Clonal evolution: a landmark of MPN evolution

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Myeloproliferative Neoplasms comprise a heterogeneous group of diseases characterized over the past two decades by the acquisition of somatic mutations in hematopoietic stem cells, leading to a pre-leukemic state. The natural history of the disease is marked by the sequential acquisition of genetic events that play an essential role in the phenotype, evolution and response to treatment of the disease. Indeed, disease progression is as heterogeneous as the variety of genetic abnormalities found in individual patients, some of whom lead to disease evolution with a pejorative prognosis, while others persist in a benign manner. In order to better explore these questions, it is important to better understand: (1) the genetic structure of the tumor through dynamic reconstruction of clonal architecture (2) the factors favoring the development of certain clones and their expansion, some of which are governed by Darwinian laws. These parameters could help explain the heterogeneity between MPN patients with similar genetic profiles, and prevent the emergence of clones identified as aggressive by the use of innovative therapeutic strategies targeting new pathways to prevent early transformations in poor prognosis MPN subtypes.

KEYWORDS

myeloproliferative neoplasms, additional mutation, clonal evolution, inflammation, selection

Introduction

BCR-ABL1-negative myeloproliferative neoplasms (MPN) include essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (MF) ([1], [2]). They represent chronic hematological malignancies whose major risk is the occurrence of thrombosis, but they can also evolve into secondary myelofibrosis or acute leukemia in 10 to 30% of patients ([3]). There is a considerable heterogeneity in the disease evolution among patients, which is not clearly understood and cannot be accurately predicted. In the current state of knowledge, molecular abnormalities appear to be one of the main parameters associated with this heterogeneity. MPN are characterized by the acquisition in the hematopoietic stem cells of mutations that activate not only the JAK2/STAT5 pathway

but also STAT3 and, either in parallel or consecutively, PI3K-AKT and MEK-ERK pathways. These mutations, considered as priming the phenotype and called "driver mutations", affect the *JAK2*, *MPL* or *CALR* genes (4–9). Over the course of the disease, some patients may acquire one or multiple additional mutations that can be part of the MPN clone or not. Therefore early stages of MPN are usually tumors with a simple molecular profile that may acquire a much more complex clonal architecture over time. An important question is to understand how these tumors evolve at the genetic level which was reviewed elsewhere (10). The aim of this review is to better describe how the tumor develops a complex molecular profile and to highlight the main events and environmental factors that reshape the genetic structure of the MPN tumor cells over time.

An oligoclonal disease

Even before the discovery of the $JAK2^{V617F}$ mutation (4), it has been acknowledged that MPN are clonal (11, 12). An important question has long been to determine the timing of the acquisition of the mutations that trigger the disease. Indeed, several studies have shown that it is possible to detect mutations associated with myeloid hemopathies in the blood of healthy subjects, defining the notion of clonal hematopoiesis of undetermined potential (CHIP), among which the $JAK2^{V617F}$ mutation is quite frequently found (13, 14). Moreover, several studies have reported the possible detection of $JAK2^{V617F}$ mutation several years before the clinical development of an MPN (15, 16), suggesting that this mutation does not give an intense proliferative advantage as previously thought, and that it may take decades for the disease to develop. While one study reported that the $JAK2^{V617F}$ mutation could already be present in cord blood (17), the probable acquisition of this mutation during childhood or the prenatal period was suggested in several MPN patients (18, 19). In these studies, the latency period between the acquisition of $JAK2^{V617F}$ mutation and the diagnosis of MPN was several decades. It therefore appears that clones harboring MPN driver mutations can arise and likely take many years to expand to a clinically detectable level, possibly depending on the influence of intrinsic and extrinsic factors.

