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Gene Rearrangements in Bone Marrow Cells of Patients with Acute Myelogenous Leukemia

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Key Words
Acute myelogenous leukemia · Clonality · Residual disease · Southern blot

Abstract
At diagnosis, clonal gene rearrangement probes [retinoic acid receptor (RAR)-α, major breakpoint cluster region (M-bcr), immunoglobulin (Ig)-JH, T cell receptor (TcR)-β, myeloid lymphoid leukemia (MLL) or cytokine genes (GM-CSF, G-CSF, IL-3)] were detected in bone marrow samples from 71 of 153 patients with acute myelogenous leukemia (AML) (46%): in 41 patients with primary AML (pAML) (58%) and in 30 patients with secondary AML (42%). In all cases with promyelocytic leukemia (AML-M3) RAR-α gene rearrangements were detected (n = 9). Gene rearrangements in the Ig-JH or the TcR-β or GM-CSF or IL-3 or MLL gene were detected in 12, 10, 16 and 12% of the cases, respectively, whereas only few cases showed gene rearrangements in the M-bcr (6%) or G-CSF gene (3%). Survival of pAML patients with TcR-β gene rearrangements was longer and survival of pAML patients with IL-3 or GM-CSF gene rearrangement was shorter than in patients without those rearrangements. No worse survival outcome was seen in patients with rearrangements in the MLL, Ig-JH or M-bcr gene. In remission of AML (CR), clonal gene rearrangements were detected in 23 of 48 cases (48%) if samples were taken once in CR, in 23 of 26 cases (88%) if samples were taken twice in CR and in 23 of 23 cases (100%) if samples were studied three times in CR. All cases with gene rearrangements at diagnosis showed the same kind of rearrangement at *relapse* of the disease (n = 12). Our data show that (1) populations with clonal gene rearrangements can be regularly detected at diagnosis, in CR and at relapse of AML. (2) Certain gene rearrangements that are detectable at diagnosis have a prognostic significance for the patients' outcome. Our results point out the significance of gene rearrangement analyses at diagnosis of AML in order to identify 'poor risk' patients – independently of the karyotype. Moreover, the persistence of clonal cells in the further course of AML can be studied by gene rearrangement analysis.

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Introduction

AML results in accumulation of leukemic blasts through clonal proliferation of an abnormal progenitor cell [1]. Clonal cell populations can be identified by cytogenetics, polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH) or Southern blot analysis [2, 3]. Clonal bone marrow (BM) cells, rearranged in the T cell receptor (TcR), in immunoglobulin (Ig), in growth factor genes (GM-CSF, G-CSF, IL-3), in the major breakpoint cluster region (M-bcr) or in the myeloid lymphoid leukemia gene (MLL), can be detected in about 50% of the cases with acute myelogenous leukemia (AML) [4–8]. Gene rearrangements of the retinoic acid receptor-α (RAR-α) gene are typical for promyelocytic leukemia (AML M3). Gene rearrangements can be used to study the presence of clonal-gene-rearranged cells in the course of the disease [9]. About 70% of patients with AML in complete remission (CR) relapse within the following 2 years. Therefore, residual leukemic cells must have survived [9, 10]. There are only few data available about the incidence and prognostic significance of gene rearrangements in the genes listed above [11, 12]. In this study we present data about the incidence and prognostic significance of certain gene rearrangements detected at diagnosis and in the course of AML. This may contribute important data about the biological features of AML cells as well as for the development of treatment strategies to restore or keep stable remissions in AML.

Materials and Methods

Patients

BM samples from 153 AML patients at diagnosis, 81 of them with primary AML (pAML) and 72 with secondary AML (sAML) after myelodysplasia (MDS) were studied (all samples were obtained from patients at the University of Munich). Mean age of the patients was 53 years, male to female ratio was 0.9:1. Moreover, 68 patients in CR and 25 patients at relapse according to cytological and cytochemical criteria were examined. The diagnosis of AML was made according to the French-American-British (FAB) classification. All patients had never been treated previously and entered the study at the time of initial therapy. Patients were treated according to approved therapy standards of the EORTC. CR was determined to be achieved when the BM was normocellular, containing <5% blasts, and when neutrophil granulocytes in peripheral blood had recovered to 1,500/μl and platelets to 100,000/μl according to the criteria of the Cancer and Leukemia Group [13]. Relapse was diagnosed when the BM contained at least 25% leukemic blasts, or when leukemic cell infiltration occurred at any other site. As a control, BM cells obtained from 5 healthy BM donors were studied.

Cell Preparation

BM cells were obtained after informed consent by aspiration from the posterior iliac crest and were collected in preservative-free heparin. Mononuclear cells were obtained from BM cells by Ficoll density gradient (density 1.077, Seromed) centrifugation and were then washed in Hanks' balanced salt solution with $NaHCO_3$ (Seromed).

