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ORIGINAL ARTICLE

RAS pathway mutations as a predictive biomarker for treatment adaptation in pediatric B-cell precursor acute lymphoblastic leukemia

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RAS pathway mutations have been linked to relapse and chemotherapy resistance in pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL). However, comprehensive data on the frequency and prognostic value of subclonal mutations in well-defined subgroups using highly sensitive and quantitative methods are lacking. Targeted deep sequencing of 13 RAS pathway genes was performed in 461 pediatric BCP-ALL cases at initial diagnosis and in 19 diagnosis-relapse pairs. Mutations were present in 44.2% of patients, with 24.1% carrying a clonal mutation. Mutation frequencies were highest in high hyperdiploid, infant t(4;11)-rearranged, *BCR-ABL1*-like and B-other cases (50–70%), whereas mutations were less frequent in *ETV6-RUNX1*-rearranged, and rare in *TCF3-PBX1*- and *BCR-ABL1*-rearranged cases (27–4%). RAS pathway-mutated cells were more resistant to prednisolone and vincristine *ex vivo*. Clonal, but not subclonal, mutations were linked to unfavorable outcome in standard- and high-risk-treated patients. At relapse, most RAS pathway mutations were clonal (9 of 10). RAS mutant cells were sensitive to the MEK inhibitor trametinib *ex vivo*, and trametinib sensitized resistant cells to prednisolone. We conclude that RAS pathway mutations are frequent, and that clonal, but not subclonal, mutations are associated with unfavorable risk parameters in newly diagnosed pediatric BCP-ALL. These mutations may designate patients eligible for MEK inhibitor treatment.

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INTRODUCTION

The prognosis of children with B-cell precursor acute lymphoblastic leukemia (BCP-ALL) has improved considerably over the past decades, and nowadays about 80% of the patients are cured.[1] However, especially medium- and high-risk cases still show dissatisfying rates of relapse despite intense chemotherapy.[2–4] Relapsed ALL is treated with the same drugs as the initial leukemia, but cells of relapsed cases are more resistant.[5] New, targeted approaches are therefore warranted.

Activating mutations in *KRAS*, *NRAS* and *HRAS* are among the most frequent mutations in cancer.[6–9] The RAS GTPases are convert extracellular growth signals into a complex intracellular response. Upon activation of a growth factor receptor, RAS-guanidine exchange factors (e.g. SHP2 encoded by *PTPN11*) activate RAS by enabling binding of GTP, which activates the RAF and PI3 kinases and RALGDS. The RAF–MEK–ERK kinase axis is crucial for mediating the oncogenic effects of RAS, demonstrated by the efficacy of MEK inhibitors in RAS-mutated cancers.[10–12] Clinical trials are ongoing, and benefits were reported for RAF-mutated melanomas, ovarian cancer and thyroid cancer.[13–18]

RAS mutations have been initially reported in about 15% of pediatric BCP-ALL.[19–21] However, these studies were often restricted to *N*- and *KRAS*, certain subtypes or high-risk groups, or were technically limited in sensitivity (therefore overlooking subclonal mutations), and did not compare mutation frequencies between cytogenetic subtypes.[22–26] Mutations were reported in the context of recent genomic studies but not studied for their prognostic impact in isolation. Therefore, the clinical significance of RAS mutations is still debated, and it is unknown whether the recent inclusion of minimal residual disease (MRD) levels as risk criterion influences the prognostic effect of RAS pathway mutations in contemporary protocols. Recent studies found that mutations in *NRAS*, *KRAS*, *FLT3* and *PTPN11* are more frequently observed at relapse (34–38%), and in part confer a poor prognosis.[26–29] We previously observed in a small cohort of 26 patients that RAS pathway mutations are more frequent in *ex vivo* prednisolone-resistant cases, which are sensitized to prednisolone by RAS pathway inhibition.[30] Backtracking has shown that RAS-mutant relapse-forming clones may exist as small subclones at initial diagnosis.[27,31,32] However, these retrospective analyses rely on selected cases, and the predictive value of (sub)clonal RAS pathway mutations at initial diagnosis of BCP-ALL treated in contemporary protocols is unknown.

Here we report deep next-generation sequencing of 13 RAS pathway genes together with a risk-stratified survival analysis in a clinically and biologically well-characterized cohort of 461 initial diagnosis patients with BCP-ALL, treated according to a contemporary, MRD-based ALL treatment protocol (DCOG ALL10). In

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addition, we report links between RAS mutation status, clonality, *ex vivo* cellular drug resistance and *ex vivo* response to MEK inhibition.

MATERIALS AND METHODS

A detailed description of all methods can be found in the online Supplementary Data.

Patient material 2 and patient-derived xenografts

This study comprised children with newly diagnosed BCP-ALL with an age range of 0–18 years. These studies were conducted in accordance with the Declaration of Helsinki; written informed consent was obtained from parents or guardians and approval given by institutional review boards. Mononuclear cells were isolated using density gradient centrifugation with Lymphoprep (Axis Shield, Oslo, Norway) as described previously.[33] Animal experiments were approved by the animal ethics committee (EMC 2863 (103-12-08)). In some cases, xenografts of primary patient material were established in three 7–12-week-old female NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice per patient (Charles River, Leiden, Netherlands). Leukemic cells were isolated and subsequently used for sequencing, western blot and trametinib (GSK1120212) cytotoxicity assays. For all samples leukemic blast percentage was at least 90%, and in relapse samples blast percentage was at least 80%. Subtypes were determined by karyotype, fluorescence *in situ* hybridization and/or fusion-gene specific PCR. *BCR-ABL1*-like cases were identified using microarray gene expression profiling by means of a 110 probe set classifier.[34,35] The composition of analyzed groups is described in a flow diagram (Supplementary Figure S1).

