The prognostic value of *MLL-AF9* detection in patients with t(9;11)(p22;q23)-positive acute myeloid leukemia

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Background and Objectives. Translocation (9;11) is the most common t(11q23) in acute myeloid leukemia (AML). A considerable number of patients with this cytogenetic abnormality relapse and die of their disease. We evaluated the clinical significance of minimal residual disease (MRD) monitoring in t(9;11)(p22;q23)-positive AML patients using real-time quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) analysis.

Design and Methods. We identified 34 newly diagnosed patients with t(9;11)(p22;q23)-positive AML treated within three multicenter trials of the AML Study Group. MRD could be investigated by RQ-PCR in 19 patients during and after therapy. Because of the relatively low sensitivity of the RQ-PCR (10^{-3} to 10^{-4} at the cellular level), samples from RQ-PCR-negative patients were also analyzed by nested polymerase chain reaction (nPCR; sensitivity 10^{-4} to 10^{-5} at the cellular level).

Results. RQ-PCR monitoring revealed two groups of patients: group 1 (n=11) had negative RQ-PCR in all samples collected in hematologic complete remission whereas group 2 (n=8) had at least one positive RQ-PCR in samples collected in complete remission during therapy. Group 1 had a significantly lower cumulative incidence of relapse (p=0.004) and better overall survival (p=0.003) compared to group 2. nPCR did not add information to that gained from RQ-PCR. Molecular relapse was detected in two patients by RQ-PCR four and six weeks, respectively before hematologic relapse occurred. Quantitative *MLL-AF9* levels at diagnosis or during and after therapy had no prognostic impact.

Interpretation and Conclusions. Early achievement of sustained RQ-PCR negativity appears to be a prerequisite for long-term hematologic complete remission in t(9;11)-positive AML. Furthermore, RQ-PCR might be useful for early detection of relapse. Additional patients need to be studied to corroborate these findings.

Key words: minimal residual disease, MLL-AF9, real-time PCR.

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Karyotype is one of the key determinants of outcome in acute myeloid leukemia (AML).[1] The most frequent balanced chromosome aberrations are t(8;21)(q22;q22), inv(16)(p13q22), t(15;17)(q22;q11~21), and t(11q23).[2,3] The most common t(11q23) in *de novo* and therapy-related AML (t-AML) is t(9;11)(p22;q23) that results in fusion of the *MLL* gene at 11q23 and the *AF9* gene at 9p22. At the molecular level, t(9;11)(p22;q23) have different fusion types resulting from various breakpoints within the *MLL* and *AF9* breakpoint cluster regions.[4-9] All fusion types cause expression of an aberrant chimeric mRNA consisting of a 5´-*MLL* portion and a 3´-*AF9* portion. The following fusion products have been identified: *MLL* exon 7/exon 8-*AF9* site A (7A/8A type; the exon 7-site A fusion is a splice variant of the exon 8-site A fusion), *MLL* exon 6-*AF9* site A (6A type), MLL exon 6-*AF9* site B (6B type), *MLL* exon 6-*AF9* site D (6D type), and *MLL* exon 7/exon 8-*AF9* site C (7C/8C type).[4-9] Although several groups have shown that t(9;11)-positive *AML* is associated with a better outcome than that of AML with other 11q23 abnormalities,[2,10,11] a considerable number of patients with t(9;11) relapse and die of their disease. Therefore, early identification of patients who have a high risk of relapse is of particular interest. We recently established a real-time quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) assay for the quantification of four different *MLL-AF9* fusion transcripts.[9] Here, we present the results of minimal residual disease (MRD) monitoring by RQ-PCR and nested RT-polymerase chain reaction (nPCR) in a large cohort of t(9;11)-positive AML patients enrolled in three multi-institutional clinical trials.

Design and Methods

Patients and samples

Thirty-four consecutive patients with t(9;11)-positive AML who had been entered into the multicenter treatment trials AML HD93 (n=10),[12] AML HD98-A (n=20),[13] and AML HD98-B (n=4)[14] of the AML Study Group were identified by chromosome banding and by fluorescence *in situ* hybridization at the central reference

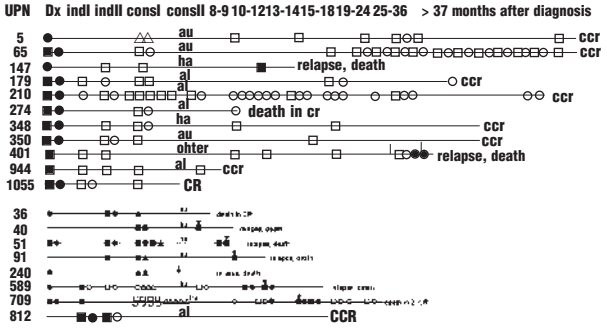

Figure 1. RQ-PCR results at diagnosis and during follow-up. The first cohort (UPN5-1055) belongs to MRD group 1; the second cohort (UPN36-812) belongs to MRD group 2. Dx, diagnosis; ind I, first induction; ind II, second induction; cons I, first consolidation; cons II, second consolidation; au, autologous SCT; ha, high-dose cytarabine; al, allogeneic SCT; CCR, continuous complete remission. Square, bone marrow; circle, peripheral blood; triangle, leukapheresis product; arrow, time of hematologic relapse; close symbols, positive RQ-PCR; open symbols, negative RQ-PCR.

