Brazilian Journal of Medical and Biological Research (2008) 41: 610-614
ISSN 0100-879X Short Communication

Association of loss of heterozygosity with cytogenetic abnormalities in acute myeloid leukemia and myelodysplastic syndrome

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Deletions on chromosomes 5 and 7 are frequently seen in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). It is assumed that these deletions indicate loss of tumor suppressor genes on these chromosomes and until these tumor suppressor genes are identified, the functional consequences of these deletions and the molecular basis of these myeloid disorders cannot be completely understood. We evaluated loss of heterozygosity (LOH) in 44 patients (18 MDS and 26 AML, diagnosed according to WHO classification criteria) at diagnosis, using a four-microsatellite marker panel: an intragenic marker on the 7th intron of gene *IRF-1* of the 5q31.1 region and three markers located inside the 7q31.1 region and correlated the LOH with karyotype abnormalities. The microsatellites chosen corresponded to chromosome regions frequently deleted in MDS/AML. The samples with Q (peak area) less than or equal to 0.50 were indicative of LOH. The percent of informative samples (i.e., heterozygous) for the intragenic microsatellite in gene *IRF-1* and in loci D7S486, D7S515 and D7S522 were 66.6, 73.7, 75.5, and 48.8%, respectively. Cytogenetic abnormalities by G-banding were found in 36% (16/44) of the patients (2 of 18 MDS and 14 of 26 AML patients). We found a significantly positive association of the occurrence of LOH with abnormal karyotype ($P < 0.05$; chi-square test) and there were cases with LOH but the karyotype was normal (by G-banding). These data indicate that LOH in different microsatellite markers is possibly an event previous to chromosomal abnormalities in these myeloid neoplasias.

Key words: Myelodysplastic syndrome; Acute myeloid leukemia; Cytogenetic abnormalities; Loss of heterozygosity

Publication supported by FAPESP.

Received January 31, 2008. Accepted July 2, 2008

Loss of heterozygosity (LOH) describes the homozygous state of a distinct chromosomal region and points to the presence of closely located inactivated tumor suppressor gene (TSG) that may be involved in the malignant transformation (1,2). Loss of heterozygosity in tumor cells has been analyzed using microsatellite markers (2).

Cytogenetic abnormalities are important to identify malignant cells and to indicate prognosis in myeloid malignancies (3). Translocations, duplications, deletions, insertions, and inversions involve the repositioning, addition or omission of millions of nucleotides and are associated with genomic instability in cancer cells.

Myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) are myeloid malignant disorders of hematopoietic stem cells characterized by numerous cytogenetic abnormalities. Alterations on chromosome 5, which include interstitial deletions of the long arm or complete loss of the entire chromosome (monosomy 5), are frequently observed in MDS and AML patients (3). Deletion of chromosome 7q is also common in MDS and AML. Loss of whole chromosome 7 or deletion of the long arm are detected in up to 20% of patients with MDS or AML and the

highest frequency is noted in MDS and AML arising after cytotoxic therapy (3). It is assumed that these deletions indicate loss of possible TSG in these chromosomes and until these TSG are identified, the functional consequences of these deletions and the molecular basis of these myeloid disorders cannot be completely understood.

Chromosome abnormalities are important markers of genetic instability but are considered to be late events during MDS transformation and AML evolution. The question is what are the initial steps in MDS and AML related to these abnormalities? Could LOH be a sign of future alterations in these cases?

The aim of this report is to evaluate LOH in MDS and AML patients using a four-microsatellite marker panel: an intragenic marker on the 7th intron of gene interferon regulatory factor 1 (*IRF-1*) of the 5q31.1 region and three markers located inside the 7q31.1 region and to correlate the LOH with karyotype abnormalities.

Karyotype analysis was performed according to standard techniques (4). When possible, 20 G-banded metaphases were analyzed and classified according to the International System for Human Cytogenetic Nomenclature (5). Favorable karyotype in MDS was considered to be del(20q), -Y, normal and del(5q) isolated; unfavorable were complex karyotype (at least three abnormalities in metaphase) and abnormalities of chromosome 7 (6); intermediate were those not classified as favorable and unfavorable. Favorable karyotype in AML was: inv(16)/t(16;16), t(15;17) and t(8;21); intermediate was normal, +8, +6, -Y and del(12p), while unfavorable were alterations of chromosomes 3, 9, 11, 20, 21, del(5q), del(7q), -5, -7 and complex karyotype (7).

