

MYELOID NEOPLASIA

Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms

Pontus Lundberg,¹ Axel Karow,¹ Ronny Nienhold,¹ Renate Looser,¹ Hui Hao-Shen,¹ Ina Nissen,² Sabine Girsberger,³ Thomas Lehmann,³ Jakob Passweg,³ Martin Stern,^{1,3} Christian Beisel,² Robert Kralovics,^{4,5} and Radek C. Skoda^{1,3}

¹Experimental Hematology, Department of Biomedicine, University Hospital of Basel, Basel, Switzerland; ²Department of Biosystems Science and Engineering, Swiss Federal Institute of Technology, Zurich, Switzerland; ³Division of Hematology, University Hospital Basel, Basel, Switzerland; ⁴CeMM Research Center for Molecular Medicine, Austrian Academy of Sciences, Vienna, Austria; and ⁵Division of Hematology and Blood Coagulation, Department of Internal Medicine I, Medical University of Vienna, Vienna, Austria

Key Points

- The total number of somatic mutations was inversely correlated with survival and risk of leukemic transformation in MPN.
- The great majority of somatic mutations were already present at MPN diagnosis, and very few new mutations were detected during follow-up.

Myeloproliferative neoplasms (MPNs) are a group of clonal disorders characterized by aberrant hematopoietic proliferation and an increased tendency toward leukemic transformation. We used targeted next-generation sequencing (NGS) of 104 genes to detect somatic mutations in a cohort of 197 MPN patients and followed clonal evolution and the impact on clinical outcome. Mutations in calreticulin (*CALR*) were detected using a sensitive allele-specific polymerase chain reaction. We observed somatic mutations in 90% of patients, and 37% carried somatic mutations other than *JAK2* V617F and *CALR*. The presence of 2 or more somatic mutations significantly reduced overall survival and increased the risk of transformation into acute myeloid leukemia. In particular, somatic mutations with loss of heterozygosity in *TP53* were strongly associated with leukemic transformation. We used NGS to follow and quantitate somatic mutations in serial samples from MPN patients. Surprisingly, the number of mutations between early and late patient samples did not significantly change, and during a total follow-up of 133 patient years, only 2 new mutations appeared, suggesting that the mutation rate in MPN is rather low. Our data show that comprehensive mutational

screening at diagnosis and during follow-up has considerable potential to identify patients at high risk of disease progression. (*Blood*. 2014;123(14):2220-2228)

Introduction

Myeloproliferative neoplasms (MPNs) are a group of stem cell disorders characterized by aberrant hematopoietic proliferation and an increased tendency toward leukemic transformation. MPNs comprise 3 major subgroups: polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). An acquired mutation in *JAK2* (*JAK2* V617F) is present in the majority of MPN patients.¹⁻⁴ Although *JAK2* mutations have been shown to be the phenotypic drivers in MPN, there is evidence of clonality and mutational events preceding the acquisition of *JAK2* V617F.⁵⁻⁸ An increasing number of mutations in genes distinct from *JAK2* have been identified in patients with MPN. These include mutations in epigenetic modifiers, such as *TET2*,⁸ *DNMT3A*,⁹ *ASXL1*,¹⁰ and *EZH2*,¹¹ and genes involved in hematopoietic signaling (reviewed in Vainchenker et al¹²). Very recently, recurrent mutations in the calreticulin gene (*CALR*) have been reported in ET and PMF by 2 next-generation sequencing (NGS) whole-exome studies.^{13,14} In addition, novel recurrent mutations occurring at low frequencies have been also found in *CHEK2*, *SCRIB*, *MIR662*, *BARD1*, *TCF12*, *FAT4*, *DAP3*, and

POLG.^{14,15} Mutations in *TP53*, *TET2*, *SH2B3*, and *IDH1* are more frequently observed in leukemic blasts from transformed MPN patients, suggesting a role for these gene mutations in leukemic transformation.¹⁶⁻¹⁹ However, so far only mutations in *ASXL1* and *NRAS* have been shown to be of prognostic value in patients with PMF.^{15,20}

Using targeted NGS to search for mutations in 104 cancer-related genes, we have defined the mutational profile of a cohort of 197 MPN patients and dissected the temporal order of acquisition and clonal architecture of mutational events. We further analyzed the impact of the somatic mutations on clinical outcome. We provide evidence that most somatic mutations were present already at MPN diagnosis. In addition, we show that somatic mutations in *TP53* and *TET2* are associated with decreased overall survival and increased risk for leukemic transformation. Importantly, mutations in *TP53* were present for several years in the chronic MPN phase at a low allelic burden, whereas after loss of the wild-type (WT) *TP53* allele, the clone rapidly expanded, resulting in leukemic transformation.