laboratory located at our institution.[15-17] The *MLL-AF9* fusion type was determined as previously described.[9] Two hundred and five samples (bone marrow, n=100; peripheral blood, n=90; leukapheresis product, n=15) from 28 patients were available for RQ-PCR analysis. The remaining six patients could not be analyzed, three because no material was available and three because the *MLL-AF9* fusion type was not detectable by RQ-PCR. Bone marrow and peripheral blood from 19 patients could be analyzed during and after therapy (Figure 1). The clinical characteristics of the patients are summarized in Table 1. Approval was obtained from the institutional review boards of the participating institutions. All patients gave informed consent for sample cryopreservation according to the Declaration of Helsinki.

Therapy of patients with t(9;11)-positive AML

The AML HD93 trial[12] and the consecutive AML HD98-A trial[13] included younger AML patients (16 to 60 years). In both studies, patients underwent double induction therapy with ICE (idarubicin 12 mg/m^2 on days 1, 3, and 5; cytarabine 100 mg/m^2 continuously on days 1 through 7; etoposide 100 mg/m^2 on days 1 through 3), followed by first consolidation therapy with one course of HAM (cytarabine 3 g/m^2 every 12 hours on days 1 through 3; mitoxantrone 12 mg/m^2 on days 2 and 3). For second consolidation therapy, t(9;11)-positive patients were assigned to autologous stem-cell transplantation (SCT) or, if an HLA-compatible sibling donor was available, allogeneic SCT.

The AML HD98-B trial included elderly AML patients (61 years and older).[14] Randomized induction therapy consisted of two courses of age-adjusted ICE (idarubicin 12 mg/m^2 on days 1 and 3; cytarabine 100 mg/m^2 continuously on days 1 through 5; etoposide 100 mg/m^{2}on days 1 and 3) or the same chemotherapy with the addition of all-trans retinoic acid (ATRA; 45 mg/m^2 on days 3 through 5, 15 mg/m^2 on days 6 through 28), followed by first consolidation therapy with a course of age-adjusted HAM (cytarabine 0.5 g/m^2 every 12 hours on days 1 through 3; mitoxantrone 10 mg/m^2 on days 2 and 3) with or without ATRA (15 mg/m^2 on days 3 through 28). Subsequently, patients were randomized between a second course of intensive consolidation therapy with intravenous IE (idarubicin 12 mg/m^2 i.v. on days 1 and 3; etoposide 100 mg/m^2 i.v. on days 1 through 5) and 12 monthly courses of outpatient maintenance therapy with oral IE (idarubicin 5 mg p.o. on days 1, 4, 7, 10 and 13; etoposide 100 mg p.o. on days 1 and 13).

RNA isolation, cDNA synthesis, and RQ-PCR

Preparation of total RNA, cDNA synthesis, identification of the *MLL-AF9* fusion type, and RQ-PCR were performed as previously described.[9] The assessment of RQ-PCR runs and the criteria adopted to report assays and patients' samples as positive or negative have been described elsewhere.[9] The sites of the primers and probes are shown in italic letters in Figure 2, the corresponding sequences are published elsewhere.[9] Each PCR run included a standard curve of a serial dilution from 10^6 to 10^2 plasmids, negative controls, and the samples from patients. All samples and negative controls were analyzed in duplicate and each standard dilution in triplicate. Representative examples of the standard curves of the four different *MLL-AF9* fusion types are shown in Figure 3. The *MLL-AF9* copy number was normalized to the housekeeping gene porphobilinogen deaminase (*PBGD*). The ratio of the *MLL-AF9* copy number and the *PBGD* copy number, multiplied by 10,000, was named the *MLL-AF9* copy ratio. To confirm RNA specificity, *no template controls* (cDNA synthesis with no reverse transcriptase) were carried out if sufficient RNA was available. All the *no template controls* analyzed were negative for *MLL-AF9* and *PBGD*. The sensitivity of RQ-PCR, determined at the cellular level, was 10^{-5} to 10^{-4} (detection of one *MLL-AF9* positive cell in 103 or 104 MLL-AF9 negative cells, respectively) for the 6A and 7A/8A fusion types, as previously reported.[9]

Nested RT-PCR

To verify RQ-PCR-negative samples, we established nPCR assays for the detection of the 7A/8A, 6A, and 6B types. The following primers were used for the detection of the 7A/8A fusion type: external primers, f-MLL6 (5´-GTC CAG AGC AGA GCA AAC AG AAA-3´) and r-AF9B (5´-ATG CCT TGT CAC ATT CAC ATT CAC CAT TC-3´); internal primers, f-MLL7 (5´-CCA CCT CCG GTC AAT AAG CA-3´) and r-AF9A3 (5´-GCA GAT CTT TCA TTA TAG ACC TCA AAG G-3´). For the detection of the 6A and 6B fusion

Table 1. Patients' characteristics.