Ex vivo cytotoxicity assays

Sensitivity towards chemotherapeutics was evaluated as previously described.[36] In brief, freshly isolated primary ALL cells were incubated with a concentration range of prednisolone, vincristine, daunorubicin, L-asparaginase, 6-mercaptopurine and 6-thioguanine. After 4 days, cell viability was evaluated by adding MTT and measuring formazan conversion with optical density measurement. LC_{50} values were calculated (concentration at which 50% conversion activity was measured relative to no-drug control cells) and compared between groups using Mann–Whitney *U*-tests. *Ex vivo* sensitivity towards trametinib was measured similarly (5 μM–0.6 nM).

Sequencing and code availability

DNA and RNA were isolated using Trizol reagent (Life Technologies, Bleiswijk, Netherlands), or using DNeasy (Qiagen, Hilden, Germany) in two cases (used only for trametinib sensitivity assay) and the cell line 697. For TruSeq Custom Amplicon sequencing (Illumina, San Diego, CA, USA), sequencing libraries were prepared from 100 to 250 ng genomic DNA. Successful library preparation was confirmed using the Labchip GX genomic analyzer (Caliper Life Sciences Benelux N.V., the Netherlands). Samples were then pooled equimolarly and sequenced on an Illumina MiSeq in paired-end reads of 250 bp each. Forty-nine amplicons of 425 bp covered mutational hotspot regions in 13 RAS pathway genes (Supplementary Table S1). The analysis script will be provided upon request.

Sequence reads were aligned to the 1000 genomes human reference sequences (version b37, GATK resource bundle; Broad Institute, Cambridge, MA, USA) using BWA v0.7.10[37] and GATK indel realigner v.3.3-0. Single-nucleotide variants were called with Freebayes v0.9.18–24,[38] Varscan v.2.3.7,[39] Bcftools v1.0[40] and GATK v3.3-0.[41] The resulting variant call format files were annotated using snpEff and snpSift v.4.1a[42] and dbNSFP v.2.7.[43] For reliable detection of high-confidence mutations, variants were filtered based on several criteria: For each sample, variants were excluded if they were reported by only one caller, coverage was <100 reads, or <20 reads supported the variant allele. Overall, variants were excluded if variant allele frequency (VAF) never exceeded 2% or distribution was unequal between runs. Furthermore, variants were only considered if they were reported in the COSMIC V73 GRCh37 database,[44] non-synonymous, unlikely to be germline variants and not known SNPs (see Supplementary Methods for details). Read depth per amplicon is summarized in Supplementary Table S1. An estimate of cases missed due to insufficient coverage is given in Supplementary Table S2. In a comparison with 25 samples that were sequenced previously,[30] all variants could be identified by both platforms, indicating a low false-positive rate. Only Exon 3 of *NRAS* was sequenced using Sanger sequencing in 248 ALL10 samples since primer design for this exon in a multiplex amplicon setting failed. Chromatograms were visually inspected for the presence of mutations in codons 59 to 63 as well as analyzed by the R package sangerseqR in combination with the tools above to call and annotate detected variants.

Clinical characteristics and statistics

Clinical characteristics were compared using Fisher's exact test in R (version 3.2.1). We analyzed cases from the Dutch Childhood Oncology Group (DCOG) and Cooperative Study Group for Children with ALL (COALL) ($n=432$). We restricted the RAS pathway-mutated group to those carrying a verified MAPK pathway activating mutation (codons 12, 13 and 146 of *NRAS* or *KRAS* and *FLT3* or *PTPN11*). Event-free survival and cumulative incidence of relapse and non-response after induction therapy were evaluated in 244 eligible cases treated within one protocol (DCOG ALL10), stratified for risk group. The COALL97/03 cohort consisted of patients treated in the consecutive COALL 06–97 and COALL 07-03 protocols ($n=131$). For details see Escherich *et al.*[45] Treatment intensity in arms LR-R and LR-S is comparable to DCOG ALL10 standard-risk treatment; LR-I, HR-R and HR-S treatment is comparable to DCOG ALL10 medium-risk treatment. In the COALL97/03 cohort, no patient was treated with an intensity comparable to that of the DCOG ALL10 high-risk arm. Cumulative incidence of relapse and non-response was estimated using a competing risks model and compared using Gray's test. Event-free survival probabilities were estimated using the actuarial Kaplan–Meier method and compared using the log-rank test. Hazard ratios were calculated in SPSS v21 using Cox's proportional hazard model. Outcome analyses were performed in R 3.2.1, using the packages cmprsk[46] version 2.2-7, mstate version 0.2.7[47] and survival version 2.38-3.[48]

RESULTS

Frequency and clonality of RAS pathway mutations in newly diagnosed pediatric BCP-ALL

Targeted amplicon deep sequencing was used to identify RAS pathway mutations in samples from 461 children with BCP-ALL at initial diagnosis. Mutational hotspots in 13 key members of the RAS pathway were analyzed with 49 amplicons. The median read depth per amplicon was 1085 reads per sample (IQR: 527–2647) (Supplementary Table S1 and Supplementary Figure S2).

Most recurrent lesions were activating mutations in *NRAS* and *KRAS* (79%), followed by mutations in *PTPN11* and *FLT3* (9.3% and 10.1%, respectively; Figure 1a and Supplementary Figure S3). Mutations in *BRAF*, *NF1*, *CBL* and other genes occurred sporadically. Overall, variants in RAS pathway genes were observed in 44.2% of pediatric BCP-ALL cases. Clonal mutations (VAF $\geqslant$ 25%) were found in 24.1% of all patients (median: 40.5% VAF; Supplementary Figure S4) and subclonal mutations (VAF $<$ 25%) were exclusively found in 20.1% of patients (median: 4.3% VAF).

