RAS diseases in children

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ABSTRACT

RAS genes encode a family of 21 kDa proteins that are an essential hub for a number of survival, proliferation, differentiation and senescence pathways. Signaling of the RAS-GTPases through the RAF-MEK-ERK pathway, the first identified mitogen-associated protein kinase (MAPK) cascade is essential in development. A group of genetic syndromes, named "RASopathies", had been identified which are caused by heterozygosity for germline mutations in genes that encode protein components of the RAS/MAPK pathway. Several of these clinically overlapping disorders, including Noonan syndrome, Noonan-like CBL syndrome, Costello syndrome, cardio-facio-cutaneous (CFC) syndrome, neurofibromatosis type I, and Legius syndrome, predispose to cancer and abnormal myelopoiesis in infancy. This review focuses on juvenile myelomonocytic leukemia (JMML), a malignancy of early childhood characterized by initiating germline and/or somatic mutations in five genes of the RAS/MAPK pathway: *PTPN11, CBL, NF-1, KRAS* and *NRAS*. Natural courses of these five subtypes differ, although hematopoietic stem cell transplantation remains the only curative therapy option for most children with JMML. With whole-exome sequencing studies revealing few secondary lesions it will be crucial to better understand the RAS/MAPK signaling network with its crosstalks and feed-back loops to carefully design early clinical trials with novel pharmacological agents in this still puzzling leukemia.

Introduction

Discoveries made about 50 years ago revealed the transforming power of the Harvey and Kirsten murine sarcoma retroviruses by a set of genes named *RAS* genes for their role in forming rat sarcomas. Subsequently, the contribution of *RAS* genes to cancer pathogenesis has been studied extensively. While the somatic dysregulation of RAS is a primary driver of cancer, there is a class of developmental disorders caused by germline mutations (as opposed to the somatic mutations found in cancer) in genes that encode components of the RAS signaling pathway. This chapter summarizes hematologic disturbances and cancer predisposition of these genetic disorders named "RASopathies". It will focus on juvenile myelomonocytic leukemia (JMML), an infant leukemia with initiating germline and somatic mutations in genes of the RAS signal transduction pathway.

RAS and the RAS/mitogen-activated protein kinase signaling cascade

RAS turned out to be an essential cellular hub for a wide variety of signaling pathways including the three human *RAS* genes, *KRAS, NRAS* and *HRAS* (reviewed by Stephen *et al.*[1]). RAS proteins act as molecular switches by cycling between an active guanosine triphospate (GTP)-bound (RAS-GTP) and an inactive guanosine diphosphate (GDP)-bound (RAS-GDP) conformation (Figure 1).[2] RAS-GTP concentrations are tightly regulated by the competitive action of guanosin exchange factors (GEFs) and GTPase activating proteins (GAPs).

RAS is activated by extracellular stimuli such as growth factor binding to receptor tyrosine kinases (RTKs) followed by RTK autophosphorylation and the creation of docking sites for adaptor molecules (e.g. GRB2). These molecules recruit and activate GEFs (e.g. SOS1) which displace GDP from RAS allowing RAS to bind to GTP. RAS-GTP binds to and activates a large number of effector pathways. The RAS-mediated mitogen-activated kinase (MAPK) pathway is one of several important downstream cascades. Activated RAS binds to RAF (RAF1, also known as CRAF, BRAF), the first MAPK of the signaling cascade. RAF phosphorylates MEK1 and/or MEK2, which in turn activate ERK1 and/or ERK2. Active ERKs serve as regulators of a large number of downstream processes both in the cytosol and nucleus.

Genetic syndromes of the RAS/mitogen-activated kinase pathway

In the last decade, a group of human genetic syndromes was discovered to result from germline mutations in genes of the RAS/MAPK pathway (Figure 1).[2,3] These mutations induce the activation of the pathway, and thus, these disorders share common clinical features including reduced growth, facial dysmorphism, cardiac defects, ectodermal anomalies, variable cognitive deficits and susceptibility to certain malignancies. Based on the shared pathogenic mechanism with up-regulated RAS/MAPK signaling and the clinical overlap, these genetic diseases have been grouped as "neuro-

cardio-facio cutaneous syndromes (NCFCS)" or "RASopathies" (Table 1).[4-22]

Noonan syndrome (NS) is the most common of these disorders occurring in 1 of 1000-2500 births (Table 1). It is characterized by a typical facial appearance, congenital heart defects and a variety of abnormalities in other organ systems. At present, heterozygous germline mutations in 6 genes are recognized in NS with *PTPN11* mutations contributing for approximately half of the cases (Table 1). Other RASopathies consist in the two NS-like syndromes caused by mutations in *CBL* or *SHOC2*, Costello syndrome, cardio-facio-cutaneous syndrome (CFC), neurofibromatosis type 1 (NF-1) and Legius syndrome (Table 1).