UPN	Sex	Age	FAB	Study	Karyotype	AML type	*MLL-AF9* fusion type	MRD group	Relapse (months)*	Current status
5	F	16	M4	AML HD93	46,XX,t(9;11)(p22;q23)[18] 47,XX,+8,t(9;11)(p22;q23)[2]	*de novo*	6B	1	no	alive in CR
30	M	36	M4	AML HD93	47,XY,t(9;11)(p22;q23),+der(9)t(9;11)(p22;q23)[17]	t-AML	n.m.	–	yes (13.6)	dead
36	M	47	M5b	AML HD93	46,XY,t(9;11)(p22;q23)[16]	de novo	7A/8A	2	no	dead in CR[1]
40	F	41	M4	AML HD93	46,XX,t(9;11)(p22;q23)[18]	t-AML	7A/8A	2	yes (7.2)	dead
51	F	39	M1	AML HD93	47,XX,t(9;11)(p22;q23),+21[12] 47,XX,del(7)(p11),t(9;11)(p22;q23),add(12)(q24),+21[3]	*de novo*	7A/8A	2	yes (8.6)	dead
65	M	48	M5a	AML HD98-A	46,XY,t(9;11)(p22;q23)[11]	*de novo*	6A	1	no	alive in CR
91	M	24	M4	AML HD93	46,XY,t(9;11)(p22;q23)[4] 46,XY[1]	*de novo*	7A/8A	2	yes (8.6)	dead
147	F	49	M1	AML HD93	46,XX,t(9;11)(p21;q23)[10]	t-AML	7A/8A	1	yes (11)	dead
179	M	65	M5b	AML HD98-B	46,X,-Y,+8[2] ; 45,X,-Y[9] FISH: t(9;11)	*de novo*	7A/8A	1	no	alive in CR
180	M	17	M5	AML HD93	51,XY,+8,+9,t(9;11)(p22;q23),+14,+19,+21[12] 46,XY,t(9;11)(p22;q23),add(16)(q24)[3]	*de novo*	6D	–	yes (10.8)	alive in CR[2]
193	M	27	M5	AML HD93	46,XY,t(9;11)(p22;q23)[14]	*de novo*	n.m.	–	yes (12)	dead
210	F	52	M5b	AML HD98-A	47,XX,+8,t(9;11)(p22;q23)[10]	*de novo*	6B	1	no	alive in CR
240	F	17	M5	AML HD93	47,XX,+8,t(9;11)(p21;q23)[7]	*de novo*	6A	2	yes (3.6)	dead
258	M	62	M5	AML HD98-B	46,XY,t(9;11)(p13;q23)[5] 47,XY,+8,t(9;11)(p13;q23)[8]	*de novo*	6B	–	yes (25.0)	dead
261	F	23	M5a	AML HD98-A	46,XX,t(9;11)(p22;q23)[14]	t-AML	6A	–	n.k.	dead
274	F	48	M5b	AML HD98-A	46,XX,t(9;11)(p22;q23)[18]	t-AML	7A/8A	1	no	dead in CR[3]
335	F	60	M5	AML HD98-B	46,XX,t(9;11)(p22;q23)[12]	t-AML	7A/8A	–	yes (8.2)	dead
348	F	33	M5a	AML HD98-A	47,XX,+8,t(9;11)(p22;q23)[9] 46,XX[2]	*de novo*	6B	1	no	alive in CR
350	F	36	M5a	AML HD98-A	46,XX,t(9;11)(p22;q23)[14]	*de novo*	6B	1	no	alive in CR
375	M	22	M5	AML HD98-A	46,XY,t(9;11)(p22;q23)[15]	t-AML	7C/8C	–	–	early death
401	M	62	M5b	AML HD98-B	no metaphases; RT-PCR: *MLL-AF9* fusion	*de novo*	6A	1	yes[2] (31.3)	dead
587	M	35	M4	AML HD98-A	46,XY,t(9;11)(p22;q23)[14]	*de novo*	7C/8C	–	yes (8.1)	dead
589	F	31	M5b	AML HD98-A	46,XX,t(9;11)(p22;q23)[3] 47,XX,t(9;11)(p22;q23),+13[8]	t-AML	7A/8A	2	yes (11)	dead
649	F	51	M5	AML HD98-A	46,XX,t(9;11)(p22;q23)[10] 47,XX,+8,t(9;11)(p22;q23)[4]	t-AML	6B	–	–	early death
686	F	48	n.k.	AML HD98-A	46,XX[13] FISH: t(9;11)	*de novo*	7A/8A	–	yes[2] (6.5)	dead
691	M	27	M5b	AML HD98-A	46,XY,t(9;11)(p22;q23)[10] 47,XY,+8, t(9;11)(p22;q23)[5]	t-AML	6A	–	no	alive in CR
709	M	51	M4	AML HD98-A	46,XY,t(4;11;9)(q27;q23;p22)[20]	*de novo*	6A	2	yes (13.5)	dead in 2. CR
716	F	41	M0	AML HD98-A	46,XX,t(9;11)(p22;q23)[4]	t-AML	6A	–	yes (13.5)	dead
799	M	56	M5	AML HD98-A	47,XY,+8,t(9;11)(p22;q23),inv(16)(p13q22), del(17)(p11)[9]	de novo	6A	–	–	early death
812	F	42	n.k.	AML HD98-A	46,XX,t(9;11)(p22;q23)[20]	*de novo*	7A/8A	2	no	alive in CR
944	M	49	M5	AML HD98-A	46,XY,t(9;11)(p22;q23)[23]	*de novo*	6A	1	no	alive in CR
1019	F	54	n.k.	AML HD98-A	46,XX,t(9;11)(p22;q23)[15]46,XX[15]	n.k.	n.m.	–	n.k.	n.k.
1052	F	32	n.k.	AML HD98-A	49,XX,+add(8)(q22),add(9)(p13),t(9;11)(p22;q23), +add(11)(p13),add(12)(p11),+14[17]	n.k.	7A	–	n.k.	n.k.
1055	M	46	n.k.	AML HD98-A	46,XY,t(9;11)(p22;q23)[16]	n.k.	7A/8A	1	no	alive in CR