Most cases with RAS pathway mutations were high hyperdiploid or those with rare or negative for *BCR-ABL1, ETV6-RUNX1, TCF3-PBX1, MLL*-rearrangement and high hyperdiploidy (51–67 chromosomes), including non-*BCR-ABL1*-like 'B-other' and *BCR-ABL1*-like cases (Figure 1b and Supplementary Table S3). Mutation frequencies were high in *BCR-ABL1*-like (49.4%), B-other (41.8%), high hyperdiploid (72.6%) and t(4;11)-rearranged (*MLL-AF4*, 73.3%) cases, with clonal mutations observed in 31.6%, 25.4%, 41.9% and 20%, respectively (Figure 1b). Comparison of each subtype to the remaining BCP-ALL cases showed significantly more mutations in cases with *MLL*-rearrangement and high hyperdiploid karyotype (odds ratio 3.8 and 5.7, respectively), and significantly less mutations in cases with *ETV6-RUNX1*-, *TCF3*- and *BCR-ABL1*-rearrangement (odds ratios <0.3; Supplementary Table S4). Interestingly, within the *BCR-ABL1*-like group RAS pathway mutations were mutually exclusive with ABL/JAK class tyrosine kinase fusions, but not with high *CRLF2* expression (Supplementary Figure S5). *ETV6-RUNX1*-rearranged cases showed

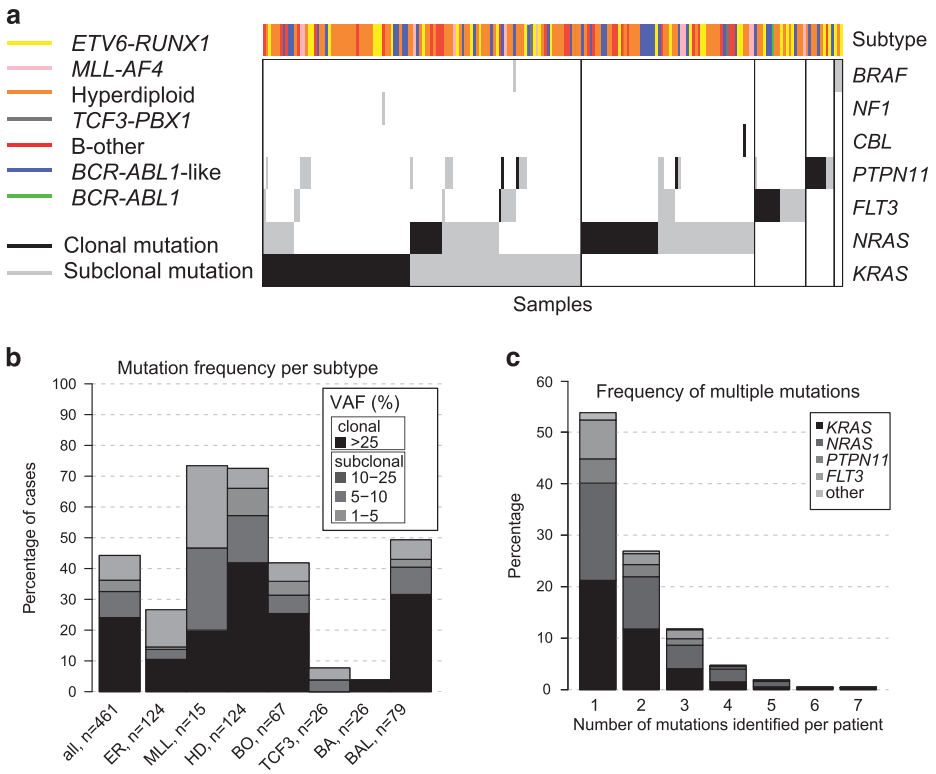

Figure 1. Frequency and distribution of RAS pathway mutations in pediatric BCP-ALL. (**a**) Overview of all clonal or subclonal mutations found in pediatric BCP-ALL cases at initial diagnosis. Top bar represents the cytogenetic subtype. Black boxes represent clonal mutations (variant allele frequency (VAF) ⩾ 25%) and gray boxes represent subclonal mutations (VAF < 25%). (**b**) Frequency of clonal and subclonal RAS pathway mutations overall and within the cytogenetic subtypes. BA, *BCR-ABL1*-rearranged; BAL, *BCR-ABL1*-like; BO, B-other; ER, *ETV6-RUNX1*-rearranged; HD, high hyperdiploid; MLL, t(4;11)-rearranged; TCF3, *TCF3-PBX1*-rearranged. (**c**) Co-occurrence of RAS pathway mutations: bar heights indicate the frequency of mutated cases carrying the number of RAS pathway mutations indicated on the *x*-axis. Segmentation of each bar indicates the distribution of mutated genes.

Table 1. Incidence of clonal RAS pathway mutations among BCP-ALL patients with clinical risk factors

Risk parameter	*Incidence of clonal mutations among*		*Statistics (clonal vs wild type)*		
	Risk parameter: Yes	*Risk parameter: No*	*Fisher's P*[a]	*Odds ratio*[b]	*95% CI*
Age ⩾ 10	22/75 (29%)	90/357 (25%)	0.88		
Male	55/227 (24%)	57/205 (24%)	0.56		
High WBC (>50/nl)	34/99 (34%)	78/331 (24%)	0.24		
Down syndrome	2/16 (13%)	105/305 (27%)	0.10	0.3	0.03–1.38
CNS+	0/1 (0%)	24/248 (10%)	1		
PPR	7/17 (41%)	51/181 (21%)	0.15	2.22	0.65–7.41
MRD high d33 ALL10	16/48 (33%)	35/147 (18%)	**0.01**	2.99	1.27–7.05
MRD high d79 ALL10	2/8 (25%)	47/171 (20%)	1		

Abbreviations: CNS+, non-traumatic puncture and >5 WBC/μl CSF with identifiable leukemic cells; patients with a traumatic lumbar puncture were not included; d33, at the end of induction therapy (day 33); d79, at the end of consolidation therapy (day 79); PPR, prednisone poor responder, that is, ⩾1000 leukemic blasts/μl in peripheral blood on day 8 of induction therapy; MRD high, minimal residual disease ⩾ 10^{-3}; WBC, white blood cell count. [a]Fisher's exact test *P*-values < 0.05 are printed in bold font. [b]Odds ratios are only given if *P*-values in Fisher's exact test were < 0.2.

an intermediate frequency (26.6%, 10.5% clonal), and *TCF3-PBX1*- or *BCR-ABL1*-rearranged cases had the lowest frequencies with 8% (0% clonal) and 4% (4% clonal) of cases being affected, respectively.

