The role of *RAS* mutations in *MLL*-rearranged leukaemia: a path to intervention?

Marcela B. Mansur[a], Anthony M. Ford[b], Mariana Emerenciano[a*]

[a] Molecular Cancer Study Group, Research Centre, Instituto Nacional de Câncer (INCA), Rua André Cavalcanti 37, 2º floor, Rio de Janeiro, RJ, 20231050, Brazil.

[b] Centre for Evolution and Cancer, The Institute of Cancer Research (ICR) - London, Brookes Lawley Building, 15 Cotswold Road, Sutton, Surrey, SM2 5NG, UK.

*** Corresponding author:**

Dr Mariana Emerenciano, Research Centre, Instituto Nacional de Câncer (INCA); Rua André Cavalcanti 37, 2º floor, Rio de Janeiro, RJ, Brazil; Postcode: 20231050; Telephone: +55 21 3207 6544; E-mail: memerenciano@inca.gov.br

Abbreviations: ALL, acute lymphoblastic leukaemia; iALL, infant ALL; MLL, myeloid/lymphoid leukaemia or mixed lineage leukaemia, also known as KMT2A; RAS, rat sarcoma; BCP-ALL, B-cell precursor ALL; EFS, event-free survival; OS, overall survival; WBC, white blood cell count; CNS, central nervous system; HRAS, Harvey rat sarcoma virus; KRAS, Kirsten rat sarcoma virus; NRAS, neuroblastoma RAS; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase or phosphatidylinositide 3-kinase; mTOR, mechanistic target of rapamycin; MEK, serine/tyrosine/threonine kinase also known as MAP2K or MAPKK; MAF, minor allele frequency; AF4, ALL-1 fused gene on chromosome 4 also known as AFF1; PCR, polymerase chain reaction; iAMP21, intrachromosomal amplification of chromosome 21; UKALL, United Kingdom Acute Lymphoblastic Leukaemia protocol; T-ALL, T-cell acute lymphoblastic leukaemia; FISH, fluorescence in situ hybridization; ETP-ALL, early T-cell precursor ALL.

ABSTRACT

Childhood acute lymphoblastic leukaemia (ALL) with *MLL* rearrangement (*MLL*-r) is an aggressive disease still associated with a high mortality rate. Recent investigations have identified co-operating mutations in the RAS pathway and although the functional consequences of these mutations are not yet fully understood, aberrant regulation of RAS pathway signalling at both transcriptional and protein levels is observed. Studies investigating the efficacy of specific inhibitors of this pathway, e.g. MEK-inhibitors, have also achieved encouraging results. In this context, this mini-review summarizes the available data surrounding *MLL*-r infant ALL with *RAS* mutation in relation to other well-known features of this intriguing disease.

Keywords:

Acute lymphoblastic leukaemia

MLL

RAS

Prognosis

Targeted therapy

1. Introduction

It is no longer a surprise when a new study reports that children with the most common type of childhood cancer, acute lymphoblastic leukaemia (ALL), have a survival rate of 85% or more [1]. Indeed the survival rates have increased for girls and boys of varied ethnic groups and age groups except in infants (≤1 year-old). Among these very young children, even those enrolled in the most recent and specific therapeutic protocols, only ~50% achieve long-term event-free survival (EFS) [2]. The main cause of this dismal outcome in infants with ALL (iALL) is the high prevalence of rearrangement of the mixed-lineage leukaemia gene (*MLL*, also known as *KMT2A*). The presence or absence of an *MLL* rearrangement (*MLL*-r) is paramount both to provide a realistic prognosis and to determine a high-risk treatment strategy. These features have been recognised for many years but, unfortunately, have not yet been converted into significantly better therapeutic strategies and improved outcomes. Recent studies consistently show that *KRAS* and/or *NRAS* mutations (*RAS*mut) are recurrent within patients with *MLL*-r, with *MLL-AF4*+ B-cell precursor ALL (BCP-ALL) being the most targeted subset. This is of particular translational interest since the RAS signalling pathway offers an alternative therapeutic strategy for *MLL*-r ALL patients.