*MRD group 1, negative RQ-PCR in all examined samples during therapy in complete remission ; MRD group 2, at least one positive RQ-PCR in samples collected in complete remission during therapy. UPN, unique patient number; F, female; M, male; FAB, French-American-British Cooperative Group; t-AML, therapy-related acute myeloid leukemia; MRD, minimal residual disease; CR, complete remission; FISH, fluorescence in situ hybridization; 6A, MLLexon6/AF9siteA; 6B, MLLexon6/AF9siteB; 6D, MLLexon6/AF9siteD; 7A/8A, MLLexon8/AF9siteA+MLLexon7/AF9siteA; 7C/8C, MLLexon8/AF9siteC+MLLexon7/AF9siteC; n.k., not known; n.m., no material available. *months after diagnosis; [1]due to autologous SCT; [2]extramedullary relapse; [3]due to graft-versus-host disease after allogenic SCT.*

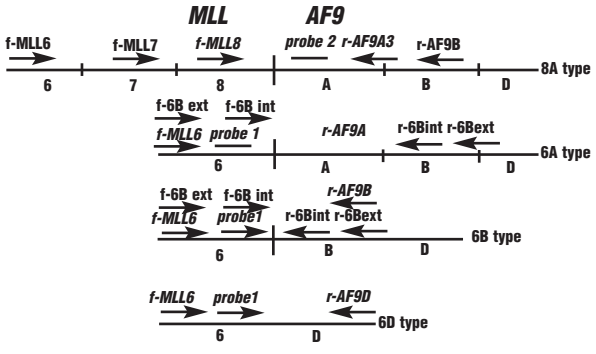

Figure 2. Sites of primers and probes for nPCR and RQ-PCR. *MLL* exons and *AF9* sites are indicated below, *MLL-AF9* fusion types are indicated next to each schematic mRNA map.

types, the following primers were used: external primers, f-6Bext (5´-CCA GAG CAG AGC AAA CAG AAA A-3´) and r-6Bext (5´-CGA TCT GCT GCA GAA TGT GTC-3´); internal primers, f-6Bint (5´-CCG CCC AAG TAT CCC TGT A-3´) and r-6Bint (5´-GTA TGC CTT GTC ACA TTC ACC A-3´). The sites of the primers are shown in non-italic letters in Figure 2 (except r-AF9A3, this primer was used for both nPCR and RQ-PCR).

The total reaction volume of 50 µL contained 2 µL cDNA or PCR product from the first round, 0.025 U/µL AmpliTaq Gold DNA polymerase (Applied Biosystems), dNTP (0.2 mM each; Roche Diagnostics GmbH, Mannheim, Germany), 1×PCR buffer, 2.5 mM $MgCl_2$, and primers (0.2 µM each). Reaction conditions for the first and second round amplifications of the 7A/8A fusion type were as follows: 95°C for 10 min,

