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Chromosomal breakpoints and changes in DNA copy number in refractory acute myeloid leukemia

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Comparative genomic hybridization (CGH) was used to detect changes in DNA copy number in 25 cases of refractory acute myeloid leukemia (AML). CGH detected changes in DNA copy number in nine AML (36%). Losses (82%) were more frequent than gains (18%). No high-level amplifications were detected in any of the cases. Losses involved minimal overlapping regions at 5q14q32, 7q31.2q32 and 12p12. The most frequent gain was detected at 8q. CGH gave normal results in all cases with a normal karyotype or a translocation as the sole aberration. The absence of high-level DNA copy gains suggests that, in contrast to other malignancies, gene amplification is not an important mechanism for drug resistance in AML. In addition to 5q and 7q, known to be associated with disease refractoriness, 12p may be another region related to poor prognosis.
Keywords: CGH; refractory AML; 5q; 7q; 12p

Introduction

Resistance to antileukemic drugs is a major cause of treatment failure in patients with acute myeloid leukemia (AML).[1–5] About 20–40% of AML patients never achieve remission even with intensive chemotherapy.[4] About half of this group dies in a state of hypoplasia, mainly from infections, and the other half does not respond to chemotherapy. Furthermore, about 50–70% of the patients who achieve remission will relapse due to regrowth of leukemic cells that have survived chemotherapy, and the prognosis is then poor. A short duration of the first remission is a very poor prognostic sign. Based on results from 150 patients, Hiddemann *et al*[6] defined refractoriness against standard first-line chemotherapy as a nonresponse to induction therapy or an early relapse within 6–12 months of first remission. Also, the immunophenotypic features may have diagnostic and prognostic value in AML. The most consistent finding has been a correlation between CD34 expression and a poor response to therapy. However, there are cases with CD34 expression despite their favorable outcome.[7–9] Drug resistance at the gene level has been thought to be one of the most important determinants of clinical outcome.[10] Chromosomal aberrations such as monosomy 5 or 7 (or deletions of their long arms), or t(9;22), have been associated with a poor response to therapy and considered to indicate a poor prognosis.[11–13]

Several mechanisms of drug resistance have been demonstrated in preclinical drug trials. Much less is known about the reasons for the poor responsiveness of patients receiving treatment for AML. Nevertheless, there is growing evidence that changes at the gene level (amplifications, mutations and losses) contribute to this kind of drug resistance. Amplification and overexpression of the multidrug resistance gene (*PGY1*), located at 7q21, is a source of resistance to chemotherapy in AML.[14,15] Amplification of the oncogene *MYC* in AML has also been associated with a poor response to chemotherapy.[16] Complete or partial loss of the short arm of chromosome 17, which contains the *TP53* tumor suppressor gene, as well as mutations of the *TP53* gene have been reported in refractory AML.[17,18]

Comparative genomic hybridization (CGH) can be used to demonstrate changes in DNA copy number and to map them to chromosomal regions.[19] Recently, CGH has been used for studying hematologic malignancies.[20–25] Moreover, CGH is a means of screening for chromosomal areas that contain amplified or deleted genes associated with drug resistance.[20,22,26] We performed CGH on 25 cases of refractory leukemia to detect aberrations responsible for drug resistance.

Materials and methods

Patients

Twenty-five patients diagnosed with *de novo* AML with poor prognosis, based either on complete refractoriness against induction therapy or on a short duration of first remission, were included in the study. All patients, except five (Nos 1, 10, 14, 20 and 25), died from refractory leukemia; four of the five survivors received a bone marrow transplant from an HLA-identical sibling donor. Table 1 shows the clinical and laboratory data of the patients.

Standard cytogenetics

BM aspirates were studied at the time of diagnosis by standard G-banding methods. Karyotype abnormalities were described according to the specifications of the International Standing Committee on Human Cytogenetic Nomenclature (ISCN, 1995).[27]

Comparative genomic hybridization

CGH was performed, on the same material obtained for karyotype analysis at diagnosis, using direct fluorochrome-conjugated DNAs by methods described previously.[19] Briefly, the tumor DNA was labeled with fluorescein isothiocyanate (FITC)-dUTP (DuPont, Boston, MA, USA), and reference genomic DNA was labeled with Texas red-dUTP (DuPont) by nick translation to obtain DNA fragments ranging from 600 to 2000 base pairs. The hybridization mixture consisted of 400 ng labeled tumor DNA, 400 ng labeled reference genomic DNA, and 10 μg unlabeled Cot-1 DNA dissolved in 10 μl of hybridization buffer (50% formamide, 10% dextran sulfate, 2 × SSC). The hybridization mixture was denatured at 75°C