2. *MLL*-associated childhood acute lymphoblastic leukaemia

Childhood ALL characterized by *MLL*-r is a disease associated with aggressive clinical features. It is remarkable that 80% of iALL cases harbour an *MLL*-r, whereas *MLL*-r is only occasionally observed in older children with ALL (~5%) [3-5]. The genetic lesion leads to an extremely aggressive subset of leukaemia, frequently associated with early age onset, high white blood cell count (WBC), hepatosplenomegaly and central nervous

system (CNS) involvement. While children diagnosed with other subtypes of leukaemia experience good prognosis (80-90% overall survival rates, OS), children with *MLL*-r present a high mortality rate (~50%) [2, 6]. Worldwide, this rare group of patients remains a major challenge for paediatric oncology.

2.1. A spectrum of MLL rearrangements in childhood acute lymphoblastic leukaemia

Chromosomal abnormalities involving the *MLL* gene are very heterogeneous including reciprocal translocations, complex rearrangements, internal duplications, inversions and deletions, among others. The majority of the rearrangements are cytogenetically unbalanced [7, 8]. To date, more than 80 different direct *MLL*-r and about 120 reciprocal *MLL*-r have been reported and characterized at the molecular level [8]. Despite this broad cytogenetic spectrum, the most frequent partners in childhood ALL are limited to three: *AF4/AFF1*, *AF9/MLLT3* and *ENL/MLLT1*, with the *MLL-AF4* fusion being associated with a very early age at diagnosis, pro-B ALL phenotype and very poor outcome.

The distribution of chromosomal breakpoints has also been the subject of investigation. The breakpoint cluster region located between *MLL* exon 9 and intron 11 is responsible for 93.5% of breaks. The localization of breakpoints varies according to age, type of leukaemia and *MLL* partner gene [9], indicating that the underlying molecular mechanisms that drive the rearrangements are also different. Of note, we previously showed that the OS of children with breakpoints in *MLL* intron 11 was worse compared to other locations [6].

2.2. The lack of multiple genetic abnormalities in MLL-r ALL

Studies evaluating iALL have demonstrated that twin pairs with a monochorionic placenta and concordant leukaemia share identical genomic *MLL*-r [10, 11]. In non-twined siblings, the genomic breakpoint sequence is detectable in neonatal blood spots [12]. Collectively, these findings provide strong evidence for an *in utero* origin of this disease and also, given the short latency period, suggest that *MLL*-r may be the only required genetic hit necessary to induce overt leukaemia or that very few additional mutations are required. In agreement, data obtained from genome-wide studies has consistently shown that secondary genetic alterations are rarely found in *MLL*-r ALL [13-15]. More recently, this deficit of additional mutations, when compared to other types of human cancer, led to the conclusion that iALL with *MLL*-r display one of the lowest somatic mutation rates [7]. This frank discovery supports the repeated assertion that this single genetic lesion is sufficient for malignant transformation [16, 17].

3. A renaissance for *RAS* mutation investigation in *MLL*-r leukaemia

The genes that encode RAS proteins have been recognised as powerful drivers of cancer for more than three decades. The first screening of *RAS*mut in *MLL*-r ALL samples dates from 1998, when Mahgoub and colleagues hypothesized that this pathway might play a role in this leukaemia subtype. Despite an analysis of 13 samples, *RAS*mut were absent in that series [18]. The debate remained subdued in the literature until 2006, when Liang *et al*. reported that 10 of 20 *MLL*-r ALL samples harboured a *RAS*mut [19].

Despite the very low frequencies of copy number abnormalities in *MLL*-r leukaemia observed through genome-wide analysis [13, 15], consecutive experimental models showed that MLL fusion proteins synergistically cooperate with activation of

RAS in leukaemogenesis [20-22]. These data helped renew the search to determine the frequency of *RAS*mut in *MLL*-r ALL. Driessen *et al*. screened 109 iALL samples for *NRAS* and *KRAS* and found that the mutations were significantly more frequent (23.7% *versus* 7.8%) in infants with *MLL-AF4* [23]. Similarly, Prelle and colleagues investigated 80 paediatric leukaemia samples and observed similar results, finding *RAS*mut in 26% of *MLL-AF4* cases and in 10% of patients with other *MLL*-r [24]. In conjunction these studies led to important conclusions regarding frequency of *RAS*mut in *MLL*-r ALL: that they are recurrent and the mutations are especially associated with the *MLL-AF4* subset.

