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After 95 years, it's time to eRAsE JMML

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Abstract

Juvenile myelomonocytic leukaemia (JMML) is a rare clonal disorder of early childhood. Constitutive activation of the RAS pathway is the initial event in JMML. Around 90% of patients diagnosed with JMML carry a mutation in the *PTPN11*, *NRAS*, *KRAS*, *NF1* or *CBL* genes. It has been demonstrated that after this first genetic event, an additional somatic mutation or epigenetic modification is involved in disease progression. The available genetic and clinical data have enabled researchers to establish relationships between JMML and several clinical conditions, including Noonan syndrome, Ras-associated lymphoproliferative disease, and Moyamoya disease. Despite scientific progress and the development of more effective treatments, JMML is still a deadly disease: the 5-year survival rate is ~50%. Here, we report on recent research having led to a better understanding of the genetic and molecular mechanisms involved in JMML.

Keywords: JMML, RALD, RAS signalling.

Introduction

Juvenile myelomonocytic leukaemia (JMML) is a rare, malignant, clonal disorder of early childhood. It is characterized by the massive production of monocytic and granulocytic cells, which are thought to be derived from pluripotent hematopoietic cells (1-3). The condition was first described in 1924 (4, 5). It accounts for 2-3% of cases of childhood leukaemia (with a median age at diagnosis of 2 years), and the incidence rate is around 1.2 per million per year (6, 7). At present, hematopoietic stem cell transplantation (HSCT) is the most effective means of stopping the uncontrolled production of monocytic cells. However, the 5-year event-free survival rate after HSCT is only 44 to 53% (8-10). Hence, extension of the therapeutic armamentarium is essential. In this context, genetic and molecular research is now providing unexpected, essential insights into the physiopathology of JMML. A number of studies of patients with JMML have evidenced abnormalities in the RAS pathway in general and mutations in the *PTPN11*, *NRAS*, *KRAS*, *NF1* and *CBL* genes in particular. It is noteworthy that *NRAS* and *KRAS* mutations have been linked to a non-malignant, non-infectious leukoproliferative disease that resembles the autoimmune lymphoproliferative syndrome (ALPS) caused by mutations affecting the FAS/FASL pathway; hence, the condition has been referred to as "Ras-associated lymphoproliferative disease" (RALD) (11, 12). Here, we review recent progress in determining the genetic and molecular bases of JMML.

Classification of JMML

The clinical features of JMML vary markedly from one patient to another. Several clinical studies have shown that in the absence of treatment, the progression of JMML disease is highly variable - including blast crisis, acute leukaemia or even spontaneous partial or

complete remission (13-17). In order to facilitate the diagnosis of JMML, the World Health Organization (WHO) established three categories of clinical features (Table 1); to be diagnosed with JMML, a patient has to present with all the features in category 1 and one feature from category 2. If no category 2 features are found, two category 3 features are required (18). To improve diagnostic accuracy, a standardized review of the morphology, cytogenetic and molecular features of 260 paediatric patients with myelodysplastic syndromes and JMML is ongoing (NCT00662090). The clinical symptoms mentioned in the official guidelines are fever, pallor, infection, cutaneous lesions, diarrhoea, and haemorrhagic manifestations (19). A hypercellular bone marrow may also be observed, with a predominance of either granulocytic or erythroid cells (20). Accumulation of monocytes and granulocytes leads to the tissue invasion of various organs, including the spleen, liver, lung, skin and gut (7, 20). Hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) is frequently observed in JMML, although no associated mutations in the genes coding for the GM-CSF receptor have been found (21, 22). This hypersensitivity might result from continuous activation of the GM-CSF receptor and the RAS-RAF-MEK-ERK signalling pathway (23). More than 90% of patients with JMML carry a mutation in RAS pathway genes, including RAS (NRAS, KRAS), PTPN11, NF1, and CBL (24, 25) (Figure 1).

RAS mutations: a possible continuum from RALD to JMML, caused by a second genetic event?

Most of the RAS gene mutations described in human cancers affect HRAS (on chromosome 11), NRAS (on chromosome 1) or KRAS (on chromosome 12). Phosphorylation of the receptor tyrosine kinase exposes intracellular docking sites and thus enables the recruitment of guanosine exchange factor (GEF). The latter catalyses the replacement of GDP by GTP, to

obtain the active form of RAS. The interaction between activated RAS and RAF is followed by the phosphorylation of mitogen-activated protein kinase (MEK1) and/or MEK2, and, ultimately, the activation of ERK1 and/or ERK2 (Figure 2A). When *RAS* is mutated at key residues (G12, G13, Q61), the protein's conformation prevents the conversion of RAS-GTP to RAS-GDP - thereby leading to continuous activation of the downstream pathway (Figure 2B). *RAS* mutations are found in 20-30% of all human malignancies and in 20-25% of patients with JMML. Most of the germline or somatic *RAS* mutations have been found in **exon 1, exon 2 (codons 12 and 13) and exon 3 (codon 61)** (Figure 1) (15, 26-29). Heterozygous somatic *KRAS* or *NRAS* mutations are considered as the initial drivers in JMML. However, compound heterozygous or homozygous *RAS* mutations and secondary mutations are also associated **with the progression and aggressiveness JMML**, and malignant transformation into acute myeloid leukaemia (AML) (29-32). **Furthermore, loss of the wild-type *NRAS* locus during aggressive disease progression has been observed in a patient with JMML originally associated with a heterozygous *NRAS* mutation (33).** Another interesting case of JMML (described by Kato et al. in 2013) carried a heterozygous *KRAS* mutation at the time of diagnosis. However, disease progression was accompanied by a second hit (duplication of the *KRAS* gene, probably due to acquired uniparental disomy) that resulted in aberrant activation of the RAS-MAPK pathway (31). In 2011, two groups reported on a novel clinical entity related to *RAS* mutations (11, 34). Takagi et al. described two unrelated patients carrying a missense mutation in the *KRAS* gene (G13D) and presenting with non-conventional clinical features of **both JMML and ALPS** (34). The researchers found that the patients' T cells were resistant to intrinsic apoptosis (which is usually triggered by IL-2 starvation) *in vitro* but were sensitive to FAS-dependent apoptosis. Both patients presented with severe autoimmune manifestations (immune cytopenia, lymphadenopathy, and hepatosplenomegaly), which are usually

characteristics of ALPS. Similar observations were also reported by Niemela et al. in two patients respectively harbouring a somatic *KRAS* G13C mutation and a somatic *KRAS* G12D mutation (11). These two reports led to the definition of a new entity named RAS-associated ALPS-like disease (RALD). The above-mentioned findings have been confirmed in other cases of RALD (12, 35-41). It is noteworthy that some patients develop leukemia after a non-malignant clinical course of several years; this phenomenon is not yet well understood at the molecular level. Our group investigated a patient with RALD who developed severe JMML after 10 years of non-malignant lymphomyeloproliferation (39). We have now observed two additional RALD cases who subsequently developed leukemia (unpublished observations). These findings suggest that (i) RALD might be the first step in the development of leukemia, and (ii) RALD and JMML might form a clinical continuum conditioned by additional genetic or epigenetic events (39). A transition to acute disease in patients with RALD was not observed in another study of RAS-related diseases (36). However, since leukemia may develop more than 20 years after the manifestations RALD, some of these patients might still develop a malignant disease later in life. Alternatively, several subgroups of RALD may exist. More recently, a RALD patient carrying a *KRAS* G13C mutation presented with severe autoimmunity, lymphadenopathy, pancytopenia, neurological involvement and (for the first time in RALD) histiocytosis – a condition frequently observed in the context of leukemia (42). This observation also supports the existence of a potential continuum that starts at RALD and finishes (after additional genetic hits) with leukemia. Thanks to next-generation sequencing, several research groups have identified additional mutations in *SETBP1* (an oncogene which binds SET and inhibits the tumour suppressor PP2A), *RRAS* (encoding a small GTPase), *SH2B3* (encoding a protein recruited by JAK2), *GATA2*, *RUNX1* (both transcription factors involved in haematopoiesis), *ASXL1*, *EZH2* (both from the polycomb group of genes), *DNMT3A* (encoding

a DNA methyltransferase), *ZRSR2* (encoding a splicing factor), *WASP* (involved in dynamic changes in actin polymerisation), *PDE8A* (encoding a protein involved in the cAMP pathway), *RAC2* (encoding a GTPase) and *JAK3* in patients with JMML already carrying a RAS mutation (in *NRAS* or *KRAS*) or a *PTPN11* or *NF1* mutation. Based on this finding in RAS-JMML, one can hypothesize that the acquisition of the second mutation leads to a malignant form of disease and would thus explain the continuum between RALD and leukemia (25, 30, 43-45). It remains to be determined whether other mechanisms or events (besides additional mutations) can lead to disease progression or malignant transformation. Epigenetic phenomena are interesting candidates in this respect. Even though a hypermethylated profile is more frequent in patients harbouring a *PTPN11* mutation than in patients harbouring *CBL* or *RAS* mutations (45, 46), some *KRAS*- and *NRAS*- mutated patients were classified in a high methylation group (46, 47). Methylation events might therefore have a key impact in the progression from RALD to leukaemia.

