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Molecular pathogenesis of atypical CML, CMML and MDS/MPN-unclassifiable

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Abstract According to the 2008 WHO classification, the category of myelodysplastic/myeloproliferative neoplasms (MDS/MPN) includes atypical chronic myeloid leukaemia (aCML), chronic myelomonocytic leukaemia (CMML), MDS/MPN-unclassifiable (MDS/MPN-U), juvenile myelomonocytic leukaemia (JMML) and a "provisional" entity, refractory anaemia with ring sideroblasts and thrombocytosis (RARS-T). The remarkable progress in our understanding of the somatic pathogenesis of MDS/MPN has made it clear that there is considerable overlap among these diseases at the molecular level, as well as layers of unexpected complexity. Deregulation of signalling plays an important role in many cases, and is clearly linked to more highly proliferative disease. Other mutations affect a range of other essential, interrelated cellular mechanisms, including epigenetic regulation, RNA splicing, transcription, and DNA damage response. The various combinations of mutations indicate a multi-step pathogenesis, which likely contributes to the marked clinical heterogeneity of these disorders. The delineation of complex clonal architectures may serve as the cornerstone for the identification of novel therapeutic targets and lead to better patient outcomes. This review summarizes some of the current knowledge of molecular pathogenetic lesions in the MDS/MPN subtypes that are seen in adults: atypical CML, CMML and MDS/MPN-U.

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Introduction

The 2008 World Health Organization (WHO) classification of haematopoietic and lymphoid tissue tumours includes five subcategories of myeloid neoplasms: (i) myeloproliferative neoplasms (MPNs) (ii) myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB* and *FGFR1* (iii) myelodysplastic syndrome (MDS) (iv) myelodysplastic/myeloproliferative neoplasms (MDS/MPNs) or "overlap MDS/MPN", and (v) acute myeloid leukaemia (AML) [1].

According to Vardiman et al. [1], MDS/MPN are clonal myeloid disorders that characterized, at the time of their initial presentation, by the simultaneous presence of both myelodysplastic and myeloproliferative features, thus preventing them from being classified as either myelodysplastic syndrome (MDS) or myeloproliferative neoplasms (MPNs). These disorders comprise chronic myelomonocytic leukaemia (CMML), atypical chronic myeloid leukaemia (aCML, *BCR-ABL1*-negative CML), juvenile myelomonocytic leukaemia (JMML), and a "by exclusion" subcategory, myelodysplastic/myeloproliferative neoplasms unclassifiable (MDS/MPN-U). The best characterized of the latter conditions is the "provisional" entity defined as refractory anaemia with ringed sideroblasts (RARS) associated with marked thrombocytosis (RARS-T). Of these subtypes, CMML is by

far the most common and as a group, many MDS/MPN cases, and particularly those with aCML, have a poor prognosis and limited therapeutic options [2–6].

Diagnosis based on the 2008 WHO classification is primarily based on leukocyte counts and morphology. Additional disease features regarding genetic data are obtained using classic cytogenetic analysis, fluorescence in situ hybridization (FISH) and/or polymerase chain reaction (PCR), principally to exclude *BCR-ABL1* [7]. The molecular characterization of these conditions has advanced considerably with the use of next generation sequencing technologies, and it has become clear that there is considerable heterogeneity between cases at the molecular level. Furthermore, as detailed below, there are no molecular markers known that are absolutely specific for any MDS/MPN subtype.

This review focuses on current knowledge and challenges related to the pathogenesis of atypical CML, CMML and MDS/MPN-U, as well as the relationship between molecular findings, clinical phenotype and prognosis.

Cytogenetic findings in atypical CML, CMML, and MDS/MPN-unclassifiable

Conventional cytogenetic evaluation and high-resolution single polymorphism genotyping arrays (SNP-A) reveal chromosomal abnormalities in almost two-thirds of patients with aCML, CMML and MDS/MPN-U [8]. The most commonly detected abnormalities are aneuploidies (+8, +9, −7) or deletions (7q−, 13q−, 20q), [9, 10], though none are specific to any disease subtype. A small subset of patients (~1 %) present with reciprocal translocations, which have led to the identification of diverse tyrosine kinase fusion genes which are important to recognise because of the potential for targeted therapy [11–16]. Although the initial working diagnosis for many of these cases may be an MPN or MDS/MPN subtype, often with prominent eosinophilia and lymphadenopathy, many can be correctly assigned to the category "Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB* and *FGFR1*" following cytogenetic and molecular investigation. Under the current classification scheme, however, cases with involvement of other tyrosine kinases may be classified under another disease subtype, e.g. CMML [17].