Immunophenotyping

Immunophenotyping by flow cytometry (Cytoron Absolute, Ortho Diagnostic Systems) was carried out on mononuclear BM cells in order to estimate the percentage of cells positive for a given antibody. The following antibodies were used: myeloid markers: CD15, labeled with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (Sigma), and CDw65 labeled with FITC (Ortho), CD13 and CD33, labeled with FITC or PE (Becton Dickinson); T cell markers: CD2 and CD3 labeled with PerCP (Becton Dickinson); CD4 and CD5 and CD7, labeled with FITC (Ortho), and CD8, labeled with PE (Ortho); B cell markers: CD10 and CD22, labeled with PE (Ortho); CD19 and CD20, labeled with PerCP (Becton Dickinson), and blast antibody: CD34 (class III clone 581), labeled with PE (Immunotech). Moreover, blast phenotypes at diagnosis were regularly evaluated using a combination of different leukocyte antibodies according to consensus protocols [14]. In the further course of AML, the patients' typical antibody combinations, including CD34 antibodies in combination with pan myeloid antibodies (e.g. CD13, CD33), pan leukocyte antibodies (CD45), were used to detect and quantify positive cells. To avoid unspecific or epitope-class-specific variations of antibody binding, the same antibodies of the same company were always used. Data were evaluated using a special software (Immunocount 2) from Ortho Diagnostic Systems.

Southern Blot Analysis

At diagnosis of AML, DNA from mononuclear BM cells of patients was studied for gene rearrangements by Southern blot analysis. Gene probes used were known to detect gene rearrangements in a great proportion of AML cases. If a gene rearrangement was detectable at diagnosis, the respective gene probe was used to study the persistence or re-emergence of gene-rearranged cells in the further course of the disease, in CR and at relapse. DNA was prepared according to standard procedures [15]; 10 μg were cut by restriction enzymes (*Bam*HI, *Hin*dIII or *Bgl*II from Gibco BRL); the resulting fragments were electrophoretically separated on a 0.6% agarose gel, blotted onto nitrocellulose and hybridized to several digoxigenated probes: a 2.5-kb Ig-JH fragment [clone H24 (J3-6)], a 0.73-kb TcR-β (cβ1) fragment, a 2.0-kb M-bcr (5′bcr) fragment, a 0.8-kb GM-CSF fragment, a 1.8-kb G-CSF fragment, a 1.0-kb IL-3 fragment, a 0.74-kb MLL fragment or a 1.64-kb RAR-α fragment in cases with promyelocytic leukemia [16–21]. The reaction was developed with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and nitroblue tetrazolium chloride (Boehringer, Mannheim, Germany). Bands occurring in addition to the germline band were interpreted as 'clonal bands' due to clonal leukemic cells.

Statistics

Statistical tests (Kaplan-Meier analysis, log rank test, Mann-Whitney U test) were performed on a personal computer with a special software ('Stasy', from Pic GmbH, and NCSS 6.0.4, from Jerry Hintze). Differences were considered as significant in cases with $p < 0.05$. For survival analysis, patients were evaluated at the time of BM transplantation.

Table 1. Southern blot analysis of BM samples from patients at diagnosis of AML using a panel of different gene probes

Gene rearrangements	AML M0		AML M1		AML M2		AML M3		AML M4		AML M5		AML M6		AML M7		n.d.		Total	
	p (1)[1]	s (1)	p (9)	s (15)	p (23)	s (19)	p (7)	s (2)	p (21)	s (18)	p (16)	s (5)	p (1)	s (0)	p (0)	s (0)	p (3)	s (12)	p (81)	s (72)
Ig-JH	/[2]	1/1[2]	1/7	0/10	0/16	3/14	0/3	0/1	5/18	1/14	2/16	1/5	0/1	/	/	/	0/3	0/4	8/64 13%	6/49 12%
TcR-β	0/1	0/1	0/6	0/11	5/20	1/12	1/4	0/1	2/15	1/13	0/15	0/5	0/1	/	/	/	0/2	1/6	8/65 12%	3/49 6%
GM-CSF	/	0/1	1/7	0/13	2/14	4/14	0/4	0/1	3/13	5/17	2/15	0/1	0/1	/	/	/	0/1	2/11	8/55 15%	11/61 18%
IL-3	/	/	0/4	1/11	1/12	1/9	0/1	0/1	2/13	1/12	3/14	0/4	0/1	/	/	/	0/1	2/6	6/46 13%	5/43 12%
MLL	/	/	2/4	0/5	0/10	0/8	0/1	0/1	1/12	1/8	3/14	0/4	/	/	/	/	0/1	1/5	6/43 14%	2/31 6%
RAR-α	/	/	/	/	0/5	0/1	5/5	2/2	/	0/1	/	/	/	/	/	/	1/1	0/1	6/11 55%	2/5 40%
M-bcr	/	0/1	0/6	0/14	1/14	1/14	1/3	0/1	2/15	0/13	2/15	0/4	0/1	/	/	/	0/1	0/6	6/56 11%	1/53 2%
G-CSF	/	0/1	0/5	0/9	0/12	1/8	0/3	0/1	1/12	0/13	1/15	0/4	0/1	/	/	/	0/1	0/3	2/49 4%	1/39 3%
Gene rearrangement detectable	0/1 0%	1/1 100%	4/9 44%	1/15 7%	9/23 39%	11/19 58%	7/7 100%	2/2 100%	16/21 76%	9/18 50%	13/16 81%	1/5 20%	0/1 0%	/	/	/	1/3 33%	6/12 50%	41/81 51%	30/72 42%

A total of 153 cases with AML were studied at diagnosis for gene rearrangements by Southern blot analysis using a panel of different probes (Ig-JH, TcR-β, GM-CSF, IL-3, MLL, RAR-α, M-bcr, G-CSF). Cases, divided in FAB groups, without and with gene rearrangements after hybridization with the respective gene probes are shown.

n.d. = Not determined.