In addition to driver mutations, mutations considered "additional" can also be detected, targeting other genes involved in various cellular processes, all involved in the regulation of gene expression: intracellular signalling pathways, epigenetics (DNA methylation, post-transcriptional histone modifications), transcription factors, RNA splicing. Thus, from the early 2010s it appeared that MPN were less likely monoclonal diseases with accumulation of mutations in a single clone, but more likely oligoclonal with the coexistence of several molecularly distinct clones (20, 21). However, it was rapidly shown that the clonal origin of mutations is complex, as 2 different patterns can be distinguished: on the one hand, patients who first acquire a mutation in a driver gene and then additional mutations, and on the other hand, patients who acquire driver mutations within cells that have already acquired a mutation in genes such as *TET2* or *DNMT3A*.

The presence of additional mutations has been shown to have a significant impact on patients' prognosis. Indeed, the

accumulation of several mutations in itself represents a poor prognostic factor in MPN patients, the number of mutations at diagnosis being associated with the risk of transformation (3, 22). Moreover, certain mutations are particularly associated with a poor prognosis. A group of high-molecular-risk mutations affecting the *ASXL1*, *EZH2*, *SRSF2* and *IDH1/2* genes has been defined (23), to which have been added mutations in the *TP53* (24), *NRAS/KRAS* (25, 26) and *NFE2* (27) genes. There are now several prognostic scores for MPN that have incorporated molecular data. Recently the presence of some mutations was shown to have an impact on treatment sensitivity. Indeed, the risk of developing resistance to hydroxyurea seems higher in patients with mutations in *TP53* (28) or genes regulating RNA splicing (29), and mutations in the RAS pathway are more frequently associated with resistance to ruxolitinib (25, 26). Confirmation of the deleterious role of the co-occurrence of $JAK2^{V617F}$ mutations with some of these mutations has been provided by mouse models. For example, the transduction of $JAK2^{V617F}$ in the bone marrow cells of *TP53* knockout mice increased the occurrence of leukemia in recipient mice (30).

Similarly, while deletion of the *EZH2* gene does not induce a marked phenotype in mice, crossing *EZH2* knockout mice with $JAK2^{V617F}$ transgenic mice showed an accelerated onset of myelofibrosis and a very marked shortening of the lifespan of the mice (31). Same observations were described with the inactivation of ASXL1 and IDH2 mutations (32, 33). Conversely, combined with SRSF2 mutants, JAK2 mutated MPN do not accelerate the evolution of the disease whereas patients seem to evolve (23, 34) highlighting the limits of those mouse models. The use of human xenotranplantation models would probably help resolving those discrepancies even though still technologically challenging. Overall, these observations demonstrate that certain additional mutations expressed in the $JAK2^{V617F}$ clone modify cell fate and can accelerate the transformation of MPN, confirming that clonal evolution is an important milestone linked to clinical evolution.

Clonal evolution in MPN: an example of multistep process in cancer evolution

Although it was quickly accepted that ET, PV and MF can clinically evolve from one entity to another, the concept that MPN could evolve at the genetic level took 50 years to establish. Indeed, studies of X chromosome inactivation patterns on female samples identified the clonal nature of the disease before the first molecular markers and additional mutations were discovered (12, 35). After the description of the first acquired mutations in MPN, one of the questions that quickly arose was how these mutations are acquired and distributed within the tumor. It is now well established that the driver mutation occurs in HSCs, giving rise to the MPN phenotype, while additional mutations acquired within the so-called driver clone contribute to the evolution of the disease, as shown by mouse models with $JAK2^{V617F}$ an additional mutations (31, 33, 34, 36). A linear pattern of mutation acquisition in the same cells is quite rare and a branching pattern of mutations is most often observed in

MPN. Altogether, this leads to the frequent presence of various co-existing clones within the same tumor (10, 18, 19, 37–39). A novel and possibly more accurate vision of MPN could be envisaged to evaluate the prognosis of patients no longer based on the numbers and types of mutations but on the numbers and types of clones present at one time point in the tumor (Figure 1). Furthermore, the clonal architecture of MPN is a dynamic process, with clones appearing or disappearing and clones that can increase or decrease in length during disease evolution. Therefore, patients' prognosis may rely on the clonal evolution of the disease and longitudinal study of clonal architecture could be of high clinical relevance.