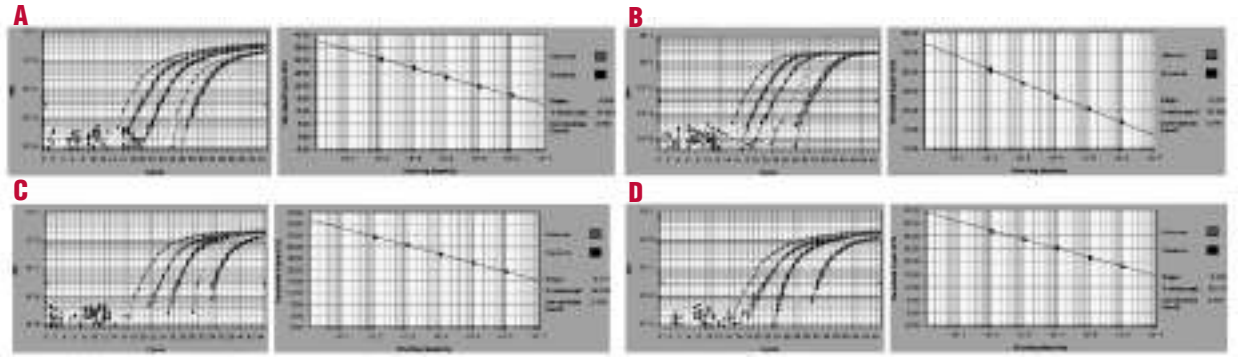

Figure 3. Amplification plots and corresponding standard curves for the *MLL-AF9* fusion transcripts 8A (A), 6A (B), 6B (C), and 6D (D).

followed by 40 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension step for 10 min at 72°C. The conditions for the first round amplification of the 6A and 6B types were: 95°C for 10 min, followed by 45 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, and a final extension step for 10 min at 72°C; the second round amplificaction conditions were: 95°C for 5 min, followed by 40 cycles of 95°C for 30 seconds, 56°C for 1 min, 72°C for 1 min, and a final extension step for 10 min at 72°C. Sensitivity was determined at the cellular level with the cell line dilutions used for RQ-PCR.

Statistical analysis

The definition of complete remission followed recommended criteria.[18] Overall survival end-points, measured from entry into one of the prospective studies, were death (failure) and alive at last follow-up (censored).[18] Cumulative incidence of relapse, cumulative incidence of death, their standard errors (SE), and differences between groups were estimated using Gray's method.[19] The influence of *MLL-AF9* copy ratios at diagnosis on overall survival and cumulative incidence of relapse was evaluated in univariable Cox models. Correlations between co-variates were perfomed by non-parametric Spearman's correlation. The Kaplan-Meier method was used to estimate the distribution of cumulative incidence of relapse and overall survival. Survival distributions and cumulative incidence of relapse were compared using the log-rank test. The statistical analyses were performed with the statistical software package R, version 1.9.0.[20]

Results

MLL-AF9 copy ratios at diagnosis and correlation with clinical parameters

At diagnosis, samples from 25 patients (bone marrow, n=15; peripheral blood, n=22) were available for RQ-PCR analysis. The *MLL-AF9* copy ratio in peripheral blood and bone marrow ranged from 490 to 12,559 (median, 2,829) and from 496 to 31,911 (median, 5,207), respectively (Table 2). We found no correlation between the *MLL-AF9* copy ratio in bone marrow and peripheral blood and white cell count (p=0.17 and p=0.86), lactate dehydrogenase (LDH; p=0.59 and p=0.75), and bone marrow blast count (p=0.74 and p=0.24) using Spearman´s rank correlations. There was no correlation between the *MLL-AF9* copy ratio in bone marrow and peripheral blood, evaluated as continuous variables in a univariable Cox model, and overall survival (p=0.43 and p=0.29) or cumulative incidence of relapse (p=0.36 and p=0.31).

MRD monitoring

Samples from 19 patients collected in complete remission were available for RQ-PCR analysis during therapy, i.e., after first or second induction or after first consolidation therapy (Figure 1). In 17 cases, bone marrow (median samples per patient, 2; range, 1 to 6) and peripheral blood (median samples per patient, 1; range, 0 to 5) could be analyzed; in two cases (UPN5 and UPN240), only leukapheresis products collected after first consolidation were available. Eleven patients were RQ-PCR-negative in all samples analyzed in complete remission during therapy (group 1), whereas eight patients were RQ-PCR-positive in at least one sample collected in complete remission (group 2), with *MLL-AF9* copy ratios ranging from 5 to 5,286 in bone marrow (median, 108; n=13), from 18 to 5,424 in leukapheresis products (median, 1,184; n=5), and of 19, 44, and 3,891 in three peripheral blood samples (Figure 1, Table 2).

Group 1 (n=11): eight patients are in continuous complete remission with a median follow-up of 40.6 months (range, 5.7 to 131 months), and one patient (UPN274) died in complete remission due to graft-versus-host disease after allogeneic SCT. The remaining two patients relapsed 11 (UPN147) and 31 (UPN401) months after diagnosis and died. The *MLL-AF9* copy ratio of UPN147 at relapse was 5858. UPN401 had iso-

Table 2. MLL-AF9 copy ratios of all analyzed patients.