Protein tyrosine phosphatase, non-receptor type 11 (PTPN11)

The *PTPN11* gene encodes the tyrosine phosphatase SHP-2. This protein has four main domains: two N-terminal variant Src homology region 2 (SH2) domains (N-SH2 and C-SH2), a catalytic protein-tyrosine phosphatase (PTP) domain, and a C-terminal tail containing phosphorylation sites (48). In the inactive form of SHP2, the N-SH2 domain interacts with the PTP domain and inhibits its activity. In the active form of SHP2, disruption of this inhibition promotes the activation of the PTP domain. Although the relationship between RAS and SHP2 has not been fully characterized, some aspects of SHP2's role in the RAS pathway are clear. In 2015, Bunda et al. showed that SHP2 dephosphorylates RAS and thus activates the RAS-MAPK

pathway via the interaction between RAF and RAS (49, 50) (Figure 3). Moreover, it is well known that SHP2 binds to the adapter GRB2 associated with the guanine nucleotide exchange factor Son of Sevenless (SOS) (50)(Figure 3). A mutation in the N-SH2 or PTP domains might prevent the inhibition of PTP activity and thus induce constitutive activation of the RAS-MAPK pathway (Figure 3). *PTPN11* mutations are found in 50% of patients with Noonan syndrome - a congenital disorder characterized by unusual facial features, short stature, a heart defect, and skeletal malformations. It is known that patients with Noonan syndrome have a predisposition to JMML (51-53). Accordingly, Tartaglia et al. demonstrated the presence of *PTPN11* mutations in 34% of non-syndromic patients with JMML; most of the mutations were located in exon 3 (encoding the N-SH2 domain) or exon 13 (encoding the PTP domain) (Figure 1) (54-57). Mutations in *PTPN11* induce hypersensitivity to GM-CSF among hematopoietic progenitors, which thus leads to uncontrolled cell differentiation and proliferation (58-60). Yang et al. showed that c-JUN (JUN) is overexpressed in cells carrying a *PTPN11* mutation; this strengthens the c-JUN (JUN) /PU.1 (SPI1) interaction and thereby promotes monocytic differentiation (61). In fact, PU.1 (SPI1) is a master regulator in immune cell differentiation. Interestingly, PU.1 (SPI1) regulates the expression of miR-223 and miR-22 during myelopoiesis and monocyte/macrophage differentiation, respectively (62, 63). Mulero-Navarro et al. observed elevated miR-223 expression in patients with JMML harbouring a *PTPN11* mutation (64). These findings suggest that targeting PU.1 (SPI1) might decrease miR-223 or miR-22 levels and thereby control myelopoiesis or monocytic differentiation (64). On the same lines, GAB2 might also be a valuable therapeutic target. The GAB2 protein interacts with SHP2, leading to activation of the PI3K/AKT pathway. When *PTPN11* is mutated, formation of a complex between GAB2 and the mutant SHP-2 results in hyperactivation of the PI3K/AKT pathway (Figure 3). Mice with a *Ptpn11*^{E76K/+}/*Gab2*^{-/-} or *Ptpn11*^{D61G/+}/*Gab2*^{-/-} genotype had less

aggressive disease (with regard to splenomegaly, myeloid cell infiltration, the sensitivity of myeloid progenitors to growth factor, and myeloid differentiation) than mice with *Ptpn11*^{E76K/+} alone - again suggesting that GAB2 is a potential therapeutic target (65, 66). Another aspect of PTPN11's activity impacts the centrosome; SHP2 might compromise the mitotic checkpoint and thus contribute to the development of malignancy (67). The literature indicates that *PTPN11* mutations are associated with a poor prognosis in patients with JMML (68). Moreover, experiments in murine models harbouring E76K, D61G, D61V or D61Y *PTPN11* mutations confirmed the aggressive nature of the JMML disease observed in patients with *PTPN11* mutations (60, 65, 67, 69, 70). In order to better understand the mechanisms induced by *PTPN11* mutation, it is important to consider both cell-intrinsic and cell-extrinsic functions. Recently, the microenvironment's role in disease development has been considered. Tarnawsky et al. showed that the severity of the monocytosis differs according to whether the *PTPN11* E76K mutation is expressed in foetal progenitors or adult progenitors (71). This finding suggests that the microenvironment has an essential role in disease onset and/or progression. Furthermore, *PTPN11* mutation induces the overproduction of the CCL3 chemokine; in turn, this contributes to monocyte recruitment and drives the development of myeloproliferative neoplasms (72). There is a striking relationship between the *PTPN11* mutation and the mutant cells' methylation profile. *PTPN11* mutated cells are more likely to present a hypermethylation profile than *CBL* or *RAS* mutated cells. These hypermethylation profiles might be linked to the aggressive clinical course observed in patients with JMML bearing *PTPN11* mutations (45, 46, 73, 74). Liu et al. showed that the phosphorylation status of c-AMP response element-binding protein (CREB) influences the expression of c-JUN (JUN) (a coactivator of PU.1 (SPI1) in monocytic differentiation, as described above). The researchers observed an association between low expression of CREB and low expression of the

transcription factor EGR1. CREB controls the transcriptional activation of EGR-1, which in turn upregulates PTEN expression. The downregulation of EGR-1 is also linked to elevated expression of the RNA miR-183. Liu et al. therefore suggested that miR-183 contributes to the low levels of EGR1 observed in patients with JMML. These observations are in line with the downregulation of PTEN seen in another cohort of patients with JMML (75, 76).

Casitas B-lineage lymphoma (CBL)

The *CBL* genes encode E3 ubiquitin-protein ligases involved in the transport and degradation of the **receptor tyrosine kinase**. Three mammalian homologues of *CBL* genes have been described: *c-CBL* (*CBL*, first identified in haematopoietic tumours), *CBL-b* (*CBLB*), and *CBL-3* (*CBLC*). The first two genes were shown to be of importance in the immune receptor signalling pathway (77). The *CBL* genes share a number of important domains: a tyrosine kinase-binding domain (TKB), a RING finger domain (which recruits enzymes for ubiquitination), and a proline-rich domain (enabling interaction with SH3-domain-containing proteins). The TKB domain is composed of a four-helix bundle, a Ca²⁺ binding EF hand and an SH2 domain. A short linker domain connects the TKB domain and the RING finger domain (78). *c-CBL* has a role in thymocyte development, whereas *CBLB* is involved in peripheral T cell regulation (77, 79, 80). As in most patients with a germline *PTPN11* mutation, germline mutations in *CBL* are associated with a Noonan-syndrome-like phenotype and a high risk of developing JMML (81, 82). Around 10-15% of patients with JMML are found to carry homozygous or heterozygous *CBL* mutations. **Disease progression has been linked to homozygosity for *CBL* mutations or loss of heterozygosity (LOH) for *CBL* via a uniparental disomy mechanism** (83-86). Most *CBL* mutations are located in exons 8 and 9 (which correspond to the RING finger and the linker

domains (83, 84)) and especially at position 371 (Y371C) (83, 87) (Figure 1). *CBL* mutations abolish the interaction with ZAP70 and thereby prevent the downregulation of T-cell receptor signalling (88). Furthermore, CBL reportedly has a role in the JAK/STAT pathway. Indeed, *CBL* mutations result in hyperactive GM-CSF signalling *via* JAK2 and LYN - probably due to the constitutive phosphorylation of CBL by LYN (89). It was also recently reported that CBL regulates JAK2's stability (and thus activity) *via* SH2B3, suggesting the presence of a link between CBL and JAK2 in hematopoietic cells (90) (Figure 4). Furthermore, CBL can regulate GRB2, which is involved in RAS/MEK/ERK activation (23, 91). As with *PTPN11* mutations, *CBL* mutations could prevent CBL-GRB2 binding and thus continuous activation of RAS/MEK/ERK signalling (Figure 4). Interestingly, three recent case reports found an association between *CBL* mutations on one hand and the development of JMML and Moyamoya disease (a cerebrovascular disease characterized by occlusion of the cerebral arteries). **Moyamoya phenomena were observed in patients lacking strict clinical features of JMML. In this context, it was suggested that patients with Moyamoya should be screened for mutations in the *CBL* gene (92-94). In a recent report, no secondary mutations were found in JMML patients with germline *CBL* mutations (25, 30).**