Environmental factors modifying clonal evolution

From a Darwinian point of view, one of the major questions posed today, and because we know that multiple clones co-exist within the tumor, is to identify and better understand how environmental factors promote or, on the contrary, inhibit the expansion of a clone. The fitness of clones is the result of their own proliferation rate, their characteristics in terms of metabolism requirements and their ability to resist under a given selection pressure (40, 41).
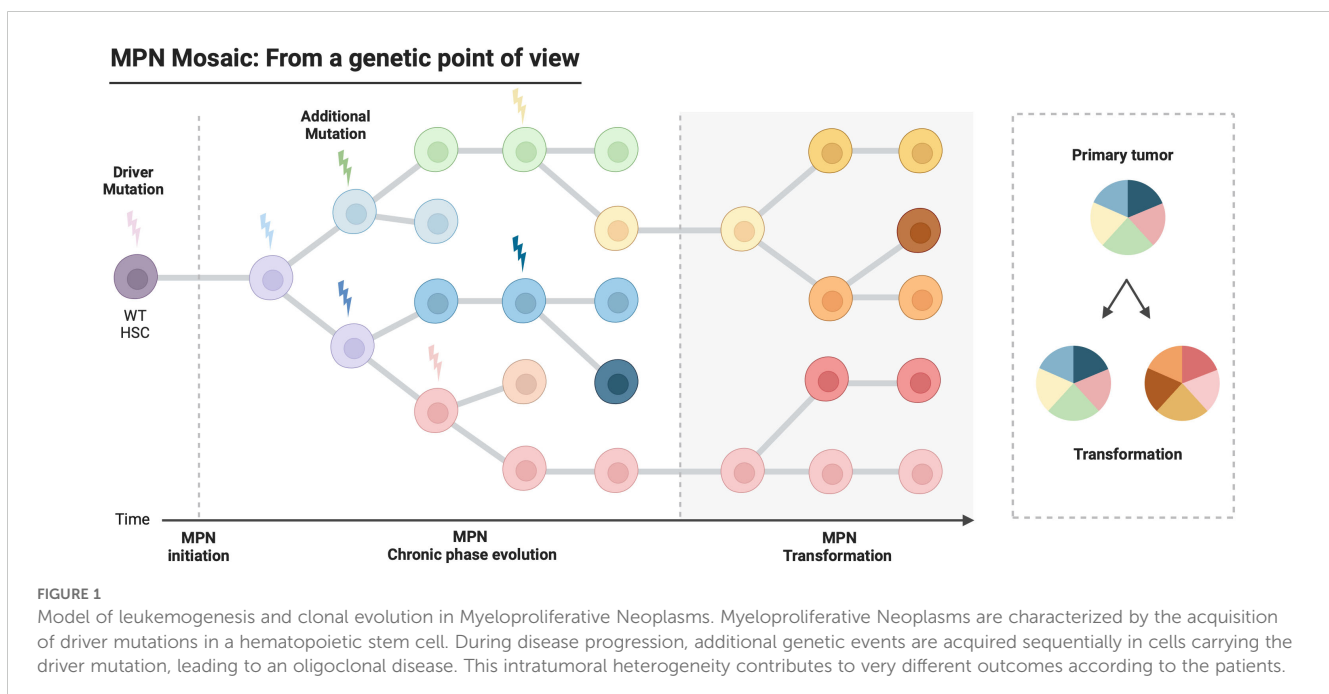
Inflammation