Molecular findings in atypical CML, CMML, and MDS/MPN-unclassifiable

Until a few years ago, the only known recurrently mutated genes in MDS/MPN were *KRAS* and *NRAS*. Approximately one-third of cases were initially thought to present with mutation in these genes, although the true frequency is now believed to be lower. Subsequently, array-based comparative genomic hybridization (aCGH), genome wide SNP-A, whole genome and full exome sequencing have enabled the identification of >40 novel somatically mutated genes in myeloid malignancies [18, 19]. Analysis of SNP-A data has revealed large regions of acquired uniparental disomy (aUPD) in almost one-third of MDS/MPN cases [8, 20]. Evaluation of these areas of aUPD revealed an association with either gain of function mutations in oncogenes or loss of function mutations in tumour suppressor genes [21–23]. Whole genome and full exome sequencing have led to the extensive characterization of additional recurrent somatic alterations and the central role some of them play in the distinctive features of disease biology [24–26]. However, none of these abnormalities are unique to MDS/MPN, but are also seen in a range of myeloid malignancies such as MPN, MDS and AML. Most of these mutations affect a range of essential, interrelated cellular mechanisms including signalling, RNA splicing, transcriptional control, DNA damage response and epigenetic regulation.

Mutations that activate signalling in aCML, CMML, and MDS/MPN-U

Mutations that activate growth factor signalling pathways constitute the largest category of somatically mutated genes in MDS/MPN. Genes affected include growth factor receptors, downstream signalling components and negative regulators. Overall, about 50 % of CMML cases have mutations in genes that result in activated signalling (Table 1) and the presence of such mutations correlates with more proliferative disease as well as hypersensitivity to granulocyte–monocyte colony-stimulating factor (GM-CSF) [27, 28].

RAS

RAS proteins are membrane-associated GTPases that control the MAP kinase cascade of serine/threonine kinases. The most frequent mutations in *KRAS* and *NRAS* genes occur at codons 12, 31 and 61 [29–31]. Although some earlier series indicate a high frequency of *RAS* mutations in CMML and aCML, more recent studies have indicated that the true frequency of mutations is in the region of 10–15 % [25, 27]. Though these mutations may confer a competitive advantage to haematopoietic stem cells [32], they appear to be rather secondary events at least in CMML [33] and are often associated with an MPN-like phenotype with monocytosis [34].

JAK2 and MPL

The *JAK2*V617F mutation is found infrequently in aCML cases, [35–37], but is seen in up to 8–10 % of CMML cases

Table 1 Affected pathways and genes in patients with aCML, CMML and MDS/MPN-U

Cellular pathway	Gene	Frequency				Prognosis
		aCML	CMML	MDS/MPN-U	RARS-T	
Signalling	*KRAS*	10 %	7–11 %			
	NRAS	10–30 %	4–16 %	10 %		
	JAK2	4–8 %	10 %		60 %	
	MPL				5–20 %	
	CBL	8 %	10–20 %	>10 %		
	KIT	>5 %	>5 %			
	FLT3	5 %	1–3 %	3 %		
	CSF3R	<10 %	4 %	Rare		
	SETBP1	25 %	6–15 %	10 %		
	NOTCH2		Rare			
	PTPN11	Rare	Rare			
	NF1	Rare	Rare			
RNA splicing	*SF3B1*		6 %		80 %	Favourable
	SFSR2		36–46 %		~1 %	Unfavourable
	U2AF35		5–15 %			Unfavourable
	ZRSR2		8–10 %		~1 %	
Transcription	*RUNX1*	6 %	15–20 %	14 %		Unfavourable
	CEBPA	4 %	4–20 %	4 %		Unfavourable
	NPM1	1 %	1–6 %	3 %		Unfavourable
	WT1		~1 %	~1 %		Unfavourable
	TP53	Rare	>1 %	Rare		Unfavourable
Cohesin complex	*STAG2*		~10 %			Unfavourable
DNA methylation	*DNMT3A*	Rare	5–10 %	4 %	17 %	Unfavourable
	TET2	30 %	50–60 %	30 %	26 %	
	IDH1/2	Rare	1–6 %	5–10 %		Unfavourable
Histone modifications	*ASXL1*	20–30 %	43–44 %		10	Unfavourable
	EZH2	13 %	6–10 %	10 %		Unfavourable
	SUZ12	Rare	>5 %	Rare		
	EED	Rare	>5 %	Rare		
	UTX		8–9 %			

[27, 36] and in almost 60 % of patients with RARS-T [38–40]. JAK2 is a non-receptor tyrosine kinase that plays essential role in transducing signals from several cytokine receptors that are critical for normal myelopoiesis, notably the erythropoietin receptor (EpoR), the thrombopoietin receptor (TpoR, encoded by the *MPL* gene), and the granulocyte colony-stimulating factor receptor (G-CSFR). Activated JAK2 phosphorylates specific tyrosine residues on itself (autophosphorylation) and other proteins (trans-phosphorylation), thus activating specific signalling cascades involving MAPK, PI3K and STAT proteins [41]. The V617F mutation results in activation of JAK2 signalling in the absence of class I receptor stimulation, leading thus to constitutive activation of signal transduction pathways and altered transcriptional activity [42].

Indirect dysregulation of JAK2 signalling can arise by several mechanisms, the most common being activating mutation of *MPL* gene, with the *MPL*W515L/K mutations being seen in almost 25 % of RARS-T patients [43].