Frequent secondary aberrations in clonal RAS pathway mutant cases were chromosome 21 aberrations, dic(9;20) chromosomes, 9p-deletion, *PAX5* amplifications and *CDKN2A/B* deletions. Opposed to that, significantly less *BTG1* deletions and *ETV6* deletions were detected in cases with clonal RAS pathway mutations. Cases with subclonal RAS pathway mutations also more frequently carried chromosome 21 aberrations and additionally more often expressed high *CRLF2* and harbored less mutations in *IKZF1* and *PAX5* (Supplementary Table S5).

All except three *NRAS* mutations and three quarters of the *KRAS* mutations affected the codons 12 and 13 (Supplementary Figure S6).

Although clonal RAS pathway mutations were mutually exclusive (Figure 1a), additional subclonal mutations were present in 41% of cases with a clonal mutation and in 49% of cases with a

Table 2. Clinical outcome of patients with clonal or subclonal RAS pathway mutations

Mutation status	n	*EFS*			*CIR*	
		5-year EFS in % (s.e.)	*Cox's hazard ratio*[a]	*Log-rank* P_{cl}	*5-year CIR in % (s.e.)*	*Gray's test* P_{cl}
ALL10[b]						
Wild type	135	92 (2)			8 (2)	
Mutated	109	86 (3)			11 (3)	
ALL10[b]						
Wild type	135	92 (2)			8 (2)	
Subclonal	59	88 (4)			11 (4)	
Clonal	50	84 (5)			10 (4)	
SR						
Wild type	49	96 (3)			4 (3)	
Subclonal	15	93 (6)			7 (7)	
Clonal	10	69 (15)	4.57 (CI: 1.02-20.5)	**0.027**	21 (14)	
MR						
Wild type	75	88 (4)			12 (4)	
Subclonal	42	85 (6)			12 (5)	
Clonal	31	97 (3)		0.058	0	**0.01**
HR						
Wild type	11	100			0	
Subclonal	2	N/A[c]			N/A[c]	
Clonal	9	56 (16.6)		**0.015**	33 (17)	**0.044**

Abbreviations: CIR, cumulative incidence of relapse and non-response; EFS, event-free survival; HR, high risk; MR, medium risk; n/a, not applicable; P_{cl}, *P*-value for comparison clonal vs wild-type cases; SR, standard risk. Only *P*-values < 0.1 are shown. [a]Only shown if significantly different, reference group: wild-type cases. [b]All tests stratified for risk arms of this protocol (SR, MR and HR). [c]5-year follow-up only reached by one patient. Values of $P < 0.05$ are printed in bold.

subclonal mutation (46.2% of all mutated patients; Figure 1c). Most of these additional RAS pathway mutations were present at a VAF $< 10\%$ (Supplementary Figure S4).

Clinical characteristics and outcome

Clinical characteristics were compared between BCP-ALL cases with clonal RAS pathway mutations ($n = 110$, *KRAS, NRAS, PTPN11* or *FLT3*) and wild-type cases ($n = 235$). Age, white blood cell count, gender, Down syndrome, CNS status at diagnosis and prednisone window response at day 8 of therapy did not differ significantly (Table 1). In DCOG ALL10 patients clonal mutations were enriched among cases with high MRD levels ($\geqslant 10^{-3}$) after 4 weeks of induction treatment (33% in of MRD-high vs 18% in MRD-low cases, $P = 0.01$). This had an impact on mutation frequencies in the actual treatment arms: 13.5% of cases in the standard-risk arm carried a clonal RAS pathway mutation, compared with 21.9% in medium risk and 40.9% in the high-risk treatment arm (Supplementary Table S6, $P = 0.02$).

In contrast, subclonal mutations were not associated with poor risk features. These mutations were significantly more common among younger children ($P = 0.04$), those with low white blood cell counts (< 50 cells/nl, $P = 0.02$), and those in the medium-risk treatment arm ($P = 0.01$; Supplementary Table S7).

The prognostic value of RAS pathway mutations was analyzed in 244 newly diagnosed BCP-ALL patients treated according to the DCOG ALL10 protocol (for baseline characteristics and cohort composition see Supplementary Table S8 and Supplementary Figure S1). In the total cohort, clonal and subclonal RAS pathway mutations did not associate with an inferior clinical outcome (Table 2 and Figure 2a). In the DCOG ALL10 study, patients are risk-stratified by MRD response on day 33 and day 79 into standard-risk, medium-risk and high-risk treatment arms. In standard- and high-risk-treated cases clonal RAS pathway mutations were associated with a significantly worse event-free survival compared with wild-type cases, caused in part by a higher incidence of relapse and non-response (Table 2 and Figure 2b, red lines). Significantly fewer relapses occurred in medium-risk-treated patients harboring clonal mutations compared with wild-type cases, which resulted in a trend for better event-free survival. Subclonal mutations were not predictive for outcome (Figure 2b, blue lines).

Univariate analysis of RAS pathway status in these three risk arms revealed that clonal but not subclonal mutations in RAS pathway genes were predictive for an unfavorable outcome in the standard-risk-treated group (hazard ratio 4.6, $P = 0.047$), which remained prognostic in a multivariate analysis including WBC and age (hazard ratio 5.4, $P = 0.032$; Supplementary Table S9). Univariate analysis of RAS pathway mutations in the medium-risk and high-risk groups did not reveal statistical significant associations with event-free survival in this DCOG ALL10 study cohort.

The impact of RAS pathway mutations on clinical outcome was also evaluated in the COALL97/03 cohort (Supplementary Figure S7). These patients had been stratified into treatment arms according to white blood cell count, age, immunophenotype and *ex vivo* drug response. Small group sizes are limiting this analysis and differences were not statistically significant, but the trends support our results of the DCOG ALL10 study. In standard-risk-treated cases, those with clonal but not subclonal mutations more often suffered from a relapse than did non-mutated cases, whereas this prognostic impact was absent in the medium-risk-treated group (Supplementary Figure S7).