Interestingly, germline mutation in genes involving the RAS/MAPK pathway may predispose to autoimmune disease as is known for the respective somatic mutations (see below). In a series of 42 patients with RASopathies, 52% were shown to have autoantibodies against various tissues.[23] Other investigators suggested a predisposition to the development of systemic lupus erythematosus in NS.[24]

Malignancies in patients with germline mutations in RAS/MAPK pathway genes

Besides NF-1, an extensively studied familial tumor syndrome, there is a clear correlation between germline mutations in the genes of the RAS/MAPK pathway and specific neoplasms (reviewed by Kratz *et al.*[25]). Since the RAS signaling pathway is a major contributor to tumorigenesis (Table 2) this is not surprising. From a literature review, the estimated rate of cancer was 11% for Costello syndrome, and 4% each for CFC syndrome and NS (excluding transient myeloproliferative disorder).[25] In Costello syndrome, more than 70% of the malignancies are rhabdomyosarcomas.[26] The patient age at the time of cancer diagnosis is comparable to that expected for sporadic cancers, thus, there is an early-childhood peak.

The most common hematopoietic disorder in NS is a transient myeloproliferative disorder (MPD)[25-27] estimated to occur in up to 10% of all children with NS.[28] While the
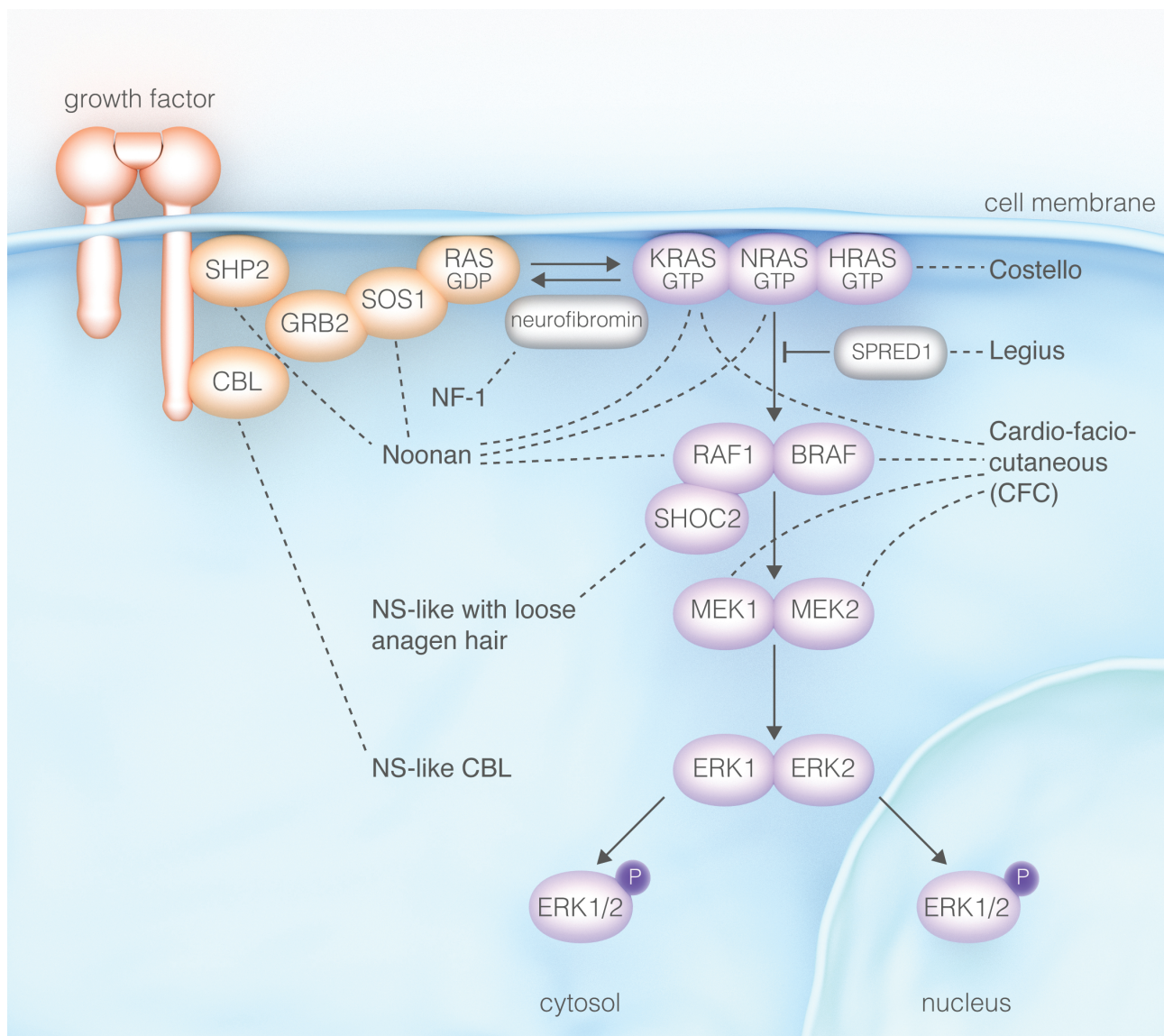

Figure 1. Genetic syndromes of the RAS-MAPK pathway: "Rasopathies" (modified from Tidyman *et al.*[2]).

abnormal myelopoiesis is benign in the vast majority of these cases, some children develop JMML. The following part of this chapter will focus on MPD in NS, and on JMML, a unique leukemia characterized by initiating genetic events in one of 5 genes of the RAS signal transduction pathway.