CBL

CBL mutations have been reported in approximately 10 % of cases of CMML and aCML but are possibly less frequent in MDS/MPN-U [22, 27, 44–46]. CBL is a well-characterized protein that plays both positive and negative regulatory roles in tyrosine kinase signalling. In its positive role, CBL binds to activated signalling complexes and serves as an adaptor by recruiting downstream signal transduction components. However, the CBL RING domain has E3 ligase catalytic activity and ubiquitinylates activated target proteins on lysine residues. This negative regulatory role of CBL is best characterized for receptor tyrosine kinases, in which lysine ubiquitinylation serves as

a signal that triggers internalization of the receptor/ligand complex and subsequent recycling or proteasomal degradation in endosomes. Furthermore, CBL also targets other proteins for degradation, most notably STAT5, a key downstream component of JAK2 signalling. *CBL* mutations are almost always missense changes in exons 8 and 9 that inactivate the E3 ligase activity. The loss of catalytic activity but retention of other functions gives rise to a gain of function and hypersensitivity to multiple cytokines [22, 46].

KIT

KIT mutations are found infrequently in CMML and aCML cases ($\sim$1–4 %) [27, 33] and usually in cases with overt or undiagnosed systemic mastocytosis with an associated clonal haematological non-mast cell lineage disease [47].

The *KIT* proto-oncogene encodes a type III receptor tyrosine kinase (KIT). The ligand for KIT is stem cell factor (SCF), a haematopoietic cytokine, which plays an important role in haematopoietic cell survival, proliferation and differentiation [48, 49]. Activated KIT affects downstream a number of signal transduction pathways, including those involving RAS/MAPK/ERK1-2, PI3K, the SRC family and PLC-γ signal. Aberrant activation of KIT similarly leads to up regulation of target signalling pathways [50]. The *KIT*D816V mutation is usually associated with systemic mastocytosis with or without eosinophilia when present in CMML cases [51, 52].

FLT3

Activating mutations or internal tandem duplications in *FLT3* (*fms-like tyrosine kinase III*) gene have been described infrequently in CMML [27, 53], in 5 % of cases with aCML and 3 % of patients with MDS/MPN-U [54]. Furthermore, occasional cases with *FLT3* fusions have been described in patients with aCML that are responsive to FLT3 inhibitors [55, 56]. FLT3 is a transmembrane tyrosine kinase that belongs to the class III receptor tyrosine kinase family, which also includes KIT, PDGFRB and FMS. FLT3 is expressed by immature hematopoietic cells and is important for the normal development of stem cell and immune system [57, 58]. *FLT3* internal tandem duplication generates a CMML phenotype in mice, yet *FLT3* mutations are very rare in CMML (about 3 %) compared to AML [59].

CSF3R

Oncogenic mutations in the receptor of the granulocyte colony-stimulating factor 3 (*CSF3R; G-CSFR*) have been reported to be frequent in chronic neutrophilic leukaemia (CNL), a rare MPN subtype sharing overlapping features with aCML [6, 60, 61]. Maxson et al. [60] reported that $\sim$50–60 % of patients with CNL or aCML harbour mutations in the *CSF3R*, whereas Pardanani et al. [61] and Wang et al. [6] showed that *CSF3R* mutations were essentially restricted to CNL and absent in aCML and MDS/MPN-U.

CSF3R is the transmembrane receptor of the granulocyte colony-stimulating factor 3 and is believed to play an essential role in the growth and differentiation of granulocytes [62, 63]. Under normal circumstances CSF3 (better known as G-CSF) binds to CSF3R and promotes growth and survival of myeloid precursor cells, which ultimately differentiate into neutrophils. Deletion of *CSF3R* leads to neutropenia in murine models [64]. CSF3/CSF3R signalling affects downstream the JAK/STAT signalling pathway and the SRC family kinase signalling through activation of LYN tyrosine kinase [65–67]. Mutations in *CSF3R* fall into 2 types: nonsense or frameshift mutations leading to premature truncation of the cytoplasmic tail of the receptor (truncation mutations) and point mutations in the extracellular domain of *CSF3R* (membrane proximal mutations). The most common *CSF3R* mutation in CNL/aCML is T618I, a membrane proximal mutation that strongly activates the JAK/STAT pathway and along with the other membrane proximal mutations, is sensitive to JAK2 inhibitors such as ruxolitinib in model systems [60, 68]. On the other hand, *CSF3R* truncation mutations (which are often seen in combination with membrane proximal mutations) may be sensitive to SRC kinase inhibitors, such as dasatinib [60, 68, 69]. Whether JAK2 or SRC inhibitors are of benefit to patients with *CSF3R* mutations remains to be investigated in appropriate clinical studies. The fact that *CSF3R* mutations occur frequently in CNL and probably, occasionally in aCML, identifies a novel diagnostic criterion for these diseases and suggests the need for a careful pathological analysis to distinguish putative "*CSF3R* positive aCML" from CNL [70]. Whether this distinction is of any clinical significance remains to be determined.

Mutations in *CSF3R* have been previously reported in patients with severe congenital neutropenia (SCN), on a background of inherited *ELANE*, *HAX1* and *G6PC3* gene mutations, especially when this disease progresses to AML [71, 72]. Furthermore, Kosmider et al. [73], reported variant *CSF3R* somatic mutations, other than the ones found in CNL/aCML, in 4 % of CMML cases, in which they preferentially occur in the context of an *ASXL1* gene mutation and they are being associated with poor prognosis.