[1] Number.

[2] n/n = n of n cases with gene rearrangements.

Results

Results at Diagnosis of AML

Patients' Characteristics. BM samples of 68 cases with undifferentiated leukemia (M0: n = 2; M1: n = 24; M2: n = 42), of 9 cases with promyelocytic leukemia (M3), of 60 cases with monocytic leukemia (M4: n = 39; M5: n = 21), 1 case with megakaryocytic leukemia (M7) and 15 morphologically not classified leukemias were studied. Eighty-one patients presented with pAML, 72 with sAML. Male to female ratio was 0.9:1. On average, the patients were 53 years old. Mean Hb values were 9 ± 2 g/dl, WBC counts 26 ± 66 × 10^9/l and platelet counts 73 ± 72 × 10^9/l. The patients presented on average with 56 ± 46% BM blasts. Most of the patients (n = 135) received standard chemotherapy according to study protocols and achieved CR in 58% of the cases. The remaining cases (n = 18) were treated supportively. Two-year survival of treated patients with pAML was 46% and that of sAML patients 17%.

Incidence of Gene Rearrangements. Southern blot analysis could be performed in 153 patients at diagnosis of AML; gene rearrangements could be detected in 71 (46%) of the cases (table 1). Twelve percent (14 of 113) of the DNA samples studied showed clonal Ig-JH gene rearrangements with 43% (6 of 14) of them detectable in the FAB M4 group (table 1). Ten percent (11 of 114) of the examined cases showed clonal TcR-β gene rearrangements, with 55% of the rearrangements being detectable in the FAB M2 group. Sixteen percent (19 of 116) of the cases presented with GM-CSF gene rearrangements, with most of the cases belonging to the FAB M2 or M4 groups. IL-3 gene rearrangements were found in 12% (11 of 89) of the cases, preferentially in cases with FAB types M4 or M5. MLL gene rearrangements were detected in 11% (8 of 74) of the cases, with most of the cases classified as AML M2,

Table 2. Clinical outcome of patients studied at diagnosis of sAML or pAML who presented with or without gene rearrangements

Gene rearrangement	Survival[1] months m ± σ	p value log rank test	Months to relapse[2] m ± σ	p value log rank test	Response to chemotherapy[3] n/n	p value U test
RAR-α gene rearranged	26 ± 27 (n = 3)	n.s.	20 ± 13 (n = 2)	n.s.	5/7 (71%)	n.s.
RAR-α gene not rearranged	21 ± 31 (n = 6)		16 ± 19 (n = 7)		9/10 (90%)	
M-bcr gene rearranged	5 ± 5 (n = 5)	n.s.	23 ± 19 (n = 4)	n.s.	4/6 (67%)	n.s.
M-bcr gene not rearranged	10 ± 10 (n = 64)		12 ± 12 (n = 32)		60/98 (61%)	
MLL gene rearranged	10 ± 10 (n = 4)	n.s.	21 ± 26 (n = 4)	n.s.	7/9 (78%)	n.s.
MLL gene not rearranged	10 ± 12 (n = 41)		17 ± 17 (n = 21)		39/66 (59%)	
IL-3 gene rearranged	4 ± 4 (n = 9)	<0.001	5 (n = 1)	<0.05	3/9 (33%)	n.s.
IL-3 gene not rearranged	10 ± 11 (n = 48)		14 ± 14 (n = 27)		48/76 (63%)	
GM-CSF gene rearranged	6 ± 6 (n = 12)	0.06	20 ± 24 (n = 6)	n.s.	9/16 (56%)	n.s.
GM-CSF gene not rearranged	10 ± 10 (n = 64)		12 ± 9 (n = 32)		56/92 (61%)	
G-CSF gene rearranged	8 ± 4 (n = 3)	n.s.	8 ± 4 (n = 2)	n.s.	1/3 (33%)	n.s.
G-CSF gene not rearranged	15 ± 16 (n = 35)		14 ± 14 (n = 28)		52/82 (63%)	
TcR-β gene rearranged	20 ± 18 (n = 5)	0.06	28 ± 28 (n = 4)	0.06	9/11 (82%)	n.s.
TcR-β gene not rearranged	9 ± 10 (n = 68)		13 ± 11 (n = 36)		57/95 (60%)	
Ig-JH gene rearranged	16 ± 18 (n = 9)	n.s.	20 ± 27 (n = 4)	n.s.	8/11 (73%)	n.s.
Ig-JH gene not rearranged	11 ± 14 (n = 65)		15 ± 15 (n = 39)		61/98 (62%)	
Gene-rearranged cases	11 ± 15 (n = 42)	n.s.	18 ± 18 (n = 22)	n.s.	40/63 (64%)	n.s.
Not-gene-rearranged cases	11 ± 13 (n = 57)		13 ± 12 (n = 30)		29/56 (52%)	