Transient myeloproliferative disorder in infants with Noonan syndrome

The transient MPD in Noonan syndrome[25-27] is diagnosed in the neonatal period or early infancy. In contrast to JMML, NS/MPD is thought to be of polyclonal origin. It generally resolves spontaneously over months or years. However, leukocytosis and tissue invasion by monocytes and immature myeloid cells can have deleterious effects. Up to 30% of children with NS and severe MPD suc-cumb to myeloproliferation in combination with their multiple other clinical problems (the EWOG-MDS, unpublished observation, 2014). To ameliorate the deleterious effects of abnormal myelopoiesis, mild cytoreductive therapy, such as treatment with 6-mercaptopurine, can be helpful. An estimated 10% of cases of NS/MPD acquire a cytogenetic abnormality and progress to JMML. Thus, NS/MPD is a tumor predisposition syndrome and affected patients should be followed closely. Nearly all patients with NS/MPD have mutations in *PTPN11*.[29] Gain-of-function effect of these mutations on the protein product SHP2 is predicted to be intermediate between that expected in NS without myeloproliferation (lower gain of function) and somatic mutations in JMML (higher gain of function) (see below).[30] In very rare cases, the underlying germline mutation in NS/MPD affects the *KRAS* gene.[11]

Table 1. Summary of syndromes of the RAS/MAPK pathway, named RASopathies or neuro-cardio-facial cutaneous syndromes (adapted from Roberts *et al.*[3] and Aoki *et al.*[4]).

Syndrome	Phenotype	Mendelian inheritance in man	Gene(s) mutated	Reference
Neurofibromatosis type 1 (NF-1)	Café-au-lait spots; intertriginous freckling; neurofibromas and plexiform neurofibromas; Iris Lisch nodules; osseous dysplasia; optic pathway glioma; mild neurocognitive impairment	16220	*NF1*	[5]
Noonan syndrome (NS)	Facial dysmorphism (improves with age: high forehead, hypertelorism, downslanting palpebral fissures, epicanthal folds, ptosis, low-set and posteriorly rotated ears); feeding difficulties in infancy (75%); congenital heart defect (in 80%: pulmonic stenosis, hypertrophic cardiomyopathy, atrial septal defect); cryptorchism (60-80% of males); bleeding tendency (60%); spinal abnormalities (30%); intellectual impairment (20%); lymphatic abnormalities (20%); sensorineural hearing loss (10%).	605275	*PTPN11, KRAS, SOS1, RAF1, NRAS PTPN11*[8]	Clinical picture[6,7] *SOS*[9,10] *KRAS*[11] *RAF*[12,13] *NRAS*[14]
NS-like syndromes	*CBL-syndrome:* In comparison to NS: less often congenital heart disease; in addition to NS: delayed brain myelination, hypoplasia of the cerebellar vermis, café-au-lait spots.	613563	*CBL*	[15,16]
	NS-like disorder with loose anagen hair: NS-like facial features and hairless and darkly pigmented skin with eczema or ichthyosis.	607721	*SHOC2*	[17]
Costello syndrome (CS)	Coarse facial features, benign nasal and perianal cutaneous papilloma; premature aging and hair loss, loose skin; developmental delay, moderate intellectual disability; congenital heart defect (most commonly pulmonic stenosis); predisposition to solid tumors (rhabdomyosarcoma, neuroblastoma, bladder carcinoma)	218040	*HRAS*	[18,19]
Cardio-facio-cutaneous (CFC) syndrome	Shares features with NS and CS; ectodermal abnormalities with sparse curly hair and sparse or absent eyelashes.	115150	*BRAF, MEK1, MEK2, KRAS*	[20,21]
Legius syndrome (NF-1-like syndrome)	Shares many phenotypical features with NF-1, Lisch nodules and central nervous tumors are, however, absent.	611431	*SPRED1*	[22]

Juvenile myelomonocytic leukemia

Juvenile myelomonocytic leukemia is a clonal hematopoietic disorder of early childhood characterized by hepatosplenomegaly and organ infiltration due to excessive proliferation of cells of the monocytic and granulocytic lineages. It accounts for approximately 2.4% of all childhood hematologic malignancies.[31]

Clinical presentation

There is generally marked splenomegaly and hepatomegaly. Occasionally, spleen size is normal at diagnosis, but it rapidly increases thereafter. Dry cough, tachypnea and interstitial infiltrates on chest X-ray are signs of peribronchial and interstitial pulmonary infiltrates. Gut infiltrates may lead to diarrhea or gastrointestinal infections. Skin lesions other than petechiae are common and often present as eczematous eruptions, erythematous maculopapules or indurated raised lesions. In addition, multiple juvenile xanthogranulomas may be seen. Unlike acute monoblastic leukemia, JMML rarely involves the central nervous system.

