Brief report

Multiplex reverse transcriptase–polymerase chain reaction screening in childhood acute myeloblastic leukemia

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To determine the incidence of leukemia-specific rearrangements, 60 cases of childhood acute myeloblastic leukemia and transient myeloproliferative disorder were screened with a novel multiplex reverse transcriptase–polymerase chain reaction (RT-PCR) assay, and the results were correlated with the cytogenetic findings. The RT-PCR assay detects 28 different fusion genes and more than 80 different fusion transcript variants. RNA was isolated from methanol/acetic acid–fixed cells that had been routinely prepared for cytogenetic analysis. Nine different fusion transcripts were found in 40% of the cases, whereas 78.3% of the cases had abnormal karyotypes. Two cases with a t(6;11) and an *MLL/AF6* gene fusion were missed cytogenetically. Conversely, cytogenetic analysis revealed 10 other well-defined chromosome rearrangements. Although cytogenetic analysis reveals a much broader range of abnormalities, multiplex RT-PCR serves as quality control and provides the essential information for minimal residual disease studies. Moreover, discrepant findings lead to the detection of new rearrangements on the molecular genetic level. (Blood. 2001;97:805-808)

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Introduction

Approximately 50% of adult and 80% of childhood acute myeloblastic leukemias (AMLs) harbor nonrandom karyotype abnormalities that define subentities with unique biological and clinical features.[1-4] (Interactive database, http://www.infobiogen.fr/services/chromcancer/.) Cytogenetic analysis provides a comprehensive overview of overall quantitative and qualitative karyotype abnormalities and reveals clonal changes and secondary abnormalities. The cloning of translocation-associated breakpoints has led to the identification of a variety of genes that normally regulate and control cell division, growth, differentiation, and apoptosis.[5,6] The fusion of such genes either leads to their abnormal activation or generates novel chimeric genes with neoplastic properties.[5,6] More than 50 leukemia-specific fusion genes have been defined already.[4-6] The resulting hybrid transcripts provide the essential basis for the development of reverse transcriptase–polymerase chain reaction (RT-PCR) techniques for the molecular genetic detection of such rearrangements.[7-15] So far, the majority of RT-PCR screening programs have searched for each of the most common fusion transcripts individually.[7-15] This is particularly true for those transcripts found in AML, whereas the clinically most important acute lymphocytic leukemia (ALL)–specific abnormalities have been combined in several types of multiplex assays.[16,17] However, the steadily increasing number of detectable abnormalities makes the conventional screening approaches for single specific fusion transcripts more and more impractical and obsolete.

With that in mind, Pallisgaard et al[18] have recently presented a multiplex RT-PCR assay that facilitates the detection of 29 fusion genes and more than 80 breakpoint and splice variants. We have used a similar modified assay (Hemavision; DNA Technology,

Aarhus, Denmark, for Bio-Rad Laboratories, Hercules, CA) to screen all childhood AML cases that were collected at our institution during a 5-year period. Our particular aim was to compare the results obtained by this assay with those from conventional cytogenetic analysis and to assess the diagnostic specificity and value of both techniques.

Study design

Patients

Between 1993 and 1998, 67 children with AML or transient myeloproliferative disorder (TMD) were diagnosed in Austria and registered at our institution. Cytogenetic analysis was performed on bone marrow aspirates or peripheral blood samples that were obtained from 64 of these patients. Sufficient material for the multiplex RT-PCR assay was available from 60 patients. This study was approved by the ethics committee of the Children's Cancer Research Institute, and informed consent was obtained from the patients or their parents.