Paired samples of bone marrow and buccal smears were obtained, after informed consent, from 44 patients (18 MDS and 26 AML) at diagnosis. This study was approved by the Institutional Review Ethics Committee. The diagnoses of AML and MDS were made according to the World Health Organization (Table 1) (8).

Buccal smear collection was carried out with sterile cytology brushes and served as normal controls. Patients were instructed to brush the inside of both sides of the cheek for 30 s, and the brush was kept in a sterile saline solution for extraction. Genomic DNA was extracted from buccal smears by the Puregene DNA isolation kit (Gentra Systems, USA) according to manufacturer recommendations.

Bone marrow samples were obtained for cytogenetic analysis and for DNA extraction by standard procedures (4). Bone marrow DNA was extracted by conventional cell lysis and proteinase K method. DNA was purified by phenol-chloroform extraction and precipitated with sodium acetate and ethanol (9). The concentration and quality of DNA were measured by the GeneQuant PRO RNA/DNA calculator (GE Healthcare, USA).

PCR was performed on 25 ng DNA from bone marrow aspirates and buccal smears, in a 25 µL containing 1X buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol of each primer, and the forward primer was 5' end fluorescently labeled (FAM), 0.625 U (0.12 µL) AmpliTaq Gold DNA polymerase (PE Applied Biosystems, USA) (9).

Amplification was performed in a Perkin Elmer 9700 Thermal Controller (PE Applied Biosystems) after an initial denaturation at 94°C for 12 min. Twenty-five cycles were performed using the following temperature and time profile: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 70°C for 30 s, and a final extension of 72°C for 25 min to minimize stutter bands. The PCR products were analyzed on a 6% polyacrylamide gel in 1X Tris-borate-EDTA buffer in the 377 automated DNA sequencer (PE Applied Biosystems). One microliter of each PCR product was combined with 4 µL formamide, 0.5 µL blue dextran, and 0.5 µL of a fluorescent size marker (GS 500 ROX; PE Applied Biosystems). The mixture samples were heated to 90°C for 5 min and then cooled on ice until ready to load.

The electrophoresis conditions were 3000 W and 51°C. PCR products were quantitatively detected by fragmentation analysis using ABI GeneScan and ABI Genotyper software (PE Applied Biosystems).

Cytogenetic abnormalities by G-banding were detected in 36% (16/44) of the patients, specifically 2 of 18 MDS and 14 of 26 AML patients (Table 1).

The criteria selected for detecting allele loss were described by Cawkwell et al. (10). The samples with Q (peak area) less than or equal to 0.50 indicated LOH. The percent of informative samples (i.e., heterozygous) for the intragenic microsatellite in gene *IRF-1* and in loci D7S486, D7S515 and D7S522 were 66.6, 73.7, 75.5, and 48.8%, respectively. The results of microsatellite analysis are summarized in Table 2.

Allelic loss occurred in 2 of 29 (6%) informative cases for *IRF-1*. One patient (case 21) was an AML with prior MDS and the other (case 43) was an AML secondary to therapy, both patients had an unfavorable karyotype abnormality.

LOH was detected for one or more markers in 5 of 44 (11%) patients. Cases 19 and 31 presented LOH for the 3 markers (D7S486, D7S515 and D7S522). Case 21 showed LOH for markers D7S486 and D7S515 and cases 5 and 23 presented LOH for marker D7S515. Among the 2 patients who presented deletion 5q (cases 15 and 21), only case 21

Table 1. Classification of patients in the present study according to WHO criteria (8) and results of karyotyping and microsatellite analyses.