Inflammation and driver mutations in MPN

Chronic inflammation is a hallmark of MPN which has been well described (42) and MF development is associated with the most severe level of inflammation (43). Among various mechanisms of inflammation, the most widely described in MPN is due to cytokine dysregulation. Indeed, a large amount of literature has been written on this topic, and many cytokines have been implicated. TGF-β was one of the first cytokine identified as hypersecreted in HSPCs from MF patients (44, 45). Its role in the development of myelofibrosis is well established in several models, including murine models overexpressing the thrombopoietin which rapidly develop myelofibrosis (46). The mechanism through which the TGF-β secreted by megakaryocytes in the bone marrow niche participates in myelofibrosis development was recently shown (47). More recently, the number of inflammatory cytokines identified as upregulated in MPN has been steadily increasing. For instance, IL-8 is overexpressed in MPN, via the transcription factor *NFE2* which is frequently mutated in these diseases (48) and lipocalin-2 plays a pro-inflammatory but also a direct pro-fibrotic role in MPN (49). Interestingly, the $JAK2^{V617F}$ mutated cells were shown to be hypersensitive to TNFa (50), one of the most important inflammatory cytokines. Also, the IL-6 pathway is hyperactivated in CALR-mutated MPN, which is an important inflammatory pathway associated to myelofibrosis development (51–53). In addition, several inflammatory pathways are interdependent and may contribute to deregulation of key pathogenic processes involved in MPN. For example the IL-4/IL-13 axis that activates the TGF-β pathway has been shown to be deregulated in MPN, explaining in part the deregulation of this pathway (54). Very recently, overproduction of IL-1β by the $JAK2^{V617F}$ clone was implicated in bone marrow fibrosis in mouse models but was also shown to be a major factor favoring the clonal expansion of $JAK2^{V617F}$-mutated cells (55, 56) providing a link between inflammation, fibrosis and clonal selection.

All these data given, it is still rather unclear whether inflammation is a cause or a consequence of MPN development (57). From a genetic point of view, although various models have

FIGURE 1

Model of leukemogenesis and clonal evolution in Myeloproliferative Neoplasms. Myeloproliferative Neoplasms are characterized by the acquisition of driver mutations in a hematopoietic stem cell. During disease progression, additional genetic events are acquired sequentially in cells carrying the driver mutation, leading to an oligoclonal disease. This intratumoral heterogeneity contributes to very different outcomes according to the patients.

been proposed, the question remains as to whether the presence of the mutation induces an inflammatory phenotype, or whether pre-existing inflammation is responsible at least in part for the clonal evolution (58). In the era of molecular characterization of tumors, a number of new data allow us to better answer the question. Several studies in various *in vivo* models of MPN showed strong evidence that constitutive activation of the JAK2/STAT5 pathway due to the acquisition of a driver mutation induces a major inflammatory phenotype, which is highly implicated in the disease (59–61).

Recently, studies combining at the single cell level the genotyping of specific genes with the analysis of transcriptome (60, 62) as well as chromatin accessibility showed that *JAK2^{V617F}* mutated HSPCs exhibited specific proinflammatory signatures with accessibility to DNA motifs binding NF-kB or JUN/FOS factors. These approaches also allowed to highlight epigenetic modifications specific to mutated erythroid or megakaryocytic progenitors, demonstrating that the consequences of the mutations are different according to the differentiation stage and cellular context (63).

Finally, inhibitors of the JAK-STAT pathway such as ruxolitinib are widely used to treat MPN with important efficacy on symptom burden (64, 65). The main explanation for such efficacy seems to be linked to their potent anti-inflammatory effect (57, 64, 66). Some other anti-inflammatory therapies have also demonstrated activity against the mutated cells such as anti-IL1b therapies (52) and Interferon alpha has been reported to be effective at least in part through its anti-inflammatory property (56, 67).