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Table 1 Clinical and laboratory data at diagnosis of 25 cases with refractory acute myeloid leukemia

Patient No. (sex/age)	*Code*	*Date of diagnosis*	*Blasts in BM (%)*	*FAB*	*WBC ($\times 10^9$/l)*	*Blasts in PB (%)*	*Duration of first CR (days)*	*Survival (days)*	*Last status*
1 (F/52)	960933	16-09-96	40	M1	3	60	0	108	Alive
2 (M/48)	890644	24-07-89	42	M4	73	51	0	113	Dead
3 (M/47)	950392	30-05-95	40	M4	152	56	0	57	Dead
4 (F/47)	920391	16-04-92	50	M4	51	60	0	189	Dead
5 (M/64)	950689	23-05-95	30	M2	123	60	0	225	Dead
6 (F/39)	911322	13-12-91	57	M4	88	12	0	226	Dead
7 (F/64)	890529	04-07-89	47	M2	2	3	0	251	Dead
8 (M/21)	920104	28-01-92	45	M2	53	73	0	265	Dead
9 (M/32)	881023	18-10-88	60	M2	6	47	0	278	Dead
10 (M/50)	950979	16-11-95	33	M2	20	6	46	380	BMT, CR
11 (F/68)	950160	08-03-95	90	M1	18	90	47	510	Dead
12 (M/50)	910986	06-09-91	40	M4	8	3	73	164	Dead
13 (M/23)	890581	21-07-89	40	M6	154	8	98	416	Dead
14 (F/49)	880924	22-09-88	40	M2	140	52	139	2589	BMT, CR
15 (M/56)	940425	27-05-94	60	M4	102	10	152	296	Dead
16 (F/58)	940732	20-09-94	30	M2	128	38	164	366	Dead
17 (F/42)	900278 901218	22-03-90	90	M2	29	84	197	398	Dead
18 (F/53)	890363	06-05-89	45	M4	27	12	198	398	Dead
19 (M/56)	900402	25-04-90	90	M1	153	92	299	577	Dead
20 (M/32)	880436	06-05-88	45	M6	5	90	304	560	BMT, CR
21 (F/40)	890827	15-09-89	85	M4	54	78	365	688	Dead
22 (M/66)	940819	17-10-94	50	M6	11	42	0	267	Dead
23 (M/51)	930132	16-11-91	99	M1	39	96	344	520	Dead
24 (F/25)	880389	26-04-88	40	M5	207	77	0	201	Dead
25 (M/21)	900417	02-05-90	95	M1	226	99	0	1734	BMT, CR

BM, bone marrow; CR, complete remission; BMT, allogeneic bone marrow transplantation from an HLA-identical sibling donor; PB, peripheral blood; WBC, white blood cell count.

for 5 min and hybridized to a slide preparation with normal metaphase spreads denatured in 70% formamide/2 × SSC at 68°C for 2 min. Hybridization was performed at 37°C for 48 h. The slides were washed three times in 50% formamide/2 × SSC (pH 7), twice in 2 × SSC, and once in 0.1 × SSC at 45°C, followed by 2 × SSC, 0.1 M NaH_2PO_4–0.1 M Na_2HPO_4–0.1% NP40 (pH 8), and distilled water at room temperature for 10 min each. After air-drying, the slides were counterstained with 4′,6-diamidino-2-phenylindoledihydrochloride (DAPI) (Sigma, St Louis, MO, USA), and then mounted with an antifading medium (Vectashield, Vector Laboratories, Burlingame, CA, USA).