Patient No.	WHO classification	Cytogenetics	LOH
1	Refractory cytopenia with multilineage dysplasia (RCMD)	Without metaphases	
2	Refractory cytopenia with multilineage dysplasia (RCMD)	46,XX[20]	
3	Refractory anemia with excess blasts-1 (RAEB-1)	46,XY[20]	
4	Refractory anemia with ringed sideroblasts (RARS)	46,XY[20]	
5	Refractory cytopenia with multilineage dysplasia (RCMD)	46,XX[20]	D7S515
6	Refractory anemia with excess blasts-2 (RAEB-2)	46,XY[20]	
7	Refractory anemia with ringed sideroblasts (RARS)	46,XX[20]	
8	Refractory anemia (RA)	Without metaphases	
9	Refractory anemia (RA)	46,XY[20]	
10	Refractory anemia with ringed sideroblasts (RARS)	Without metaphases	
11	Refractory anemia (RA)	46,XY[15]	
12	Refractory anemia (RA)	Without metaphases	
13	Refractory cytopenia with multilineage dysplasia (RCMD)	46,XY[20]	
14	Refractory anemia with ringed sideroblasts (RARS)	Without metaphases	
15	Refractory anemia with excess blasts-2 (RAEB-2)	46,XX,del(5)(q31)[15]	
16	Refractory anemia with excess blasts-2 (RAEB-2)	Condensed metaphases	
17	Refractory cytopenia with multilineage dysplasia (RCMD)	46,XX[20]	
18	Refractory anemia with excess blasts-1 (RAEB-1)	47,XY,+3[4]/46,XY[12]	
19	AML with multilineage dysplasia with prior MDS	45,XY,-7[16]/46,XY[4]	D7S486, 515, 522
20	AML with multilineage dysplasia with prior MDS	46,XY[15]	
21	AML with multilineage dysplasia with prior MDS	47,XX,+8[1]/47,XX,del(5)(q13q33), del(7)(q22),+8[2]/49,XX,del (7)(q22),+8,+11,+18[1]	IRF-1, D7S486, 515
22	AML with multilineage dysplasia with prior MDS	46,XX[20]	
23	AML with multilineage dysplasia without prior MDS	Without metaphases	D7S515
24	AML with multilineage dysplasia without prior MDS	46,XX[20]	
25	AML with multilineage dysplasia with prior MDS	46,XX,inc[9]	
26	AML with multilineage dysplasia with prior MDS	46,XY[20]	
27	AML with multilineage dysplasia with prior MDS	48,XX,+8,+9[10]	
28	AML with multilineage dysplasia with prior MDS	48,XY,+14,+22[14]/46,XY[1]	
39	AML not otherwise categorized/myelomonocytic	46,XY[8]	
30	AML not otherwise categorized/monocytic	46,XY,t(1;2)(p31;q34)[20]	
31	AML not otherwise categorized/AML without maturation	47,XY,+8[16]/46,XY[4]	D7S486, 515, 522
32	AML not otherwise categorized/AML with maturation	46,XY[20]	
33	AML with recurrent cytogenetic translocations/AML with t(8;21)(q22;q22)	46,XY,t(8;21)(q22;q22)[15]	
34	AML with recurrent cytogenetic translocations/AML with t(8;21)(q22;q22)	46,XY,t(8;21)(q22;q22)[20]	
35	AML with recurrent cytogenetic translocations/AML with t(15;17)(q22;q12)	46,XX,t(15;17)(q22;q11)[20]	
36	AML not otherwise categorized/AML with maturation	46,XX[20]	
37	AML with recurrent cytogenetic translocations/ AML with t(15;17)(q22;q12)	46,XX,t(15;17)(q22;q12)[20]	
38	AML not otherwise categorized/AML without maturation	47,XX,del(7)(q31),+8[3]/46,XX[8]	
39	AML not otherwise categorized/AML with maturation	46,XY[13]	
40	AML not otherwise categorized/AML with maturation	Without metaphases	
41	AML not otherwise categorized/megakaryocytic	46,XX,add(21)(q22)[15]	
42	AML not otherwise categorized/myelomonocytic	Without metaphases	
43	AML therapy related	53-56,XX,+1,+8,+9,+10,i?(11) (q13),+14,+15,+16,+17,+18,+19, ~6dmin	IRF-1
44	AML with recurrent cytogenetic translocations/AML with t(15;17)(q22;q12)	46,XY,t(15;17)(q22;q12)[20]	

WHO = World Health Organization; MDS = myelodysplastic syndrome; AML = acute myeloid leukemia; LOH = loss of heterozygosity.

presented LOH for *IRF-1*. Among the 3 patients with deletion 7q or monosomy 7 (cases 19, 21, and 38), two cases (19 and 21) presented LOH for at least one intragenic microsatellite of 7q31.1. Only 1 patient (case 5) with normal karyotype by G-banding presented LOH. It was for the marker D7S515. Otherwise, FISH centromeric probe for chromosome 7 showed monosomy in 25% of the cells. The 2 patients with complex karyotype (cases 21 and 43) presented LOH for *IRF-1*. The karyotype of case 21 was 47,XX,+8[1]/47,XX,del(5)(q13q33),del(7)(q22),+8[2]/49,XX,del(7)(q22),+8,+11,+18[1] and case 43 was 53-56,XX,+1,+8,+9,+10,i?(11)(q13),+14,+15,+16,+17,+18,+19,~6dmin.

Table 2. Analysis of loss of heterozygosity (LOH) of the *IRF-1* gene and the markers at 7q.