Ex vivo resistance to chemotherapeutic agents

Ex vivo cytotoxicity data of prednisolone, L-asparaginase, vincristine, daunorubicin, 6-mercaptopurine and 6-thiopurine were available for 211 cases. RAS pathway-mutated cells were median threefold more resistant to prednisolone compared with wild-type cases (Figure 3a; $P = 0.024$). Leukemic cells harboring clonal or subclonal *KRAS* G13 mutations were most resistant to prednisolone: these cells were median >2000-fold more resistant compared with wild-type cells ($P = 0.001$ and $P = 0.006$, respectively; Supplementary Table S10, Figure 3a and Supplementary Figure S8). Clonal *NRAS* G13 mutations showed a trend, but the

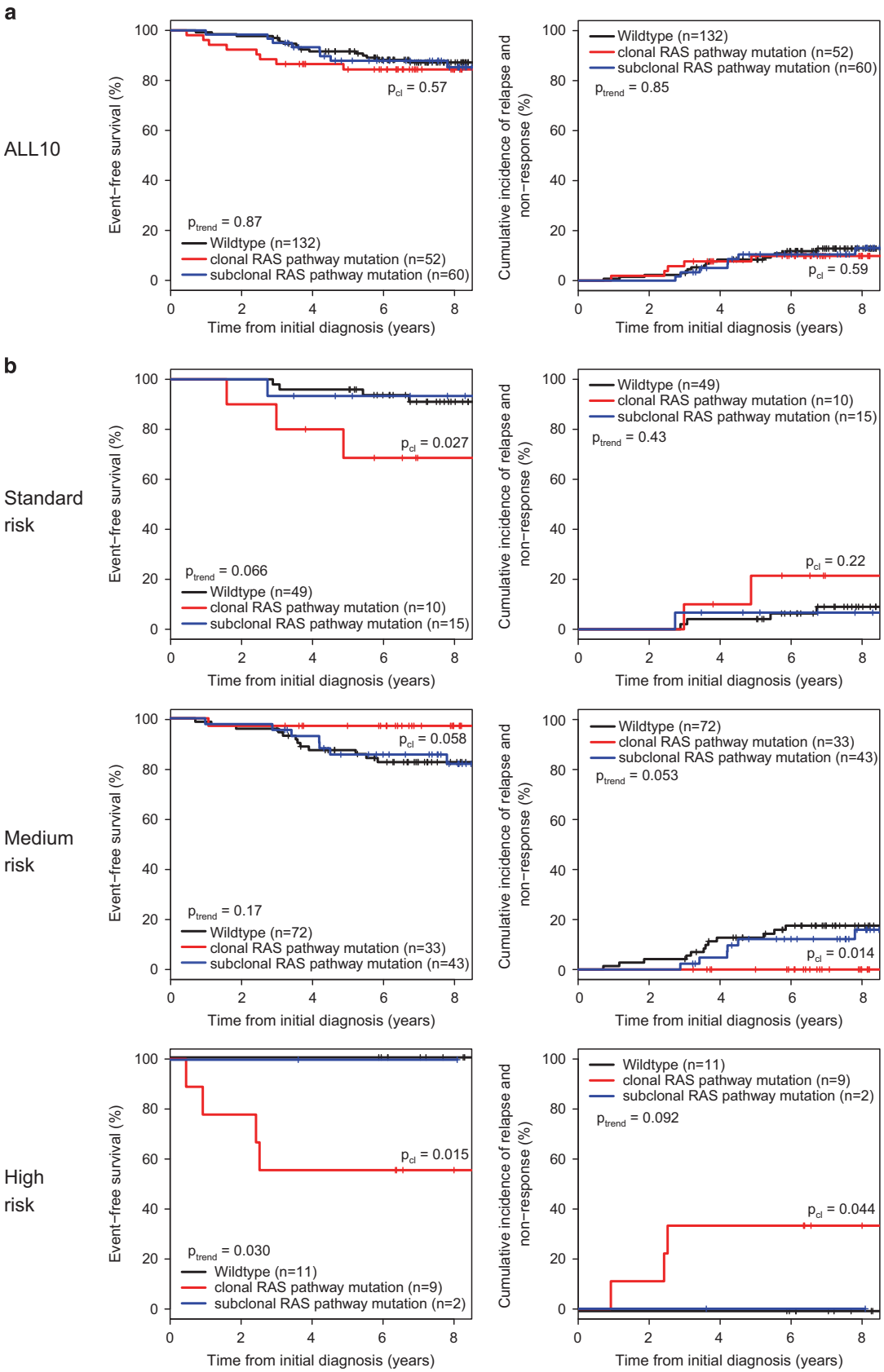

Figure 2. Clinical outcome of patients carrying clonal or subclonal RAS pathway mutations. Event-free survival (left panel) and cumulative incidence of relapse and non-response (CIR, right panel) (**a**) within the ALL10 cohort ($n = 244$) and (**b**) divided by the three risk arms of ALL10. Abbreviations: HR, high-risk group; MR, medium-risk group; P_{trend} represents the *P*-value in a log-rank (EFS) or Gray-test (CIR) across all three groups, P_{cl} represents the *P*-value in a log-rank or Gray-test comparing wild-type patients and those with a clonal RAS pathway mutation; SR, standard-risk group.