SETBP1

Recurrent mutations of *SETBP1* gene have been identified in almost 25 % of aCML patients [25], and less frequently

in MDS/MPN-U (10 %) and CMML (6–15 %), in which they are often associated with *ASXL1* and *CBL* mutations [25, 74–76], *SETBP1* is also mutated in occasional JMML cases [77, 78], 1.7–7 % of secondary AML arising from MPN or MDS [75, 76] and in Schinzel-Giedion syndrome, a rare genetic disease characterized by congenital malformations, mental retardation, and a high prevalence of epithelial tumours [25, 79]. Furthermore, *SETBP1* has been identified as a partner gene in rare chromosomal translocations involved in acute lymphoid and myeloid leukaemias [80, 81].

SETBP1 encodes SET binding protein 1, a protein that is localized predominantly in the nucleus and is expressed in haematopoietic stem/progenitor cells and also in committed progenitors [82, 83]. SET plays a role as a negative regulator of the tumour suppressor protein phosphatase 2A (PP2A) [83]. SETBP1 protects SET from proteasomal degradation, thus increasing the amount of SET available to repress the activity of PP2A [82]. The somatic mutations seen in myeloid disorders are identical to those seen in Schinzel-Giedion syndrome, and are tightly clustered and disrupt a consensus binding motif that binds an E3 ubiquitin ligase when phosphorylated. Piazza et al. and Makishima et al. [25, 84] have shown that mutant SETBP1 conferred overall diminished PP2A activity, which leads to increased self-proliferation. However, it is not clear if this is the main mechanism underlying *SETBP1* oncogenic activity. It has also been shown that SETBP1 can directly activate transcription of the *HOXA9* and *HOXA10* genes in both human and mouse myeloid progenitors, promoting increased self-renewal of progenitor cells [85]. Potentially, of relevance to this is the fact that SETBP1 shows regions of homology with several chromatin modifying factors. Finally, transcriptional profiling indicates that a significant number of TGF-β target genes are differentially expressed in aCML cells with mutated and wild-type *SETBP1* [25]. Thus, there are a number of potential mechanisms by which mutant SETBP1 might drive myeloproliferation and further work is needed to understand.

SETBP1 and *CSF3R* mutations are not mutually exclusive in aCML/CNL and CMML [60, 61, 68, 73]. In CMML, mutations on *SETBP1* gene might complement *CSF3R* mutations and associated with poor prognosis on an *ASXL1* background [73].

NOTCH pathway

Rare inactivating mutations in NOTCH2 and downstream NOTCH effectors (*NCSTN*, *APH1* and *MALM*) have been reported in CMML. Although, abrogation of NOTCH signalling leads to an MDS/MPN phenotype in murine models, its relevance to human disease is still unknown [86].

PTPN11

PTPN11 gene encodes for the SHP2 (Src-homology-2 domain containing protein tyrosine phosphatase), a non-receptor protein tyrosine phosphatase (PTPase) that mediates signalling from activated growth factor receptors to RAS and other signalling components [87, 88]. Mutations in *PTPN11* cause Noonan syndrome (NS), a developmental disorder characterized by cardiac and skeletal defects [89, 90]. NS is also associated with a spectrum of haematological disorders, including juvenile myelomonocytic leukaemia (JMML). Mutations in the *PTPN11* gene cause a gain of function of the SHP2 protein. This results in activation of the guanine nucleotide exchange factors (GNEFs), that are necessary for the conversion of GDP-RAS into GTP-RAS and subsequently to the constitutively activation of RAS. Tartaglia et al. [91] observed somatic mutations in exons 3 and 13 of the *PTPN11* gene in 35 % of the JMML patients without Noonan syndrome, however, CMML and other MDS/MPN patients are only rarely found to have *PTPN11* mutations [92, 93].

NF1

Mutations of the *NF1* gene reported from Niemeyer et al. [94] in 11 % of JMML patients, who had clinical signs of neurofibromatosis type 1. The *NF1* gene is a tumour suppressor gene encoding for neurofibromin, and is a GTPase activating protein hydrolysing GTP-RAS into GDP-RAS [95]. Primary cultures from children with JMML that carried *NF1* mutations showed a reduced neurofibromin activity, resulting in an elevated GTP-RAS activity and aberrant growth in haematopoietic cells [96]. *NF1* deletions and mutations are seen in about 5 % of myeloid malignancies, including some cases of CMML [97].

Mutations that affect RNA splicing

RNA splicing machinery components are another class of mutational targets, revealed initially through exome sequencing studies in MDS [26, 98]. RNA splicing is the mechanism through which a pre-messenger RNA (pre-mRNA) is processed by removing introns and fusing exons into a mature protein encoding mRNA (Fig. 1). The 5′-mRNA splice site of an upstream exon is fused to the 3′-mRNA splice site of the downstream exon in spliceosomes. Even small changes in the spliceosome complex can alter the specificity of splicing and lead to changes at protein level [99]. The spliceosome is a complex that consists of five small nuclear ribonucleoproteins (snRNPs) and between 100 and 300 associated proteins [100]. The spliceosome is assembled via sequential binding of snRNPs to the pre-