Multiplex RT-PCR

Total RNA was isolated from 48 methanol/acetic acid–fixed cell samples that had been stored at −20°C and from 17 cryopreserved samples according to methods described previously.[19,20] We centrifuged 200-500 μL fixed-cell suspension, and pellets were washed with ethanol. Then RNA was prepared using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. Samples were analyzed using a multiplex RT-PCR assay (HemaVision) according to the manufacturer's instructions. The assay discriminates between 28 different fusion transcripts. Reverse transcription was performed with a mixture of translocation-specific complementary DNA (cDNA) primers and PCR

Table 1. Characteristics, multiplex reverse transcriptase–polymerase chain reaction results, and cytogenetic data of 67 cases of childhood acute myeloblastic leukemia

Case	Age (y)	Sex	FAB	Multiplex RT-PCR	Fusion transcript	Karyotype
1	0.2	M	AUL	neg		46,XY,t(X;8)(p11;q23)[30]
2	14.0	M	AUL	ND		46,XX,del(5)(q13q33)[20]*
3	10.4	M	M0	MLL/AF4	MLLex7/AF4(1414)	48,XX,t(4;11)(q21;q23),+4,+mar[1]/48,XX,idem,−4,+der(4)t(4;11)(q21;q23)[19]
4	0.5	F	M0	neg		46,XX[3]/46,XX,t(6;X)(q22;p22)[17]
5	1.0	F	M0	neg		46,XX,t(X;17)(p21;q11),r(6p)[15]
6	1.4	F	M0	neg		48,XX,+8,+21c[20]
7	2.9	M	M0	neg		45,XY,−7[40]
8	1.3	M	M0	ND		ND
9	0.01	F	M1	neg		46,XX[5]/47,XX,+21[15]
10	6.4	M	M1	neg		46,XY[13]/47,XY,+?del(19)(p13),del(2)(q13-14),inc[cp11]
11	0.3	M	M1	neg		47,XY,+der(19)[40]†
12	9.0	M	M2	AML1/MGT8		46,XY[4]/46,XY,t(8;21)(q22;q22)[16]
13	7.8	M	M2	AML1/MGT8		45,X,−Y,t(8;21)(q22;q22)[5]
14	11.7	M	M2	AML1/MGT8		46,XY[1]/45,X,−Y,t(8;21)(q22;q22)[15]
15	10.1	M	M2	AML1/MGT8		45,X,−Y,t(8;21)(q22;q22)[20]
16	3.8	M	M2	AML1/MGT8		45,X,−Y,t(8;21)(q22;q22)[20]
17	4.3	F	M2	AML1/MGT8		46,XX,t(8;21)(q22;q22)[20]
18	7.9	F	M2	AML1/MGT8		46,XX,t(8;21)(q22;q22)[17]
19	8.4	F	M2	MLL/ELL	MLLex7/ELL	46,XX,t(11;19)(q23;p13)[10]
20	12.1	M	M2	neg		46,XY,del(5)(q14q34)[15]/45,idem,−Y[15]
21	15.2	F	M2	neg		46,XX[15]
22	12.0	F	M2	neg		46,XX[40]
23	15.8	F	M2	neg		46,XX[20]
24	14.4	F	M2	neg		46,XX[20]
25	3.0	F	M2	neg		46,XX[19]
26	4.0	M	M2	neg		46,XY[20]
27	9.6	F	M2	ND		ND
28	3.2	F	M3	PML/RARA	PMLex6/RARAex2	46,XX,t(15;17)(q22;q21)[20]
29	13.8	F	M3	PML/RARA	PMLex6/RARAex2	46,XX[3]/46,XX,t(15;17)(q22;q21)[17]
30	12.2	M	M3	PML/RARA	PMLex6/RARAex2	46,XY,t(4;9)(q31;q34)t(15;17)(q22;q21)[16]