A total of 153 cases with AML were studied at diagnosis for gene rearrangements by Southern blot analysis using a panel of different probes (RAR-α, M-bcr, MLL, IL-3, GM-CSF, G-CSF, TcR-β, Ig-JH). The clinical outcome of the patients (survival, time to relapse and response to chemotherapy) depending on detectable gene rearrangements is presented.

m ± σ = Mean ± standard deviation; n.s. = not significant; n/n = n of n cases studied.

[1] Only patients who died.

[2] Only patients who achieved a CR and who relapsed.

[3] Only patients who received chemotherapy.

M4 or M5. All of the 7 cases morphologically classified as AML M3 showed RAR-α gene rearrangements. Only 6% (7 of 109) of the cases presented with M-bcr gene rearrangements and only 3% (3 of 88) with G-CSF gene rearrangements.

Oligoclonality. Sixteen cases presented with more than one gene rearrangement: 4 times an M-bcr and also an Ig-JH gene rearrangement, once an M-bcr and also an IL-3 gene rearrangement, 6 times a TcR-β and also an Ig-JH gene rearrangement, 3 times a TcR-β and also a GM-CSF gene rearrangement and twice an Ig-JH and also a GM-CSF gene rearrangement. In 6 samples 3 different gene rearrangements were detected at the same time at diagnosis: once an M-bcr and also a GM-CSF and a G-CSF gene rearrangement, once an M-bcr and also a TcR-β and an IL-3 gene rearrangement, once a TcR-β and also an Ig-JH and a G-CSF gene rearrangement, once a TcR-β and an Ig-JH and an M-bcr gene rearrangement, once an Ig-JH and an M-bcr and an GM-CSF gene rearrangement and once a TcR-β and an Ig-JH and IL-3 gene rearrangement (data not shown).

Immunophenotypes of AML Cases with Gene Rearrangements. In cases with RAR-α gene rearrangements, the percentage of CD34+ cells in the BM mononuclear cells was significantly lower as compared to cases without RAR-α gene rearrangements (7 versus 35%; $p < 0.05$, data not shown), otherwise the percentages of CD15+ BM mononuclear cells were higher in the group with rearrangements (37 versus 28%, $p = 0.04$). Cases with MLL gene rearrangements showed higher counts of monocytic, CD14+ (24 versus 16%, $p = 0.04$) as well as higher counts of CD15+ BM MNC (42 versus 25%, $p < 0.03$) as com-

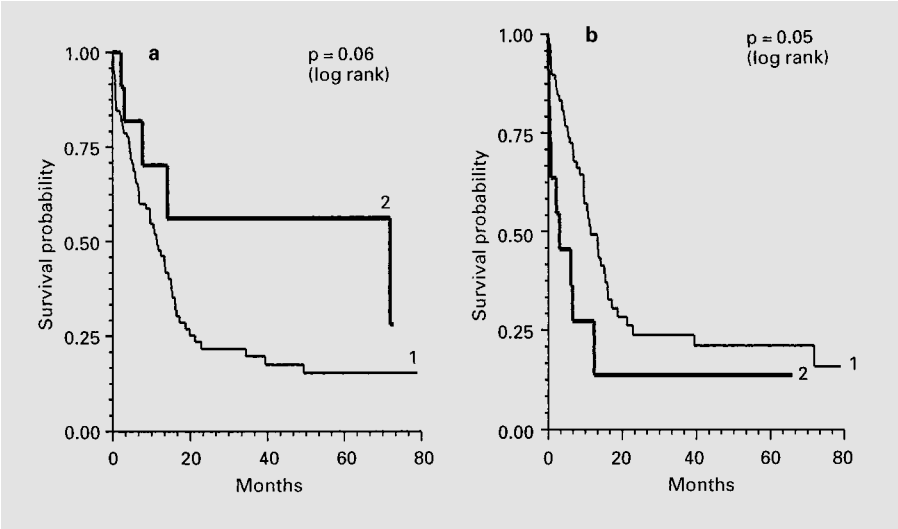

Fig. 1. DNA samples obtained from BM cells at diagnosis of AML were hybridized to a TcR-β (**a**) or an IL-3-gene-specific probe (**b**). Survival probabilities of cases without (1) or with (2) the respective gene rearrangements are shown. **a** Patients without (n = 103) or with (n = 11) TcR-β gene rearrangements. **b** Patients without (n = 78) or with (n = 11) IL-3 gene rearrangements.

pared to the group without MLL gene rearrangment. Percentages of CD34+, CD14+ or CD15+ BM mononuclear cells were not different in the groups with or without gene rearrangements for the M-bcr, IL-3, GM-CSF, G-CSF, TcR-β or Ig-JH genes (data not shown).

