Leukemia (1999) 13, 32–37
© 1999 Stockton Press All rights reserved 0887-6924/99 $12.00
http://www.stockton-press.co.uk/leu

RAS mutations and clonality analysis in children with juvenile myelomonocytic leukemia (JMML)

C Flotho[1], S Valcamonica[2], S Mach-Pascual[3], G Schmahl[1], L Corral[2], J Ritterbach[4], H Hasle[5], M Aricò[6], A Biondi[2] and CM Niemeyer[1]

[1]*Children's Hospital, University of Freiburg, Germany;* [2]*Pediatric Clinic University of Milan, Ospedale San Gerardo, Monza, Italy;* [3]*Brigham and Women's Hospital, Harvard Institutes of Medicine, Boston, MA, USA;* [4]*Children's Hospital, University of Giessen, Germany;* [5]*Department of Pediatrics, University Hospital of Aarhus, Denmark; and* [6]*Clinica Pediatrica, Università Pavia, Italy*

Juvenile myelomonocytic leukemia (JMML) is a malignant hematopoietic disorder of early childhood with excessive proliferation of the myeloid and monocytic lineage. Deregulation of the *RAS* signal transduction pathway is thought to play a key role in its pathogenesis. We examined peripheral blood or bone marrow cells of 36 children with JMML for activating point mutations in codons 12, 13 and 61 of the *NRAS* and *KRAS* proto-oncogenes by allele-specific restriction assay, single-strand conformation polymorphism and/or direct sequencing. Codons 12, 13 and 61 of *HRAS* were examined in 26 of these patients. We detected *RAS* mutations in six cases (17%) located at N12 ($n = 2$), N13 ($n = 3$) and K13 ($n = 1$). In addition, we performed clonality studies on different cell lineages in four of these patients applying the *RAS* mutation, the karyotype and X-chromosome inactivation patterns as clonal markers. Erythroid cells carried mutant *RAS*, indicating clonal origin. In EBV B cell lines, one of three patients studied harbored a *RAS* mutation, while the other two patients had polyclonal B cells with wild-type *RAS*. T lymphocytes were examined in one patient; they were polyclonal and had wild-type *RAS*. It is likely that JMML is a heterogeneous disease with respect to clonal involvement of different lineages.
Keywords: juvenile myelomonocytic leukemia; juvenile chronic myelogenous leukemia; *RAS* mutations; clonality; childhood

Introduction

Juvenile myelomonocytic leukemia (JMML) is a hematopoietic malignancy of childhood characterized by prominent hepatosplenomegaly, frequent skin involvement, leukocytosis, monocytosis and the presence of immature precursors in peripheral blood (PB).[1,2] Its annual incidence is about 1.3/million children.[3] The disorder has also been referred to as 'juvenile chronic myelogenous leukemia' (JCML) or 'chronic myelomonocytic leukemia in childhood' (CMML).[1] JMML is a clonal disorder as assessed by X-chromosome inactivation studies.[4] Hematopoiesis in JMML features an excessive proliferation of myeloid progenitor cells and a specific hypersensitivity for GM-CSF *in vitro*.[5] Because stimulation with GM-CSF is known to be associated with RAS activation,[6] mutations resulting in a deregulated RAS pathway are of particular interest in JMML.

p21RAS is a family of guanine nucleotide binding proteins located at the inner cell membrane which forward stimuli for cell proliferation and differentiation to the nucleus through a kinase cascade.[7] The RAS proteins cycle between an active, GTP-bound conformation and an inactive, GDP-bound form.[8] GTPase-activating proteins (GAPs) negatively regulate the signal output of RAS proteins by accelerating GTP hydrolysis.[9] Several GAPs connected with RAS proteins have been identified, including neurofibromin, the product of the neurofibromatosis type 1 (NF 1) tumor suppressor gene (*NF1*). NF1 is clinically diagnosed in about 15% of children with JMML.[1] In addition, truncating *NF1* mutations can be shown in a proportion of children with JMML without clinical evidence of NF1.[10] While *NF1*$^{-/-}$ knock-out mice die *in utero* at an embryonic stage, cultured fetal *NF1*$^{-/-}$ hematopoietic cells demonstrate a pattern of selective hypersensitivity to GM-CSF.[11,12]

Like decreased GAP activity, specific point mutations of the *RAS* genes can result in persistent activation of the RAS signaling pathway.[13] In this study, we wanted to determine the frequency of point mutations at codons 12, 13 or 61 of the *NRAS*, *KRAS* or *HRAS* genes in a large cohort of children with JMML. In addition, mutant *RAS* genes, karyotype analysis and, in female patients, X-chromosome inactivation clonality assays were used as markers for clonal involvement of different cell lineages in JMML patients.