Hematologic and laboratory features

Leukocytosis, anemia and thrombocytopenia are common findings in JMML. The median white blood cell count (WBC) varies from 25-33x10^9/L, and the WBC rarely exceeds100x10^9/L.[32,33] Examination of the peripheral blood smear is the most important step in establishing the diagnosis. Almost all cases show a striking monocytosis, often with dysplastic cell forms. An absolute monocyte count exceeding 1x10^9/L is required for the diagnosis. Immature monocytes, along with myelocytes, metamyelocytes and nucleated red cells, are usually evident. The median blast cell percentage in blood smears is less than 2%. Thrombocytopenia may be severe and life threatening. Megakaryocytes are reduced or absent in approximately 75% of children.[32] Bone marrow findings in JMML are not by themselves diagnostic but rather are consistent with the diagnosis.

A remarkable feature of many JMML cases is a markedly increased synthesis of hemoglobin F (HbF). Subtle immunological abnormalities like increased serum concentrations for IgG, IgM, and IgA or autoantibodies, such as antinuclear antibodies, antibodies against red cells giving rise to a positive antiglobin test are common in the absence of frank clinical autoimmune disease.[32] Chromosomal studies of leukemic cells show monosomy 7 in approximately 25% of patients, other abnormalities in 10%, and a normal karyotype in 65%.[32-34]

Diagnosis

Approximately 90% of children with JMML have largely mutually exclusive mutations in one of 5 genes:

Table 2. Disorders involving germline and somatic mutations in genes of the RAS/MAPK pathway (adapted from Aoki *et al.*[4])

Gene	Germline mutations		Somatic mutations
	Syndrome	Associated tumors	Tumors and related disorders
PTPN11	NS	Transient MPD→ JMML in rare cases	JMML, AML, B-ALL, neuroblastoma
SOS1	NS	rare	Rare
CBL	NS-like CBL-syndrome	JMML	MDS, MPN, AML, lung cancer
NRAS	NS	-	JMML, CMML, AML, ALL, MDS, MM
KRAS	NS, CFC	Transient MPD	Pancreatic carcinoma, colon carcinoma lung cancer, JMML RAS-associated ALPS-like disease, less frequent than NRAS in: CMML, AML, ALL, MDS, MM
HRAS	Costello syndrome	Rhabdomyosarcoma, neuroblastoma, bladder carcinoma, papillomata	Bladder carcinoma
NF-1	Neurofibromatosis type 1	Astrocytoma, peripheral nerve-sheath tumor, JMML, rhabdomyosarcoma, neuroblastoma, breast cancer, GI stromal tumor	Breast cancer, colon cancer, gliomas, lung cancer, ovarian cancer, and many others
SHOC2	NS-like disorder with loose anagen hair	-	-
RAF1	NS	-	Rare
BRAF	CFC syndrome	rare	Langerhans cell histiocytosis, malignant melanoma, colon cancer thyroid cancer
MEK1 / MEK2	CFC syndrome	rare	-

NS: Noonan syndrome; MPD: myeloproliferative disease; JMML: juvenile myelomonocytic leukemia; AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; B-ALL: B-cell acute lymphoblastic leukemia; ALPS: autoimmune lymphoproliferative syndrome; CMML: chronic myelomonocytic leukemia; MDS: myelodysplastic syndrome; MPN: myeloproliferative neoplasms; MM: multiple myeloma; GI: gastrointestinal; CFC: cardio-facio-cutaneous syndrome.

PTPN11, *NRAS*, *KRAS*, *NF-1* or *CBL* (Figure 2). Due to the size and complexity of the NF-1 gene, the diagnosis of NF-1 in children with JMML had in the past been based on the presence of 6 or more café-au-lait spots (and presence of an affected parent in approximately half of the patients). To identify abnormalities in the other genes, mutational screens have to be performed. Heterozygous point mutations noted in the *PTPN11* and *RAS* genes may arise at a somatic or germline level. Consequently, genetic screens in leukemic cells need to be followed by studies in non-hematopoietic tissue such as fibroblasts, cells attached to nails, hair bulbs or epithelial buccal cells.

Viral infections, leukocyte adhesion deficiency variants,[35] Wiskott Aldrich syndrome,[36] infantile malignant osteopetrosis,[35] and hemocytic lymphohistiocytosis (HLH)[37] have to be excluded in the absence of a specific mutation. Of note, there are rare cases of myeloproliferative disorders other than JMML in young children who carry balanced translocations involving the *GRAF* gene,[38]

ALK gene,[39] *FIP1L1/RARA* fusion gene,[40] or the *NUP98-HOXA9* fusion gene.[41]

Clinical course and prognostic factors

The natural course of JMML varies greatly depending on the genetic abnormality underlying the activation of the RAS/MAPK signaling pathway. However, in most series, the median survival time without hematopoietic stem cell transplantation (HSCT) is approximately one year.[32] Low platelet count, age over two years at diagnosis and high HbF at diagnosis are the main predictors of short survival.[32,34]