Digital image analysis

The hybridizations were analyzed using an Olympus fluorescence microscope and the ISIS digital image analysis system (Metasystems, Altlussheim, Germany) based on an integrated high-sensitivity monochrome charge-coupled device (CCD) camera and automated CGH analysis software. Three-color images (red for reference DNA, green for tumor DNA, and blue for counterstaining) were acquired from 8–10 metaphases per sample. Only metaphases of good quality with strong uniform hybridization were included in the analysis. Chromosomes not suitable for CGH analysis were excluded (ie chromosomes heavily bent, overlapping, or with overlying artefacts). Chromosomal regions were interpreted as overrepresented when the corresponding ratio exceeded 1.17 (gains) or 1.5 (high-level amplification), and as under-represented (losses) when the ratio was less than 0.85. All the results were confirmed using a 99% confidence interval. Briefly, intra-experiment standard deviations for every position in the CGH ratio profiles were calculated further from the variation of the ratio values of all homologous chromosomes within the experiment. Confidence intervals for the ratio profiles were then computed by combining them with an empirical inter-experiment standard deviation and by estimating error probabilities based on the *t*-distribution.

Controls

In each CGH experiment, a negative control (peripheral blood DNA from a healthy donor) and a positive control were included. The positive control was a tumor with known changes in DNA copy number (gains at 5q21qter, 9q23qter and 12q; high-level amplifications at 8q22qter and 11q14q23; losses at 2q11q22, 3p14q21, 13q11q22 and 22).

Results

Standard cytogenetic analysis

Chromosomal aberrations were detected in 15 patients (60%). Among the patients with abnormal karyotypes, four patients had balanced chromosomal translocations only (Table 2).

Comparative genomic hybridization

CGH revealed changes in DNA copy number in nine patients (36%). DNA copy losses were more frequent than gains and

Table 2 Results of karyotype and CGH analyses on 25 cases with refractory acute myeloid leukemia

Patient No.	*Karyotype*	*Changes in DNA copy number according to CGH*	
		Losses	*Gains*
1	44–45,XX,t(3;12)(q26;p12),–7,r(11),add(17)(q?),+1–2mar[10]	7q22q35	11q23qter
2	46,XY,t(3;3)(q21;q26)[10]	–	–
3	46,XY,–2,der(3) 2x2, der(5)t(1;5)(p11;q35)del(5)(q13q35), der(7)t(3;7)(q25;q22)i(8)(q10),+1–2 mar,inc[10]	3p21pter,3q11q21,5q14–q32,7q31.2qter,8p	1p,2p13p23,8q
4[a]	45,XX,del(3)(q21q26),–6,–7,–22,+mar1,+mar2[12]	6q11q22.2,7p,7q21.2qter	–
5	46,XY,del(7)(q22q36)[6]/46,XY[10]	–	–
6	46,XX,del(5)(q13q33),del(7)(q?21.1;q?34), del(12)(p11.2 or p13)[15]	5q14q33,7q21q32,12p12	
7	46,XX[16]	–	–
8	46,XY[10]	–	–
9	46,XY[20]	–	–
10	46,XY[20]	–	–
11	46,XX[20]	–	–
12	43–45,XY,add(2)(p?21),i(5)(p10),–7,+8,–12, –16,–17,t(17;?)(p11.2;?),+1–3mar,inc[cp17]	3q11q21,5q,7,12p12,16,18q	5p
13	47,XY,+8,t(8;12)(p21;p11.2), del(12)(p11p1?3)[9]	12p12	8
14	46,XX,t(8;22)(p11;q11)[20]	–	–
15	46,XY[20]	–	–
16	46,XX,–2,–3,+del(4)(p1?5),add(9)(q34),–13, –14,+?15,+?15,–17,+mar,inc[cp23]	13q21q31	–
17	46,XX,t(4;12)(q12;p12–13)[15]	–	–
18	46,XX[4]	–	–
19	46,XY[15]	–	–
20	46,XY[14]	–	–
21	46,XX,del(7)(q31.2) or del(7)(q22q34), inv(16)(p13q22)[11]/46,XX[1]	7q31.2qter	–
22	45,XY,–5,–17,–20,+2 mar,inc[3]/42,XY,idem, –4,–7,add(11)(p11),–16,+3–4mar, inc[7]/88,idemx2,inc[3]	4p,4q23qter,5q12q32,7p13q11.2, 7q31qter,12q21qter,16,17	7q21.1q21.3,8
23	46,XX,t(9;22)(q34;q11),t(12;14)(q?14;q32),inc[14]	–	–
24	46,XX,del(11)(q23)[8]/46,XX[12]	–	–
25	46,XY[20]	–	–

–, no changes in DNA copy number.
[a]One marker was partially painted with chromosome 3-specific library probe. Chromosomal painting was performed on archival preparations according to El-Rifai and Knuutila[39] using whole chromosome 3-specific library probe (American Type Culture Collection, Rockville, MD, USA).