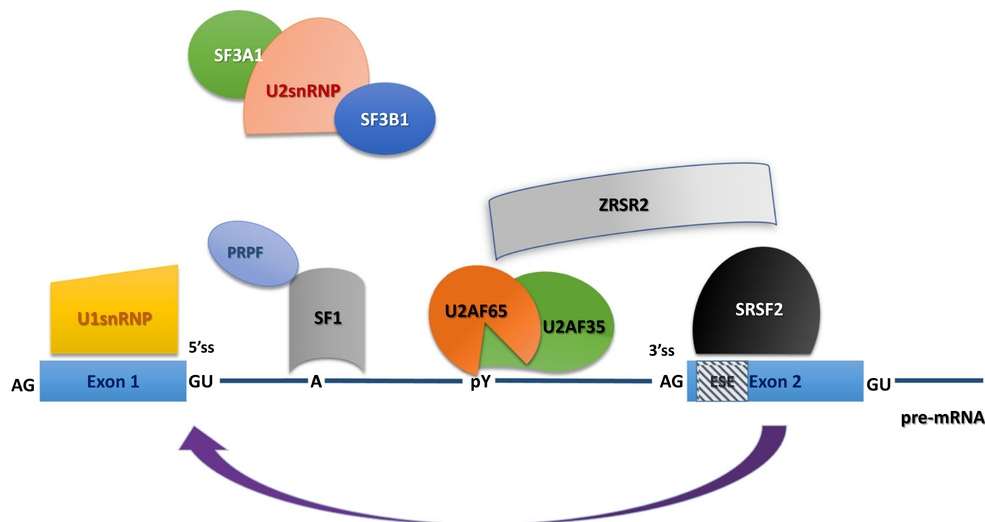

Fig. 1 Somatic mutations affect genes involved in RNA splicing. Recurrent mutations in components involved in the recognition and processing of 3′-mRNA splice sites are very common in MDS/MPN. These mutations can be either gain of function through exon skipping or alternative splicing or loss of function by intron retention of target genes. The spliceosome is assembled via sequential binding of snRNPs to the pre-mRNA and splicing is initiated by the recognition of 5′splice site by U1 snRNP, and the U2-auxiliary factor (U2AF) to the 3′ splice site. The U2AF protein is a heterodimer consisting of a U2AF35 and a U2AF65 subunits. Furthermore, the splicing factor (SF1) is thought to bind the branchpoint sequence early, through its component SF3B1, and can bridge the 5′splice site upstream to the 3′splice site of the intron and protect the region before the splicing reaction. The complex that is formed by the U2AF35/65 heterodimer, the Zinc finger RNA binding motif and serine/arginine rich 2 (ZRSR2), and one of the serine/arginine rich splicing factors, SRSF1 or SRSF2, binds to the polypyrimidine tract, which is located between the branchpoint and the 3′splice site. SRSF1 and SRSF2 are involved in the removal of introns from the primary transcript and also influence patterns of alternative splicing, thus playing a particularly crucial role in the regulation of this process

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Remarkably, mutations in components involved in the recognition and processing of 3′-mRNA splice sites are very common in MDS and MDS/MPN. Approximately 60 % of CMML cases harbour mutations on splicing machinery components [27]. The most mutated splicing gene among CMML patients is *SRSF2* (∼50 %) [27], with a further 20 % of patients carrying mutations in other splicing complex genes including *ZRSF2, SF3B1, U2AF35, U2AF65* and *SF3A1* [98, 104–107]. The *SRSF2* mutations cluster to the hotspot residue Pro95 and are frequently associated with *TET2* mutations, particularly in CMML [27, 106, 108]. Mutations in *ZRSF2* or *SF3B1* genes affect <10 % of CMML patients [26, 109]. Furthermore, *SF3B1* mutations are seen in almost 72–75 % of patients with RARS-T [98, 110]. A characteristic feature of RARS/RARS-T is the presence of abnormal sideroblasts characterized by iron overload in the mitochondria. The *SF3B1* mutations seem to be associated with altered iron distribution characterized by coarse iron deposits compared with wild-type RARS patients, and lead the formation of morphological feature of ring sideroblasts [111], possibly as a consequence of alterations in splicing of *SLC25A37*, a crucial importer of Fe^{2+} into the mitochondria [112].

Mutations in splicing components are usually mutually exclusive [26], suggesting either functional redundancy or a combined lethal effect. Apart from the specific association between *SF3B1* mutations and ringed sideroblasts, other more subtle differences have been noted with regard to patterns of genes that are co-mutated in cases with splicing component mutations, implying that the consequences of mutations in different splicing genes are not identical [113].

Functional studies of mutant U2AF35 on model systems have confirmed global impairment of splicing

including intron retention, induction of mRNA surveillance pathways and growth impairment [26, 109, 114]. The disturbed spliceosome function could also lead to genomic instability [115], and deregulation of the epigenetic machinery [116]. It is currently unclear whether the critical effects of such mutations are indeed global or if they have molecular and cellular consequences only for a small subset of genes.