Neurofibromin 1 (NF1)

The *NF1* gene encodes neurofibromin, which is a negative regulator of the RAS pathway. The NF1 protein contains a NF1 GAP-related domain that is able to stimulate the GTPase activity of RAS proteins (95). NF1 binds to RAS with high affinity, and accelerates the conversion of RAS-GTP to RAS-GDP through its GAP activity (Figure 5). *NF1* mutations have been linked to the neurofibromatosis type 1. Mutations in *NF1* reduce the gene product's GAP activity, leading to

elevated RAS-GTP levels and thus MEK and ERK overactivation (Figure 5). The link between JMML and *NF1* was first described in 1978 (96). Around 10-15% of patients with JMML carry *NF1* LOH or compound-heterozygous mutations in *NF1* (97). Importantly, patients carrying *NF1* mutations develop JMML without any clinical signs of neurofibromatosis (19, 98, 99). *Nf1^{flox/flox}* mice develop clinical features reminiscent of JMML, such as hypersensitivity to GM-CSF, hyperproliferation, and resistance to apoptosis (100, 101). As in *KRAS*-mutated patients, some *NF1*-mutated patients may develop AML following the occurrence of a second genetic event (3, 102, 103). Gratiot et al. reported that a patient with *NF1* LOH and a mutation in *FLT3* presented with disease progression but not AML (104). The combination of an *NF1* mutation and PTEN downregulation also illustrates the importance of a “second hit” in disease progression. The loss of PTEN expression in *Nf1^{-/-}* mouse mimics the clinical manifestations of JMML, and increases the severity of the disease (105). The observed downregulation in PTEN expression in patients with JMML might be due to hypermethylation of the PTEN promoter, which is observed in 77% of patients with JMML (76). Moreover, some cases of JMML in individuals with *NF1* mutations might result from an associated histiocytic disorder called juvenile xanthogranuloma (JXG), which increases the risk of JMML by a factor of 20 (106). However, the impact of *NF1*/JXG on the risk of JMML onset is subject to debate (107-110).

Other events involved in the development of JMML

Mutations in the *NF1*, *RAS*, *PTPN11*, and *CBL* genes are responsible for 90% of the reported cases of JMML. The remaining 10% might be due to chromosomal translocations, other gene mutations, specific microRNAs (miRNA) or long non-coding RNAs (lncRNAs). Chromosomal translocations (with the creation of fusion genes, specifically) have been reported in JMML

patients harbouring monosomy 7 in most cases but no mutations in the canonical RAS pathway. Indeed, two studies identified gene fusion involving two different tyrosine kinase receptors: *ALK* (111) and *ROS1* (45), known to activate the RAS/MAPK signalling. It was suggested that *ALK* or *ROS1* rearrangements were associated with constitutive tyrosine kinase receptor activation, culminating with the RAS/MAPK activation in JMML (45, 111). Gene fusions such as *FIP1L1/RARA* or *HCMOGT-1/PDGFRB* reportedly also contribute to JMML disease (112, 113). Thanks to NGS approaches, other driver mutations involved in the pathogenesis of JMML have been recently identified, such as *RRAS*, *RRAS2* (member of the RAS GTPase family), or *SH2B3* mutations (25, 114). Other studies have focused on the regulation of miRNA (such as *let-7*) in JMML disease. In 2010, Steineman et al. reported that *let-7* mutations were absent in patients with JMML (115). However, LIN28B (a protein with a role in the inhibition of *let-7*) is overexpressed in patients with JMML, thus leading to the upregulation of *let-7* targets such as the RAS proteins (45, 116). Recently, an evaluation of the miRNA expression profile in patients with JMML revealed the upregulation of miR-630 and the downregulation of miR150-5b. Interestingly, miR150-5b targets STAT5b - an important transcription factor in the control of myeloid proliferation and differentiation. In JMML, miR150-5-b downregulation might overactivate STAT5b (117). Other non-coding transcripts (such as lncRNAs) might be associated with JMML. A recent study of 44 patients with JMML and 6 controls found that 300 genes (including 15 upregulated genes) were differentially expressed. The five most upregulated genes were *lnc-THADA-4*, *lnc-SYF2-2*, *lnc-BASP1-3*, *lnc-ACSL1-1* and *lnc-CROCC-1*, and the five most downregulated genes were *lnc-SUPT3H*, *lnc-SOX6-1*, *lnc-HMCN1-2*, *lnc-ACVR2A-2* and *lncRAN-1*. A correlation was established between these lncRNAs and a set of genes involved in myeloid cell development, dendritic cell maturation, skin-wound-related neutrophil functions, systemic lupus erythematosus, and the

AML phenotype. Moreover, the dysregulation of these lncRNAs appeared to be correlated with criteria observed in some cases of JMML, such as the level of foetal haemoglobin, monosomy 7, and mutations in certain genes. These interesting results suggest that the lncRNA expression profile might be a good marker for stratifying patients with JMML, and thus for developing more specific treatments (118). Furthermore, it is well known that chromosomal aberrations impact the development of JMML. This feature has been observed on chromosome 7 and on chromosome 8, and both are associated with a poor prognosis (13, 119, 120). Surprisingly, a recent study described a case of spontaneous remission in a patient with monosomy 7 and a germline *PTPN11* mutation; this was the first case in which monosomy 7 was not associated with a poor prognosis (17). Nevertheless, chromosomal aberration is strongly associated with a poor prognosis and is still considered to be a key diagnostic criterion for JMML.

Epigenetics and JMML

In the last 20 years, several studies have suggested that epigenetic modifications are involved in JMML disease progression. The involvement of epigenetics in the development of leukaemia in children with an *NF1* mutation was first suggested in 1996 (2). Technological progress and continually enhanced databases have facilitated investigations of the methylation profile in patients with JMML. By analyzing a panel of 14 genes in a cohort of 127 patients, Olk-Batz et al. observed frequent hypermethylation in 4 genes (*BMP4*, *CALCA*, *CDKN2B*, *RARB*) associated with a poor prognosis; these results were confirmed in a cohort of 92 other patients with JMML (121, 122). An analysis of the *RASA4* (GAP) CpG island (a target for methylation in AML) showed that *RASA4* hypermethylation was related to a poor

prognosis and to disease relapse after HSCT (74). Additional work on epigenetics and JMML supports the notion whereby abnormal methylation has a role in JMML progression, and so epigenetic changes may be additional prognostic factors (73, 123, 124). Moreover, the secondary mutations affecting genes involved in the methylation process (25, 30) also constitute links between an abnormal methylation profile to disease progression. In 2017, a genome-wide DNA methylation analysis of two different cohorts of JMML patients identified three distinct subgroups with low, intermediate and high levels of methylation (LM, IM, and HM, respectively) (46, 47). The highest methylation level was correlated with the lowest 4-year event-free survival rate, the highest frequency of relapse after treatment, and with various clinical and genetic features. The HM, LM and IM groups had higher proportions of patients with *PTPN11*, *NRAS/CBL* and *KRAS* mutations, respectively (45, 47). Interestingly, seven patients with spontaneous remission clustered together with an LM profile (46). All this evidence suggests that methylation changes influence JMML disease, and therefore opens up new therapeutic avenues.