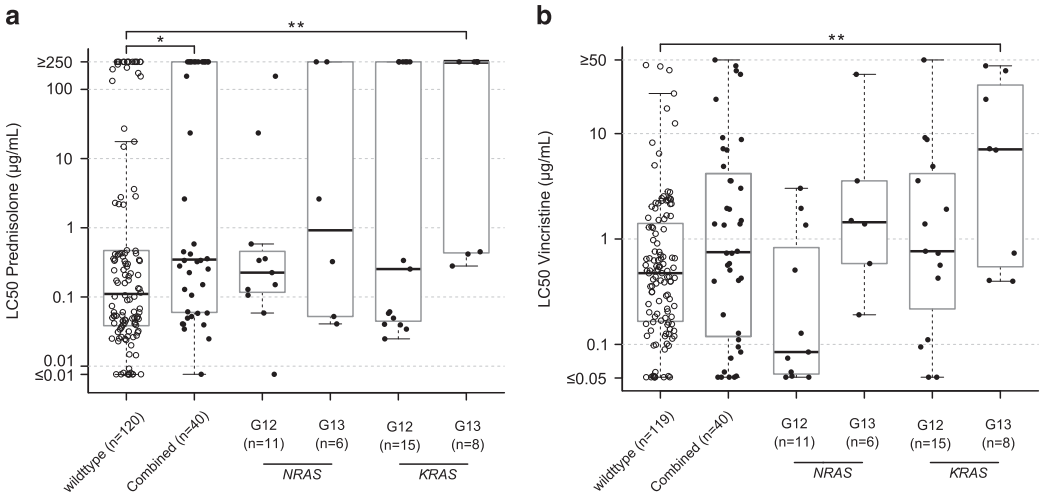

Figure 3. RAS pathway mutations and *ex vivo* cytotoxicity of chemotherapeutic agents. *Ex vivo* sensitivity of 211 primary patient samples towards (**a**) prednisolone and (**b**) vincristine, distinguished by RAS mutation status. Only clonally mutated cases are considered. Only *KRAS*- and *NRAS*-mutated groups are shown due to low recurrence of other mutations (see also Supplementary Data). Combined: All cases with a clonal mutation in *NRAS*, *KRAS*, *PTPN11*, *FLT3*. Groups were compared by Mann–Whitney U-test, $*P < 0.05$, $**P < 0.01$. LC_{50}-values were evaluated by MTT assays as reported previously.

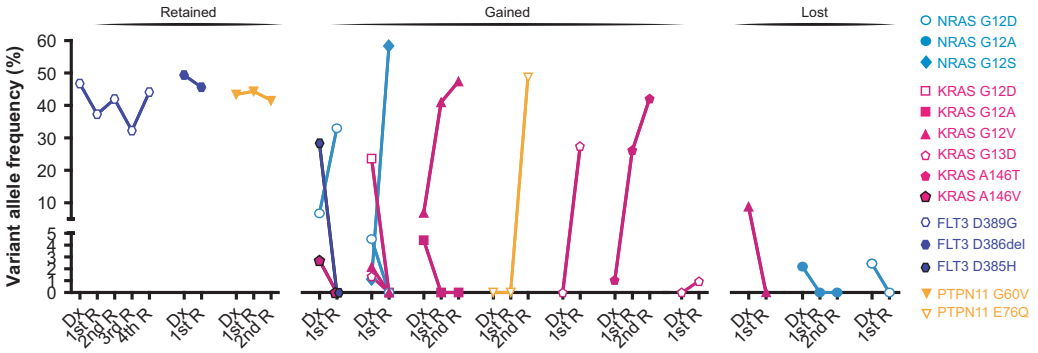

Figure 4. Evolution of clonal and subclonal RAS pathway mutations between initial diagnosis and subsequent relapse in 13 BCP-ALL cases. Variant allele frequency of all RAS pathway mutations found at initial diagnosis and/or relapse(s) is shown for cases with RAS pathway mutations detected at either time point (13 of 19). Colors distinguish affected genes; symbols distinguish observed variants. Dx, initial diagnosis sample; R, relapse sample.

difference was not significant ($P = 0.18$). *NRAS* and *KRAS* G12 mutations did not significantly affect resistance towards prednisolone, although several cases were highly resistant. Mutations in *PTPN11* and *FLT3* were not associated with cellular prednisolone resistance (Supplementary Table S10 and Supplementary Figure S8A).

Ex vivo cytotoxicity of the tubulin-inhibitor vincristine was also reduced in RAS pathway-mutated cells (Figure 3b): Cases with a clonal *KRAS* or *PTPN11* mutations were significantly more resistant to vincristine compared with wild-type cases (15-fold, $P = 0.005$ and 5-fold, $P = 0.041$, respectively). A trend was observed for clonal *NRAS* G13 mutations (3-fold, $P = 0.074$). No significant difference was observed for *KRAS* G12 mutations, and an inverse trend was found in cases carrying an *NRAS* G12 mutation (5-fold decrease, $P = 0.057$, see also Supplementary Figure S8B).

In *K/NRAS* G13-mutated cases the glycine (G) was replaced by an aspartic acid (D) in 13 of 15 cases. In contrast, the type of amino acid being substituted at the G12 position was more variable (see also Supplementary Figure S6). As visualized in Supplementary Figure S9 the substituting amino acid was not predictive for *ex vivo* prednisolone or vincristine resistance.

In contrast, wild-type cases tended to be more resistant towards L-asparaginase *ex vivo* than RAS pathway-mutated cases; however, this trend was not significant (Supplementary Table S10). No consistent association of mutation status with *ex vivo* drug response was observed for daunorubicin, 6-thioguanine and 6-mercaptopurine (Supplementary Figure S10).

RAS pathway mutations at relapse

RAS pathway mutations were detected in 13 out of 19 cases with matched initial diagnosis and relapse samples (Figure 4). Ten mutations were found at diagnosis, and 10 at relapse (53%). From diagnosis onwards, the evolution of RAS pathway mutations followed three distinct patterns: In three cases, a clonal RAS pathway mutation was detected at initial diagnosis and at relapse ('Retained'). In seven cases, a subclonal mutation (four patients) or no mutation (three patients) was observed at diagnosis, but at relapse a RAS pathway mutation was detected with higher VAF ('Gained'). In six of these relapses the mutation was clonal,and in one it was subclonal. One of these cases was remarkable in that five different subclones were observed at diagnosis but only one of these mutations (NRAS G12S) was found at relapse. Loss of one and selection of a second clone was observed in two cases, where they constituted the major clone in all subsequent relapses. In three cases, the initially observed RAS pathway mutation was not detected at relapse ('Lost'). For all 'lost'mutations VAF at initial diagnosis was lower than 10%. Remarkably, in 9 out of 10 relapse