Applying microarray analyses on archived material, Bresolin *et al.* demonstrated that JMML cases could be separated into a group with an acute myeloid leukemia (AML)-like and a group with a non-AML-like gene expression profile.[42] There was a significant difference in the 10-year probability of survival after diagnosis between AML-like and non-AML-like patients (7% *vs.* 74%; $P<0.001$).
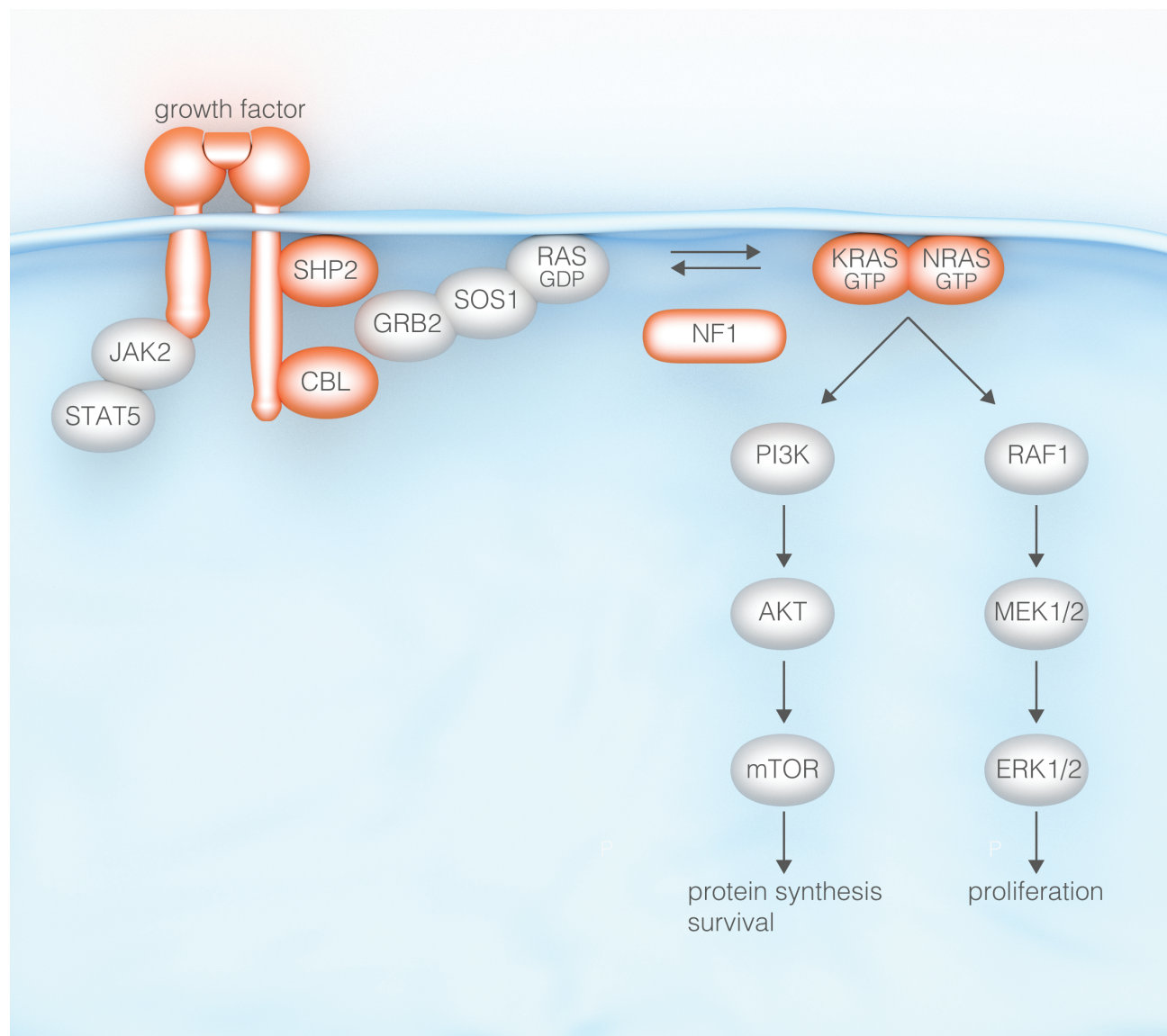
Figure 2. The RAS signaling pathway and genes mutated in juvenile myelomonocytic leukemia (in black).

Olk-Batz *et al.* studied the association of CpG island hypermethylation in JMML leukemic cells with known clinical risk factors.[43] There was a strong association of interior prognosis with older age and elevated HbF at diagnosis. In multivariate models, DNA methylation retained an independent prognostic factor. These data may suggest that patients with a high-methylation phenotype may be good candidates for complementary approaches, e.g. pre-HSCT window therapies with DNA methyltransferase inhibitors.