3.1. Frequencies of RAS mutations obtained through next-generation sequencing

The aforementioned studies used conventional Sanger sequencing to determine the frequency of *RAS*mut in *MLL*-r leukaemia. The advent of next generation sequencing (NGS) technologies has allowed the delivery of massive and accurate genome information [25]. Taking advantage of such revolutionary technology, Andersson *et al*. performed a detailed paired-end genome-wide analysis on diagnostic and matched remission samples of 22 iALL with *MLL*-r. They observed that 100% of the mutant alleles in tyrosine kinase-phosphoinositide 3-kinase (PI3K)-RAS pathways were expressed. The authors confirmed the data in a validation cohort to show that 16 of 47 (34%) infant *MLL*-r cases and 11 of 23 (48%) cases positive for *MLL-AF4* harboured an activating mutation in *RAS* pathway genes [7].

In 2016, Trentin and colleagues also used NGS technology to screen *RAS*mut in *MLL-AF4* positive paediatric and iALL patients, the most frequently targeted subgroup. Using ultra deep sequencing, they described *RAS*mut in 63.9% diagnostic samples of

patients with *MLL-AF4* positive ALL [26]. To date, this is the highest reported frequency of *RAS*mut in *MLL*-r cases, and this result is fully explained by the sample cohort (restricted to *MLL-AF4* subgroup) as well as the sensitivity of the sequencing method applied. Nevertheless, 36 out of 49 reported mutations (73.5%) were present in minor clones (mutant allele frequency, MAF < 10%).

In summary, the frequency of *RAS*mut in infants and children with *MLL*-r BCP-ALL varies from 25-60% of cases, depending on selection criteria and sequencing method applied. A consensual assumption is that this prevalence is markedly increased in patients with the *MLL-AF4* fusion (Figure 1). Despite these differences, the striking conclusion is that this pathway is indeed an important target of disruption in patients with *MLL*-r ALL.

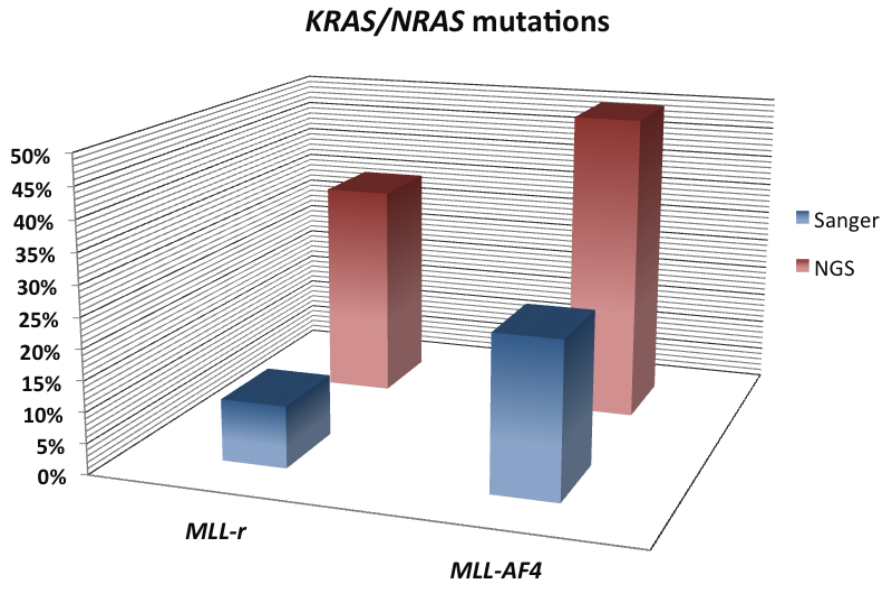

Figure 1. Frequency of *KRAS/NRAS* mutations in cases with any *MLL*-rearrangement or cases with the specific *MLL-AF4* gene fusion. The frequencies are shown according to the screening method. When analysed by Sanger sequencing, *RAS* mutations are expected in 10% or 25% of cases with *MLL*-r or *MLL-AF4*, respectively. Using next generation sequencing, *RAS* mutations are expected in 35% or 50% of cases with *MLL*-r or *MLL-AF4*, respectively.