constituted 82% of the changes detected (Table 2, Figure 1). Among the patients with abnormal CGH results, the most frequent changes in DNA copy number were losses at 5q detected in four patients (44%), at 7q in seven patients (78%), and at 12p in three patients (33%), with minimal common overlapping regions at 5q14q32, 7q31.2q32 and 12p12, respectively. In one patient, the deletion at 12p12 detected by CGH was not revealed in the karyotype analysis. Losses were also detected at 3p21pter, 3q11q21, 4p, 4q23qter, 6q11q22.2, 8p, 13q21q31, 16, 17, and 18q. Gains at 8q were detected in three patients (33%). Gains at 1p, 2p13p23, 5p, and 7q21.1q21.3 were also seen. No high-level amplifications were found in any of the cases and gains were always accompanied by losses.

Changes in DNA copy number were not observed in AML with only balanced translocations or a normal karyotype (14 patients). Additionally, CGH did not reveal changes in DNA copy number in two cases (Nos 5 and 24) with chromosomal imbalances present only in 33% of the metaphases analyzed karyotypically. CGH provided valuable information for cases (Nos 1, 3, 4, 12, 16, 22) who had monosomies with marker chromosomes by showing the sum of DNA changes in them. Cases with marker chromosomes and monosomies of chromosomes 5, 7 or 12 by karyotype analysis had only partial deletions of these chromosomes according to CGH, suggesting rearrangement of the rest of the material in the markers. DNA copy losses were detected at 3q11q21 in two cases (Nos 3 and 12) but in one of them no deletion of chromosome 3 was seen by karyotype analysis. In one case (No. 4) CGH did not reveal the del(3)(q21q26) detected by karyotype analysis.

Controls

All negative controls were normal at cut-off values of 0.85 for deletions and 1.17 for gains. The positive controls showed the same aberrations that had been detected originally.

Summary of the results

Overall, the most common sites of aberration were 3q (20%), 5q (20%), 7q (32%), 12p (20%) and 8q (16%).

Discussion

Our results show that aberrations in chromosomes 5 and 7 are associated with chemotherapy drug resistance as indicated by previous cytogenetic studies. However, important findings related to drug resistance in this study were: (1) normal chromosomes in several patients; (2) the absence of high-level