31	1.8	M	M3V	PML/RARA	PMLex6/RARAex2	46,XY,t(15;17)(q22;q12),del(17)(p11),t(17;17)(q11;p13)[30]
32	10.3	F	M3V	PML/RARA	PMLex6/RARAex2	46,XX,t(15;17)(q22;q21)[20]
33	14.0	F	M4	MLL/AF6	MLLex7/AF6	46,XX[20]
34	12.7	F	M4	MLL/AF6	MLLex6/AF6	46,XX[6]/46,del(11)(q23)[20]/46,XX,idem,del(9)(q22),[2]
35	1.8	F	M4	MLL/AF1q	MLLex6/AF1q	46,XX[2]/46,XX,t(1;11)(q21;q23),t(12;12)(q13;p12)[18]
36	11.9	M	M4	CBFB/MYH11	type A	46,XY,inv(16)(p13q22),t(16;17)(p10;q10)[20]
37	0.8	M	M4	neg	MLL/?	46,XY,der(11)add(11)(q23)[20]‡
38	8.8	M	M4	neg	MLL/?	54,XY,+Y,+6,+7,+8,+8,t(11;17)(q23;q25),+19,+20,+21[20]
39	2.1	F	M4	neg	MLL/?	46,XX,t(11;17)(q23;q21)[20]
40	12.2	F	M4	neg		46,XX[23]/46,XX,t(3;5)(q26;q13-14)[2]
41	13.4	M	M4	neg		46,XY[8]/45,X−Y[1]90,idemx2[10]
42	16.2	M	M4	neg		46,XY[2]/46,XY,t(7;14)(p15;q32)[19]
43	1.2	M	M4	neg	MLL/new gene	46,XY[13]/46,XY,inv(11)(q12q23)[7]§
44	2.8	M	M4	ND		46,XY[17]/46,XY,inv(8)(p11q13)[3]
45	11.5	M	M4	ND		46,XY[9]/46,XY,del(5)(q13q31),t(7;11)(p15;p12)[9]
46	2.0	F	M4Eo	CBFB/MYH11	type A	46,XX[12]/47,XX,+8[2]/46,XX,inv(16)(p13q22)[6]
47	11.9	M	M4Eo	ND		46,XY,inv(16)(p13q22),t(15;16)(q22;p13)inv(16)(p13q22)[20]
48	14.0	M	M5a	MLL/AF9	MLLex6/AF9A	46,XY,t(9;11)(p21;q23)[4]/46,XY,idem,del(13)(q14)[16]
49	16.4	F	M5a	MLL/AF9	MLLex6/AF9B	46,XX,t(9;11)(p21;q23)[20]
50	14.6	M	M5a	MLL/AF9	MLLex7/AF9A	46,XY,t(9;11)(p21;q23)[20]
51	4.5	M	M5a	MLL/AF9	MLLex7/AF9A	47,XY,t(9;11)(p21;q23),+8[20]
52	7.3	M	M5a	MLL/AF10	MLLex6/AF10(883)	46,XY[2]/46,XY,t(10;11)(p12;q23),t(10;12)(q24;p13)[2]/47,XY,idem,+del(1)(p13)[16]
53	10.8	F	M5b	neg		46,XX[25]
54	6.0	M	M6	neg		46,XY[3]/46-48,XY,inv(1)(p21p31),+del(1)(q21),der(1)t(1;8)(p13;q10),−6,−7,del(7)(q33), del(8)(q12),−9,−10,−21,+r,+1−3mar[cp13]
55	0.8	M	M7	neg		46,XY[20]
56	1.9	M	M7	neg		48,XY,del(6)(q21q24),+21,+21c[20]
57	0.7	M	M7	neg		47,XY,t(1;22)(p13;q13.1),t(3;19)(p23;p13),t(3;15)(q22;q22),+19[8]/46,XY,idem,−20[2]
58	1.7	F	M7	neg		47,XX,inv(9)(p11q13)c,+21c[1]/47,XX,idem,der(4)t(1;4)(q23;p15),[17]/47,XX,idem,iso(7)(q10)[2]
59	1.4	F	M7	neg		47,XX,+21c[7]/47,XX,der(7)t(1;7)(q22;p22),+21c[11]/47,XX,dup(1)(q22q44),+21c[2]
60	1.2	M	M7	neg		47,XY,t(2;4)(q37;q28),+21c[20]
61	1.9	M	M7	neg		47,XY,+21c[18]/48,XY,+8,+21c[1]/49,XY,+8,+12,+21c[1]
62	1.9	M	M7	neg		47,XY,+21c[16]/48,XY,+8,+21c[4]