3.2. The subclonality of RAS mutations

Although using different design strategies, the studies that addressed the question whether *RAS*mut in *MLL*-r ALL were either clonal or subclonal arrived at the same conclusion: the mutations are present in minor clones at diagnosis. Driessen *et al*. sequenced PCR-amplified DNA fragments cloned from three patient samples and found that in all of them the percentage of mutated fragments was lower than 50%, suggesting the subclonal nature of the mutations [23]. Using pyrosequencing, a quantitative sequencing method, we observed that in 19 out of 20 *MLL*-r iALL cases the percentage of *RAS*mut alleles at diagnosis was lower than expected for a clonal alteration [27]. In concordance, the observation that 65% of the activating tyrosine kinase-PI3K-RAS mutations found in 22 *MLL*-r cases had MAFs <30% led Andersson *et al*. to conclude that these mutations were present in minor clones. The authors also suggested that an activating mutation in RAS signalling pathways is not crucial for the establishment of the leukaemia, but rather contributes to growth advantage. Of note, regardless of the MAFs, all cases that were also analysed by RNA-seq expressed the activating mutant allele [7].

Irrefutable data about clonality came from studies that evaluated matched diagnosis and relapse samples and revealed a highly heterogeneous pattern of clonal evolution, with some cases showing the same *RAS* mutation at diagnosis and relapse and other cases showing gain or loss of *RAS* mutations at relapse [7, 26, 28, 29]. This profile supports the subclonal nature of *RAS* mutations and suggests that the treatment pressure can either positively or negatively select the *RAS* mutated clone at relapse.

3.3. Contribution of RAS mutations to MLL-driven leukaemogenesis

Although the functional consequences of *RAS* mutations in *MLL*-r patients, especially those only present in minor clones, are not yet fully understood, functional studies have been conducted in the past few years that attempt to evaluate the role played by *RAS*mut in *MLL*-driven leukaemias. It has been shown that these mutations result in over expression of RAS pathway signalling at both transcriptional and protein levels and that a potential collaboration between those two abnormalities may strongly contribute to the leukaemogenic process.

In that context, the transcription of *Elk-1*, a major effector of Ras signalling, was activated by the MLL-AF4 family fusion oncoproteins: MLL-AF4, MLL-LAF4 and MLL-AF5q31. Interestingly, when either the MEK-inhibitor U0126 or a dominant negative mutant of *Ras* (*HRas* S17N) were used, this transcriptional activation was abrogated. This data strongly links activation of Ras signalling to *MLL*-r leukaemogenesis [20].

Another investigation using a xenograft model with *MLL*-fusion mediated leukaemogenesis evaluated the cooperation between *MLL*-fusions (*MLL-SEPT6* and *MLL-ENL*) and *RAS* mutations (*NRAS* G12V). In summary, the authors suggested that the crosstalk between *MLL*-r and *RAS*mut may occur, at least partially, due to the aberrant expression of *Hoxa9*, a critical and direct *MLL* transcriptional target [21].

Similarly, by developing an oncogenic aggressive murine model, Tamai *et al*. were able to generate an *MLL-AF4*+ *KRas* G12D transgenic mouse that developed B-cell lymphoma and/or leukaemia in a 6-month latency period that resembled *MLL*-r leukaemia in humans. Corroborating the study outlined above, leukaemogenesis was most likely accelerated by *Hoxa9* overexpression, as a result of *MLL-AF4* and *KRas*mut cooperation [22].

More recently, it was observed that *KRAS* G12V either alone or combined with *MLL-AF4* was unable to initiate leukaemia, however did enhance haematopoietic engraftment in immunodeficient mice and increased significantly the ability of cord blood-derived cells to infiltrate the CNS, both hallmarks of *MLL-AF4*+ BCP-ALL. Altogether, their results indicate that *KRAS* plays an important role in *MLL-AF4*-driven leukaemias maintenance, but not in disease initiation [30].