Correspondence: C Flotho, Universitäts-Kinderklinik, Mathildenstrasse 1, 79106 Freiburg, Germany; Fax: 49 761 270 4518
Received 16 April 1998; accepted 14 September 1998

Materials and methods

Patients

Thirty-six children with JMML were studied. Clinical data are given in Table 1. NF1 was known in three patients. Chromosomal analyses in leukemic cells were available in 32 of the 36 children and showed a normal karyotype in 23 children, monosomy 7 in four children and complex aberrations in five children.

Cell cultures

Adherence depleted mononuclear cells (MNC) from PB were cultured in a serum-deprived semi-solid methylcellulose system[14] at 5×10^4 cells/ml in the presence of erythropoietin (2 U/ml). After 14 days of culture at 37°C in a 4% CO_2 atmosphere, colonies derived from erythroid burst-forming units (BFU-E) were harvested. EBV transformed B lymphocytes were grown in RPMI 1640 (Gibco, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin.[15]

Fluorescence-activated cell sorting (FACS)

After lysis of erythrocytes, PB cells were maintained in RPMI 1640/10% FCS. Per 10^6 cells, 10 μl phycoerythrin-conjugated monoclonal anti-CD3 (Laboserv, Giessen, Germany) was added. Cells were incubated for 15 min, washed twice and sorted on a FACStar Plus (Becton Dickinson, Heidelberg, Germany). The purity of $CD3^+$ cells was greater than 90%.

Table 1 Clinical characteristics of 36 children with JMML

Patient	*Age at diagnosis (years)*	*Spleen size (cm)*	*Liver size (cm)*	*Peripheral blood*			*HbF (%)*	*Karyotype*	*Survival (years)*	
				WBC (per μl)	*Monocytes (per μl)*	*Blasts (%)*				
1	3.2	6	8	48 300	3900	3	2.8	complex	1.8	(BMT)
2	1.8	5	2	51 600	8300	0	18	normal	4.7+	(BMT)
3	3.9	1	0	5700	1700	1	6.5	–7	5.3+	(BMT)
4	0.4	6	2	34 100	12 300	6	ND	normal	3.8+	(BMT)
5	4.7	4	5	28 800	1400	10	14	normal	1.5	(BMT)
6	0.3	6	4	37 000	5600	4	ND	normal	0.3	(BMT)
7	4.1	6	5	10 500	1300	1	50	normal	0.4	
8	3.7	5	7	55 000	14 300	5	60	normal	0.8	
9	7.2	10	6	9700	2500	0	0.4	normal	0.8	(BMT)
10	0.3	1	2	19 000	2700	0	ND	complex	2.1+	(BMT)
11	6.0	*	0	7000	3400	0	ND	ND	11.4	
12	3.9	2	3	10 400	2700	2	12	complex (7q–)	0.8	
13	5.1	0	0	99 700	22 900	0	32	normal	6.3	(BMT)
14	1.5	2	2	12 600	2600	0	ND	ND	4.1+	
15	0.3	*	*	39 600	8700	5	ND	ND	4.3+	
16	8.9	4	5	14 700	7500	8	0	–7	5.5+	(BMT)
17	4.4	3	6	17 000	1400	9	56	normal	1.3	(BMT)
18	0.5	5	3	50 600	2500	2	1.4	normal	1.4	(BMT)
19	1.1	4	4	51 000	11 200	0	0.4	normal	3.2+	(BMT)
20	6.4	8	9	140 000	64 400	3	1.9	complex	1.0	
21	0.2	8	5	27 400	2700	0	43	normal	2.7+	
22	4.2	7	3	100 000	16 000	1	ND	normal	0.9	(BMT)
23	2.1	8	5	25 600	5100	1	ND	ND	3.8+	
24	0.3	5	4	103 000	17 500	4	49	normal	4.4	(BMT)
25	0.5	4	3	15 600	6600	0	9.4	–7	3.8+	(BMT)
26	1.1	3	5	22 500	7200	4	0.8	–7	3.8	(BMT)
27	1.6	3	3	26 000	3400	5	32	normal	1.3	
28	0.8	3	4	42 600	11 000	3	21	normal	0.2	
29	5.5	0	3	48 000	4500	26	54.1	normal	1.0	
30	2.4	10	6	27 500	4100	5	1	normal	8.7+	(BMT)
31	3.0	9	9	25 600	1500	0	10	normal	1.7	
32	2.1	6	3	43 600	6500	3	40	complex	1.3	(BMT)
33	2.2	7	7	29 000	2900	0	28	normal	1.5	(BMT)
34	3.5	1	0	28 200	1100	0	67	normal	1.2	
35	1.8	2	2	22 200	2400	0	1	normal	7.2+	
36	0.4	13	8	139 000	36 100	40	38	normal	2.5+	(BMT)

