Frequencies and prognostic impact of RAS mutations in MLL-rearranged acute lymphoblastic leukemia in infants

Emma M.C. Driessen,[1] Eddy H.J. van Roon,[1] Jill A.P. Spijkers-Hagelstein,[1] Pauline Schneider,[1] Paola de Lorenzo,[2] Maria Grazia Valsecchi,[2] Rob Pieters,[1] and Ronald W. Stam[1]

[1]Pediatric Oncology/Hematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands; and [2]Interfant-99 Trial Data Center, Department of Clinical Medicine and Prevention, University of Milano-Bicocca,Monza, Italy

 Haematologica 2013;98. doi:10.3324/haematol.2012.067983

Online Supplementary Table S1. Sequences of primers.

Primer	Sequence (5'-3')
NRAS Exon 1 Fw	GTTTTCCCAGTCACGACGACTGAGTACAAACTGGTGG
NRAS Exon 1 Rv	CAGGAAACAGCTAGTACTGCATAACTGAATGTATACCC
NRAS Exon 2 Fw	GTTTTCCCAGTCACGACCAAGTGGTTATAGATGGTGAAACC
NRAS Exon 2 RV	CAGGAAACAGCTATGACAAGATCATCCTTTCAGAGAAAATAAT
KRAS Exon 1 Fw	GTTTTCCCAGTCACGACGGTGAGTTTGTATTAAAAGGTACTGGTG
KRAS Exon 1 Rv	CAGGAAACAGCTATGACCCTGTATTGTTGGATCATATTCGTCC
KRAS Exon 2 Fw	GTTTTCCCAGTCACGACGGATTCCTACAGGAAGCAAGTAGTAA
KRAS Exon 2 Rv	CAGGAAACAGCTATGACCTATAATGGTGAATATCTTCAAATGATTTAGT
BRAF Exon 15 Fw	GTTTTCCCAGTCACGACTCATAATGCTTGCTCTGATAGGA
BRAF Exon 15 Rv	CAGGAAACAGCTATGACGGCCAAAATTTAATCAGTGGA
M13 Fw	CAGGAAACAGCTATGAC
M13 Rv	GTTTTCCCAGTCACGAC

A PCR mixture 25 µl containing 2.5 units of Amplitaq Gold polymerase (Applied Biosystems), PCR Buffer II (Applied Biosystems), 1.5 mM MgCl2, 0.3 mM deoxyribonucleotide triphosphates (dNTPs) (Promega, Madison, WI, USA), 1 µM of forward and reverse primer, and ~40 ng of gDNA as a template was used. Cycling conditions were: polymerase activation at 94°C for 5 min, following 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and one additional hold at 72°C for 10 min. NRAS Exon 1 primers target the entire exon 1, with the exception of the first 2 nucleotides. NRAS Exon 2 primers target the entire exon 2, with the exception of the first15 nucleotides. KRAS 1 and KRAS 2 primers target the entire exons 1 and 2.

Online Supplementary Table S2. Sequence references.

Primer	References
NRAS Exon 1	ENSE00001364464
NRAS Exon 2	ENSE00001450282
KRAS Exon 1	ENSE00001189804
KRAS Exon 2	ENSE00000936617
BRAF Exon 15	ENSE00002324725

www.ensembl.org, release 59, date: 22th of April 2011.

Online Supplementary Table S3. Patients' characteristics infant ALL patients.

	N. of patients (%)
Sex (n=108)	
Male	43 (39.8)
Female	65 (60.1)
Age (median, range, days, n=108)	164 (1-360)
WBC counts at diagnosis x 10^9/L (median, range, n=101)	263 (1.4-1332)
Infant ALL (n=97 samples)	
pro-B	64 (66.0)
common B	8 (8.3)
pre-B	21 (21.7)
B-lineage not classified	1 (1.0)
Biphenotypic	1 (1.0)
T-cell	2 (2.0)
MLL-rearrangement (n=109)	
t(4;11)	38 (34.9)
t(9;11)	11 (10.1)
t(11;19)	28 (25.7)
other 11q23*	14 (12.8)
germline-MLL†	18 (16.5)
AF4/ MLL (n=38)	
positive	23 (60.5)
negative	15 (39.5)

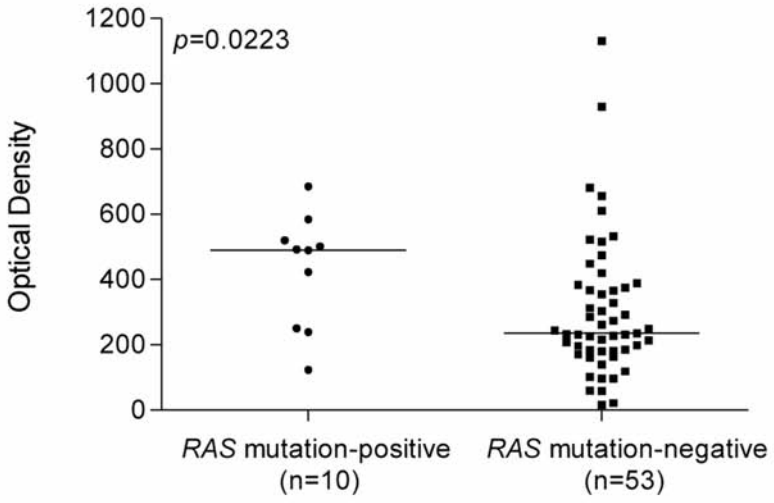
**11q23; MLL-rearranged infant ALL patients, with unknown or rare partner gene (including one t(1;11)-, one t(3;11)-, and three t(10;11)-positive patients), common partner genes (t(4;11), and t(11;19)) were excluded by PCR, † Germline-MLL; infant ALL patients without MLL-rearrangement Abbreviations: WBC; white blood cell.*