Inflammation and additional (non-driver) mutations in MPN

The identification of circulating somatic mutations in the absence of clinical or biological evidence for any hematological disorder defines CHIP (ref 2014), which has been associated with an inflammatory phenotype. *TET2* is one of the most frequently mutated genes in both MPN and CHIP. The *TET2* protein represses IL-6 induction indirectly through the recruitment of histone deacetylases on its promoter (68). This is in line with the inflammatory phenotype observed during *TET2* KO (69). The presence of *TET2* mutations in persons with CHIP has been associated with an excess of various cardiovascular events (70) and a mechanism involving the inflammasome has been proposed (71). Similarly in pathological situations, *TET2* mutations are strongly associated with inflammation in MPN and MDS (72, 73). *DNMT3A*-mutated HSPCs, on the other hand, have been shown to be selected in an inflammatory context (74). Single-cell studies capturing *DNMT3A* R882H mutations and coupling it to chromatin accessibility showed an inflammatory phenotype of mutated HSPCs compared to their wild-type counterpart (75), a finding in agreement with the anti-inflammatory properties of this gene (76) and explaining the described association between inflammatory diseases and *DNMT3A* clonal hematopoiesis (77). Finally, in MPN, *JAK2/DNMT3A* double mutants have been shown to cooperate to induce myelofibrosis via aberrant inflammation and self-renewal of HSPCs (78). Similarly, increased expression of IL-8 found in MPN (43) has been shown to be caused by overexpression of *NFE2* (48), itself regulated by the histone demethylase *JMJD1C* (79). It would thus be interesting to study whether in an *NFE2* mutated context,

which has been shown to give a poor prognosis in MPN (27), IL-8 expression is increased and involved in clonal specific progression. Finally, after the acquisition of *TP53* mutations clonal dominance of mutated cells is dependent on an inflammatory environment which participates to the transformation toward acute leukemia (60). This field of research is important since specific causes of mutation-independent inflammation could become useful new targets in MPN therapy for a subset of patients.

Altogether, because the genetic evolution of MPN appears to be partly shaped by inflammation, one can consider that MPN is a human inflammation model for cancer development (58) (Figure 2).

Treatments

MPN are chronic diseases that, in a large proportion of patients, require cytoreductive therapies on the long term. However, the correlation of a given treatment with the risk of transformation in MPN patients is difficult to establish and still controversial. It has been strongly suggested that ^{32}Phosphorus or alkylating agents such as pipobroman are associated to a higher rate of MDS/AML transformation and can promote the expansion of a subpopulation of more aggressive clones. Still, few studies have investigated the clonal composition of tumors developed after treatment with these agents (80, 81). The most frequently used cytoreductive treatment in MPN is hydroxyurea (HU), which has long been suspected to promote blastic transformation and also the selection of *TP53*-mutated cells in MPN (28, 29, 81). However, no definitive demonstration of a major role for HU in *TP53*-mutant clonal selection has emerged until now. To the contrary, the increase of *TP53*-mutations allelic frequencies under treatment with MDM2 inhibitors was recently reported in a phase II clinical trial in MF patients (82). An important element reinforcing the idea of environmental selection pressure by drugs was the regression of these clones at treatment discontinuation. We were able to confirm *in vitro* the potential for MDM2 inhibitors to promote the expansion of *TP53*-mutated clones. Furthermore, using single cell DNAseq analysis we observed that the selection pressure occurred in *TP53* and *JAK2^{V617F}* doubly mutant cells (37).

Another demonstration that treatments used in MPN could change the clonal composition of the tumor came with the discovery that Interferon alpha (IFNa) was able to specifically target *JAK2^{V617F}* or *CALR* mutated clones (83–86). However, identification of resistance to IFNa in some patients with several mutations suggested that the presence of additional mutations could interfere with this process (87). Indeed, the role of *DNMT3A* mutations in the clonal resistance to IFNa has been recently shown (88, 89), although the mechanism remains only partially explained (90). In an MPN setting, Usart and al elegantly demonstrated very recently that loss of *Dnmt3a* increased self-renewal of of *Jak2v617f* mutant HSCs and mitigates their attrition by IFNa (91).