WBC, white blood count; HbF, fetal hemoglobin; –7, monosomy 7; 7q–, deletion on long arm of chromosome 7; ND, no data available. Survival is given in years after diagnosis; +, patient alive; BMT, bone marrow transplantation. Spleen and liver size are given in cm below costal margin; *, enlarged (size below costal margin not evaluated).

DNA preparation

In 13 cases, DNA was extracted from total bone marrow (BM) ($n = 3$) or PB ($n = 10$) cells. In 24 cases, cell populations from PB ($n = 13$) or BM ($n = 11$) were separated on a Ficoll density gradient (Pharmacia, Freiburg, Germany). DNA was extracted from MNC (above the Ficoll fraction) ($n = 4$) and myeloid cells (below the Ficoll fraction, on top of red cells) ($n = 20$). DNA preparation followed a standard protocol.[16]

PCR amplification

For allele-specific restriction analyses (ASRA), one PCR primer of each pair was designed to contain one or more base mismatches adjacent to the codon of interest, creating a restriction site in wild-type, but not in mutant fragments.[17] For single-strand conformation polymorphism (SSCP) analyses, fully matched primers were used. The opposite primer of each pair was placed into intron sequences to avoid pseudogene amplification. Primer sequences, thermal cycling conditions and restriction enzymes used will be provided to interested researchers upon request. DNA from THP-1 cells (point mutation at codon 12 of *NRAS*)[18] and from RC2A cells (point mutation at codon 12 of *KRAS*)[19] served as positive controls. K562 cells were used as negative control.[17]

Mutation analysis

Two different methods were used for the identification of *RAS* mutations: ASRA ($n = 26$) and SSCP ($n = 10$). For ASRA, restriction digests were incubated in 20 μl reaction buffer (as provided by the enzyme manufacturer) containing 3–7 μl PCR product (depending on DNA content as estimated by band intensity on the gel) and 4–10 U of restriction endonuclease (New England Biolabs, Schwalbach, Germany) for 3 h at the appropriate temperature. To ensure complete digestion, each assay was subjected to a second incubation with fresh enzyme. The fragments were separated on 7–12% polyacrylamide gels. SSCP analyses were performed according to the method previously described by Orita *et al*.[20] 1 μl of the amplified PCR product was subjected to 18 additional cycles of PCR in a 25 μl solution containing 200 μmol/l dATP, dGTP

and dTTP and 1 μl ^{32}PdCTP (Amersham, Buckinghamshire, UK) in addition to the reagents used for the first PCR. Gels were run at 4°C.

Direct sequencing

If a putative mutation was seen in ASRA or SSCP, direct sequencing was performed to define the base exchange and exclude false-positive results. Sequence analysis without prior ASRA or SSCP was used for *NRAS* codon 61 in 26 cases, *KRAS* codons 12 and 13 in 11 cases and *KRAS* codon 61 in 37 cases. The dideoxynucleotide chain termination method was performed using digoxigenin-labeled primers (MWG Biotech, Ebersberg, Germany) and a cycle sequencing kit (Thermo Sequenase; Amersham). After ethanol precipitation, 2 μl PCR product was added to 1 nmol primer in a total volume of 8 μl Sequenase reaction mix. Sequencing primers were: 5′-AGGATCAGGTCAGCGGGCTACCACT for *NRAS* codons 12/13; 5′-CCTCTATDGTKGGGTCATATTC for *KRAS* codons 12/13 (K = wobble G/T, D = wobble A/G/T; this primer was intended for use with all three *RAS* genes); 5′ TTGGCAAT-AGCATTGCATTCCCTGT for *NRAS* codon 61; and 5′-TGTTGTTGAGTTGTATATAACACCT for *KRAS* codon 61. Thermal conditions were: initial denaturing (95°C; 3 min) and 30 cycles of primer annealing (60°C; 1 min) and denaturing (95°C; 1 min). Reaction products were separated on 5% polyacrylamide gels, directly blotted on to a nylon membrane, and visualized using alkaline phosphatase-conjugated anti-digoxigenin and a detection kit based on a NBT^2/X-phosphate color reaction (Boehringer, Mannheim, Germany).