Prognostic Significance of Gene Rearrangements. Statistical evaluation of our data revealed that AML patients studied at diagnosis who presented with gene rearrangements in the TcR-β gene were characterized by a favorable diagnosis (survival/time to progress) whereas gene rearrangements in the IL-3 gene indicated a poor prognosis (table 2, fig. 1). Gene rearrangements in the GM-CSF, MLL or Ig-JH gene were not prognostically relevant, although a trend to a poor prognosis was seen in patients with GM-CSF gene rearrangements (table 2). As only few cases exhibited rearrangements in the G-CSF gene, a meaningful log rank test could not be performed. Hematological parameters, such as WBC, Hb values or platelet counts were not different in the groups compared (data not shown). The clinical outcome of cases with pAML or sAML as compared to cases without gene rearrangements did not differ (data not shown). In pAML patients with gene rearrangements in the TcR-β or RAR-α genes, the differences concerning the clinical outcome of the rearranged and nonrearranged group were even more evident than in the pooled group (pAML and sAML, data not shown). Response to chemotherapy did not differ significantly in the groups of patients presenting with various gene rearrangements as compared to patients without such rearrangements (table 2).

Concordance of Gene Rearrangements and Karyotypes. In many cases studied by Southern blot analysis, a karyotype was available. We detected RAR-α gene rearrangements in 5 cases without a t(15;17) and MLL gene rearrangements in 4 cases without 11q23 aberrations (data not shown). In all of the cases with a t(15;17), however, RAR-α gene rearrangements were detected as well, whereas in 18 cases with 11q aberrations, no MLL gene rearrangements were seen. In 4 of 9 cases with 5q aberrations either GM-CSF or IL-3 gene rearrangements were seen, whereas a G-CSF gene rearrangement was detected in a case with a 7q aberration and also in 4 cases without 7q aberrations. All of the gene rearrangements in TcR-β (n = 6), Ig-JH (n = 7) or M-bcr genes (n = 4) studied in parallel did not show any chromosomal aberrations at the respective gene loci (7q35 or 14q32 or 9q34; data not shown). Our data point out that gene rearrangements in a gene locus do not necessarily mean an aberration detectable at the chromosomal level and vice versa. Moreover, in 23 of 43 cases (53%) studied, clonal gene rearrangements were demonstrated although cytogenetically normal karyotypes were described.

In summary, our results demonstrate that gene rearrangements in the genes studied can be regularly demonstrated at diagnosis of AML, with some rearrangements occurring preferentially in certain morphological subtypes. Moreover, our results have clinical significance: patients with gene rearrangements in the TcR-β gene were characterized by a favorable prognosis, whereas patients with IL-3 or GM-CSF gene rearrangements were characterized by poor outcomes.

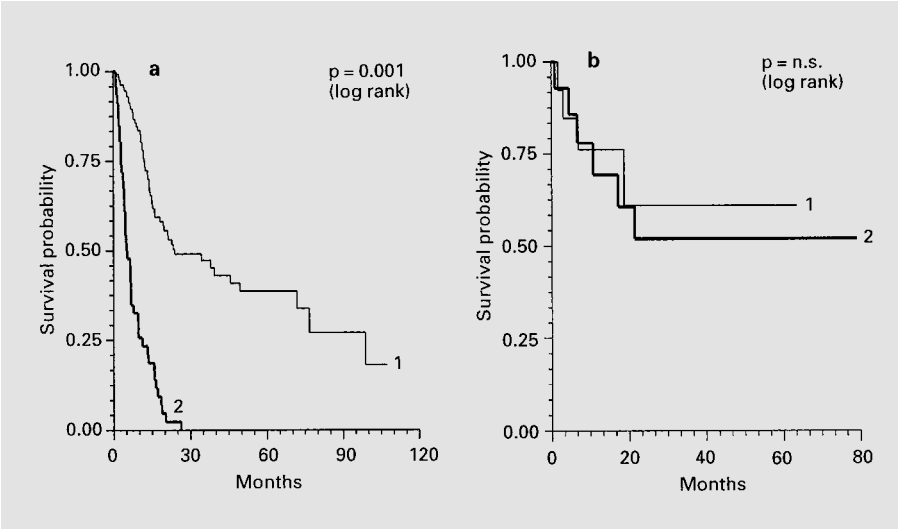

Fig. 2. a Survival probabilities of patients who achieved a hematological remission in the first 3 months after chemotherapy (1; n = 133) compared to those of patients without remission (2; n = 46) are shown. **b** DNA samples obtained from BM cells in the first 3 months after chemotherapy were hybridized to the same gene probes which had shown gene rearrangements at diagnosis of the disease. Survival probabilities of cases with (1; n = 13) or without (2; n = 14) a molecular response to chemotherapy are shown.

Table 3. Concordance of hematological and molecular response in AML patients responding or not responding to chemotherapy

		Hematological response achieved	
		yes (n = 21)	no (n = 6)
Disappearance of the gene	yes (n = 13)	**10**	3
Rearrangement after chemotherapy	no (n = 14)	11	**3**

A total of 27 BM samples obtained from patients with AML could be studied in the first 3 months after chemotherapy to study the frequency of a hematological response, a molecular response and their concordance. Cases with or without hematological and/or molecular response are listed.