Treatments

Since the first description of JMML in 1924, a number of treatments have been introduced and then improved by progress in basic science. **At present**, the sole curative treatment for JMML is allogenic HSCT. Depending on the patient's mutation, the value of HSCT can be questioned; indeed, spontaneous **disease** regression has been observed in patients with **mutations in *CBL*, *NRAS* or** (less frequently) *KRAS* (15, 19, 20, 125). Prior to HSCT, treatment with mercaptopurine (6-MP) regimen is used to stabilize the disease. However, various studies have shown that 6-MP might favour the generation of a "second hit" involved in disease

progression (31, 126). Engraftment failure is a major complication of HSCT in patients with JMML. Various combinations of busulfan (BU), melphalan (MEL), cyclophosphamide (CY) and fludarabine (FLU) have been tested as conditioning regimens. Shakashita et al. found that the BU/L-PAM/CY and BU/L-PAM/FLU combinations were associated with a higher overall survival rate than total body irradiation (TBI) or BU alone (19). A recent Phase II clinical study compared the risk/benefit balance (i.e. treatment toxicity vs. remission) for BU/CY/MEL vs. BU-FLU; the outcome was worse with BU-FLU than with BU/CY/MEL (127). On the same lines, a Phase IV clinical trial of BU/CY (rather than the usual TBI/CY) is ongoing (NCT01339988) (Table 2). A Phase I/II clinical trial that started in 1999 and is due to end in 2020 is studying BU/MEL/CY prior to HSCT (NCT00167219) (Table 2). Splenectomy is sometimes considered for the improvement of engraftment because splenomegaly may potentially interfere by trapping grafted progenitors. However, it has been shown that splenectomy had no influence (whether negative or positive) on the outcome of transplantation (8). Cord blood transplantation (CBT) might be an interesting alternative to HSCT. Hiramoto et al. reported on a case of JMML with *PTPN11* mosaicism. After the failure of treatment with 6-MP, cytarabine monotherapy and a cytarabine/etoposide combination, the patient underwent CBT after a BU-FLU-MEL conditioning regimen. Eighteen months later, and despite the presence of chronic graft-versus-host disease (treated with tacrolimus and prednisolone), the patient showed full donor chimerism (128). A study published in 2013 reported on CBT in 110 patients with JMML between 1995 and 2010. There was no difference between HSCT and CBT with regard to overall survival and disease-free survival. The only significant difference between the two types of transplantation was the time needed to find an appropriate donor (shorter for CBT), which is critical in emergency cases of JMML (10). With a view to improving treatment outcomes, a variety of molecules targeting the main signalling pathways involved in JMML

have been tested *in vitro*, in mouse models, and in clinical trials: pictilisib, idelalisib and rapamycin for the PI3K/AKT/MTOR pathway, AZD1480, momelotinib and ruxolitinib for the JAK/STAT pathway, and trametinib, PD0325901 and AZD6244 for the RAF/MEK/ERK pathway (Table 3). Overall, these studies highlighted a reduction in aberrant proliferation, differentiation, spleen size and leukocytosis, and longer survival (66, 90, 129-135) (Table 3). Moreover, a combination of drugs targeting two different pathways might be more effective than a single drug (130, 132, 136) (Table 3). However, it is important to note that the efficacy of single drugs or drug combinations depends on the patient's mutation status (130, 137) (Table 3). Targeting the GM-CSF receptor is another potentially valuable therapeutic option, as demonstrated by the inactivation of the GM-CSF receptor common beta-chain in murine models of JMML or by using CSF2A (GM-CSF receptor alpha chain)-specific chimeric antigen receptor T cells (100, 138). Given that DNA hypermethylation of certain genes is known to contribute to the aggressiveness of JMML, azacytidine (a DNA hypomethylating agent) has been tested as a potential treatment. In 2009, administration of azacytidine to a patient with JMML prior to transplantation was associated with a reduction in splenomegaly, monocytosis, and BMP4 gene promoter methylation (139). Some years later, a retrospective study of 9 patients with JMML having received low-dose azacytidine prior to HSCT highlighted two full remissions and two partial remissions; however, a number of adverse events (severe neutropenia, skin rash, and gastrointestinal problems) were noted (140). To investigate the molecular efficiency of azacytidine, Leoncini et al. profiled DNA methylation in patients with JMML before and after azacytidine treatment. The methylation level was lower after treatment in some patients, although it was still above values found in healthy individuals. Thus, Leoncini et al. suggested that JMML patients with a high methylation level may benefit from azacytidine treatment (141, 142). In 2015, these promising results prompted the

initiation of a clinical trial (scheduled end date: late 2019) of azacytidine treatment in paediatric patients with JMML or myelodysplastic syndrome (Table 2).

Future considerations

Juvenile myelomonocytic leukaemia remains a challenging disease to assess and treat. The standard of care is currently HSCT, although relapse rates are still high. Alternative treatment options are therefore urgently required. Better disease models are essential for addressing this aspect. Various research studies have shown that reprogrammed induced pluripotent stem cells (iPSCs) from JMML patients recapitulate the hallmarks of JMML; hence, these cells are powerful new tools for studying molecular mechanisms and assessing drug efficacy (143-145). Indeed, iPSC experiments can highlight differences in drug sensitivity as a function of the primary mutation (137). Therefore, iPSCs constitute an efficient model for investigating the pathophysiology of JMML. Many research groups are looking for novel, specific, more tolerable and potentially more personalized treatments that target the various signalling pathway impacted by RAS-related mutations and secondary mutations found in JMML cells. In order to better understand the pathophysiological mechanisms in JMML and develop effective treatments, efforts are likely to focus on epigenetics and RNA profiles. Most ongoing studies are looking at DNA methylation levels in bulk JMML cells; one promising avenue might be the investigation of methylation and transcriptomic profiles at the single-cell level. Indeed, given the cellular dynamism and multiple interactions in the immune system, epigenetic modifications of one or several subtypes of immune cells (and thus changes in RNA levels) may create an immune imbalance and promote JMML development and progression. A more detailed epigenetic map and transcriptomic profiles may help to (i) characterize the

pathological pathways at play, (ii) identify new biomarkers, and (iii) identify specific therapeutic targets. For instance, a nanoparticle-based drug delivery approach to the specific modification of JMML cells could be envisaged. Along with epigenetic and transcriptomic profiles, immunometabolic analyses constitute another promising, complementary axis. Over the last few years, it has become clear that the interplay between metabolic process and immunity is of particular relevance in the field of cancer (146). Metabolic remodelling might be a key part of the immune dysfunction observed in JMML. Epigenetic, transcriptomic and immunometabolic analyses at the single-cell level will enable us to rethink current treatments for JMML and thus offer patients new, personalized treatments with a molecular rationale.

Conclusion

Over the last 95 years, scientists have gained a better understanding of the genetic events and mechanisms that lead to JMML. In turn, this knowledge has led to improvements in patient care. Despite this progress, JMML is still a deadly disease. The only curative treatment is HSCT, although the 5-year event-free survival rate is only 52%. Genetic analyses of patients with JMML have highlighted the involvement of five key genes: *PTPN11*, *NRAS*, *KRAS*, *NF1* and *CBL*. Most (if not all) mutations in these genes trigger the constitutive activation of the RAS-MEK-ERK pathway and thus lead to uncontrolled myeloid cell activity. Although innovative treatments (such as MEK inhibitors) have been tested with a view to blocking this pathway, none of the currently available drugs combines lasting efficacy, specificity, and low toxicity. Several studies have shown that besides *PTPN11*, *NRAS*, *KRAS* and *NF1* mutations, some patients with JMML display recurrent somatic mutations, high levels of gene methylation or non-coding RNA variants. The possible continuum between RALD and JMML suggests that studies of patients with RALD will be essential for identifying “second hits”, determining the

underlying disease mechanisms, and thus choosing appropriate treatments that stop disease progression by specifically impacting dysregulated pathways in the mutant cells.

Practice Points

- 90% of patients with JMML carry a mutation in RAS pathway genes (including *NRAS*, *KRAS*) *PTPN11*, *NF1*, and *CBL*), although driver mutations have also been found in *RRAS*, *RRAS2* and *SH2B3*
- RALD and JMML form a clinical continuum in some patients, conditioned by additional genetic or epigenetic events
- Methylation status is essential for understanding the pathophysiology of JMML

Research Agenda

- Understand the continuum between RALD and JMML
- Develop iPCS techniques in the context of JMML
- Analyze epigenetics, transcriptomics and immunometabolism at the single-cell level

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Conflict of Interest Statement

None.

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Legends

Figure 1: mutations in *PTPN11*, *NRAS*, *KRAS*, *NF1* and *CBL* genes are involved in JMML.

Figure 2: the effect of *RAS* mutations on signalling pathways in JMML.

A- Upon stimulation, a docking site for adaptor molecules is created; this allows the recruitment of a guanine exchange factor (GEF). The latter replaces the RAS-bound GDP by a GTP, thus generating the active form of RAS. The RAS-GTP initiates a cascade of phosphorylations on RAF, MEK and, lastly, ERK. The phospho-ERK then translocates into the nucleus. In order to stop activation of the RAS signalling pathway, a GTPase-activating protein (GAP) stimulates the activity of the RAS GTPase domain, so that RAS-GTP is converted into RAS-GDP. B- Mutation of RAS (*) modifies the conformation of the RAS protein and prevents the conversion of RAS-GTP to RAS-GDP – inducing the constitutive activation of the RAS signalling pathway.

Figure 3: the effect of *PTPN11* mutations on signalling pathways in JMML.