Human androgen receptor gene (HUMARA) clonality assay

The HUMARA assay was performed in two female patients as previously described.[21]

Fluorescence in situ hybridization (FISH)

The repetitive alphoid probe p7t1[22] was used after nick translation with biotin-16-dUTP. Preparations were pretreated with RNase, washed in SSC and dehydrated in a graded ethanol series. After probe and target DNA denaturation hybridization (20 h) was performed with a mixture containing 60% formamide, 2 × SSC; 5% dextran sulfate, 500 μg/ml salmon sperm DNA and 2 μg/ml DNA probe. The reaction was visualized using an avidin-conjugated fluorescein isothiocyanate (FITC) vector.[23] Amplification was done with additional layers of biotinylated goat anti-avidin and avidin-FITC. Signal evaluation followed the criteria published by Hopman *et al*.[24] From each specimen 500 nuclei were scored.

Gene sequence data

Information on human gene sequences was obtained from EMBL database, Heidelberg, Germany. Sequence information on the 3′ terminus of *NRAS* intron 2 came from a previous cloning experiment.

Results

Point mutations in codons 12, 13 or 61 of the *RAS* genes were detected in six of the 36 (17%) children with JMML. Three children with *RAS* mutations were girls, and three were boys. Because the three children with JMML and NF1 had wild-type *RAS* genes, the frequency of *RAS* mutations among children with JMML without the clinical diagnosis of NF1 was six of 33 (18%). Two children had a mutation in codon 12 of *NRAS*, three children in codon 13 of *NRAS*, and one child in codon 13 of *KRAS*. None of the 26 patients analyzed had a mutation in one of the three *HRAS* codons studied. Of the six children with *RAS* mutations, five had a normal karyotype and one (patient 25) had monosomy 7.

The *RAS* mutations identified provided an opportunity to investigate the clonal derivation of different lineages. The erythroid lineage was analyzed in two patients. Point mutations detected in PB myeloid cells were also present in erythroid cells of BFU-E derived colonies (Table 2). In concordance with this finding, erythroid cells from one of the two patients studied were clonally derived by X-chromosome inactivation studies (Table 2). The involvement of the lymphoid lineage was assessed in three patients. In patient 20 (mutation at *NRAS* 12), EBV-transformed B cells showed wild-type *RAS*. In concordance, the HUMARA assay demonstrated clonal involvement of granulocytes, but not of EBV B cells (Table 2). From patient 25 (mutation at *KRAS* 13), EBV-transformed B lymphocytes, sorted CD^{3+} T lymphocytes and CD3$^-$ cells (a mixture of granulocytes and MNC) were analyzed. While mutant *RAS* was seen in CD3$^-$ cells, *RAS* was normal in B and T lymphocytes (Figure 1). On a cytogenetic level, FISH analyses confirmed monosomy 7 in CD3$^-$ cells and demonstrated disomy 7 in CD3$^+$ lymphocytes and in the EBV-transformed B cell line (data not shown). These results were consistent with the HUMARA assay which showed polyclonal derivation of CD3$^+$ T lymphocytes and EBV-transformed B lymphocytes (Table 2). Thus, in patients 20 and 25, lymphoid cells were not part of the leukemic clone. In contrast, EBV-transformed B lymphocytes of patient 35 (mutation at *NRAS* 13) harbored the same *RAS* mutation that had previously been demonstrated in unfractionated cell lysates.

While in five patients the *RAS* mutations were presented at the time of diagnosis, hematopoietic cells of patient 20 acquired the mutation during the clinical course. The girl presented at the age of 4.8 years with severe thrombocytopenia without hepatosplenomegaly, monocytosis or spontaneous growth of myeloid progenitors. There was no *RAS* mutation in PB myeloid cells. The child was seen again 1.5 years later with a clinical and hematological picture characteristic of JMML. She had developed leukocytosis, monocytosis and a marked enlargement of liver and spleen. Spontaneous growth of myeloid progenitors was present. A nucleotide substitution at codon 12 of *NRAS* was detectable in PB myeloid cells, but was absent in EBV-transformed B cells.