Intraclonal cooperation and competition

Since the discovery that MPN are oligoclonal diseases, the question has arisen of the distribution of mutations within the
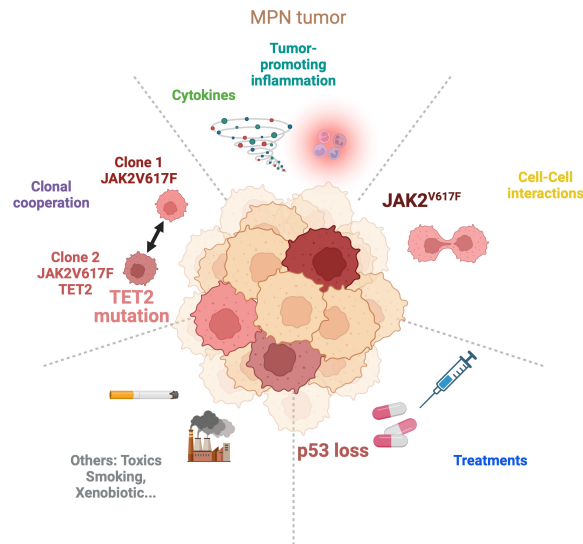
FIGURE 2
Main clonal selection factors in Myeloproliferative Neoplasms. The selection pressures exerted by the tumor are multiple and still poorly described and characterized. A distinction is made between extrinsic selection factors (toxins, treatments) and intrinsic selection factors (clonal cooperation, cell-cell interactions, microenvironment), the latter being poorly studied. The genetic evolution of tumors over time combines multiple factors, the real modeling of which remains a challenge.

tumor, thus defining subclones. Depending on the study, between one and more than ten different clones can now be identified, giving an idea of the complexity of clonal hierarchy in MPN (37–39, 92). These clones probably interact with each other and may influence tumor evolution over time. Therefore it may be envisioned that the interactions between these clones may be synergistic defining positive clonal cooperation, or on the contrary these interactions may prove to be deleterious between clones, demonstrating a rather competitive character (93). Even though most interactions among subclones are neutral, an ecological interaction between clones has been reported in solid tumor models with detectable interactions that should be positive or negative, resulting in a phenotypic switch (94). Such cooperative or antagonistic interactions, due for instance to metabolite exchanges, could apply to MPN where multiple clones of various sizes coexist. In particular, the presence of a CHIP clone aside from the MPN clone could, directly or indirectly, regulate tumor development. The impact of such process on clinical evolution has been suggested (39) but the mechanisms explaining these effects remain to be studied.

Understanding the extent of cooperation and communication among MPN clones will be an important step in the future. Imaging technologies such as mass cytometry applied to bone marrow or spleen of patients with MPN could provide an accurate phenotypic characterization of diverse genotypic cell types. This has given greater granularity to early observations of clonal cooperation, and relationships between cells can now be described in detail (95–97). Multi-omic studies associated to the genotype of each clone within the same sample that can be mapped spatially to the tumor will help to further define functional examples of tumor heterogeneity.

Clonal cell-microenvironment interactions and communication

The differential interaction of clonal cells harboring different mutations with other cells in the microenvironment has been clearly demonstrated, but few studies showed a direct effect of these exchanges on clonal selection. In glioma models for example, it was shown that wild-type *IDH* tumors cause greater neuronal cell interaction and signaling than mutated *IDH* tumors (98). In lymphoid malignancies, it has been shown recently that *TET2* and *IDH2* double mutant cells in T follicular helper cells were able to alter cross-talk with germinal center B cells that promotes B cell clonal expansion while decreasing Fas-FasL interaction and reducing B cell apoptosis (99). Likewise, in myeloid malignancies, *Asxl1-/-* and *nrasG12D/+* leukemic cells induced Host-derived wild-type T cells overexpressed programmed cell death protein 1 (PD-1) and T-cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) receptors, leading to a predominant exhausted T-cell phenotype (100). In hematopoietic models of CHIP, Partial transplantation with *Dnmt3aR878H/+* bone marrow altered function of neutrophils and Tregs exacerbates CHIP-driven inflammation (101). Same model caused MSCs to upregulate the senescence markers SA-β-gal, BCL-2, BCL-xL, Cdkn1a (p21) and Cdkn2a (p16), ex vivo and *in vivo*. This effect was cell contact-independent and can be replicated by IL-6 or TNFα, which are produced by *Dnmt3aR878H/+* HSPCs (102). In the medullary microenvironment of MPN, interaction between neuronal cells and death of mesenchymal stromal cells has been demonstrated, promoting the expansion of *JAK2^V617F* clones (103, 104). Although soluble factors are involved in this process (IL-1b, CXCL12 and