3.4. Prognostic value of RAS mutations in patients with MLL-r ALL

The occurrence of *MLL*-r is the strongest prognostic marker to independently predict dismal outcome in iALL, the EFS rate in this leukaemia subgroup being considerably poor, ~28-36% [2]. Besides *MLL*-r, other molecular markers, such as *RAS*mut, have been selected as prognostic predictors in infants.

In 2013, Driessen and colleagues showed that *RAS*mut iALL cases exhibit a high WBC at diagnosis and glucocorticoid resistance *in vitro*, two factors linked to disease aggressiveness. Moreover, in terms of 5-year OS and EFS, the presence of *RAS*mut was independently associated with dismal prognosis [23]. A year later, our own group published data ratifying the prognostic value of *RAS*mut in a Brazilian iALL series of cases. Although not independently, the presence of *RAS*mut was a predictor of adverse outcome. Moreover *RAS*mut was also found to be associated with the occurrence of *MLL-AF4* translocation (OR 5.78; 95% CI 1.00 – 33.24) in those cases [31].

Another study defining the genomic landscape of iALL with *MLL*-r, observed a trend toward poorer OS and EFS in patients carrying *RAS*mut, however in contrast to previous investigations, they found no statistical significance for this data. [7]. One

229 potential critique is the number of patients included in the survival analyses, only 33
230 and 31 cases were evaluated for 10-year OS and EFS, respectively. Even so, it is
231 important to highlight that other studies also with small cohorts were still able to
232 observe statistically significant results on their survival analyses.

233 The role of *RAS* mutations on *MLL*-r patients prognosis has also been recently
234 evaluated by Trentin *et al*., who showed that, in agreement with previous reports,
235 patients harbouring *RAS* mutations had worse outcomes than those with *RAS* wild-type
236 [26]. Similar to the study mentioned above, the number of infant patients included was
237 fairly small (n=22) and no significance was found.

238

239 **4. Other high-risk groups with *RAS* mutations (hypodiploid ALL, iAMP21, T-**
240 **ALL)**

241 In addition to *MLL*-r iALL, there are other high-risk groups of ALL in which *RAS*mut
242 are recurrently found and we discuss here three entities: hypodiploid ALL,
243 intrachromosomal amplification of chromosome 21 (iAMP21) and T-cell acute
244 lymphoblastic leukaemia (T-ALL).

245 By definition, hypodiploid ALL have fewer than 44 chromosomes and can be
246 subdivided into three categories according to the number of chromosomes present: near-
247 haploid (24–31 chromosomes), low-hypodiploid (32–39) and high-hypodiploid cases
248 (40–43 chromosomes). Patients exhibiting 44-45 chromosomes are classified as near-
249 diploid and, unlike the other categories of hypodiploid ALL, do not present a poor
250 prognosis. Overall, the genetic profile of hypodiploid ALL is still poorly defined, but it
251 is well recognised that this leukaemic subtype is characterised by whole-chromosomal
252 losses and extremely sombre outcomes [32]. Considering the paucity of studies

253 evaluating the genetic basis of hypodiploid ALL, Holmfeldt *et al*. delineate the genomic
254 landscape of 124 paediatric patients diagnosed with this high-risk subtype. By using
255 next generation sequencing, they described activation of both RAS- and PI3K-signalling
256 pathways as the main molecular events in these cases. Particularly regarding near-
257 haploid and low-hypodiploid subgroups, they showed a considerable recurrence of
258 *KRAS* and *NRAS* abnormalities (copy number alterations and mutations) in 17.6% of the
259 hypodiploid cases, with *NRAS* being the most affected gene. As expected, mutations
260 were found mainly in codons 12 and 13 of both *RAS* genes. Evaluating the impact of
261 those *RAS* abnormalities on patient survival, no significant differences were observed
262 when comparing patients with and without mutations [33].