		Diagnosis		*After Induction I*		*After Induction II*		*After Consolidation I*		*After Consolidation II*		
UPN	MRD Group	BM	PB	BM	PB	BM	PB	BM	PB/*LP*	BM	PB	Months after diagnosis
5	1		861						*0,0*	0		9
										0		13
										0		19
										0	0	25
										0		32
										0		69
65	1	31911	6131					0	0	0		15
										0	0	19
										0	0	24
										0	0	27
										0	0	33
										0	0	37
										0	0	40
										0	0	43
										0	0	47
										0	0	56
										0	0	63
										0		69
147	1		1177			0		0		**5858**		11
179	1	3227	6711	0			0	0, 0		0		15
											0	17
											0	38
210	1	8149	10487		0	0	0	0, 0, 0, 0, 0	0, 0	0	0	7.5
											0	9
											0	9.5
											0	10
											0	10.5
											0	11
											0	12
										0	0	13
											0	14
											0	15
											0	16
											0	18
											0	21.5
										0		29
											0	33
											0	35
											0	50
											0	56
274	1	1625	2806					0	0		0	9
348	1	3677	2604			0		0	0	0		9
										0		17
350	1	8409	12559			0	0	0		0		14
401	1	13692		0		0				0		9
										0		12
										0	0	15
										0	0	33
											112	34
											3638	35
944	1	2839		0		0		0		0		5.4
1055	1	4876	3669	0	0			0				
36	2		1408			108	19		*202*			
40	2							34, 33		**3494**		7.5
51	2	5207	5226			816	3891	5286, 1094, 1788	*1184*	51		8
										265		9
91	2					37		5	*18*	**3290**		9
240	2		490						*3559, 15424*			
589	2		2979	55	0	0	0		0, *0, 0, 0*	0	0	7.5
										120	82	10
										1620	**1110**	11
709	2	16831	6321	693				0, 0, 0, 0	0, 0, 0, 0, *0, 0, 0, 0*		0	9
											0	0
										0	0	10
											97	12
										17866	4818	13.5
										60	59	14.7
										0	0	15
											0	17
										0		19
											0	20.5
812	2			461	44	14	0					
180	–		1925									
258	–	6371										
261	–		6905									
335	–		2136									
649	–	496	932									
686	–		2042									
691	–	944	884									
716	–	11037	6563									
799	–		2852									

MRD group 1, negative RQ-PCR in all examined samples during therapy in complete remission; MRD group 2, at least one positive RQ-PCR in samples collected in complete remission during therapy. UPN, unique patient number; MRD, minimal residual disease; BM, bone marrow; PB, peripheral blood; LP, leukapheresis product. Bold numbers: MLL-AF9 copy ratio at relapse.

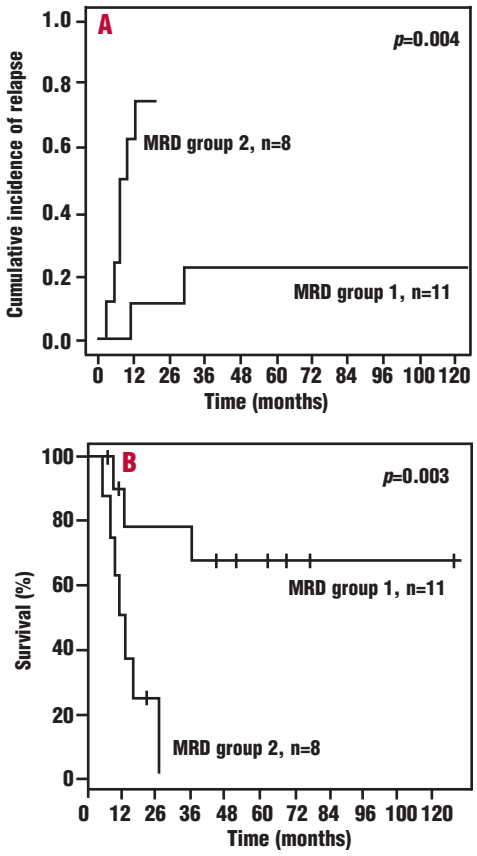

Figure 4. Outcome of patients according to MRD group. (A) cumulative incidence of relapse; (B) overall survival.

lated central nervous system relapse; the *MLL-AF9* copy ratio in cerebrospinal fluid was 13,752; bone marrow or peripheral blood were not available at the time of relapse.

Group 2 (n=8): six patients were RQ-PCR-positive at each time point examined during therapy, and two patients (UPN589 and UPN709) were RQ-PCR-positive after first induction therapy and became negative after second induction or first consolidation therapy (Figure 1, Table 2). In the latter two patients, molecular relapse was detectable four weeks (*MLL-AF9* copy ratios in bone marrow and peripheral blood, 120 and 82, respectively) and six weeks (*MLL-AF9* copy ratio in peripheral blood, 97) before hematologic relapse occurred. Six patients in group 2 relapsed at a median of 8.6 months after diagnosis (range, 3.6 to 13.5 months) with *MLL-AF9* copy ratios in bone marrow ranging from 265 to 17,866 (n=5; median, 3,290) at relapse. Five of the six patients died of their disease; one patient (UPN709) received re-induction therapy followed by allogeneic SCT, became RQ-PCR-negative and died 23 months after diagnosis from sepsis after graft failure. Of the remaining two patients, one (UPN36) died six months after diagnosis, in complete remission, from complications of autologous SCT, and one (UPN812) underwent allogeneic SCT in first complete remission and has now been in continuous complete remission for 17 months. No material for RQ-PCR was available during follow-up for this latter patient.

