 +21[16]	NB	NP	-7
35	AM		(-)	
36	45,XY,inv(3)(q?23q?26),-7[9]	NB	NP	-7
37	45,XX,-7[17]	NB	NP	-7
38	46,XY[9]	NL	(-)*	NL
39	46,XX,-7, +21[20]	NB	NP	-7
40	46,XX[12]	NL	(-)	NL
41	NP		NP	
42	46,XY[10]	NL	(-)	NL

NP: not performed; AM: absence of metaphase; NL: normal karyotype; B: balanced rearrangements; NB: unbalanced rearrangements; -7: monosomy 7; C: complex karyotype; O: other clonal abnormality
 * this sample used peripheral blood

observed by conventional karyotyping in 25/32 (78.1%) cases.

FISH investigations (Table 2) for chromosome 7 monosomy were carried out in 11 patients; two (18.2%) were positive (Patients 1 and 25).

Altogether, the clonal abnormalities detected by conventional karyotyping and FISH accounted for 27/32 (84.4%) of the cases. Monosomy 7 was observed in 10/27 (37%), complex karyotypes in 12/27 (44.4%) and other abnormalities in 5/27 (18.5%) including deletion of the long arm of chromosome 7, chromosome 8 tetrasomy, t(3;21) and structural alterations involving chromosomes 1 and 21 (Figure 1). The frequency of unbalanced abnormalities was 26/27 (96.3%); a single case presented a balanced abnormality. Monosomy 7 was more prevalent in patients with secondary MN after AA (66.6%), whereas in the other cases the incidence of complex karyotypes was higher (52.2%). The description of the karyotypes according to the ISCN 2009 and the classification of the abnormalities are shown in Table 2.

Figure 2 exemplifies a complex karyotype, Figure 3 shows a karyotype with a balanced translocation and Figure 4 is a FISH image showing a nucleus with monosomy 7.

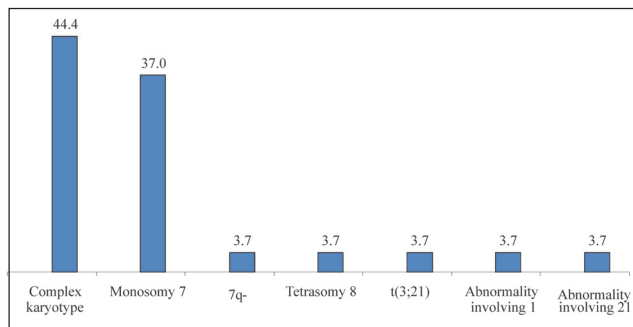


Figure 1 – Incidence of chromosome abnormalities (%) in secondary myeloid neoplasms (n = 27)

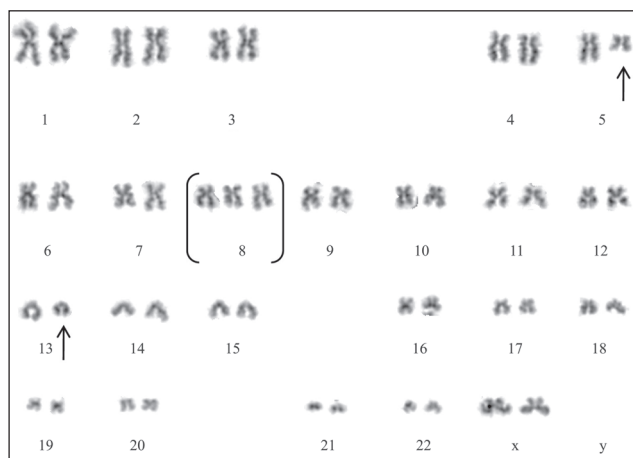


Figure 2 – Therapy-related complex karyotype (Patient 14) showing del(5q), trisomy 8 and del(13q). The therapy for follicular lymphoma (previous disease) included cyclophosphamide and fludarabine phosphate

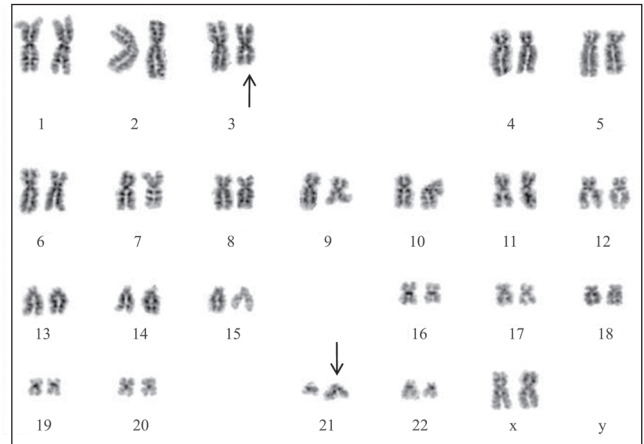


Figure 3 – Therapy-related anomaly (Patient 9) showing t(3;21) (q22;q26). The therapy for plasma cell myeloma (previous disease) included melphalan

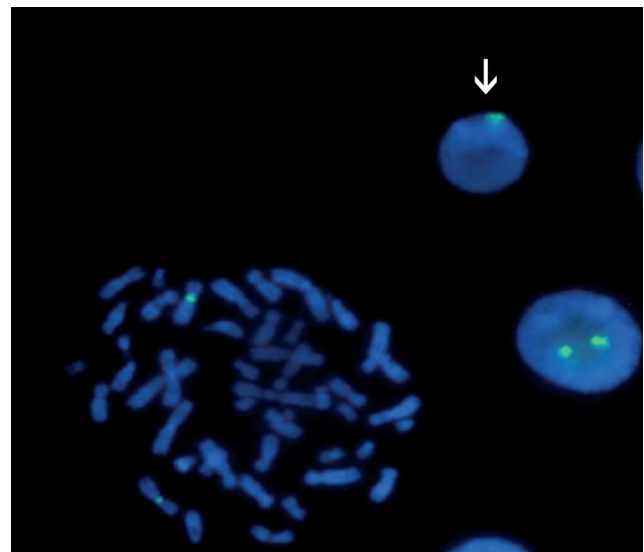


Figure 4 – FISH study using centromeric probe for chromosome 7. Therapy-related abnormality (Patient 25) one interphase cell (only one green signal) demonstrating monosomy 7. The therapy for rhabdomyosarcoma (previous disease) included cyclophosphamide, etoposide, doxorubicin and carboplatin.