Online Supplementary Table S4.

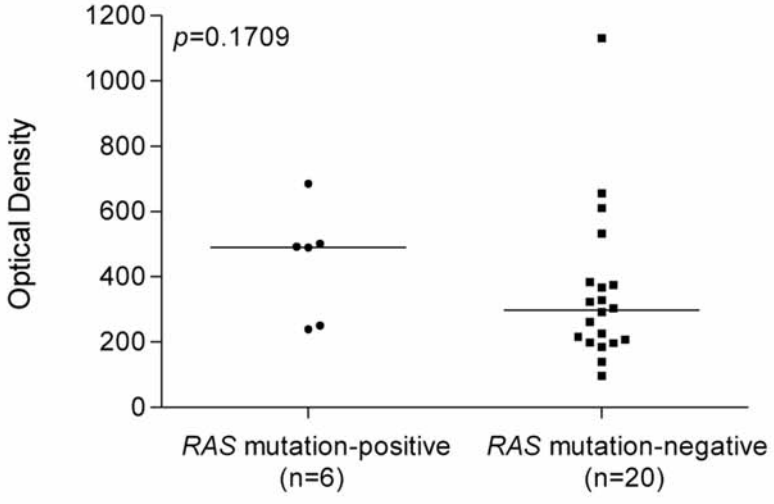
Patient	Clonality based on Sanger sequencing	N. of mutated clones (analyzed clones)	Mutated clones (%)	Expected mutated clones if clonal* (%)	*P*
3	subclonal	5 (16)	31.5	48	0.069
6	subclonal	12 (43)	27.9	49.5	0.003
7	clonal	9 (43)	20.9	48.5	<0.001

In order to confirm subclonality of the RAS mutations as implied by our Sanger sequencing results, we used TOPO® TA Cloning (Invitrogen Life Technologies, Breda, the Netherlands) to sequence single PCR-amplified DNA fragments in 3 patient samples (i.e. patient #3, #6, and #7). For this, multiple PCR fragments from the three patient samples positive for the KRAS1 (Gly13Asp) mutation were individually ligated into the TA cloning vector (pCR®2.1), and the constructs were transformed into competent E. coli *cells using heat shock. Transformed cells were then spread LB agar plates containing X-Gal, 100 g/mL ampicillin, and incubated overnight at 37°C. Next, multiple single colonies (each containing multiple copies of a single PCR fragment) were picked for plasmid isolation using the QiaPrep Spin Miniprep Kit (Qiagen, Venlo, The Netherlands). Finally, the inserts of all plasmids were sequenced by Sanger sequencing as described in the Design and Methods section of the manuscript. In all patients the number of mutated DNA fragments was lower than the expected percentage in case the mutation would have been clonal (*corrected for the percentage blasts (96-99%) in the patient samples). The results were statistically analyzed using the one-sided Z-test. Although the results of patient #3 did not reach statistical significance (which may be due to the relatively low number of clones analyzed) these results demonstrate that RAS mutations in infant ALL patients can indeed be subclonal. However, results derived from conventional Sanger sequencing must be interpreted with care, as the RAS mutation in patient #7 appeared clonal in our initial sequencing data, but clearly turned out to be subclonal when analyzing single PCR fragments.*

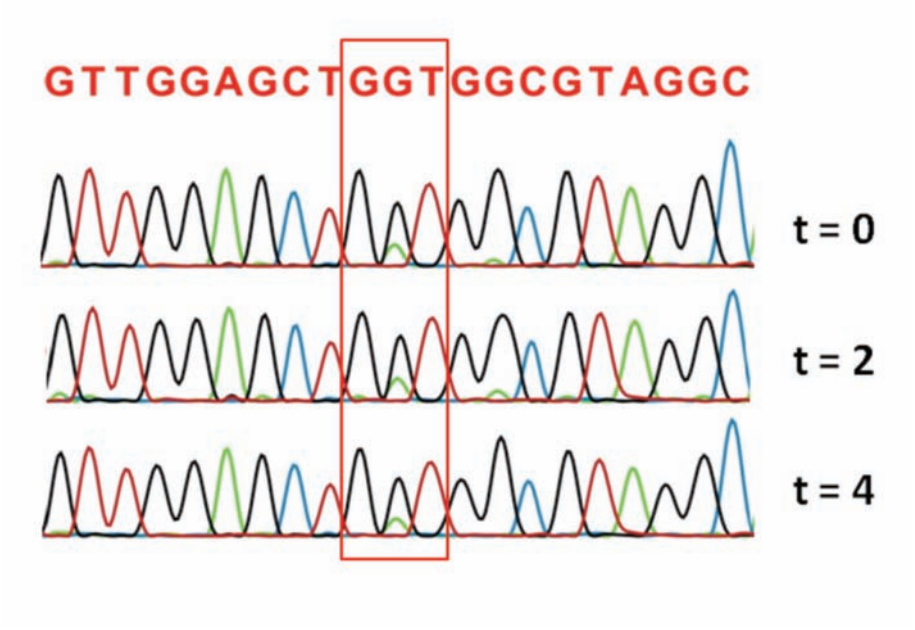
A ***MLL*-rearranged infant ALL patients.**