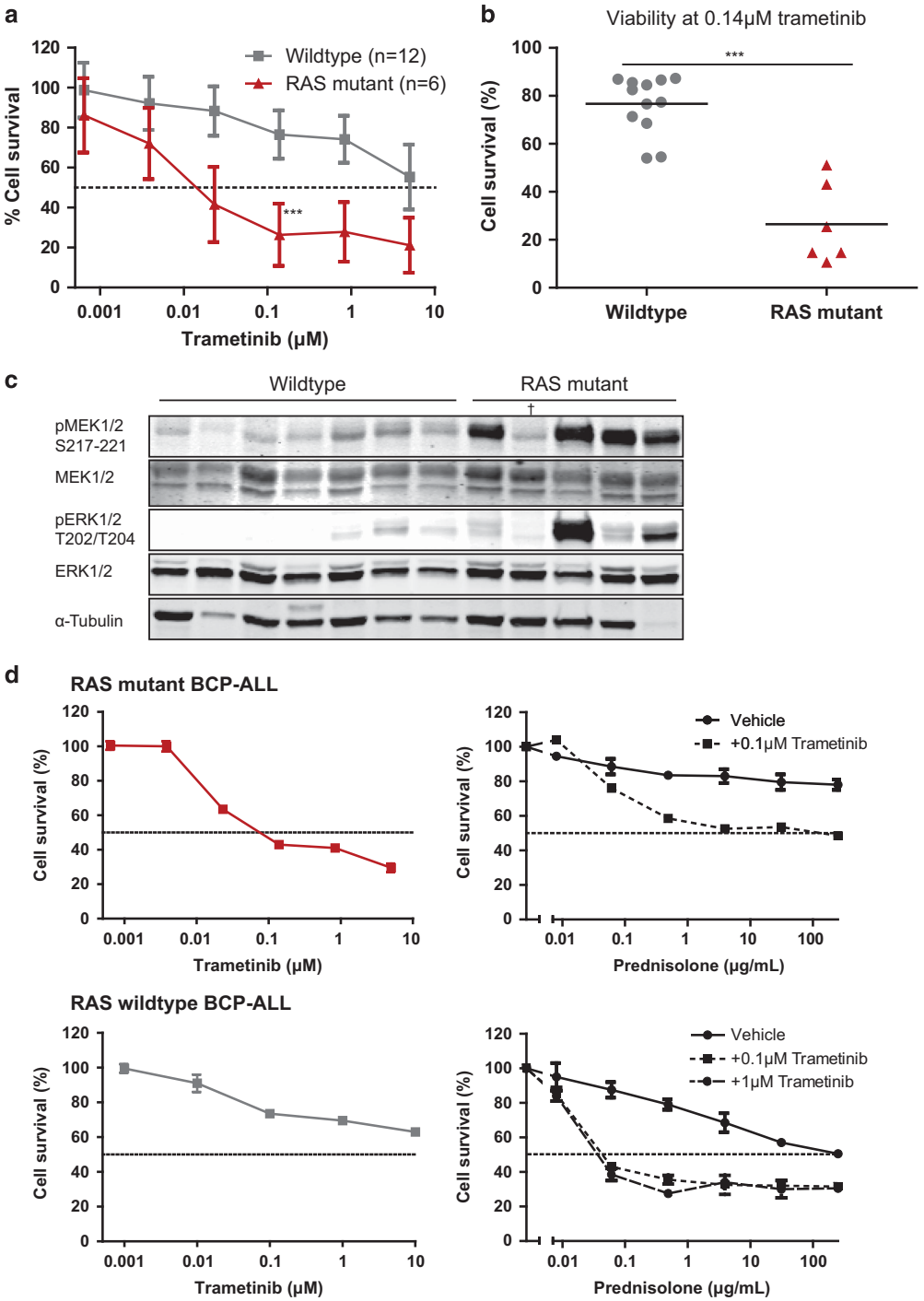

Figure 5. The MEK inhibitor trametinib effectively kills RAS mutant primary BCP-ALL cells. (**a**) Sensitivity towards the MEK inhibitor trametinib in primary or xenograft-derived BCP-ALL cells. Mean and standard deviation are shown. (**b**) Relative cell survival at 0.14 μM trametinib split up per case. Gray circles represent wild-type cases; red triangles represent RAS pathway mutant cases. Bars represent group mean. Groups were compared by Mann–Whitney *U*-test, ***$P = 0.001$. (**c**) Western blot analysis of phospho-ERK (T202/T204) and phospho-MEK (S217/S221) in a subset of samples tested in (**a**). †Sample isolated after thawing, cells previously tested positive. (**d**) *Ex vivo* response to trametinib (left panels) and sensitization towards prednisolone (right panels) in one NRAS G12D-mutant and one RAS pathway wild-type case.

cases a single, clonal mutation was observed (VAF of 27% or higher).

MEK inhibitors as therapeutic option

The MEK inhibitor trametinib was cytotoxic to RAS mutant but not to wild-type leukemic cells in a range of concentrations (Figure 5a and Supplementary Figure S11A). This effect is also illustrated by individual data points at a fixed concentration of 0.14 μM (Figures 5b, $P = 0.001$). High levels of MEK1/2 and ERK1/2 phosphorylation indicated an activated RAS pathway in mutant but not in wild-type cells (Figure 5c). The cytotoxicity of increasing trametinib concentrations corresponded with effective reduction of ERK1/2 phosphorylation (Supplementary Figure S11B). In

addition, we found that intrinsic resistance to prednisolone could be reversed by trametinib irrespective of RAS mutation status (Figure 5d), which corroborates our previous findings.[30]

DISCUSSION

The RAS pathway is the most frequently mutated pathway in cancer, and may serve as treatment target.[7,8,49] Recent reports suggest that RAS pathway mutations are recurrent in pediatric BCP-ALL.[20,27,30,31,50] However, frequency and prognostic value of subclonal mutations at initial presentation were unknown. In this study we addressed the clinical value of mutations in 13 key members of the RAS pathway using deep targeted sequencing, which allowed detection of subclones down to 1% VAF. Our study cohort contained 461 newly diagnosed cases and represents all subtypes and risk groups of BCP-ALL, with a distribution comparable to the general pediatric BCP-ALL population.