Discussion

In this study we detected *RAS* mutations in six of 33 (18%) children with JMML without clinical evidence of NF1. These results are in agreement with findings by Kalra *et al*[25] who reported mutant *N*- or *KRAS* in the absence of NF1 in five of 24 (20%) patients classified as JCML and four of 27 (15%) patients classified as monosomy 7 syndrome (some of which had clinical features of JMML). In a study by Sheng *et al*,[26]

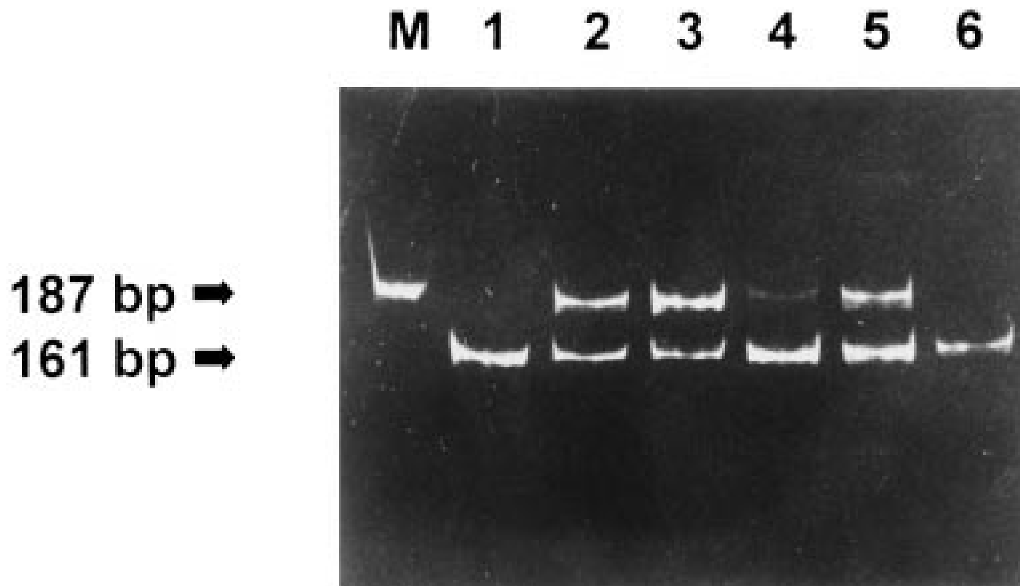

Figure 1 Allele-specific restriction analysis of *RAS* mutations in different hematopoietic cell lineages of patient 25 (*KRAS* codon 13: GGC→GAC). The 161 bp band represents normal *RAS* alleles. The 187 bp band represents mutant *RAS* alleles. Lanes: M, 187 bp standard; 1, wild-type control; 2, granulocytes; 3, BFU-E-derived colonies; 4, CD3⁺ cells; 5, CD3⁻ cells; 6, EBV-transformed B cells.

Table 2 Synopsis of molecular and cytogenetic findings in different cell lineages from four patients with *RAS* mutations

		Myeloid granulocytes	*Erythroid BFU-E derived erythroblasts*	*Lymphoid*	
				CD3⁺ T cells	*EBV B cells*
Patient 20:	*NRAS* 12 GGT→GTT (Gly→Val)	mutant	ND	ND	wild-type
	HUMARA assay	clonal	ND	ND	polyclonal
Patient 22:	*NRAS* 12 GGT→GAT (Gly→Asp)	mutant	mutant	ND	ND
Patient 25:	*KRAS* 13 GGC→GAC (Gly→Asp)	mutant	mutant	wild-type	wild-type
	HUMARA assay	clonal	clonal	polyclonal	polyclonal
	Cytogenetics	monosomy 7	ND	disomy 7	disomy 7
Patient 35:	*NRAS* 13 GGT→GAT (Gly→Asp)	mutant	ND	ND	mutant

BFU-E, erythroid burst-forming units; ND, not done.