263 iAMP21 accounts for 2% of paediatric ALL and was identified more recently as
264 a distinct cytogenetic subgroup characterised by the presence of additional copies of
265 *RUNX1*. In fact, the international classification of iAMP21 is accepted as the presence
266 of 3 or more extra copies of *RUNX1* on a single abnormal chromosome 21, i.e. 5 or
267 more *RUNX1* signals per cell. Patients with iAMP21 have a very dismal outcome when
268 treated with standard therapy and the relapse rate is very high. Recently however,
269 protocols such as the United Kingdom acute lymphoblastic leukaemia protocol
270 (UKALL), has been treating those patients in the more intensive/high-risk treatment
271 arm (in spite of other risk factors) and the initial results seem very promising [34]. The
272 mutational landscape of the RAS pathway was also recently investigated in a series of
273 44 diagnostic samples of iAMP21 ALL. The study revealed a very high frequency
274 (60%) of RAS pathway abnormalities (mutations involved *NRAS*, *KRAS*, *FLT3*,
275 *PTPN11*, *BRAF* and *NF1*). Moreover these mutations were genetically heterogeneous
276 and resulted in some clonal heterogeneity, with mutations co-existing within a gene or

individual patient sample in different patterns. Unfortunately, the prognostic impact of those *RAS* mutations was not evaluated in these series of iAMP21 cases [35].

T-ALL is an aggressive haematological malignancy characterised by high WBC count, presence of mediastinal mass, CNS involvement, ~20% relapse rate and older age for the paediatric group. A significant number of cases fall into the high-risk group [36, 37]. In terms of genomic profile, *NOTCH1* mutations and *CDKN2A/B* deletions are the main abnormalities affecting more than 50% of the T-ALL cases. In light of currently available genomic data, it is possible to estimate that for each T-ALL case ~10 genomic abnormalities are present, however the contribution and the role of all these lesions for the pathogenesis and prognosis of T-ALL is not yet fully understood [38]. The occurrence of *RAS* mutations in T-ALL has been reported in the literature for the last few decades [39], especially in the early T-cell precursor ALL (ETP-ALL) subgroup [40]. However, in the past, most studies were experimental and used murine models to investigate the role of *RAS* mutations in leukaemogenesis [41]. More recently, Oshima *et al*. aiming to identify the mutational landscape of relapsed ALL observed a high frequency of RAS pathway (*NRAS*, *KRAS* and *PTPN11*) mutations (44%). Particularly for the T-ALL cases they found 12% of *KRAS* mutations and 27% of *NRAS* in those relapse samples. They also revealed that ALL relapse emerges from subclonal populations sharing only part of the mutations present in the dominant leukaemic clone found at diagnosis [29]. A recent study attempting to identify genes that could predict the ultra-high-risk group of relapse T-ALL, showed that *RAS* mutations were significantly enriched in this subgroup. Moreover, all relapsed patients with *RAS*mut evolved to death, resulting in a significantly worse EFS in this particular subgroup ($p=0.0059$) [42].

In summary, a variety of studies investigating different high-risk ALL subgroups showed that mutations affecting the RAS pathway are major genetic events present in a significant fraction of high-risk cases. Despite the fact that some studies, including our own in paediatric T-ALL [39], failed to show *RAS*mut either as an independent prognostic factor or as OS and EFS statistically significant results, we should not rule out the importance of these *RAS* mutations in the leukaemogenesis of these aggressive subsets of ALL.

5. New therapeutic strategies using targeted therapy

Treating aggressive leukaemias classified as high-risk cases, such as *MLL*-r iALL, remains a major challenge in paediatric haematology worldwide, therefore the development of new therapeutic strategies is imperative. In this regard, many international collaborative efforts have been initiated and are currently in progress through the use of novel targeted therapies based on iALL molecular biology.

Particularly for *MLL*-r iALL, currently there are four main lines of new targeted therapy under investigation: Clofarabine, FLT3-inhibitors, epigenetic modifiers and MEK-inhibitors. Clofarabine is a nucleoside analogue and, since infant leukaemic cells are known to be sensitive to this class of drug, its implementation in the current leukaemia protocols might prove to be beneficial in the treatment of those high-risk patients. FLT3-inhibitors, such as Lestaurtinib (CEP-701), Midostaurin (PKC412) and Quizartinib (AC220), act against the high-levels of *FLT3* expression. The inclusion of these inhibitors in the clinic could potentially benefit 80% of *MLL*-r iALL cases, since this is the frequency of patients that exhibit *FLT3* overexpression. Epigenetic modifiers seem to be the most appealing class of drugs to treat *MLL*-r iALL, since this leukaemia

subtype is characterised by globally aberrant methylation profiles. These modifiers can be subdivided into 4 classes: demethylating agents (Azacitidine, Decitabine), histone deacetylase inhibitors (Vorinostat, Panobinostat), DOT1L-inhibitor (EPZ-5676) and BET protein inhibitors (OTX-015). A promising international collaborative trial for iALL involving the use of epigenetic modifiers is underway and will involve the Interfant, COG, and JPLSG groups [43].