Patient No.	*IRF-1*	Markers at 7q		
		D7S486	D7S515	D7S522
1	Homoz.	1.0	Homoz.	Homoz.
2	0.98	0.92	1.0	Homoz.
3	0.91	0.91	0.83	1.0
4	1.0	Homoz.	1.0	1.0
5	0.94	0.78	**0.23**	Homoz.
6	1.0	0.91	0.83	Homoz.
7	Homoz.	Homoz.	Homoz.	Homoz.
8	0.98	1.0	0.91	1.0
9	0.96	0.91	0.91	Homoz.
10	1.0	1.0	Homoz.	Homoz.
11	Homoz.	Homoz.	0.91	0.83
12	0.97	0.77	0.91	Homoz.
13	0.96	1.0	1.0	Homoz.
14	0.96	1.0	Homoz.	1.0
15	Homoz.	Homoz.	1.0	0.82
16	0.95	Homoz.	0.83	0.91
17	0.96	0.91	Homoz.	Homoz.
18	Homoz.	Homoz.	Homoz.	Homoz.
19	Homoz.	**0.35**	**0.0**	**0.23**
20	1.0	Homoz.	1.0	0.83
21	**0.34**	**0.28**	**0.21**	Homoz.
22	Homoz.	1.0	0.62	0.55
23	Homoz.	0.91	**0.23**	0.83
24	0.99	Homoz.	Homoz.	0.91
25	1.0	0.83	Homoz.	Homoz.
26	0.99	1.0	0.78	Homoz.
27	1.0	1.0	0.83	Homoz.
28	Homoz.	1.0	1.0	Homoz.
29	1.0	0.99	0.92	0.91
30	0.94	Homoz.	0.92	Homoz.
31	Homoz.	**0.0**	**0.37**	**0.0**
32	0.94	Homoz.	0.83	Homoz.
33	1.0	0.91	0.83	0.77
34	0.98	0.71	Homoz.	Homoz.
35	0.94	0.83	0.91	0.97
36	Homoz.	1.0	0.68	Homoz.
37	0.97	1.0	Homoz.	Homoz.
38	0.93	0.91	1.0	0.91
39	Homoz.	0.91	0.91	0.99
40	Homoz.	0.85	0.91	1.0
41	Homoz.	Homoz.	1.0	Homoz.
42	Homoz.	0.91	0.91	Homoz.
43	**0.45**	Homoz.	0.86	1.0
44	1.0	0.91	0.83	1.0

Homoz. = homozygosity. Numbers in bold = Q (peak area) ≤ 0.50 indicating LOH.

It is important to note that there was a significantly positive association of the occurrence of LOH with abnormal karyotype ($P < 0.05$; chi-square test).

LOH on chromosome 7q has been frequently reported in several types of human cancer including myeloid neoplasia (1). Using a panel of three microsatellite markers at band 7q31, we found 5 of 44 (11%) patients with one or more allelic loss. Among these 5 cases, three presented cytogenetic abnormalities involving chromosome 7 (cases 5, 19, and 21). Case 5 presented monosomy 7 by FISH and LOH for D7S515; case 19 showed monosomy 7 and LOH of the three markers, as expected; case 21 presented LOH of D7S486 and D7S515, but the deletion was at band 7q22. Koike et al. (11) studied the LOH on the long arm of chromosome 7 in AML patients using a panel of 15 markers and found a higher incidence of LOH (27%) than in the present study probably due to the different panel with more markers (fifteen). In fact, Basirico et al. (12) studied the LOH of 7q using a panel of 11 polymorphic microsatellite markers at band 7q21-36 and correlated the findings with conventional karyotype. Sixteen patients (16/50) showed allelic loss, but only three presented cytogenetic abnormalities related to chromosome 7. Our results suggest that LOH in different microsatellite markers on the long arm of chromosome 7 is an event previous to chromosomal abnormalities in these myeloid neoplasias, with karyotype abnormalities occurring later.

An important finding among the present cases was the correlation between LOH of *IRF-1* and complex karyotype with a lot of trisomies, deletions, isochromosome and double minutes concomitantly. An interesting aspect that remains to be determined is if *IRF-1* gene expression is decreased in these cases of LOH. Unfortunately, immunohistochemistry for *IRF-1* performed in these cases was not successful, and there were no more samples available for additional molecular studies. Notwithstanding, *IRF-1* has been implicated as a mediator for interferon signaling, when induced by TNF-α, interferons, viral infections and retinoids. *IRF-1* has been considered to be a TSG that plays an essential role in cell growth control and surveillance against malignant development (13). The findings of the present study support the view of *IRF-1* as an important TSG.

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