Patients in group 1 had a significantly lower cumulative incidence of relapse (p=0.004) and a significantly longer overall survival (p=0.003) compared with those in group 2 (Figure 4). The median overall survival in group 1 was not reached, and the cumulative incidence of relapse at one year was 0.11 (SE 0.11); the corresponding values for patients in group 2 were 10.5 months, and 0.63 (SE 0.20). The proportion of patients who received allogeneic SCT as second consolidation therapy was higher in group 1 than in group 2 (4/11 vs. 1/8), whereas autologous SCT was more frequent in group 2 (3/11 vs. 4/8) (Figure 1). The differences in cumulative incidences of relapse and overall survivals between the two groups remained statistically significant (p=0.007 and p=0.001, respectively) even if patients were censored at the time of allogeneic SCT.

Nested RT-PCR

The sensitivity of the nPCR assay was 10^{-5} for the 7A/8A fusion type and 10^{-4} for the 6A fusion type. All RQ-PCR-negative patient samples were also negative by nPCR.

Comparison of bone marrow and peripheral blood for MRD detection

We investigated the influence of the material analyzed on the results of RQ-PCR. We were able to analyze 40 paired bone marrow and peripheral blood samples that had been collected at identical time points during or after therapy. Thirty-eight pairs showed identical results with regard to RQ-PCR positivity or negativity. In the two remaining pairs, the bone marrow samples were RQ-PCR-positive with *MLL-AF9* copy ratios of 55 (UPN589, after first induction) and 14 (UPN812, after second induction), whereas the corresponding peripheral blood samples were RQ-PCR-negative (Figure 1, Table 2).

Discussion

In AML patients with favorable genetics (*PML-RARA*-, *AML1-ETO*-, and *CBFB-MYH11*-positive AML), MRD detection using RQ-PCR has been shown to be a useful method for identifing patients with a high risk of relapse.[21-28] Moreover, early therapy of molecular relapse may improve clinical outcome in *PML-RARA*-positive acute promyelocytic leukemia.[29,30]

In order to detect MRD in patients with t(9;11)(p22;q23)-positive AML, we recently developed a RQ-

PCR assay for the quantification of four different *MLL-AF9* fusion transcripts resulting from t(9;11).[9] We present here the results of MRD monitoring using RQ-PCR in a large series of intensively treated t(9;11)-positive AML patients. Thirty-four consecutive patients with t(9;11) were identified within three multicenter treatment trials of the AMLSG.[12-14] Twenty-five of these 34 patients were studied at diagnosis. In 19 of the 34 patients, MRD monitoring could be performed during and after therapy. The *MLL-AF9* copy ratio in bone marrow and peripheral blood at diagnosis was not correlated with clinical characteristics, such as the percentage of bone marrow blasts, white cell count, and lactate dehydrogenase, or outcome, i.e., cumulative incidence of relapse, and overall survival. These findings resemble results of a recent North American intergroup study on acute promyelocytic leukemia showing that *PML-RARA* transcript numbers at diagnosis are not correlated with white cell count or clinical outcome.[21] In contrast, two groups reported a correlation between *AML1-ETO* or *CBFβ-MYH11* transcript numbers at diagnosis and outcome parameters, such as overall survival,[22] event-free survival,[22] and relapse rate.[25]

Serial MRD monitoring during therapy using RQ-PCR identified two groups of t(9;11)-positive AML patients with significantly different outcomes. Patients with negative RQ-PCR in complete remission during the treatment period (group 1) had a significantly lower cumulative incidence of relapse and significantly longer overall survival than did patients with at least one positive RQ-PCR in complete remission (group 2; Figure 4). Quantitative MRD levels had no influence on clinical outcome. These findings suggest that early reduction and continuous negativity of the *MLL-AF9* transcript level are prerequisites for long-term complete remission. This conclusion is also supported by the single study focusing on MRD detection in t(9;11)-positive AML published to date, which showed that two patients who became *MLL-AF9* nPCR-negative after one course of induction therapy remained in continuous complete remission at 15 and 22 months, whereas one of two patients who remained RT-PCR-positive in complete remission relapsed.[7] In our study, only two of 11 patients with negative RQ-PCR results in all samples analyzed in complete remission during therapy relapsed. One of these two patients (UPN401) had been treated within the AML HD98-B trial for patients above the age of 60,[14] and one may speculate that the significantly lower cytarabine dose might have contributed to relapse in this particular case.[10] On the other hand, one patient (UPN 812) from group 2 is in continuous complete remission, presumably because this patient underwent allogeneic SCT in first complete remission. We cannot exclude the possibility that the favorable prognosis of patients in group 1 was related to the higher number of them who had received allogeneic SCT as second consolidation therapy (Figure 1). However, when patients were censored at the time of allogeneic SCT, the differences in cumulative incidence of relapse and overall survival remained statistically significant (p=0.007 and p=0.001, respectively). In contrast to our findings in t(9;11)-positive AML, transcript numbers after consolidation therapy appear to have a major impact on clinical outcome in *AML1-ETO*- and *CBFB-MYH11*-positive AML as well as in *PML-RARA*-positive acute promyelocytic leukemia.[21-23,25,28] In addition, one report showed in concordance to t(9;11) that rapid reduction of *AML1-ETO* transcript levels after first induction therapy was significantly correlated with a low risk of relapse, but PCR negativity, which was a predictor of long-term complete remission in our study, was not reached.[25]