Table 1. Characteristics, multiplex reverse transcriptase–polymerase chain reaction results, and cytogenetic data of 67 cases of childhood acute myeloblastic leukemia (cont'd)

Case	Age (y)	Sex	FAB	Multiplex RT-PCR	Fusion transcript	Karyotype
63	1.3	F	M7	neg		47,XX,+21c[20]
64	0.01	M	M7/TMD	neg		47,XY,+21c[20]
65	0.02	M	M7/TMD	neg		47,XY,+21c[27]
66	0.01	M	M7/TMD	neg		47,XY,+21c[20]
67	0.01	F	M7/TMD	ND		ND

For the fusion transcript data, the interpretation table in the HemaVision kit uses the old exon nomenclature of the MLL gene; exons 6-7 correspond to exons 9-10 of the new numbering according to Nilson et al.[21]

FAB indicates French-American-British; RT-PCR, reverse transcriptase–polymerase chain reaction; M, male; AUL, acute undifferentiated leukemia; neg, negative; ND, not determined; F, female.

*FISH analysis revealed an additional t(5;11)(q35;p15).
†FISH analysis revealed a cryptic t(7;12)(q36;p12).
‡Whole chromosome painting showed a t(11;17).
§The inv(11)(q12q23) was detected by FISH analysis, and a new MLL fusion partner was cloned (Litzka et al, unpublished data, 2000).

amplification in 8 parallel nested multiplex master reactions. Each of the master solutions contains several primers that are specific for particular fusion transcripts and a pair of control primers that amplifies a ubiquitously expressed gene. Thus, each of the master reactions identifies various chromosomal aberrations that, due to the heterogeneity of the breakpoints on the genomic level and/or alternative splicing, generate different messenger RNA (mRNA) variants. To verify the presence of a specific fusion transcript in a positive multiplex reaction, a nested split-out analysis with individual translocation-specific primer sets was performed. The specific translocation and splice variant was classified by comparing the respective pattern with the interpretation table provided by the manufacturer.

Results and discussion

Cytogenetic analysis of 64 childhood AML and TMD samples found an abnormal clone in 51 (79.7%) samples of the cases and a normal chromosome complement in 13 (20.3%) samples of the cases, whereas 24 (39%) of 60 samples analyzed by the multiplex RT-PCR were positive for one of 9 different fusion transcripts (Table 1). Examples of the multiplex RT-PCR results are shown in Figure 1.

Of the 24 RT-PCR–positive cases, 22 (91.7%) cases had correlating cytogenetic findings. In 2 cases (nos. 33 and 34) with an MLL/AF6 fusion transcript, the presence of the t(6;11)(q27;q23) had been missed cytogenetically. In samples from cases Nos. 33 and 34, only normal metaphases and a clone with a del(11)(q23) were found, respectively. Due to the location of the breakpoints in the telomeric regions of the chromosomes and submicroscopic deletions that occur in approximately 20% to 30% of such cases, this cryptic translocation is difficult to identify by cytogenetic and fluorescence in situ hybridization (FISH) analysis.[22]

Of particular interest are cases with 11q23 abnormalities and rearrangements of the MLL gene, which account for 5% to 10% of acquired karyotype changes in childhood and adult acute leukemias and myelodysplastic syndromes. At least 40 different 11q23 translocations have been described cytogenetically, and 23 MLL fusion partner genes have already been identified.[4] Our group of patients included 14 (23%) cases with 11q23 abnormalities. Thirteen (20.3%) cases with 8 different structural abnormalities were identified cytogenetically. They consisted of 6 different translocations that involved chromosomes 1, 4, 9, 10, 17, and 19 as well as one inversion and one del(11)(q23). Using the multiplex RT-PCR, 10 positive cases with 6 different fusion transcripts were found.