Upon stimulation, the active form of SHP2 binds the adaptor GRB2. In turn, GRB2 binds a GEF enabling the conversion of RAS-GDP to RAS-GTP (pathway 1, in black). SHP2 can also bind and dephosphorylate RAS, allowing the latter to bind RAF and thus activate the MEK/ERK (pathway 2, in pink). GRB2 can also bind GAB2, which then interacts with SHP2 and activates the PI3K/AKT/mTOR pathway (pathway 3, in orange). Gain-of-function mutations in *SHP2* (*)

increase the protein's phosphatase activity and so continuously activate the RAS-MAPK and PI3K/Akt pathways (in green).

Figure 4: the effect of *CBL* mutations on signalling pathways in JMML.

Upon T-cell receptor activation, activated Lck phosphorylates an intracellular domain on the CD3 zeta chain, creating a docking site for Zap-70. The latter phosphorylates LAT, allowing the recruitment of several proteins that convey the T cell activation signal. In order to avoid an uncontrolled immune response, a negative signal is required. This is provided by the E3 ubiquitin ligase CBL, which promotes the ubiquitination and degradation of ZAP-70. Next, upon stimulation, JAK proteins (linked to the receptor) are autophosphorylated and thus trigger the recruitment, phosphorylation and dimerization of STAT. The dimer enters the nucleus and acts on its target genes. To regulate this signalling, CBL binds LNK and downregulates the JAK activity. CBL might also regulate the RAF/MEK/ERK pathway by degrading Grb2. When CBL is mutated (*), the negative regulation is absent - leading to continuous activation of the signal (the pathway in green).

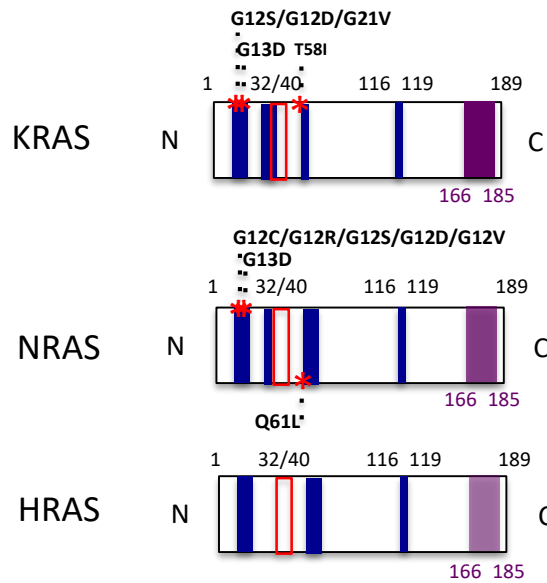
Figure 5: the effect of *CBL* mutations on signalling pathways in JMML.

To inhibit the activation of the RAS signalling pathway, a GAP allows the conversion of RAS-GTP to RAS-GDP. NF1 binds to RAS-GTP and increases the conversion of RAS-GTP to RAS-GDP. When NF1 is mutated (*), the low GAP activity leads to high levels of RAS-GTP and thus over-activation of MEK and ERK pathways (in green).

Table 1: diagnostic criteria for JMML.

Table 2: therapeutic clinical trials in JMML.

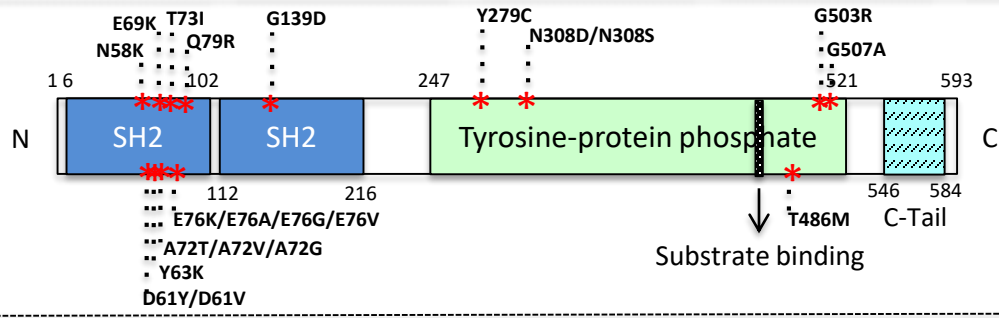
Table 3: *in vivo* and *in vitro* studies of treatments for JMML.



| | KRAS | NRAS | HRAS |
|---------------------------|------------------------------------|------------------------------------|---------------------------|
| Nucleotide binding domain | 10-18 29-35 59-60 116-119 | 10-18 29-30 57-61 116-119 | 10-17 57-61 116-119 |
| Effector region | 32-40 | | |
| Variable region | 166-185 | | |

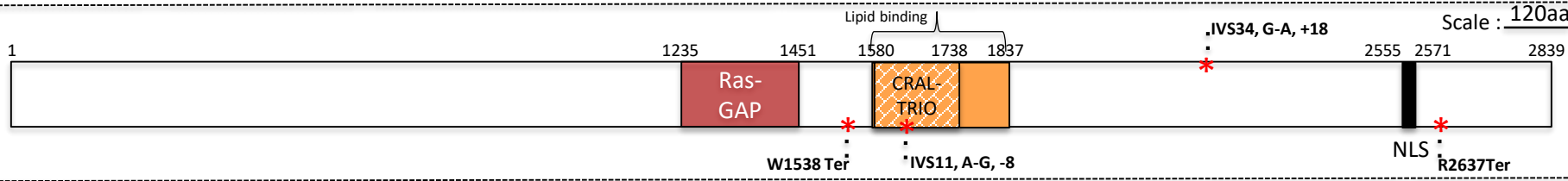
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PTPN11



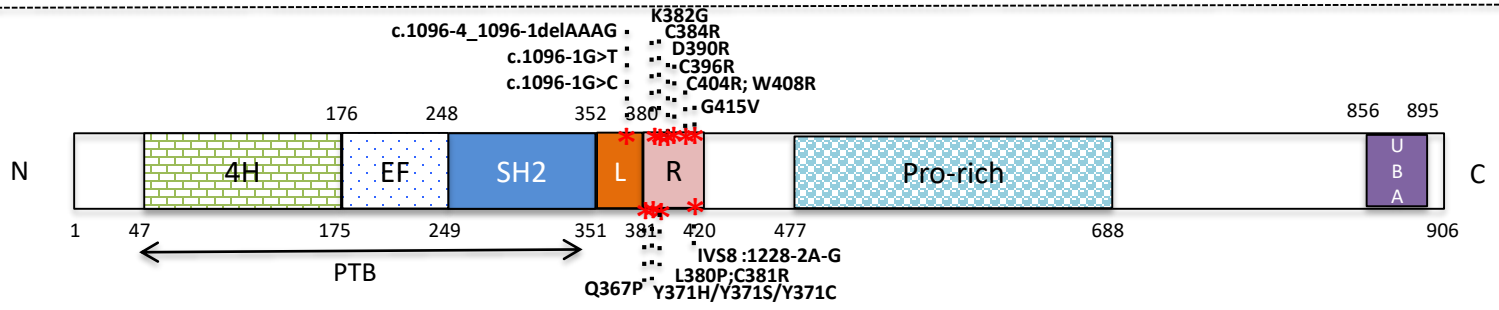
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NF1