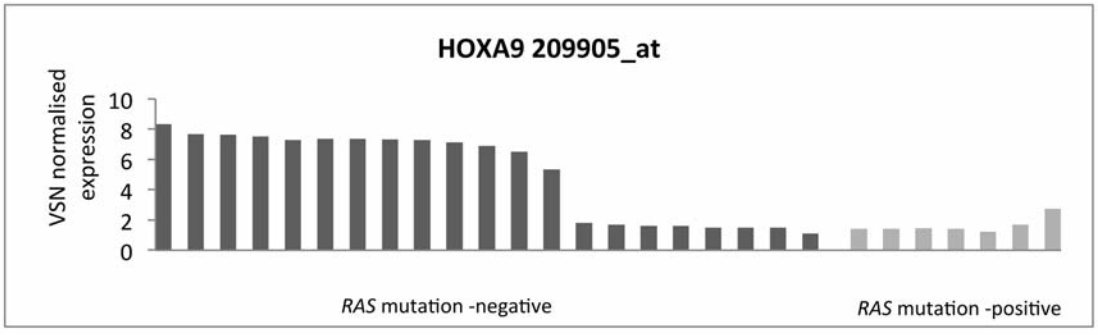
B **t(4;11)-rearranged infant ALL patients**

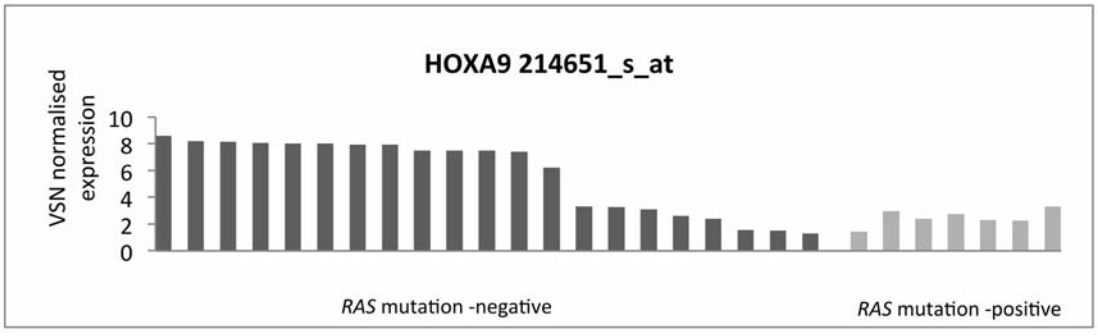


Online Supplementary Figure S1. Optical density (OD) (cell viability) of untreated primary infant ALL cells. In our *in vitro* cytotoxicity assays (i.e. MTT assays), leukemic cells are cultured in the presence of increasing drug concentrations. Cell viability is determined by measuring the OD of the blue formazan derivative of the tetrazolium MTT, which is formed only by viable cells. Here we compared the ODs of control cells (i.e. cultured in the absence of drug) between RAS-mutated and non-mutated samples derived from (A) MLL-rearranged infant ALL patients and (B) t(4;11)-positive infant ALL patients. Differences in the ODs between RAS-mutated and non-mutated samples were statistically analyzed by the Mann-Whitney U-test. The horizontal lines represent the median OD in each group.



Online Supplementary Figure S2. Representative sequences from time lapse *in vitro* prednisolone exposure experiment (*KRAS* exon1 condon12(Gly> Asp mutation). Two infant ALL samples (that appeared to have subclonal *RAS* mutations) were exposed to various concentrations of prednisolone (0 μg/mL, 0.488281 μg/mL, and 250 μg/mL). Cells were harvested from different timepoints (Day 0, Day 2, and Day 4) and sequenced for the RAS mutations.





Online Supplementary Figure S3. *HOXA9* mRNA expression (Affymetrix HU133plus2.0 microarray data) in t(4;11)-positive infant ALL patients. Differences in expression between *RAS*-mutated and non-mutated were statistically analyzed by the Mann-Whitney U-test, showing significant lower levels of *HOXA9* expression in *RAS*-mutated cases for both probesets tested: 209905_at (*P*=0.006) and 214651_s_at (*P*=0.020).