Response to Chemotherapy. Patients presenting with gene rearrangements at diagnosis were studied again within 3 months after chemotherapy in order to find out whether a clonal response (defined as a loss of the gene rearrangements) is associated with a better survival probability (fig. 2). Our data show that although a significant advantage in survival probability could be demonstrated in cases with a hematological response to chemotherapy in the first 3 months as compared to cases without response ($p < 0.001$, fig. 2a), no differences in survival could be demonstrated in cases with or without gene rearrangements (fig. 2b). Similar results were found by comparing progress-free survival times (data not shown).

Moreover, we could demonstrate that 10 patients (37%) who achieved hematological remissions also responded at the molecular level (table 3). Three patients (11%) who did not achieve hematological remissions were 'nonresponders' also at the molecular level. In 11 patients (41%), however, who responded hematologically, no molecular response could be detected, which means that a gene rearrangement could still be detected. In 3 additional patients (11%) who did not achieve hematological remissions, a molecular response could be detected which might also mean that more than one clone is involved in these patients.

In conclusion, our data showed a concordance between hematological and molecular results in 13 of 27 cases (48%). The loss of a gene rearrangement after chemotherapy in CR had no prognostic significance.

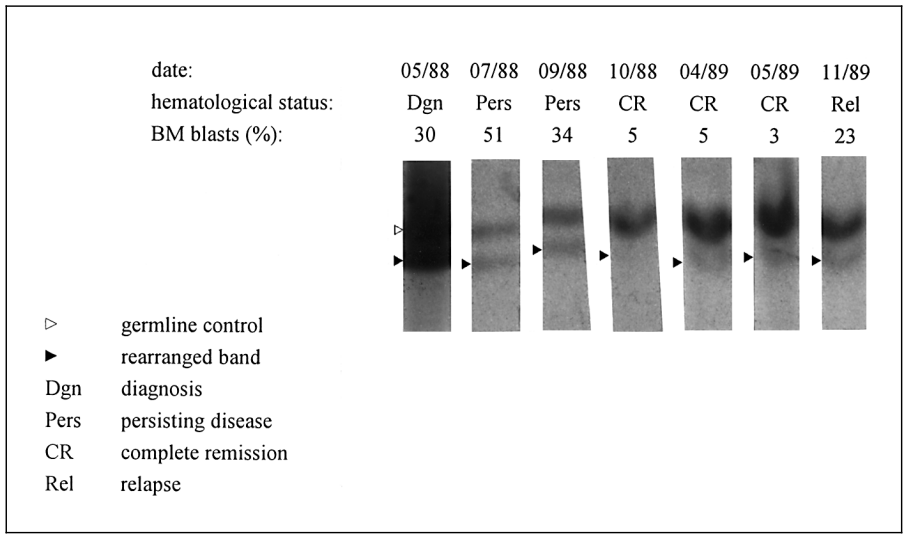

Fig. 3. DNA from BM cells obtained at different stages of AML was prepared, cut by restriction enzymes, electrophoresed on an agarose gel, blotted to nitrocellulose and hybridized to a digoxigenated GM-CSF gene probe. Rearranged bands (►) in addition to the germline bands (▷) are shown.

Results in CR of AML

The goal of molecular analysis in remission was to detect persisting gene-rearranged cells and to evaluate their clinical significance. Therefore samples obtained at every possible time point in CR were studied. In 23 of 48 cases (48%), the same gene rearrangement known from diagnosis could be detected again, if BM samples were studied *once* in CR. A statistical comparison of the two groups did not show any differences in hematological or immunological parameters or in clinical outcome (data not shown). However, the gene rearrangement could be detected in 23 of 26 samples (88%) if BM samples were studied *twice* and in 23 of 23 samples (100%) if studied *three times* in the course of CR. Figure 3 shows an example of a Southern blot analysis in the course of AML. At diagnosis of AML, BM cells of the patient shown were characterized by a clonal gene rearrangement of the GM-CSF gene. The additional band disappeared in the course of the disease while the patient responded to therapy and became stronger again as the patient progressed to relapse.

This means that clonal-gene-rearranged cells persist in CR of AML and can be consistently detected in the course of the disease by Southern blot analysis.

Results at Relapse and at Progress of AML

BM samples from 25 patients could be studied at relapse. In 12 of them, the same gene rearrangement known from diagnosis could be detected in the following genes: 4 times for the GM-CSF, twice for M-bcr, Ig-JH and RAR-α genes, once for the MLL and TcR-β genes. In the remaining 13 cases again, as at diagnosis, no gene rearrangement could be detected. A statistical comparison of cases with or without gene rearrangements at relapse did not show any differences concerning survival or progress-free survival times or response rates to chemotherapy between the two groups (data not shown). No differences could be detected when comparing hematological parameters (hemoglobin, white blood cells, platelets, BM/peripheral blood blasts) or percentages of immunologically detectable granulocytes, monocytes, T/B cells, CD34+ cells, erythropoietic or megakaryopoietic cells in the two groups (data not shown).