Cytogenetics and prognosis

The median overall survival of the 42 patients was 6 months; a little shorter (5.7 months) when the date of HSCT, a procedure used for eight patients, was delayed.

A normal cytogenetic result was associated with better survival (no median value was reached in this study) compared to an abnormal cytogenetic result with 5 months median survival (p-value = 0.03 – Figure 5). A slight tendency of poorer survival was observed when complex karyotypes was compared with other cytogenetic abnormalities (p-value = 0.057). There was no significant difference between patients with chromosome 7 monosomy

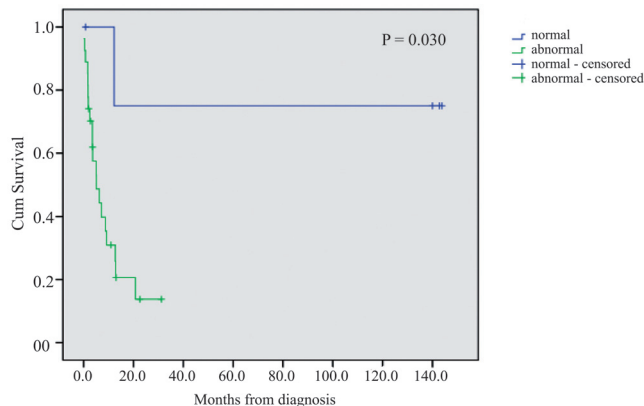


Figure 5 – Kaplan-Meier survival curves according to the cytogenetic profile, normal vs. abnormal

and with complex karyotypes (p-value = 0.123). Binary logistic regression analysis for overall survival-related factors showed that the presence of an abnormal karyotype was an independent factor for reduced survival (p-value = 0.012).

Discussion

The results obtained from our sample are concordant with those of other authors, reinforcing the importance of cytogenetics, both in the diagnosis and prognosis of secondary MN.

The high rate of clonal abnormalities (84.4%) observed here is similar to other studies that reported karyotype abnormalities in 80-95% of patients with secondary MN.⁽⁶⁾ Also, the predominance of unbalanced abnormalities (96.3%), the high frequency of complex karyotypes (44.4%) and chromosome 7 monosomy (37%) are consistent with the literature.^(6,7,9) In particular, monosomy 7 was described in patients following AA; this cytogenetic abnormality has been reported to be recurrent in secondary MN of patients with medullar failure treated with immunosuppressants and G-CSF.^(8,17,22) Curiously, the only case of balanced abnormality with t(3;21) was a patient (Patient 9) who had been treated for multiple myeloma using an alkylating agent (melphalan).

Balanced abnormalities involving the *CBFB* (16q22), *RARA* (17q21) and *AML1* (21q22) genes have been reported more frequently after the use of topoisomerase inhibitors. The presence of the t(3;21)(q26;q22) translocation that leads to the fusion of the *EVII* (3q26) and *AML1* or *RUNX1* (21q22) genes has been frequently reported in MN-t and in the blast crisis stage of CML.⁽²³⁾ In the present sample, no rearrangements of the 11q23 region were observed; the importance of investigating the integrity of the *MLL* gene by FISH must be remembered, given the fact that the *MLL* rearrangement may remain cryptic by conventional cytogenetic methods.⁽²⁴⁾

Cytogenetic abnormalities are known to be independent prognostic factors in *de novo* MDS and are incorporated in the most commonly used prognostic scores such as the

International Prognostic Scoring System (IPSS) and WHO Prognostic Scoring System (WPSS).^(25,26) Yet in secondary MN, the prognostic factors are less well studied, with variables such as age, primary disease, platelet count, hemoglobin concentration, serum protein and C-reactive protein levels, cytogenetic analysis, medullar fibrosis and CD34 cell expression in bone marrow histology, all being associated with survival.⁽²⁷⁻³⁰⁾ The prognosis of MN-t is highly reserved, with a median survival time estimated at 6 months; the cytogenetic study plays an important role, not only for prognosis, but in the follow-up after HSCT.^(7,12) In this study, abnormal karyotype proved to be an independent factor for poorer survival (p-value = 0.03), while complex karyotype alterations showed a slight tendency towards a reduced survival (p-value = 0.057) compared to the other cytogenetic abnormalities.

Secondary MNs are becoming more common; this is the price of longer survival of patients with neoplasias. The treatment of chronic lymphoproliferative diseases with purine analogs associated with alkylating agents, as well as high-intensity therapeutic regimens, followed by autologous transplantation are current examples of this scenario. Moreover, in the field of non-neoplastic hematological diseases, a clear increase in the incidence of MDS is observed in patients with AA. Despite the unfavorable prognosis of secondary MNs, karyotype analysis allows the stratification of these patients. New drugs, such as hypomethylating agents, are the subject of research as a "bridge" until a suitable donor for allogeneic HSCT can be found.

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