Mutations that affect transcription and DNA damage response

RUNX1

Mutations in the *RUNX1* (also named *AML1* or *CBFA2*) transcription factor are found in 15–37 % of CMML patients [27, 92, 117, 118] and less frequently in other MDS/MPN cases [117]. *RUNX1* is located on chromosome band 21q22.12 and encodes the alpha subunit of the core-binding factor (CBF) complex [119]. This complex activates and represses transcription of key regulators of growth, survival and differentiation pathways. *RUNX1* is one of the most frequent targets of chromosome translocations in leukaemia and somatic mutations have also been identified, especially in AML M0 subtype [120, 121], *de novo* high-risk MDS [122], and therapy-related MDS/AML [123, 124]. *RUNX1* mutations seem to be associated with poor prognosis in CMML and higher risk of AML progression [117, 118].

CEBPA

CEBPA mutations have been described in 4–20 % of CMML patients but seem to be rare events in other MDS/MPN cases [117, 125]. The *CEBPA* gene, located on chromosome band 19q13.11, encodes the transcription factor CCAAT/enhancer-binding protein-alpha which is essential for normal differentiation of granulocytes [126]. The involvement of *CEBPA* in leukaemogenesis has been confirmed in many studies, with inactivating mutations reported predominately in AML M0, M1 and M2 [127, 128]. Mutations are usually acquired but can occasionally be inherited [129].

NPM1

NPM1 (Nucleophosmin 1, also known as B23) mutations have been described in about 3 % of MDS/MPN patients [117] and in 1 % of CMML patients [27]. The *NPM1* gene, is located on chromosome band 5q35.1 and encodes a phosphoprotein which moves between the nucleus and the cytoplasm. NPM1 is involved in cellular activities related to both proliferation and growth-suppression pathways. It is thought to be involved in regulation of the ARF/p53 pathway [130, 131].

TP53

Mutations on *TP53* (tumour protein p53) are uncommon in MDS/MPN and aCML, but have been described in about 1 % of CMML cases [27, 132]. The *TP53* gene, which is located on chromosome band 17p13.1, encodes a tumour suppressor protein that has transcriptional activation, DNA binding, and oligomerization domains. TP53 plays a major role in various biologic activities, including the control of cell cycle checkpoints and apoptosis [133]. *TP53* mutations occur occasionally in MDS/MPN but are more common in high-risk/therapy related MDS, MDS-derived leukaemia, and in the context of complex chromosomal abnormalities including 17p− and they are associated with poor prognosis [134, 135]. MDS/MPN with an isolated isochromosome 17p leading to *TP53* haploinsufficiency may be a distinct disease entity with increased risk of AML progression [136].

Cohesin complex

Mutations in several members of the cohesin complex (*SMC1*, *SMC3*, *RAD21* and *STAG*) have been reported in myeloid neoplasms, including 5–10 % of CMML or MDS/MPN cases [24, 137]. Cohesin is a highly conserved multimeric protein complex composed of four core subunits, i.e. SMC1, SMC3, RAD21 and STAG proteins, along with a number of regulatory molecules. This multimeric complex is involved in sister chromatid separation during cell division, post-replicative DNA repair, and regulation of global gene expression through long-range *cis*-interactions [24, 138, 139]. Combined mutations of cohesin components are also found in 10–15 % of AML and MDS cases, in a mutually exclusive manner.

Mutations that affect the epigenetic regulation of gene expression

Epigenetics refers to changes in phenotype or gene expression that are heritable through cell division but are caused by mechanisms other than changes in the underlying DNA sequence. Mechanistically epigenetics generally refers to DNA methylation of CpG dinucleotides, a modification associated with gene silencing, or histone tail modifications that are associated with transcriptional activation or repression (Fig. 2). Mutations in genes known or suspected to encode proteins involved epigenetic regulation are very common in MDS/MPN.