Cell culture studies and induced pluripotent stem cells

The malignant transformation in JMML takes place at a stage of a committed stem cell that has the ability for myeloid, as well as early B-lymphoid, differentiation, while involvement of T-lymphoid precursors is generally absent.[44,45] When cultured in semisolid media, JMML cells give rise to an excess number of monocyte-macrophage colonies in the absence of added growth factors. This so-called spontaneous proliferation depends on endogenous production of cytokines by monocytes. In addition, JMML myeloid progenitor cells were shown to display a striking hypersensitivity to GM-CSF.[46] In fact, GM-CSF hypersensitivity became the hallmark of the disease, and represented an important diagnostic tool prior to the discovery of the molecular abnormalities of the RAS signaling pathway. Single cell profiling for changes in phosphoproteins demonstrated quite heterogeneous responses for readout of both RAS/MAPK and mTOR activation. However, for a subpopulation of CD33+CD14+CD38low JMML cells, a distinct pattern of STATA5 hyperphosphorylation in response to GM-CSF could be validated.[47,48]

Most recently, induced pluripotent stem cells (iPSCs) from malignant cells of 2 JMML patients with somatic heterozygous p.E76K missense mutations in PTPN11 have been generated.[49] In vitro differentiation of iPSCs produced myeloid cells with increased proliferative capacity, constitutive activation of GM-CSF, and enhanced STAT5/ERK phosphorylation, similar to what has been seen in primary JMML cells. Furthermore, pharmacological inhibition of MEK kinase in iPSC-derived JMML cells reduced their GM-CSF independence. Thus, iPSCs offer the possibility of modeling the disease and exploring targeted therapy.

Juvenile myelomonocytic leukemia with neurofibromatosis type 1

The association between neurofibromatosis type 1 (NF-1) and JMML has long been established. A clinical diagnosis of NF-1 can be made in up to 12% of European[32,34,50] and 4% of Japanese[51] children with JMML. Approximately half of these cases have a parent affected with NF-1. In comparison to the other JMML subtypes, NF-1 is more common among JMML patients diagnosed after the age of five years.[32] In the absence of HSCT, the clinical course of JMML in patients with NF-1 is invariably fatal.

Primary leukemic cells from children with NF-1 show a selective decrease in NF-1-like GAP activity and an increased percentage of RAS in the GTP-bound state.[52] Finding of uniparental disomy (UPD) surrounding the NF-1 locus indicated that mitotic recombination in the leukemia-initiating cell is a crucial leukemic event.[53,54] In a study on 15 children with NF-1 and JMML, two-thirds of

patients had loss of heterozygosity (LOH) at the NF-1 locus, predominantly caused by segmental UPD disomy of large parts of chromosome arm 17q, and in a minority of cases by somatic interstitial deletions, while one-third had compound-heterozygous NF-1-inactivating mutations in leukemic cells.55 In a conditional murine model system, inactivation of somatic Nf-1 induces a myeloproliferative disorder with 100% penetrance and a subacute clinical course with progressive splenomegaly, a shift in hematopoiesis from the marrow to the spleen, and hypersensitivity of myeloid cells to GM-CSF.56

Juvenile myelomonocytic leukemia with somatic PTPN11 mutations

The discovery of heterozygous germline *PTPN11* mutations in NS and in NS with transient myeloproliferation led to the prediction that children with 'non-syndromic' JMML may bear somatic *PTPN11* mutations in their leukemic cells. Subsequently, several groups showed that *PTPN11* is the most common mutated target in JMML with somatic *PTPN11* mutations noted in approximately 35% of JMML cases (Figure 2).[29,57] JMML with *PTPN11* mutation is a rapidly fatal disorder unless the patient undergoes transplantation.

PTPN11 encodes SHP-2, a widely expressed cytoplasmic protein tyrosine phosphatase involved in multiple cell signaling processes. Despite its direct function in protein dephosphorylation, SHP-2 generally plays a positive role in transducing signals initiated from receptor and cytosolic kinases. SHP-2 contains two tandem SH-2 domains at the N-terminus and a catalytic PTP domain at the C-terminus. In its inactive state, PTPase activity is repressed by inhibition of the enzymatic cleft by the N-terminal SH-2 domain.[58] Binding of the SH-2 domain to phosphorylated tyrosine residues induce a conformational shift that relieves the inhibitory interaction between the SH-2 domain and the catalytic PTP domain.

All *PTPN11* mutations identified in JMML or in transient myeloproliferation in Noonan syndrome are missense mutations in the N-terminal SH-2 (exon 3) or PTP interacting surfaces (exon 13), while other exons are commonly mutated in the germline of patients with NS.[30] Functionally, leukemia-associated SHP-2 mutants were shown to have significantly higher phospatase activity than mutants identified in NS,[29,59] although this correlation did not hold true for all the mutant SHP-2 proteins examined.[60] Approximately half of the mice reconstituted with hematopoietic progenitors transduced with leukemic *Ptpn11* mutants succumbed to a severe MPD by seven months after transplantation,[61] in contrast to a later-onset, non-lethal, chronic myelomonocytic hyperplasia observed in a mouse model bearing a common NS germline *PTPN11* mutation.[62]

Juvenile myelomonocytic leukemia with somatic RAS mutations

The cancer-associated somatic heterozygous *RAS* mutations typically introduce amino acid substitutions at positions Gly12, Gly13 or Gln61 and lock Ras in the active GTP-bound state by diminishing the intrinsic Ras GTPas activity and/or by causing resistance to GAPs. In JMML, these somatic gain-of-function mutations occur in *NRAS* and *KRAS* and are found in leukemic cells at diagnosis in 20-25% of cases.[44,50,51,63] Rarely, insertions are noted.[64] For both, *NRAS*- and *KRAS*-associated disease, acquired loss

of the wild-type *RAS* locus is followed by aggressive disease.[65,66]