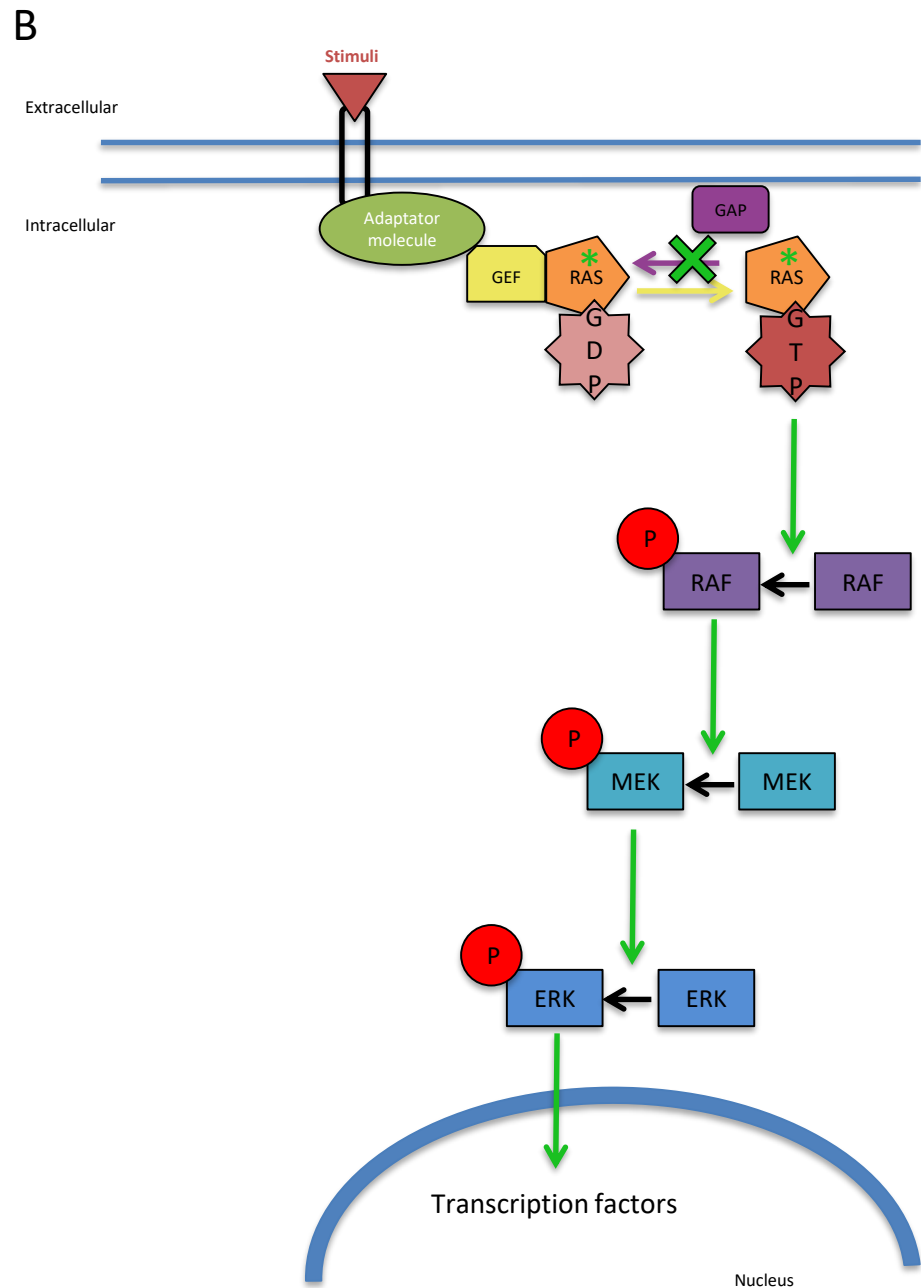
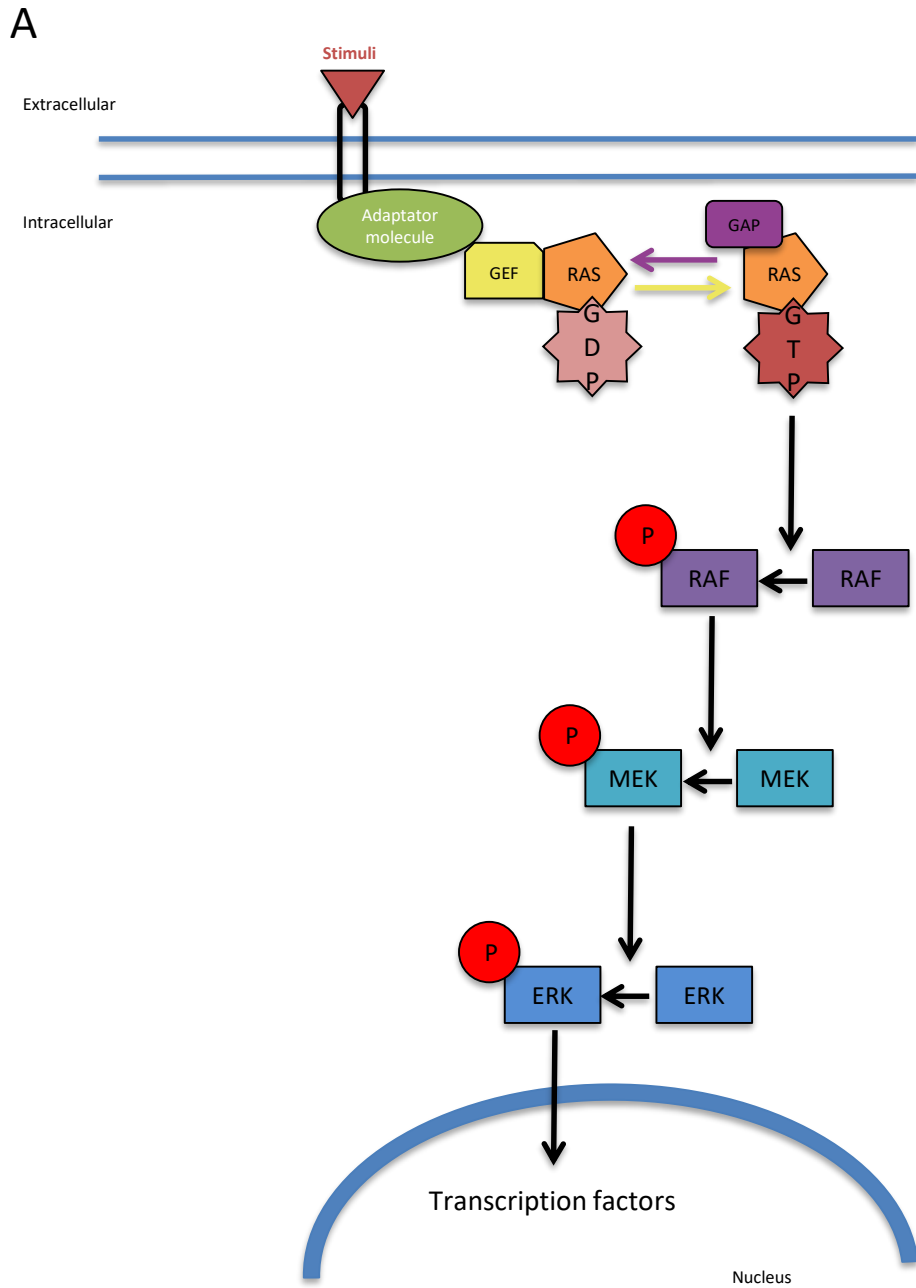