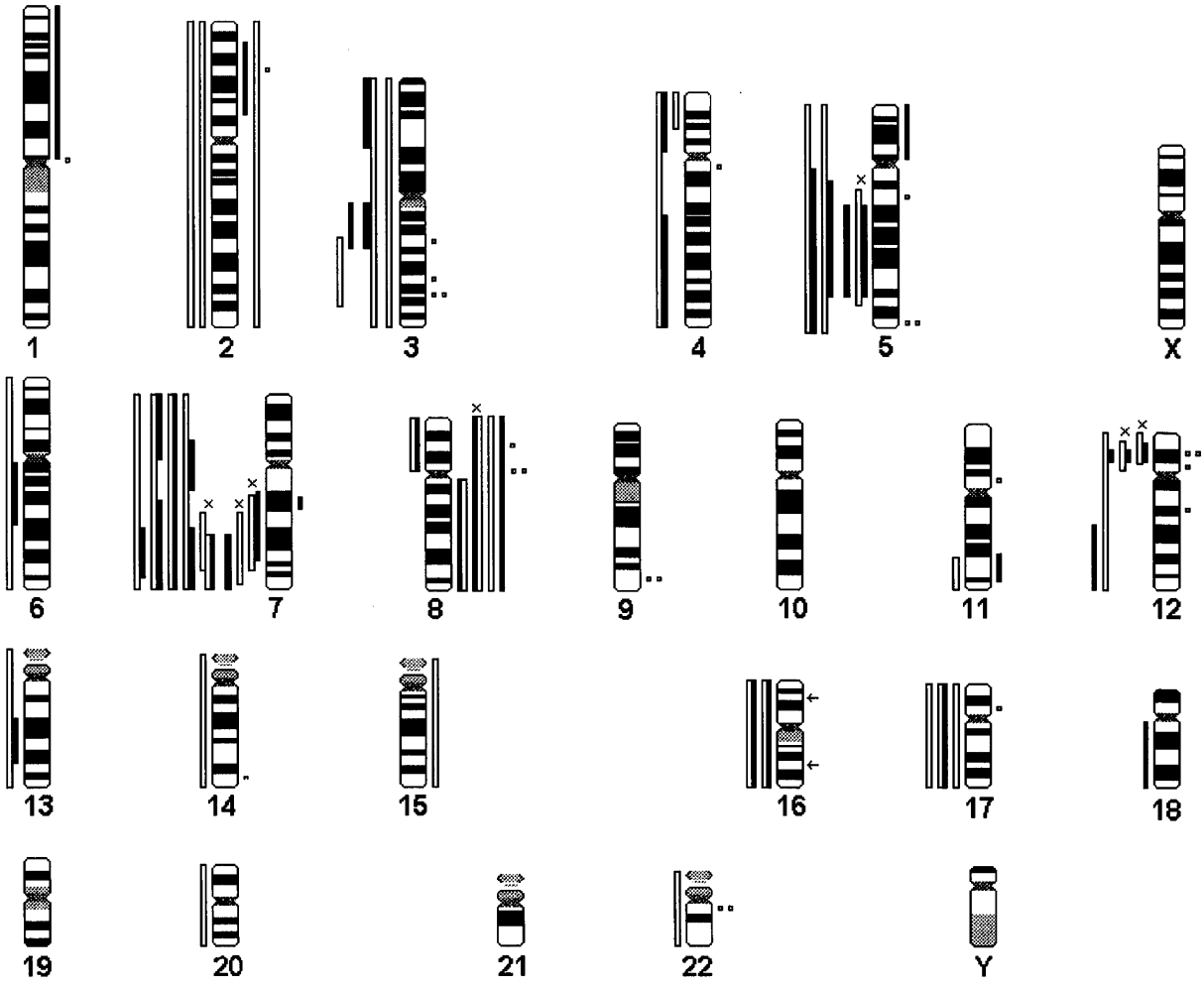

Figure 1 Summary of DNA copy number gains and losses detected by CGH and karyotype analysis. Each bar presents one patient. The empty bar represents the karyotype analysis and the solid bar the CGH analysis on the same sample. Gains are on the right side, losses on the left side. All cases had markers in their karyotype except those marked with 'x'. ◘, translocation breakpoints detected by karyotype; arrows, inversion breakpoints detected by karyotype.

amplifications; and (3) deletions or translocations at 12p. CGH provided important information about net gains and losses in patients with complex karyotypes (Nos 1, 3, 4, 12, 16, 22). Instead of complete monosomy, CGH showed that the deletions are small and interstitial in most cases, suggesting that the marker chromosomes in these AML cases contained DNA material from the deleted chromosomes.

In our cases, the common overlapping region of losses in chromosome 5 was 5q14q32, which coincides with previously reported cytogenetic data on chromosome 5q deletions.[28] Recently, a myeloid tumor suppressor locus was mapped to 5q31.1,[29,30] which is consistently lost in cases with 5q aberrations.[31]

CGH showed that the deletions in chromosome 7, in our cases, had a minimal common overlapping region at 7q31.2q32. In addition, gain at 7q21.1q21.3 with simultaneous loss at 7q was detected in case No. 22. Amplifications of the *PGY1* gene located at 7q21.1 and deletions at 7q32 imply poor response to chemotherapy[10,14,15,32] and may explain the refractoriness of the disease in these AML cases.

Although deletions at 12p12 have not been reported as signs of drug resistance, our CGH analyses revealed deletions at 12p12 in three patients (Nos 6, 12, 13) and karyoype analyses a translocation involving 12p12–13 in two other patients (Nos 1 and 17). The region includes recently described genes *GDID4, CDKN1B* and *ETV6,*[33–36] which may have relevance to the drug resistance in our patients.

Breakpoints at 3q21 or 3q26 detected in five patients (Nos 1, 2, 3, 4 and 12) by CGH and/or karyotype analyses coincide with previous cytogenetic studies reporting poor response to therapy in AML with abnormalities of chromosome 3 at 3q21 and q25–26.[37,38]

In conclusion, our results by CGH together with standard cytogenetics suggest that DNA copy number losses, but not high-level amplifications, may contribute to the refractoriness of AML. In addition to regions known to be critical for prognosis, aberrations at 12p could be related to a poor prognosis. Since some refractory cases yield normal results, it may be that submicroscopic molecular changes are also responsible for resistance to therapy. Further studies using molecular techniques are needed to identify the loci and genes responsible for drug resistance in AML.

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