Despite the fact that the multiplex RT-PCR kit allows the detection of 10 of the currently 23 cloned MLL fusion partners, we nevertheless encountered 4 cases with an involvement of the MLL gene that remained undetected in the RT-PCR analysis. In case nos. 38 and 39 at t(11;17)(q23;q21~q25) and in case no. 37 additional chromosome material at 11q23 was found by cytogenetic analysis. In all 3 cases, whole chromosome painting and FISH using MLL-specific PAC clones confirmed the presence of a t(11;17) and involvement of the MLL gene (data not shown).[23] Although the multiplex RT-PCR detects 2 t(11;17)-associated fusion transcripts, MLL/AF17 and PLZF/RARA, neither of them was present in the samples. Moreover, 2 of these cases were also negative for the only other currently known MLL fusion partner on 17q25 (MLL/MSF) (data not shown). These data suggest the presence of a cluster of
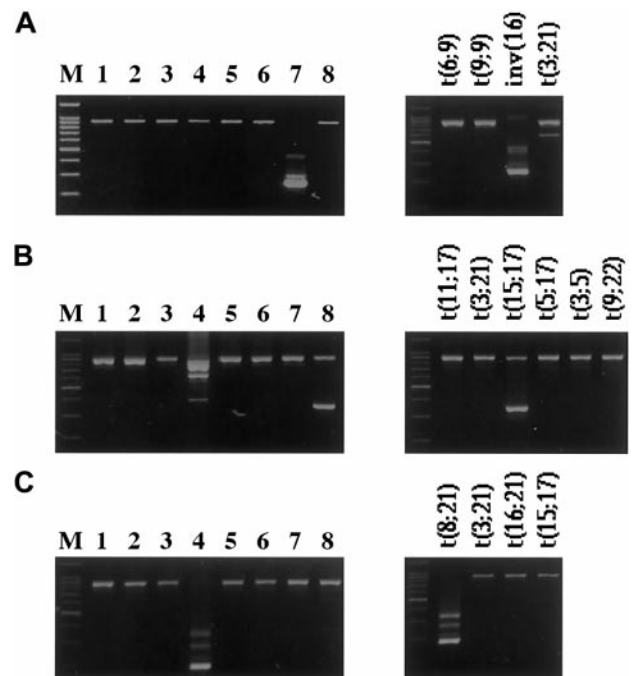
Figure 1. Examples of fusion genes detected by the multiplex RT-PCR. Amplification products of 8 parallel multiplex RT-PCR reactions (left panels) and the corresponding split-out reactions (right panels). The product with the higher molecular weight represents the internal positive control, and the lower bands represent the specific fusion genes. (A) inv(16)(p13q22)—CBFB/MYH11 fusion gene. (B) t(15;17)(q21;q22)—PML/RARA. (Note: Due to the primer combinations for the detection of different breakpoints, a few of the translocations detected by the HemaVision kit can appear in more than one master reaction, as seen in lanes 4 and 8. In the right panel of B, split-out reactions correspond to master reaction no.8.) (C) t(8;21)(q22; q22)—AML1/MGT8 (M, 100-bp DNA Ladder (Promega).

MLL fusion partners on 17q similar to those already known at 10p11.2-12 (*ABII* and *AF10*) and 19p13.1-13.3 (*ENL, ELL/MEN,* and *EEN*).[4] In the fourth case (no. 43), with a paracentric inv(11)(q12q23) and *MLL* involvement, we were able to clone a new *MLL* fusion partner (Litzka et al, unpublished data, 2000).

Two other cytogenetically detected specific chromosome rearrangements, t(7;11)(p15;p13) (case no. 45) and inv(8)(p11p13) (case no. 44), have molecular genetic equivalents in the form of *NUP98/HOXA9* and *MOZ/TIF2* fusion genes, respectively.[4] However, these fusion genes are not covered by the RT-PCR kit. In addition, 2 other translocations that are specific for particular subsets of myeloid neoplasms, t(1;22)(p13;q13) (case no. 57) and t(5;11)(q35;p15) (case no. 2), were encountered.[24,25] FISH analysis also revealed a case with a cryptic translocation t(7;12)(q36;p12) (case no. 11) in an infant in whom cytogenetic analysis had only discovered a marker chromosome 19.[26] All these translocations are currently not analyzable on the molecular genetic level because the involved genes have not been cloned yet. Finally, we observed a t(3;5)(q26;q13-14) (case no. 40) with breakpoints

that differed from those generating the RT-PCR–detectable fusion gene *NPM/MLF1*.[4]

The results of the comparative karyotype and RT-PCR analyses prove that they are complimentary techniques and are both indispensable for the evaluation of the disease-specific genetic features of myeloid malignancies. Although an increasing number of reciprocal rearrangements are detectable by molecular genetic means, karyotyping still provides the most comprehensive overview that is not obtainable by any other method. The demonstration of particular fusion transcripts by RT-PCR, on the other hand, is essential for subsequent molecular genetic follow-up and minimal residual disease studies.

Acknowledgments

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