Juvenile myelomonocytic leukemia with somatic KRAS mutations: children with JMML and heterozygous somatic *KRAS* mutation show a particularly aggressive form of JMML and generally have an urgent indication for HSCT. Similarly, mice expressing oncogenic Kras[G12D] in hematopoietic cells develop a fatal MPD with 100% penetrance that is characterized by leukocytosis, splenomegaly, and anemia, with death at approximately three months of age.[67,68]

There is a puzzling interface between KRAS-associated lymphoproliferative disease (RALD) and JMML with *KRAS* mutation.[69,70] Individuals affected by RALD show overexpansion of lymphocytes with hepatosplenomegaly, lymphadenopathy and autoimmune phenomena like autoimmune hemolytic anemia, thrombocytopenia (Evan's syndrome) and neutropenia. Lanzarotti *et al.* recently presented a case with RALD as initial presentation at five years of age and fatal JMML at 20 years of age.[71] The authors speculate that RALD and JMML are not distinct entities but a continuum with additional genetic or epigenetic events contributing to the clinical phenotype and evolution.

Juvenile myelomonocytic leukemia with somatic NRAS mutations: like KRAS disease, JMML with *NRAS* mutation is a fatal disease in most instances unless HSCT is performed early in the course of the disease. Matsuda *et al.* suggested a mild clinical course for the G12S substitution in a number of children with NRAS disease.[72] Other investigators agreed that, indeed, an occasional patient with JMML and *NRAS* mutations had spontaneous resolution of their myeloproliferative disorder despite persistence of the *NRAS* mutation in hematopoietic cells.[44,70] However, there was no specific genotype-phenotype correlation for the different *RAS* codons. Doisaki *et al.* claimed that 2 of their patients with JMML, *NRAS* mutation and a mild and self-limiting course had somatic mosaicism of their oncogenic *NRAS* mutations.[73] Future genetic and epigenetic studies will have to unravel the unusual clinical courses of these few patients with JMML and *NRAS* mutation. In our own experience, all children with spontaneous resolution of their disease had low HbF levels (see prognostic factors above).

Juvenile myelomonocytic leukemia with Noonan-like CBL syndrome

Based on the observation of UPD 11q in children affected by JMML, homozygous and rarely heterozygous *CBL* mutations were identified in up to 12% of JMML cases.[74-77] Unexpectedly, a high percentage of children with JMML and *CBL* mutations had been noted to have developmental delay, cryptorchidism and impaired growth.[15] In fact, and in contrast to adult MPN, children with JMML and *CBL* mutations were found to have a germline *CBL* missense mutation. Like in NF-1, leukemic cells displayed the germline mutation on one allele and acquired LOH on the other allele.[15,16,76] Children with this germline *CBL* disorder display a NS-like phenotpye (Table 1).[15,78]

CBL, originally discovered as the cellular homolog of the *v-Cbl* oncogene (reviewed by Kales *et al.*[79]), is a member of a family of RING finger ubiquitin ligases that is responsible for the intracellular transport and degradation of a large number of receptor tyrosine kinases but also retains important adaptor functions. It contains a tyrosine kinase binding (TKB) and zinc binding RING finger domain that mediate the E3 ubiquitin ligase activity. These two highly conserved domains are separated by a short linker sequence crucial for ubiquitin ligase activity of CBL. *CBL* mutations reported in myeloid malignancies uniformly affect either the linker region or the RING finger domain. In JMML, mutations are located throughout the linker and RING finger domain (intron 7, exon 8, intron 8, and exon 9). Loss of ubiquitination of activated receptor tyrosine kinases is thought to contribute to the transforming potential of leukemia-associated mutant CBL proteins. Approximately 150 proteins are associated with or are regulated by CBL. Among these proteins is Grb2, an adaptor molecule that binds to CBL and prevents it from binding to SOS, a known guanine nucleotide exchange factor for RAS.

Many children with germline *CBL* mutations and JMML experience spontaneous regression of their myeloproliferation despite the persistence of LOH of the *CBL* locus in hematopoietic cells. Some of these children develop vasculitis-like optic neuritis later in life. Similarly, mice that lacked *Cbl* in T or B cells developed severe vascular lesions and autoimmunity.[80,81] Of note, among children with JMML and *CBL* mutations treated with HSCT there was a high rate of partial rejection with stable mixed chimerism.[15] Since none of these children are currently known to have vasculitis, it is conceivable that stable mixed chimerism is sufficient to improve T- and/or B-cell function. Further studies will have to clarify a possible role of HSCT in JMML with *CBL* mutations. Currently, most children with JMML and *CBL* germline mutations are followed closely in the absence HSCT, which is, however, indicated if chromosomal aberrations occur or the disease progresses rapidly.