Eight cases presenting with gene rearrangements at diagnosis of sAML after a phase of MDS could be studied shortly before their progress to AML. In 3 of the 8 cases, clonal-gene-rearranged cells could already be detected 2–6 months *before* the clinical onset of AML (data not shown).

This means that disease progression can be detected early by Southern blot analysis and that relapse of AML is caused by a regrowth of the same clone as at diagnosis. Therefore, our data show that clonality analysis allows to monitor the present status of patients with AML.

Control Experiments

DNA obtained from BM or peripheral blood samples of patients with nonhematological diseases served as controls (n = 5). Those DNA samples were regularly studied for gene rearrangements in parallel with the gene probes used in every experiment. No clonal gene rearrangements were detected in the control samples.

Discussion

Gene Rearrangements at Diagnosis of AML

Incidence of Gene Rearrangements. AML is a stem cell disease developing clonally, but it is heterogeneous with respect to cell differentiation [1]. Southern blot analysis is a suitable tool to detect clonal-gene-rearranged cell populations at diagnosis of AML. Compared to cytogenetics, Southern blot analysis has the advantage that it is independent of proliferation and therefore allows the detection of clonal gene rearrangements in dividing and nondividing cells. Moreover, the detection of a clonal marker in cases without chromosome abnormalities is possible. We could detect gene rearrangements in Ig-JH, TcR-β, GM-CSF, G-CSF or IL-3 genes in 12, 10, 16, 3 or 12% of the cases, respectively, with 53% (23 of 43 cases) presenting with a normal karyotype. Gene rearrangements in a variety of different genes, such as growth factor genes (GM-CSF, IL-3, G-CSF), RAR-α, MLL, M-bcr, Ig-JH or TcR-β genes, are known to be found in a certain proportion of AML cases [5–9 and own unpubl. data]. In agreement with the literature, we could demonstrate that RAR-α gene rearrangements are specific to cases with AML-M3 [22]. Therefore, Southern blot analysis contributes important data to assure diagnosis of patients with promyelocytic leukemia, especially in those cases without cytogenetically detectable t(15;17) aberrations [2].

Concordance of Gene Rearrangements and Karyotypes and Their Prognostic Significance. Although a tendency to a better outcome could be demonstrated in patients presenting with RAR-α gene rearrangements, it was an astonishing finding that the clinical outcome of patients without RAR-α gene rearrangements was not significantly worse. This may be attributed to the low number of AML M3 cases studied (n = 9) or to the fact that, besides the M3 FAB type (13%, 9 of 71 cases), a large proportion of patients (20 of 71 cases, 28%) studied in this group presented AML M2, which has a favorable prognosis as well. Therefore, the clinical outcomes of the group without and the group with RAR-α gene rearrangements were similar. MLL gene rearrangements could be detected in 11% of the cases studied, with most of the rearranged cases occurring in FAB groups M1, M4 and M5, as already described [23]. Moreover, MLL gene rearrangements could be detected in 4 cases, which were studied in parallel by cytogenetics but did not show 11q23 aberrations. This was also observed by other authors [23].

Our data show that gene rearrangements in a gene locus do not mean a chromosomal aberration at the respective band and vice versa. Especially in cases with cytogenetically undetectable or masked chromosomal aberrations, Southern blot analysis could therefore help to identify certain subgroups. This could be of clinical significance, notably in cases with therapeutically and prognostically different outcomes, such as AML M3 cases.

Prognostic Significance of Gene Rearrangements. Statistical evaluation of our data showed that gene rearrangements in the TcR-β and the RAR-α gene mean a favorable diagnosis for patients with AML. Rearrangements in the MLL, the Ig-JH or the M-bcr genes mean an intermediate prognosis and rearrangements in the IL-3 or GM-CSF gene mean an unfavorable or at least a tendeny to an unfavorable prognosis. Concerning the RAR-α rearrangements, our data are in agreement with the literature, according to which those rearrangements are typical for AML M3 and mean a favorable prognosis [22]. Our data point out that MLL gene rearrangements do not necessarily mean an unfavorable prognosis, as already shown by others [24]. An astonishing finding was that TcR-β gene rearrangements are associated with a favorable prognosis for patients with pAML. This means that 7q aberrations do not necessarily represent an unfavorable prognosis, with prognosis depending on the gene loci involved. Whereas other groups have shown that a rearrangement in the Ig-JH gene detectable by PCR means a worse prognosis for the patients [11], our results did not reveal any prognostic significance for patients with Ig-JH gene rearrangements as compared to patients without such rearrangements. The differences between our data and those of Kyoda et al. [11] could be due to the different detection method or gene probes used or to the fact that in our patient cohort only 34 of 113 tested cases (30%) belonged to the prognostically favorable groups with FAB type M2 or M3, versus 21 of 35 cases (60%) in the group presented by Kyoda et al. An IL-3 or GM-CSF gene rearrangement means an unfavorable prognosis. This could be due to the fact that 4 of 9 patients who could be studied in parallel by cytogenetics also presented with solitary or complex 5q aberrations, which means a very unfavorable prognosis [24, 25]. Moreover, our data point out that gene loci for the IL-3 or GM-CSF gene on locus 5q31 need not be *deleted* in case of a 5q aberration, as discussed by some authors [26], but could be translocated or rearranged at a molecular level. Alternatively, the rearrangements could also have happened on the second chromosome without the 5q aberration. The prognostic significance of a G-CSF rearrangement could not be evaluated because of the fact that only 3 cases presented with such gene rearrangements. Due to low amounts of cells or DNA available in some patients, DNA could not be hybridized with all gene

probes. Samples were classified as 'not rearranged' if DNA samples were tested and not rearranged with at least one gene probe. This means that gene rearrangements might have been overlooked.