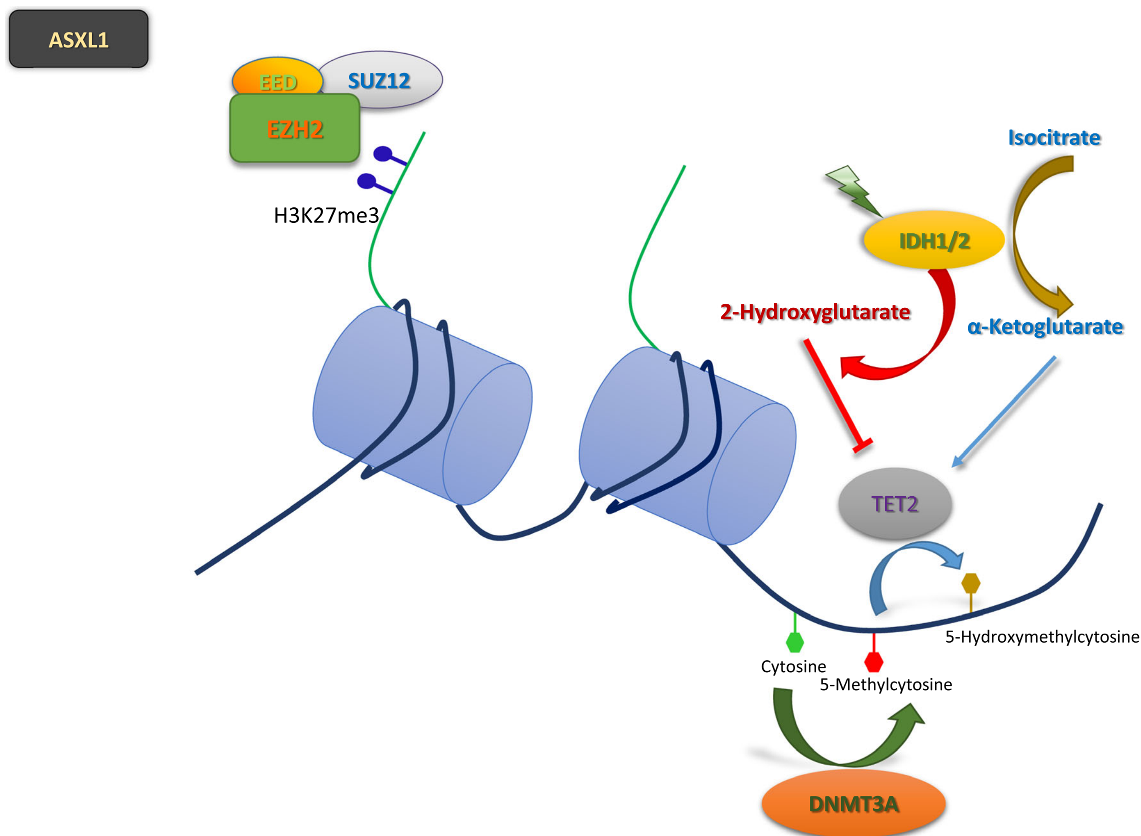

Fig. 2 Somatic mutations affect genes involved in epigenetic regulation. Recurrent mutations affecting genes involved in histone modifications and DNA methylation have been described in a range of myeloid malignancies. **a** Histone modifications. Loss of function mutations affect the PRC2 members *EZH2*, *EED* and *SUZ12*. PRC2 complex places methyl- groups on the H3K27 residues, a process associated with repression of gene transcription. *ASXL1* is member of PRC1 complex and appears to play a role in the recruitment of the PRC2 complex to its target sequences, while it seems to be involved in the control of chromatin structure and is believed to be a component that deubiquitinates histone H2AK119. Mutations of either *ASXL1* or *EZH2* lead to loss histone H3K27 methylation regulation, thus resulting in non-regulated transcriptional activation and associated with poor prognosis. **b** DNA methylation. Mutations affect genes involved in DNA methylation, i.e. cytosine modifications. Specifically, in *DNMT3A* gene, a member of the DNA methyltransferase proteins that enzymatically add a methyl group to 5′ cytosine in the CpG dinucleotides, have been described mutations of unclear function. The removal of the methyl group from the 5′ cytosine nucleotides can be actively mediated by the TET family proteins (TET1, TET2 and TET3) and TET2 participates in the conversion of 5-methylcytosine to 5-hydroxymethylcytosine. Loss of function mutations has been identified in *TET2* gene. Finally, TET2 function depends on α-ketoglutarate for this reaction, which is produced by IDH1 and IDH2. Gain of function mutations has been described in myeloid malignancies the mutants exhibit a neomorphic function characterized by aberrant production of 2-hydroxyglutarate which inhibits TET proteins directly and leads to a reduction of 5hmC levels and the presence of widespread promoter hypermethylation

DNA methylation

DNMT3A (DNA-methyltransferase 3A) is a member of the DNA methyltransferase proteins that enzymatically add a methyl group to 5′ cytosine in the CpG dinucleotides [140]. Removal of the methyl group from the 5′ cytosine nucleotides can be actively mediated by the (Ten-Eleven Translocations) TET family proteins (TET1, TET2 and TET3). Specifically, TET2 participates in the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) [141]. TET2 function depends on α-ketoglutarate (aKG) for this reaction, which is produced by Isocitrate Dehydrogenase 1 and 2 (IDH1 and IDH2) [142, 143]. IDH1/2 normally catalyzes the oxidative decarboxylation of isocitrate to aKG (the third step in the Krebs cycle), but IDH mutants exhibit a neomorphic gain of function characterized by aberrant production of 2-hydroxyglutarate (2HG). 2HG, in turn, inhibits TET proteins directly, which leads to a reduction of 5hmC levels and the presence of widespread promoter hypermethylation [41, 141]. IDH1/2 and TET2 mutations are mutually exclusive and associated with similar epigenetic defects [141].

TET2 mutations are very common and found in 40-61 % of CMML patients and around 30 % of aCML and MDS/MPN-U [18, 27, 144–150]. Mutations affecting *DNMT3A* and *IDH1/2* are found less frequently in CMML

(around 5 % and 1–6 % respectively), MDS/MPN-U (4 % and 5–10 % respectively); notably, *DNMT3A* mutations have been reported in about 17 % of patients with RARS-T [110], but are rather rare events in aCML [27, 151–153].