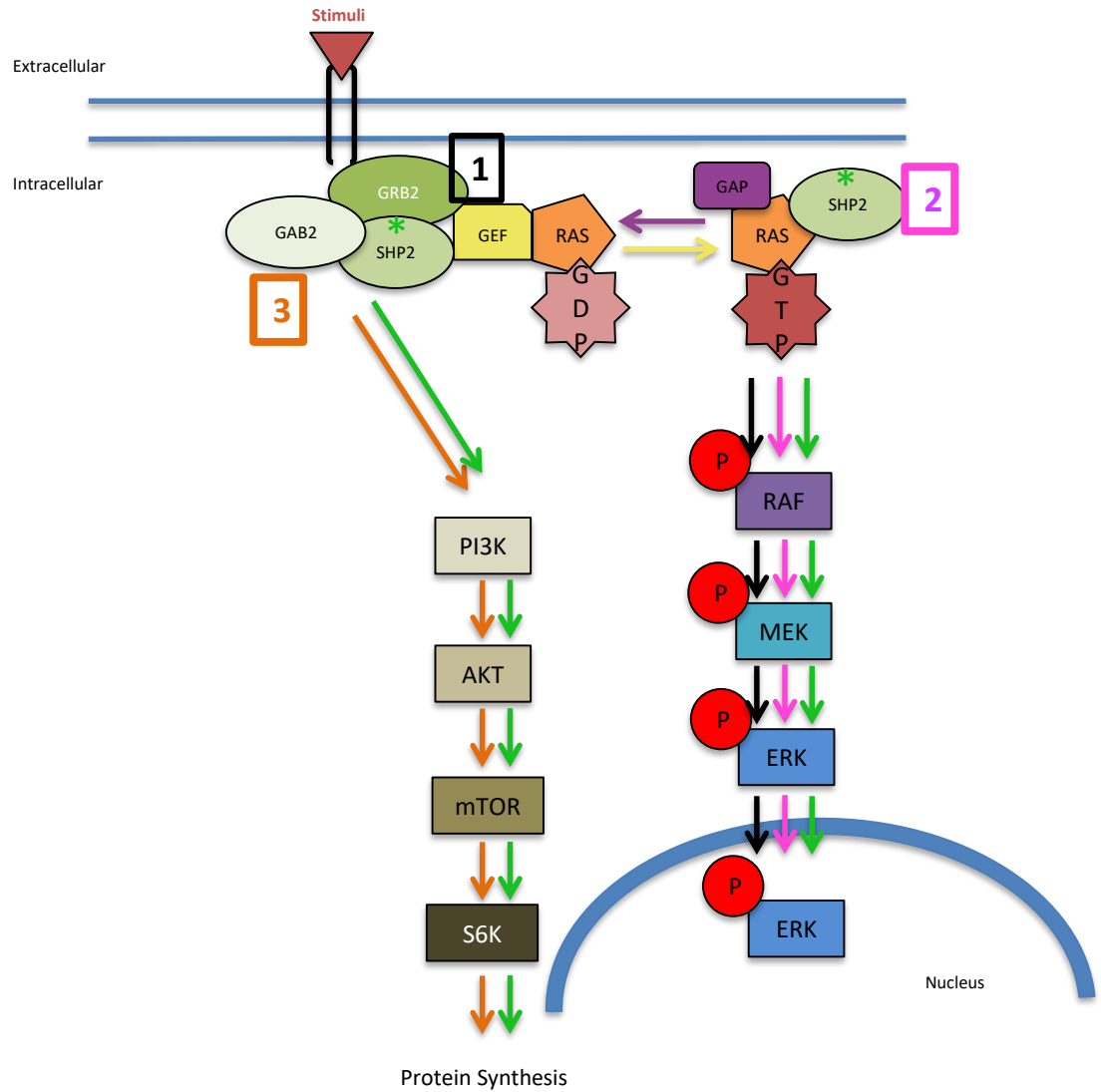
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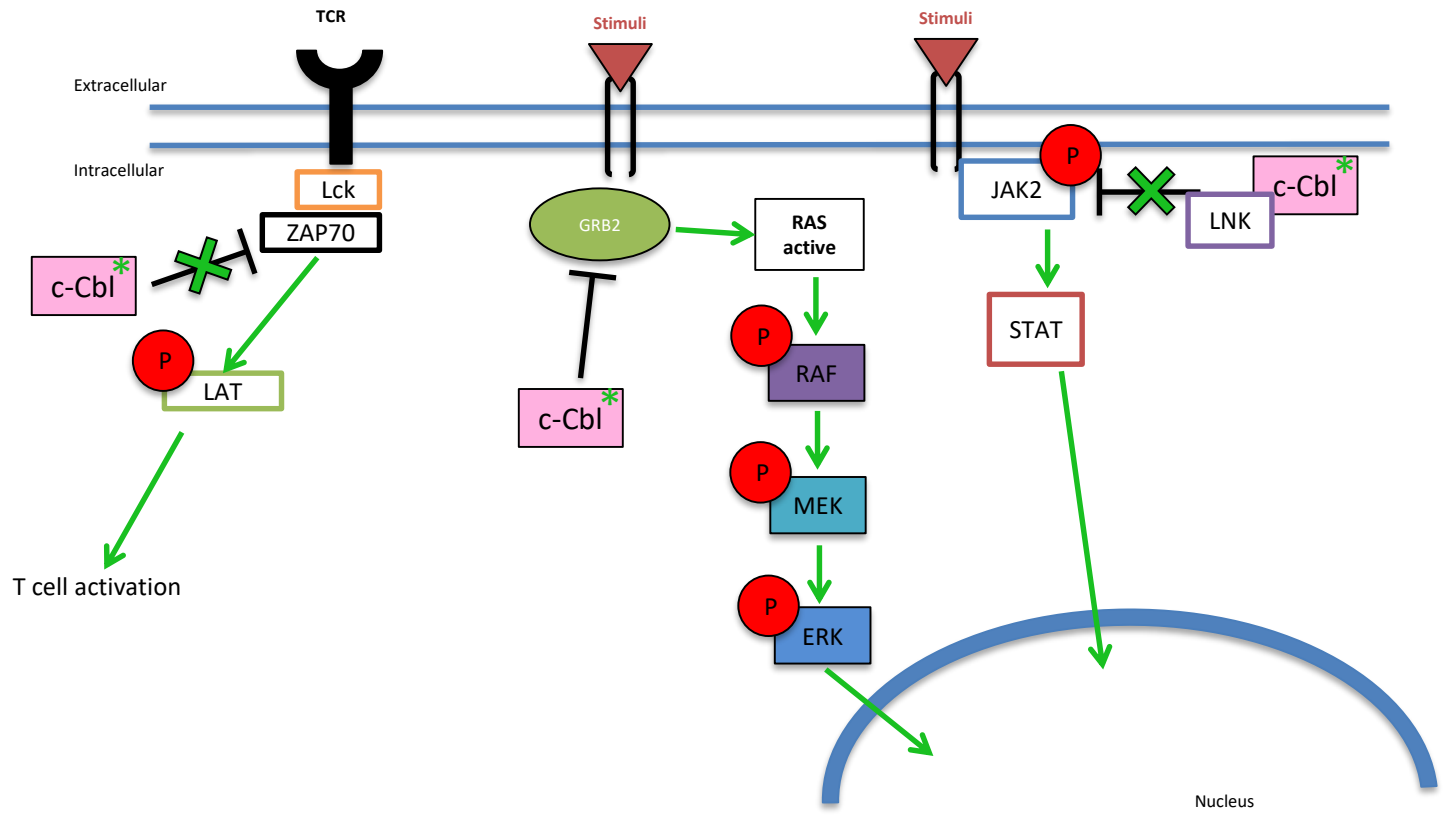
CBL