MEK-inhibitors are also a new class of drugs that have great potential to be used in combined therapy approaches in *MLL*-r iALL patients that harbour RAS pathway abnormalities (Figure 2). Currently the following MEK-inhibitors are being tested in advanced stages of clinical trial: Trametinib (GSK1120212), Pimasertib (MSC1936369B) and Selumetinib (AZD6244, ARRY-142886). In theory these types of inhibitor should result in less "off target" activity, inhibiting the pathway despite the mechanism of upstream activation. Data coming from *in vitro* and *in vivo* models of both *FLT3*-mutant and *RAS*-mutant acute leukaemias have shown very encouraging results [44, 45]. In 2016, Kerstjens *et al*. investigated the effects of MEK-inhibitors in *MLL*-r iALL cells carrying *RAS* mutations. They showed that after treatment with Trametinib, Selumetinib and MEK162 those cells exhibited an increased apoptosis and enhanced prednisolone sensitivity. At first, one may argue that considering the subclonal nature of *RAS*mut, targeting of minor clones could be a questionable approach. However, they have also observed that the use of MEK inhibitor enhances overall sensitivity to prednisolone treatment of both *RAS* wild-type and *RAS*mut *MLL*-r ALL cells [46]. Additionally, three other studies have observed the same sensitizing effect of MEK inhibitors in the response to glucocorticoids (methylprednisolone, prednisolone and dexamethasone) [47-49].

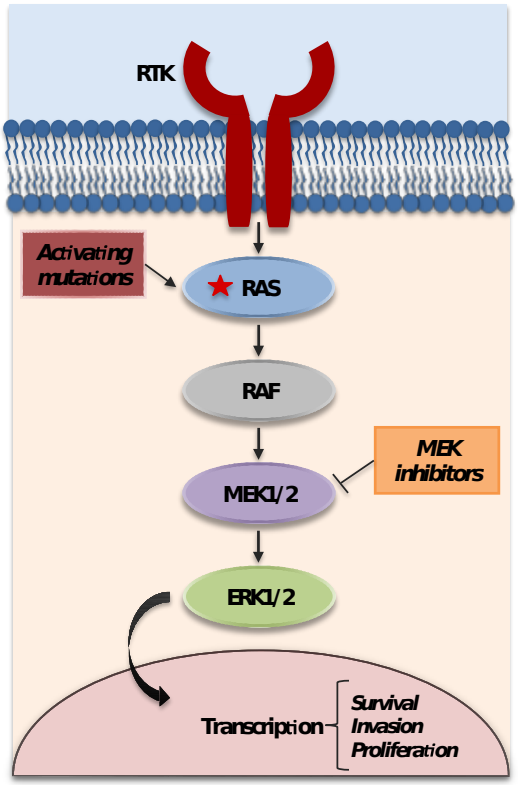

Figure 2. Schematic representation of RAS signalling pathway showing the use of MEK-inhibitors. The occurrence of *RAS* mutations results in abnormal activation of RAS pathway proteins, such as MEK1/2, which can be targeted by specific inhibitors (Trametinib, Pimasertib and Selumetinib).

6. Closing remarks

Infant ALL is a rare entity but the aggressive nature and high mortality rate of the disease, especially for those cases with *MLL*-r, challenge both researchers and clinicians to unravel its complex molecular biology. *RAS* mutations in infants and children with *MLL*-r BCP-ALL play an important role in the maintenance of disease and despite intensive current treatment regimens the majority of patients with iALL still relapse and die.

The latest international and collaborative studies investigating the efficacy of specific inhibitors of the RAS pathway are already producing encouraging results. We

hope that in the near future these combined therapeutic approaches will act synergistically both to increase survival rates and reduce treatment-related toxicities.

Conflict of interest

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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