Histone modifications

EZH2

EZH2 (Enhancer of the Zeste Homolog 2), is a well-characterized gene that encodes the catalytic subunit of the polycomb repressive complex 2 (PRC2), the highly conserved histone H3 lysine 27 methyltransferase (H3K27) that influences stem cell renewal by epigenetic repression of genes involved in cell-fate decisions. Mutations of *EZH2* gene are loss of function, are seen in about 10 % of MDS/MPN cases and appear to be associated with poor prognosis [21, 27]. Inactivating mutations affecting other PRC2 complex components *EED* and *SUZ12* are less frequent (>5 %), while mutations in the gene encoding for the H3K27 demethylase *UTX*, have also been identified in up to 9 % of CMML patients [23, 153].

ASXL1

ASXL1 (Addition of sex combs-like 1) plays a role in the recruitment of the PRC2 complex to its target sequences, as well as being a component of a complex that deubiquitinates histone H2A lysine 119 (H2AK119) [154].Mutations of *ASXL1* gene are loss of function mutations that promote myeloid transformation through loss of PRC2-mediated gene repression [155]. Clonal analyses suggest that *ASXL1* mutations could the initial driving event in CMML in many cases, and the fact that concomitant *ASXL1* and *TET2* mutations are less frequent than expected, suggests that they represent independent, though synergistic, pathogenic mechanisms in CMML. *ASXL1* is the most frequently mutated histone modifying enzyme in CMML (40 % of cases) [27, 156].

Genomic findings and clinical implications

The mutational profile of adult MDS/MPN subtypes is summarised in Table 1. It is clear from this table that our knowledge of genes mutated in CMML is good, but further systematic and collaborative studies are required to define the mutational spectrum of rarer MDS/MPN entities. Furthermore, at least with current knowledge, mutational analysis does help in diagnosing specific MDS/MPN subtypes, although analysis of *SETBP1* and *CSF3R* might help to distinguish aCML from CNL [70].

In terms of prognostic significance of molecular aberrations, again the most complete data come from analysis of CMML. In a landmark targeted resequencing study of 18 genes, Itzykson et al. [27] showed that mutations of *ASXL1* and *SRSF2* were of adverse prognostic significance on univariate analysis but only *ASXL1* remained significant on multivariate analysis in hypomethylating agents naïve and treated patients. The adverse significance of *ASXL1* mutations has been confirmed in a larger collaborative study [157]. Other studies in myeloid disorders, and particularly MDS, have implicated several genes to be adverse prognostic factors with *ASXL1*, *RUNX1*, *EZH2* and *TP53* probably emerging as the most consistent between studies [18, 113, 158]. It is likely that mutations in these genes, and probably others [18], will also emerge as adverse prognostic factors in MDS/MPN. Much larger cohorts, however, will be required to define the clinical significance of uncommon abnormalities. For rarer disease subtypes (aCML, MDS/MPN-U) this will require international collaborative studies with careful and probably centralised morphological and clinical review to accurately compare somatic genotype to phenotype. In addition, the prognostic significance of cytogenetic abnormalities detected by conventional cytogenetics or SNP-A should be considered, with trisomy 8, complex karyotype (≥3 abnormalities), and abnormalities of chromosome 7 being considered as high-risk elements to be considered alongside clinical findings [159].

In addition to providing an in-depth assessment of mutations occurrence in CMML, Itzykson et al. [33] have also studied the clonal architecture of this disease. According to the model they propose, early dominance of the mutated clone, in particular in the presence of a *TET2* mutation, accounts for the accumulation of monocytes in CMML. They also demonstrated by mutation-specific discrimination analysis of single-cell derived colonies that these mutations create a state of early clonal dominance in which secondary mutations are acquired in a linear manner with limited branching of clones from the main dominant clone. This early clonal dominance could distinguish CMML from other myeloid malignancies with similar genetic background [33]. It is currently unclear why individuals with clonal haemopoiesis driven by mutant *TET2* may develop different myeloid malignancies, e.g. MPN, MDS/MPN or MDS but important contributory factors are likely to be inherited factors along with the chance of acquisition of specific secondary phenotype-driving mutations.

Apart from mutations in specific genes the overall somatic complexity, i.e. the number of somatically mutated genes has emerged as an important factor in CMML with more mutated driver genes being associated with inferior survival [27]. Similar studies have shown that somatic

complexity is associated with adverse survival in other myeloid disorders, e.g. MDS [113] and myelofibrosis [160] and these molecular findings mirror the well-established fact that cytogenetic complexity is a poor prognostic factor [159]. Although screening for mutations in multiple genes is now feasible on a routine basis by second generation sequencing, it remains to be defined how many and which genes should be screened to provide clinically useful and cost-effective information for the appropriate clinical management of MDS/MPN patients.

Conclusions

MDS/MPN represents a class of diseases with considerable genetic and epigenetic complexity and heterogeneity. Recent technologies, including genome wide SNP-A karyotyping and next generation sequencing have revealed a substantial number of genes that are affected and have helped in the elucidation of the clonal nature of these diseases. *TET2*, *ASXL1* and *SFRS2* represent the most commonly mutated genes but it seems unlikely that a molecular definition of disease subtypes will emerge that maps to the current WHO-defined entities. The presence of combinatorial mutations suggests the need for therapeutic approaches based on the molecular profile of individual patients, while further functional genomic studies will provide insights and better understanding on the molecular complexity of this disorder.

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Conflict of interest The authors have no relevant conflicts of interest.

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