Other genetic and epigenetic lesions in juvenile myelomonocytic leukemia

Whole exome sequencing in JMML is characterized by a paucity of gene mutations.[82] The *SETBP1* and *JAK3* genes were the most frequently targeted genes mutated in up to 17% of JMML patients. Mutations were often subclonal and were involved in the progression of leukemia.[82] Genetic alterations in *SRFS2*, *TET2*, *RUNX1*, *JAK2* and *ASXL1* do not play a significant role in JMML.[51,75,83-86] Similarly, inactivation of the *TP53* tumor suppressor gene and internal tandem duplication and activation of the Fms-like tyrosine kinase 3 (*FLT3*) gene are generally absent in JMML.[87,88]

With the paucity of genetic lesions, epigenetic changes such as abnormal DNA methylation pattern are of particular interest. Through studying CpG island hypermethylation it could be shown that a high-methylation phenotype characterized an aggressive biological variant of JMML and was an independent predictor of poor outcome in multivariate models.[43]

Hematopoietic stem cell transplantation

Allogeneic HSCT is still the only curative treatment for most children with JMML, specifically for all children with JMML and NF-1, somatic *PTPN11* mutations, close to all children with somatic *KRAS* mutations, and the vast majority of children with somatic mutations in *NRAS*. HSCT results in a long-term survival of approximately half of the patients.[89-91] Even with HSCT, the malignant JMML

clone is difficult to eradicate, and the post-transplant relapse rate is high.

In a large prospective study of childhood JMML by EWOG-MDS, 100 patients were given unmanipulated HSCT after a preparative regimen with busulfan, cyclophosphamide and melphalan.[89] The 5-year cumulative incidence of relapse was 35%, only age at diagnosis of over four years independently predicted an increased risk of relapse. The 5-year event-free survival rate over all patients was 52%, and did not differ significantly between patients treated with matched family or matched unrelated donor transplant. Japanese investigators chose a preparative regimen consisting of busulfan, fludarabine and melphalan in an attempt to decrease the mortality rate and reduce the risk of graft failure.[92] In the current trial of the Children's Oncology Group (COG), busulfan, cyclophosphamide, and melphalan is randomized against busulfan and fludarabine.[93] Umbilical cord as stem cell source results in post-transplant survival similar to that observed with bone marrow or peripheral stem cells; disease-free survival after a single unmanipulated umbilical cord blood transplant was 43±5%.[91]

There is clear evidence that in JMML, graft-*versus*-leukemia (GvL) effect plays an important role.[94] Re-emerging recipient cells and frank hematologic relapse have been successfully eradicated by immediate withdrawal of ongoing immunosuppressive therapy.[95,96] However, donor lymphocyte infusion in JMML patients who relapsed following HSCT are largely unsuccessful.[95,97] Despite the generally aggressive nature of re-emergence of the malignant clone after transplant, approximately 30% of children have been cured by a second HSCT.[98]

Treatment other than stem cell transplantation

Conventional chemotherapy: intensive chemotherapy prior to HSCT does not reduce the relapse rate and nor does splenectomy.[89,97] Clinical and hematologic responses in JMML have most consistently been attributed to mercaptopurine, administered either as single agent or in combination with low-dose cytarabine. For life-threatening disease, fludarabine in combination with high-dose cytarbine, or clofarabine may give temporary relief.

Epigenetic therapy: the first case report on the DNA-hypomethylating agent azacitidine in a child with JMML, monosomy 7 and *KRAS* mutation described an impressive response with clinical and genetic complete remission.[99] A number of children have been treated off label since,[100] and a phase II study was recently initiated in Europe.

Therapy targeting the RAS/MAPK pathway: initial attempts to develop new pharmacological agents targeted post-translational modifications of RAS proteins required for localization to the inner surface of the plasma membrane. The first obligatory step in this process is addition of a farnesyl moiety catalyzed by the enzyme farnesyl-protein transferase. Since NRAS and KRAS can be geranyl-geranylated when farnesyltransferase is inhibited, the introduction of the farnesyltansferase inhibitor tipifarnib (R115777) in JMML was not successful.[101]

Since the 1990s, downstream drug targets have been studied. RAF-1 inhibitors like sorafenib have a selective sensitivity for most *BRAF* mutations in melanomas, but can otherwise paradoxically activate RAF kinase activity.[102] Selective inhibition of MEK abrogated the myeloproliferative disease in *Nf-1* and *Kras* mutant mice[103,104] suggesting that MEK inhibitors might ameliorate hematologic abnormalities in JMML. Although therapeutic windows for pan inhibitors of the MAPK pathway may be limited,1 early clinical studies in JMML are needed to gain more insight into the specific signaling network of this rare disease.

Outlook for the future

There is the legitimate hope that our increasing knowledge on signaling transduction, feed-back loops, and JMML heterogeneity will soon translate into efficacious treatment with novel drugs. However, clinical studies in this rare disorder with genetically and clinically distinct subtypes will be a major challenge. While robust model systems may help to choose the most promising pharmacological agents for clinical evaluation, physicians need to make every effort to allow their patients to be enrolled in prospective clinical trials.

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