RAS mutations were found in one of 12 (8%) children with JMML. In contrast to these data, Miyauchi *et al*[27] examining *NRAS* only detected mutations in 30% (6/20) of JMML patients. In our cohort, there was a predominance of mutant *NRAS* ($n = 5$) over *KRAS* ($n = 1$), while the distribution was equal in the study by Kalra *et al*. In both studies, all mutations were restricted to codons 12 and 13 of the first coding exons. Although *HRAS* mutations have been described in adults with CMML,[28] we did not detect mutations in *HRAS* codons 12, 13 and 61 in 26 patients studied.

We conclude that the frequency of mutations of the *RAS* gene in children with JMML without NF1 is about 20% irrespective of karyotype. NF1 and *RAS* mutations appear to be mutually exclusive[25] as no patient with NF1 and concomitant *RAS* mutation has been identified. Considering that about 15% of children with JMML have NF1,[1] and about 15% without clinical evidence of NF1 have truncating mutations of the *NF1* gene,[10] 50% of children with JMML have evidence of activation of the RAS signaling pathway by *RAS* or *NF1* mutations. One might speculate that the remaining 50% have abnormalities in the RAS pathway that are yet to be defined. So far, we have investigated two candidate genes, *HCP* and *SHC*, and found normal expression in hematopoietic cells of JMML patients (unpublished work).

One patient in our cohort had normal *RAS* at the time of first clinical presentation but showed a point mutation in *NRAS* 1.5 years later when the clinical picture was characteristic of JMML. The appearance of new *RAS* mutations during the course of myelodysplastic syndromes has also been observed by others.[29–31] The formation of the aberrant clone and the deregulation of the RAS pathway may constitute two different steps in leukemogenesis. It is tempting to speculate that activation of the RAS pathway is a prerequisite for the JMML phenotype. This view is supported by experiments showing that sublethally irradiated mice reconstituted with *Nf1*-deficient hematopoietic liver cells consistently develop a myeloproliferative disorder similar to JMML.[12]

The erythroid lineage carried the same *RAS* alteration as the respective myeloid cells, confirming the clonal origin of erythropoiesis in JMML.[4,32,33] In two of the three patients with EBV B cell lines studied, B lymphocytes had wild-type *RAS* and – in the case of the patient with monosomy 7 – normal

karyotype. Like EBV B cells, $CD3^+$ T lymphocytes carried wild-type *RAS* and polyclonality of B and T lymphocytes was confirmed by X-chromosome inactivation analysis. These data are in agreement with previous reports of non-leukemic T and B lymphocytes in JMML as assessed in mitogen-stimulated lymphocytes and EBV-transformed B cells by analysis of *RAS* mutations or loss of heterozygosity for *NF1*.[33,34]

In contrast, one of our patients demonstrated a clonal RAS mutation in EBV-transformed B cells (patient 35). This child had typical clinical features of JMML. Clonal derivation of lymphocytes in JMML has also been suggested by X-chromosome inactivation analysis in one report,[4] although definitive purification of lymphocyte subsets had not been performed. Miles *et al*[33] reported a child with NF1 with an unusual myeloproliferative syndrome which showed clonal involvement of EBV-transformed cells as assessed by *NF1* allelic loss. In addition, two cases of JMML evolving into B lymphoid blast crisis have been reported.[35,36] A study of immunoglobulin heavy chain gene rearrangements also supported clonal derivation of B lymphoblasts in some patients.[37] Taken together, these data suggest that in JMML the clonal involvement of cells of the lymphoid lineage, in particular B lymphocytes, is heterogeneous.

Acknowledgements

This work was supported by grants from the Kind-Philipp-Stiftung (Germany), the Clotten-Stiftung (Germany), the Swiss National Science Foundation, the Associazione Italiana Ricerca Cancro (Italy) and the Fondazione Tettamanti (Italy). The authors wish to thank JW Janssen (Heidelberg, Germany) for providing RC2A DNA, and G Vorbeck (Freiburg, Germany) for operating the FACS system. We are grateful to G Hilzinger and A Molz for technical assistance.

References

1 Niemeyer CM, Aricó M, Basso G, Biondi A, Cantú Rajnoldi A, Creutzig U, Haas O, Harbott J, Hasle H, Kerndrup G, Locatelli G, Mann G, Stollmann-Gibbels B, van't Veer-Korthof ET, van Wering E, Zimmermann M, Members of the European Working Group on Myelodysplastic Syndromes in Childhood (EWOG-MDS). Chronic myelomonocytic leukemia in childhood: a retrospective analysis of 110 cases. *Blood* 1997; **89**: 3534–3543.