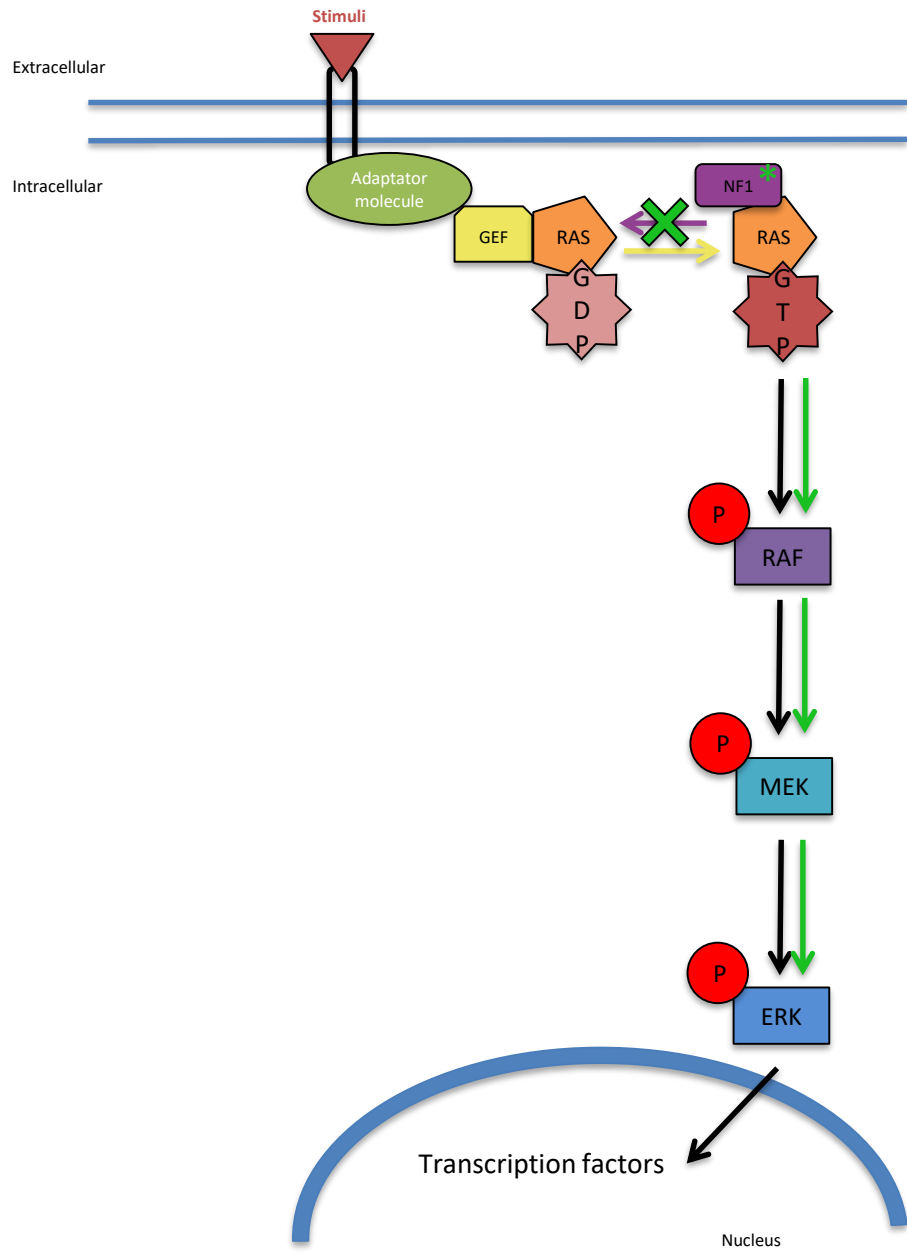


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| Category | Criteria |
|----------|--|
| 1 | <p>Peripheral blood monocyte count > 1000/μl</p> <p>Proportion of blast in peripheral blood and bone marrow <20%</p> <p>Splenomegaly</p> <p>Absence of the BCR/ABL fusion gene</p> |
| 2 | <p>Somatic mutation in <i>PTPN11</i>, <i>KRAS</i> or <i>NRAS</i></p> <p>Clinical diagnosis of NF1 or a germline mutation in <i>NF1</i></p> <p>A germline <i>CBL</i> mutation and loss of heterozygosity for <i>CBL</i></p> <p>Monosomy 7</p> |
| 3 | <p>A clonal cytogenetic abnormality, other than monosomy 7</p> <p>White blood cell count >10,000/μl</p> <p>Elevated age-adjusted foetal haemoglobin level</p> <p>Circulating myeloid precursors</p> <p>Hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF)</p> |

| Treatment | Clinical study | Title | Information |
|--|----------------|--|---|
| Trametinib (a MEK inhibitor) | NCT03190915 | A Phase 2 Study of the MEK Inhibitor Trametinib (NSC# 763093) in Children With Relapsed or Refractory Juvenile Myelomonocytic Leukemia | Started in 2017, scheduled to end in 2020 |
| Azacitidine | NCT02447666 | Study with Azacitidine in Pediatric Subjects with Newly Diagnosed Advanced Myelodysplastic Syndrome (MDS) and Juvenile Myelomonocytic Leukemia (JMML) | Started in 2015, scheduled to end in 2019 |
| Tipifamib (farnesyltransferase, to prevent RAS from binding to membranes) | AAML0122 | Phase II/III trial of a pre-transplant farnesyl transferase inhibitor in juvenile myelomonocytic leukemia: a report from the Children's Oncology Group | From 2001 to 2006 |
| Stem cell transplantation | NCT00167219 | Stem Cell Transplant for Juvenile Myelomonocytic Leukemia (JMML) | Started in 1999, scheduled to end in 2020 |
| Allogeneic stem cell transplantation followed by Gemtuzumab Ozogamicin treatment | NCT01020539 | Allogeneic Stem Cell Transplantation Followed By Targeted Immune Therapy In Average Risk Leukemia (AML/MDS/JMML) | Started in 2015, scheduled to end in 2018 |
| Treosulfan | NCT02333058 | Treosulfan-based Conditioning in Paediatric Patients With Haematological Malignancies | Started in 2015, scheduled end-date 2018 |
| Busulfan/Cyclophosphamide | NCT01339988 | Busulfan and Cyclophosphamide Instead of Total Body Irradiation (TBI) and Cyclophosphamide for Hematological Malignancies Hematocrit (HCT) | Started in 2014, scheduled end-date 2019 |

| Drug | Target | Model | Observation | references |
|--|---------------------------|---|--|---------------|
| GDC-0941 (pictilisib) | PI3K | Mx1-Cre, Kras(D12) mice, Mx1-Cre, Nf1flox/- mice | Reduction in PI3K/Akt and Raf/MEK/ER activation. Reduction in leukocytosis and anemia, longer survival, Partial correction of splenomegaly. GDC-0941 reduced the severity of MDS/MPN. | 119 |
| MK-2206 | Akt | | No effect on Raf/MEK/ERK signaling, longer survival, lower blood cell counts and reduced splenomegaly. K-2206 improved all aspects of the MDS/MPN phenotype in Nf1Δ/- mice. The combination of PD0325901 and MK-2206 increased the reduction in spleen size. | |
| GDC-0941 + Mk-2206 | Akt+PI3K | | The combination of GDC-0941 and Mk-2206 did not improve hematologic parameters in KrasD12 mice more than each reagent used alone. However, spleen size is more reduced in presence in presence of two drugs. Study claimed also that continuous drug treatment would be needed to maintain the clinical response to Akt and/or MEK inhibition. | |
| PD0325901 (901) | MEK | Mx1-Cre, Kras(D12); mice Mx1-Cre, Nf1flox/flox; GOF Shp2-expressing cells | Reduction of leukocyte number, erythropoiesis, spleen size, correction of aberrant proliferation and differentiation | 120, 121, 122 |
| AZD6244/AZD1480 | MEK/JAK | NrasG12D/G12D model | The combination of two drugs inhibited the growth of human and mouse CMML cells <i>in vitro</i> more than a single drug alone. In NrasG12D/G12D mice, combination treatment was associated with a reduction in spleen size and greater long-term survival, relative to the drugs used alone. Moreover, AZD6244 or AZD1480 were considerably better than U0126 and AG490, the old-generation inhibitors of MEK and JAK2. | 127 |
| Idelalisib, rapamycin, PD0325901, trametinib, momelitinib, ruxolitinib | MEK/JAK, PI3K/mTOR | CBL-mutant and PTPN11-mutant iPSC-derived myeloid progenitors | PI3K/Akt/mTOR activation is observed in PTPN11-mutant and CBL-mutant JMML lines. Incubation of CBL myeloid cells with JAK inhibitors (momelotinib or ruxolitinib) decreased JAK/STAT activation but did not have an effect on PTPN11 cells. Incubation of iPSC-derived myeloid cells with idelalisib or rapamycin suppressed PI3K/Akt/mTOR more in PTPN11 cells than in CBL cells. PD0325901 and trametinib (MEK inhibitors) acted on both cell lines but were more efficacious on PTPN11 cells. | 128 |
| Ruxolitinib | JAK | Mx1-Cre, Nf1flox/flox, BM cells from Cblf/f;Cbl-bf/f transplanted mice | 1) Mx1-Cre, Nf1flox/flox: Decrease in spleen weight, greater survival, a reduction of around 50% in the white blood cell count, and attenuation of MPN. Treatment of animals with ruxolitinib induces a worse anemia than in animals with MPN. The reduction in the platelet count was more pronounced in animals with MPN. 2) Cblf/f;Cbl-bf/f transplanted mice: a reduction in neutrophil counts but not in the lymphoid cell count or the red blood cell count; a decrease in the numbers of myeloid-biased MPP3 and megakaryocyte/erythroid-biased MPP2 cells but an increase in the lymphoid-biased MPP4 counts | 123, 85 |
| No drug | GMSCF | Nf1flox/flox, beta c-/-, Mx1-Cre NF1flox/flox beta c-/- | A high level of hematopoietic progenitors, no MPD profile, less severity of the MDP, less myeloid proliferation, a reducing in splenic infiltration, and no change in the spleen weight | 95 |
| No Drug, CAR T cells system | GMR | Cells | An anti-proliferation effect, and effects on colony growth and myelomonocytic differentiation. | 124 |
| Rapamycin | mTOR | Ptpn11E76K/+ mic | Rapamycin treatment was associated with a decrease in the WBC count, spleen size, the cytokine hypersensitivity of myeloid progenitors. GM-CSF-induced colony formation of myeloid blasts from JMML patients with PTPN11-activating mutations was more sensitive to rapamycin than in progenitors from a healthy donor | 62 |
| GS-9820 and idelalisib | p110δ-specific inhibitors | GOF Shp2-expressing cells; Shp2E76K/+;LysMcre+ mice | 1) a reduction in Akt and Erk phosphorylation, and thus in cell proliferation; 2) the PI3Kδ inhibitor reduced the spleen size, promoted myeloid cell maturation, and prolonged mouse life. It is noteworthy that the inhibition of PI3K p110δ did not permanently correct GM-CSF hypersensitivity in the GOF Shp2-expressing cells. | 122, 125 |
| GS-9820 and PD0325901 | PI3K/MEK | GOF Shp2-expressing cells | Reduced proliferation (in a dose-dependent manner) | |
| Azacytidine | Methylation | Patients | Patients presenting a high level of hypermethylation might benefit from azacytidine treatment | 129,130 |
| Rapamycin | mTOR | A case report | Nine months after transplantation, the patient had a relapse of JMML. After the withdrawal of immunosuppressants, the patient developed grade III GVHD (controlled with rapamycin). The patient has continued to take rapamycin for the last 6 years. The cancer is in remission, and donor engraftment in the peripheral blood is stable. | 126 |