adrenergic agonists), direct interaction also seems to play a role. It would be interesting to study the impact of direct cellular interactions between different clones of the same tumor and stromal cells, even if demonstration of indirect interactions via cytokines have already been described (105). Another evidence of cell interaction mechanism leading to evolution of myeloid disease and involving interaction of clonal cells with the microenvironment was provided by transplantation of human MF HSPCs into immunocompromised mice experiments, leading to murine AML. This study showed that the secretome-driven myeloproliferation of murine cells can occur independently of both the type of mutation and the mutational burden of transplanted HSPC patient cells (106).

Clonal evolution: a path towards transformation?

Monitoring clonal evolution is obviously of clinical interest, as the risk of transformation has been correlated with genetic changes in the tumor. The genetic basis of transformation has been well described previously (107–110). As discussed above, it has been shown that the accumulation of mutations is a clear unfavorable prognostic factor in MPN, and that the number of HMR mutations at diagnosis correlates with the risk of transformation whatever the MPN subtype, as well as high-risk mutations such as *TP53* (111, 112). However, to date, it is not exactly known why some patients with the same molecular lesions will experience leukemic progression while others will not. If we take the example of *TP53* mutations, we can find contradictory evidence in the literature. Firstly, the risk of transformation in $JAK2^{V617F}$-*TP53* double mutation models is still debated as some mouse models do not progress to secondary AML while in patients this risk of progression is clearly present (30, 60, 113, 114). The second observation is that many patients with *TP53* mutations can be followed for a long time without progressing to transformation, even without specific treatment. Finally, we know that a non-negligible proportion of transformations arise from a driver mutation-negative clone, pointing to the importance of the molecular composition of the tumor and the clones present throughout the tumor process (107) (Figure 1). Although it is well established that a second event is required to accelerate the transformation in a *TP53*-mutated context, we still do not know precisely how this event occurs in a *TP53* heterozygous MPN clone. New single cell technologies combining genotype and transcriptomics or mutliomics on longitundinal samples of MPN throughout clinical evolution will help us get through these questions.

Conclusion

The complexity of myeloproliferative neoplasms (MPNs) reflects the intricate interplay between genetic mutations, clonal evolution, and microenvironmental factors. This review has highlighted how MPNs are driven by a sequential accumulation of mutations that not only initiate but also fuel disease progression

and transformation. The dynamic nature of clonal evolution, where subclones with distinct genetic profiles compete and cooperate, adds further complexity to MPN pathogenesis. In addition, the microenvironment plays a crucial role in shaping clonal behavior, influencing both the expansion of malignant clones and their interaction with supporting cells. Lastly, acute or chronic environmental context including inflammation and selection pressure by the therapies used during the chronic course of the illness may further contribute to disease progression.

As we continue to unravel the molecular mechanisms underlying MPNs, it becomes evident that understanding clonal evolution in its full context is essential for improving therapeutic strategies. Future research, leveraging advanced technologies like single-cell sequencing and integrated multi-omics, will be key to uncovering new insights into MPN initiation, progression, and therapeutic resistance. By integrating these discoveries into clinical practice, we can better predict disease trajectories and optimize treatment approaches, ultimately aiming for more personalized and effective care for MPN patients.

Author contributions

NM: Conceptualization, Formal analysis, Validation, Writing – original draft, Writing – review & editing. SG: Validation, Writing – original draft, Writing – review & editing. BC: Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. JK: Conceptualization, Project administration, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

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