In two patients, we were able to detect molecular relapse before hematologic relapse occurred, suggesting that RQ-PCR is of potential value for early detection of relapse. In acute promyelocytic leukemia, early detection of molecular relapse and subsequent early intervention results in better clinical outcome.[29,30] It needs to be clarified in further studies whether this is also true for t(9;11)-positive AML patients. All of our patients who remained in continuous complete remission were RQ-PCR-negative in all samples analyzed during follow up, indicating that eradication of the malignant clone, as reflected by PCR negativity, seems to be critical for long-term complete remission in t(9;11)-positive AML. In contrast, previous reports have indicated that a subset of patients with *AML1-ETO*- or *CBFB-MYH11*-positive AML or acute promyelocytic leukemia may remain RQ-PCR-positive during continuous complete remission,[21,22,23] and it has been hypothesized that the residual leukemia is controlled by immunological mechanisms in such cases.[31] However, other investigators have found that molecular remission is also a prerequisite for long-term hematologic complete remission in *CBFB-MYH11*-positive AML[32] and acute promyelocytic leukemia.[33]

The relatively low sensitivity of the RQ-PCR assay, which is similar to that of conventional single-round PCR,[7] might have contributed to our finding that quantitative MRD levels were not predictive of clinical outcome. To address this problem, we verified our results with a more sensitive nPCR that can detect one *MLL-AF9*-positive cell in 10^4 to 10^5 *MLL-AF9*-negative cells, similar to a previously reported *MLL-AF9* nPCR.[7] All RQ-PCR-negative samples were also nPCR-negative, indicating that we did not miss *MLL-AF9*-positive cases due to limited sensitivity of the RQ-PCR assay. Although our most important result, the positive prognostic effect of early and sustained PCR negativity, is qualitative in nature, we favor RQ-PCR over nPCR for monitoring of MRD because RQ-PCR is less time-consuming, can be better standardized, and is associated

with a lower risk of contamination.

We also analyzed the influence of the sample source (bone marrow vs. peripheral blood) on the RQ-PCR results. In 38 of 40 cases, the results in bone marrow and peripheral blood were concordant with regard to PCR positivity or negativity, whereas in two of 40 cases the bone marrow samples were RQ-PCR-positive, and the corresponding peripheral blood samples were RQ-PCR-negative. Despite the high concordance between the RQ-PCR results derived from bone marrow and peripheral blood, we do not recommend using peripheral blood rather than bone marrow for MRD detection because the two discrepant pairs were collected during the early phase of therapy (one after first induction, the other after second induction). Thus, relying only on peripheral blood would have resulted in miscategorization of these two patients, one of whom relapsed 10 months after diagnosis and the other in continuous complete remission after allogeneic SCT, into MRD group 1. These findings contrast with results obtained in *AML1-ETO*-positive AML and acute promyelocytic leukemia indicating that MRD monitoring can be accomplished with peripheral blood instead of bone marrow in these AML subtypes.[21,24,25] Whether peripheral blood can be used to detect molecular relapse in t(9;11)-positive AML during follow-up remains to be determined.

We conclude that MRD detection using RQ-PCR might be of clinical value in patients with t(9;11)-positve AML. Our findings suggest that early achievement of molecular remission and persistent RQ-PCR negativity are prerequisites for long-term complete remission. In addition, RQ-PCR appears to have the potential for early detectioning molecular relapse early. Additional patients need to be studied to confirm these results.

KD was the principal investigator and takes primary responsibility for the paper. CS and KE performed the experiments. RFS performed the statistical analyses. CS, RFS, HD, SF, and KD contributed to conception and design of the study. CS, RFS, KE, HD, SF, and KD contributed to analysis and interpretation of data. CS and SF wrote the manuscript. CS, RFS, HD, SF, and KD contributed to revision of the manuscript. The order of authorship was a joint decision of the authors. All authors approved the final version of the article to be published.

Table 1 and Table 2 were prepared by CS. Figures 1, 2, and 3 were prepared by CS. Figure 4 was prepared by CS and RFS.

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