2 Niemeyer CM, Fenu S, Hasle H, Mann G, Stary J, van Wering E. Differentiating juvenile myelomonocytic leukemia from infectious disease. *Blood* 1998; **91**: 365–367.

3 Hasle H, Kerndrup G, Jacobsen BB. Childhood myelodysplastic syndrome in Denmark – incidence and predisposing conditions. *Leukemia* 1995; **9**: 1569–1572.

4 Busque L, Gilliland DG, Prchal JT, Sieff CA, Weinstein HJ, Sokol JM, Belickova M, Wayne AS, Zuckerman KS, Sokol L, Castleberry RP, Emanuel PD. Clonality in juvenile chronic myelogenous leukemia. *Blood* 1995; **85**: 21–30.

5 Emanuel PD, Bates LJ, Castleberry RP, Gualtieri RJ, Zuckerman KS. Selective hypersensitivity to granulocyte–macrophage colony-stimulating factor by juvenile chronic myeloid leukemia hematopoietic progenitors. *Blood* 1991; **77**: 925–929.

6 Satoh T, Nakafuku M, Miyajima A, Kaziro Y. Involvement of ras p21 protein in signal transduction pathways from interleukin 2, interleukin 3, and granulocyte–macrophage colony-stimulating factor, but not from interleukin 4. *Proc Nat Acad Sci USA* 1991; **88**: 3314–3318.

7 Seger R, Krebs EG. The MAPK signaling cascade. *FASEB J* 1995; **9**: 726–735.

8 Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: conserved structure and molecular mechanism. *Nature* 1991; **349**: 117–127.

9 Hall A. Signal transduction through small GTPases – a tale of two GAPs. *Cell* 1992; **69**: 389–391.

10 Side LE, Emanuel PD, Taylor B, Franklin J, Thompson P, Castleberry RP, Shannon KM. Mutations of the NF1 gene in children with juvenile myelomonocytic leukemia without clinical evidence of neurofibromatosis, type 1. *Blood* 1998; **92**: 267–272.

11 Bollag G, Clapp DW, Shih S, Adler F, Zhang YY, Thompson P, Lange BJ, Freedman MH, McCormick F, Jacks T, Shannon K. Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. *Nat Genet* 1996; **12**: 144–148.

12 Largaespada DA, Brannan CI, Jenkins NA, Copeland NG. Nf1 deficiency causes Ras-mediated granulocyte–macrophage colony-stimulating factor hypersensitivity and chronic myeloid leukaemia. *Nat Genet* 1996; **12**: 137–143.

13 Barbacid M. Ras genes. *Ann Rev Biochem* 1987; **56**: 779–827.

14 Sieff CA, Ekern SC, Nathan DG, Anderson JW. Combinations of recombinant colony-stimulating factors are required for optimal hematopoietic differentiation in serum-deprived culture. *Blood* 1989; **73**: 688–693.

15 Ventura M, Gibaud A, Le Pendu J, Hillaire D, Gerard G, Vitrac D, Oriol R. Use of a simple method for the Epstein–Barr virus transformation of lymphocytes from members of large families of Reunion Island. *Hum Hered* 1988; **38**: 36–43.

16 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**: 1215.

17 Todd AV, Iland HJ. Rapid screening of mutant N-ras alleles by analysis of PCR-induced restriction sites: allele-specific restriction analysis. *Leuk Lymphoma* 1991; **3**: 293–300.

18 Janssen JW, Steenvoorden AC, Lyons J, Anger B, Bohlke JU, Bos JL. Seliger H, Bartram CR. RAS gene mutations in acute and chronic myelocytic leukemias, chronic myeloproliferative disorders, and myelodysplastic syndromes. *Proc Natl Acad Sci USA* 1987; **84**: 9228–9232.

19 Janssen JW, Lyons J, Steenvoorden ACM, Seliger H, Bartram CR. Concurrent mutations in two different ras genes in acute myelocytic leukemias. *Nucleic Acids Res* 1987; **15**: 5669–5680.

20 Orita M, Suzuki Y, Sekiya T, Hayashi K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 1989; **5**: 874–879.

21 Mach-Pascual S, Legare RD, Lu D, Kroon M, Neuberg D, Tantravahi R, Stone RM, Freedman AS, Nadler LU, Gribben JG, Gilliland DG. Predictive value of clonality assays in patients with non-Hodgkin's lymphoma undergoing autologous transplant. *Blood* 1998; **91**: 4496–4503.