We identified clonal RAS pathway mutations (⩾25% VAF) in 24.1% and subclonal mutations ($<25\%$ VAF) in 20.1% of patients at initial diagnosis. This total of 44.2% is considerably higher than the 15% mutation frequency previously detected by Sanger sequencing, and shows that RAS pathway activation cooperates with several primary oncogenic lesions.[20,31] The vast majority of mutations (98%) occurred in *NRAS*, *KRAS*, *FLT3* and *PTPN11*, revealing a central role of these genes in pediatric BCP-ALL.

We observed high mutation frequencies among high hyperdiploid, *MLL-AF4*-rearranged, *BCR-ABL1*-like and B-other cases, moderate frequencies among *ETV6-RUNX1* cases, and rarely observed them in *TCF3-PBX1* and *BCR-ABL1*-rearranged cases, confirming reports of smaller sample sets.[23,27,32,51]

The *BCR-ABL1* fusion is known to require MAPK signaling, but the low mutation frequency suggests that independent RAS pathway activation is not required for this aggressive disease.[52–56] *BCR-ABL1*-like cases, who bare strong similarities with *BCR-ABL1*-positive leukemia, frequently carry RAS pathway mutations. These were mutually exclusive with tyrosine kinase fusions, suggesting divergent pathogenic mechanisms. Our data further show that RAS pathway activating mutations play a role in about half of all B-other patients. The largest group of RAS pathway-mutated cases was observed within high hyperdiploid cases. Our extensive screen extends the existing knowledge by revealing frequent subclonal events.[27,32,57,58] Our data advocate MEK inhibitors as a treatment option in high hyperdiploid cases with poor therapy response. We further identified several secondary aberrations that co-occurred less or more frequently with RAS pathway mutations. Interestingly, RAS pathway mutant cases infrequently carried BTG1 deletions, which have recently been linked to glucocorticoid resistance and may represent a distinct resistance mechanism in RAS pathway wild-type cases.[59]

In the largest data set analyzed so far ($n=211$), we confirmed the association of RAS pathway mutations with prednisolone resistance.[30,60,61] Additionally, RAS pathway mutant cells were *ex vivo* resistant to vincristine, but not to L-asparaginase, daunorubicin and thiopurines. This suggests that patients with RAS pathway mutations could profit from intensification of these drugs.

Patients with high MRD levels ($\geqslant 10^{-3}$ after induction or consolidation) are at higher risk for relapse and treated more intensely, also in DCOG ALL10.[4,62] Patients with clonal RAS pathway mutations treated in the DCOG standard-risk arm had an unfavorable clinical outcome despite negative MRD levels. Since the prognosis of clonally mutated patients is highly favorable in the medium-risk group (5-year event-free survival 97%, cumulative incidence of relapse and non-response 0%; Figure 2b), MRD-low risk patients with clonal RAS pathway mutations may benefit from medium-risk treatment, which includes intensive PEG-asparaginase and dexamethasone/vincristine pulses during maintenance therapy.[4] This is further supported by RAS-mutated patients being relatively sensitive to asparaginase *ex vivo*. The prognosis of those 20.1% of patients with subclonal mutations was similar to wild-type cases, and they generally presented with good risk features. Therefore, subclonal mutations in RAS pathway genes should not be used to assign patients to a higher risk group or treatment with an MEK inhibitor.

Evaluation of 19 cases with matched diagnosis and relapse material gave an important insight into the evolution of RAS pathway mutations: (1) clonal mutations at diagnosis were preserved at relapse, (2) subclonal mutations detected at initial diagnosis were often found at relapse and (3) a single, clonal mutation was found in 9 out of 10 relapses. These observations confirm that RAS pathway mutations are frequent at relapse in pediatric BCP-ALL.[28,29,63,64] The reduced mutational diversity at relapse suggests outgrowth of a single clone with stronger dependence on MAPK signaling, which may increase the chance of success for RAS-targeted therapy.

NRAS and *KRAS* mutations increase the risk for therapy failure at relapse, demanding alternative treatment options.[27,28] These patients may be eligible for MEK inhibitors such as trametinib. Leukemic cells with RAS pathway mutations were sensitive to trametinib, while wild-type cells were not. We further confirm our previous finding that MEK inhibition can synergize with prednisolone.[30] Interestingly, 9 of 11 RAS-mutated cases allocated to the high-risk arm had a poor *in vivo* response to prednisone after 1 week of therapy. This provides a rationale to combine MEK inhibitors with glucocorticoids. Notably, within our data no correlation was observed between the response to prednisolone and the response to trametinib.

In conclusion, clonal mutations in *NRAS*, *KRAS*, *PTPN11* and *FLT3* are associated with therapy resistance. Given that clonal mutations at initial diagnosis were retained at relapse and that subclonal mutations often expanded at relapse, RAS pathway mutations may serve as a biomarker to identify patients eligible for MEK/ERK targeted therapy. The synergistic effect between MEK inhibition and prednisolone may be of additional advantage. While our results and supremacy of MEK inhibitors require confirmation in independent cohorts, our data suggest screening (a) all cases that are eligible for treatment reduction based on MRD and (b) patients for whom treatment intensification is advised and who may benefit from adjuvant MEK inhibitor treatment. Methodically, accurate detection of any clonal RAS pathway mutation should be ensured, but detection of subclonal mutations is not required.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

ISJ and MLdB designed/performed experiments, analyzed/interpreted data and wrote the manuscript; AQH and JMB analyzed/interpreted data; IMA, NJMB, MJK and EC designed sequencing experiments; EMPS designed/performed experiments and obtained xenograft material; AB and CvdV obtained xenograft samples, HAdG-K, MAH and GE provided samples and clinical information, MLdB and RP conceptualized the study and interpreted results. All authors read, revised and approved the manuscript.

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