Immunophenotypes of AML Cases with Gene Rearrangements. In cases with a rearranged RAR-α gene, the percentage of CD34+ cells was significantly lower than in the group with a nonrearranged RAR-α gene, whereas CD15+ cell counts were higher. Cases with MLL gene rearrangements showed higher counts of monocytic cells (CD14). Those data fit well with the literature demonstrating that BM cells obtained from patients with AML-M3 do not express CD34 but CD15 antigens and that in a large proportion of patients, MLL gene rearrangements occur in cases with (myelo-) monocytic AML (AML M4, M5) [14, 27]. Moreover, our data show that studying gene rearrangements *and* immunophenotypes can help learn about biological features of proliferation and differentiation in certain subtypes of AML.

Oligoclonality in AML. In 16 BM samples, more than one gene rearrangement could be detected at diagnosis of AML. This could indicate that *one* AML clone is characterized by *multiple* clonal genetic rearrangements in these cases. Alternatively, this could mean the coexistence of *several* clones with different gene rearrangements, as already shown by others and us [28].

Gene Rearrangements in the Course of AML

Gene Rearrangements in CR. About 70% of patients with AML in CR relapse within the following 2 years [10]. Therefore, residual leukemic cells must have survived. Those cells can be detected by different methods, such as PCR, FISH or Southern blot analysis [9]. With Southern blot analysis, we could detect clonal-gene-rearranged cells in 46% of the cases if samples were studied once, in 88% of the cases if studied twice and in 100% of the cases if BM DNA was studied three times during the course of CR. This means that clonal-gene-rearranged cells regularly persist in CR, but cannot be detected at every time point due to bad quality of BM aspirates, low amounts of DNA or because of the limited sensitivity of 1–5% of Southern blot analyses [29]. These facts can also be responsible for our finding that patients *with* gene rearrangements in CR do not have a worse treatment outcome than those who lost the rearrangements.

Our data show that Southern blot analysis is a suitable method to study the persistence of clonal cells, especially in cases without chromosomal aberrations, which can be detected by PCR or FISH in a large proportion of cases [9, 30].

Gene Rearrangements on Progress of AML. We could show that gene rearrangements detectable at relapse are of the same kind as at diagnosis, pointing to the fact that the same leukemic clone induces the relapse. In our patient cohort, no favorable clinical outcome could be demonstrated for those patients *without* detectable gene rearrangements as compared to the cases with gene rearrangements at relapse. This could be explained by the fact that 3 of those rearranged 12 cases which could be studied at relapse presented with 'favorable gene rearrangements' (RAR-α or TcR-β gene) and 5 with gene rearrangements without any prognostic significance. Moreover, our data show that Southern blot analysis is a good method to detect a progress of the disease early: clonal-gene-rearranged cells could be detected 2–6 months *before* the clinical onset of AML after a phase of MDS.

This means that Southern blot analysis is an efficient method not only to ascertain a relapse, but also to detect progress of the disease early.

Although in cases with chromosomal aberrations discovered at diagnosis, residual leukemic cells can be detected more easily by PCR or FISH if the appropriate primers or probes are available [9, 30], Southern blot analysis is also a good method to detect residual cells, especially in cases without cytogenetic markers. Therefore, the persistence of clonal cells can be monitored at a molecular level and allows the detection of oligoclonal disease [28], extramedullary relapse [31] or – in combination with immunophenotyping – the detection of gene-rearranged, but differentiated leukemic cells [28, 31–33].

Conclusions

In conclusion, our results show that Southern blot analysis can be used to study clonality and evaluate the prognostic value of gene rearrangements at diagnosis of AML. Moreover, the persistence or re-emergence of clonal cells in CR or at relapse can be studied. The goal of molecular diagnosis in AML in the future should be to detect a clonal BM marker in every AML patient at diagnosis. The molecular cloning of many genes involved in cancer development could help to find new gene probes for the characterization of leukemic disease [25]. Furthermore, the value of the detection of residual disease in AML has to be studied in detail: perhaps not the *presence* but the *amount* of clonal DNA/clonal cells is predictive for relapse. Moreover, analyses of samples obtained at standardized time points (e.g. shortly after induction therapy,

after 3, 6, 9, 12 months in CR) have to be performed to evaluate the value of Southern blot analysis for screening of residual clonal cells in CR and to evaluate their clinical significance.

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