22 Willard HF, Waye JS. Hierarchical order in chromosome-specific human alpha satellite DNA. *Trends Genet* 1987; **3**: 192–198.

23 Pinkel D, Straume T, Gray JW. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. *Proc Nat Acad Sci USA* 1986; **83**: 2934–2938.

24 Hopman AH, Ramaekers FC, Raap AK, Beck JL, Devilee P, van der Ploeg M, Vooijs GP. *In situ* hybridization as a tool to study numerical chromosome aberrations in solid bladder tumors. *Histochemistry* 1988; **89**: 307–316.

25 Kalra R, Paderanga DC, Olson K, Shannon KM. Genetic analysis is consistent with the hypothesis that NF1 limits myeloid cell growth through p21ras. *Blood* 1994; **84**: 3435–3439.

26 Sheng XM, Kawamura M, Ohnishi H, Ida K, Hanada R, Kojima S, Kobayashi M, Bessho F, Yanagisawa M, Hayashi Y. Mutations of the ras genes in childhood acute myeloid leukemia, myelodysplastic syndrome and juvenile chronic myelocytic leukemia. *Leukemia Res* 1997; **21**: 697–701.

27 Miyauchi J, Asada M, Sasaki M, Tsunematsu Y, Kojima S, Mizutani S. Mutations of the N-ras gene in juvenile chronic myelogenous leukemia. *Blood* 1994; **83**: 2248–2254.

28 Gow J, Hughes D, Farr C, Hamblin T, Oscier D, Brown R, Padua RA. Activation of Ha-ras in human chronic granulocytic and chronic myelomonocytic leukaemia. *Leukemia Res* 1988; **12**: 805–810.

29 Horiike S, Misawa S, Nakai H, Kaneko H, Yokota S, Taniwaki M, Yamane Y, Inazawa J, Abe T, Kashima K. N-ras mutation and karyotypic evolution are closely associated with leukemic transformation in myelodysplastic syndrome. *Leukemia* 1994; **8**: 1331–1336.

30 van Kamp H, de Pijper C, Verlaan-de Vries M, Bos JL, Leeksma CH, Kerkhofs H, Willemze R, Fibbe WE, Landegent JE. Longitudinal analysis of point mutations of the N-ras proto-oncogene in patients with myelodysplasia using archived blood smears. *Blood* 1992; **79**: 1266–1270.
31 Nakagawa T, Saitoh S, Imoto S, Itoh M, Tsutsumi M, Hikiji K, Nakamura H, Matozaki S, Ogawa R, Nakao Y, Fujita T. Multiple point mutation of N-ras and K-ras oncogenes in myelodysplastic syndrome and acute myelogenous leukemia. *Oncology* 1992; **49**: 114–122.
32 Inoue S, Shibata T, Ravindranath Y, Gohle N. Clonal origin of erythroid cells in juvenile chronic myelogenous leukemia. *Blood* 1987; **69**: 975–976.
33 Miles DK, Freedman MK, Stephens K, Pallavicini M, Sievers EL, Weaver M, Grunberger T, Thompson P, Shannon KM. Patterns of hematopoietic lineage involvement in children with neurofibromatosis type 1 and malignant myeloid disorders. *Blood* 1996; **88**: 4314–4320.
34 Neubauer A, Shannon K, Liu E. Mutations of the ras proto-oncogenes in childhood monosomy 7. *Blood* 1991; **77**: 594–598.
35 Lau RC, Squire J, Brisson L, Kamel-Reid S, Grunberger T, Dube I, Letarte M, Shannon K, Freedman MH. Lymphoid blast crisis of B-lineage phenotype with monosomy 7 in a patient with juvenile chronic myelogenous leukemia (JCML). *Leukemia* 1994; **8**: 903–908.
36 Yamamoto M, Nakagawa M, Ichimura N, Ohtsuki F, Ohtsuka Y, Tsujino Y, Tanaka A, Kamiya T, Wada H. Lymphoblastic transformation of chronic myelomonocytic leukemia in an infant. *Am J Hematol* 1996; **52**: 212–214.
37 Mark Z, Toren A, Amariglio N, Vonsover A, Rechavi G, Brok-Simoni F. Rearrangement of the immunoglobulin heavy chain gene in juvenile chronic myeloid leukaemia. *Br J Haematol* 1995; **90**: 353–357.

Copyright © 2003 EBSCO Publishing