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Table 1. Clinical characteristics of the MPN patients at diagnosis

Diagnosis	PV	ET	PMF
Number of patients	94	69	34
% female	51	67	26
Average age at diagnosis (range), y	58 (18-87)	51 (21-86)	61 (21-86)
Average time of follow-up, mo	92	56	49
Hemoglobin (g/L) average (range)	181 (148-225)	141 (78-225)	126 (90-161)
Platelets (10 ⁹ /L) average (range)	554 (90-1487)	994 (452-1983)	635 (16-1677)
Leukocytes (10 ⁹ /L) average (range)	12 (4-39)	9 (5-17)	11 (5-27)
Neutrophils (10 ⁹ /L) average (range)	9 (2-36)	6 (3-16)	8 (3-21)
Transformation to secondary myelofibrosis	4 (4%)	1 (1%)	NA
Transformation to AML	3 (3%)	2 (3%)	2 (6%)

NA, not applicable.

Methods

Patient cohort

The collection of blood samples and clinical data was performed at the study center in Basel, Switzerland and approved by the local Ethics Committees (Ethik Kommission Beider Basel). Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki. The diagnosis of MPN was established according to the revised criteria of the World Health Organization.²¹ Table 1 provides clinical data of the patients included in our study.

Illumina library preparation and target region capture

A total of 500 ng of granulocyte DNA derived from the most recent available follow-up samples of the patients was fragmented using Fragmentase (New England Biolabs), resulting in an average fragment size of ~250. The fragmented library was purified using Agencourt AMPure XP beads. Following purification, the library was end-repaired and adenylated (both enzymes from Bioo Scientific), and after each of those steps, the library was purified using Agencourt AMPure XP beads. Finally, patient-specific barcoded adapters were ligated (NEXTflex, Bioo Scientific; 48 different ones in total) and divided into duplicate samples. Subsequently, adaptor-ligated DNA from 48 patients, each assigned with a different barcode, was pooled equimolarly in duplicate tubes.

Bait design and target capture

Capture of target regions was performed using an Agilent SureSelect custom design including the targeted exons ±50 bp of flanking regions with a total size of ~0.44 Mb. Enrichment was performed using the provided Agilent protocol and capture was performed for 72 hours. Postenrichment polymerase chain reaction (PCR) was performed for 10 cycles.

Illumina sequencing and sequencing analysis

Paired-end 100-bp cycle sequencing of the captured libraries was performed using an Illumina HiSeq2000. Demultiplexed samples were mapped and analyzed using the CLC genomics workbench. Mapping was performed using a mismatch cost of 2 and insertion and deletion cost of 3 with a length fraction 0.7 and similarity fraction of 0.8. For mutational calling, the quality-based variant detection was used, using a neighborhood radius of 5, maximum gap and mismatch count of 2, minimum neighborhood phred quality of 25, and minimum central quality of 30. Minimum coverage of called regions was set at 20, and minimum variant frequency was set to 5%. Only nonsynonymous mutations were further pursued, whereas splice-site mutations were determined using the predict splice-site effect module. Average coverage of targeted regions was performed using the coverage analysis module and including only the targeted exons and not the flanking regions. Targets consistently having no coverage are displayed in supplemental Figure 2H, available on the *Blood* Web site.

To assess copy number alterations, the RNA-sequencing analysis module was used, and expression value was calculated using reads per kilobase per

million. Statistical analysis was performed on proportions, and as references 5 normal controls were pooled. Genes deviating with >30% in expression value and with a *P* value of <.01 were considered as candidate regions.

Validation of candidate mutations

Candidate mutations observed in the Illumina screen were validated using the Ion Torrent PGM platform. Amplicons covering the regions of interest were designed with an amplicon length of 150 to 250. Sequencing adapters (IonXpress) were ligated to the amplicons using the IonXpress protocol. Final libraries were sequenced with 200-bp read length on a 318 chip. Mutation calling was performed using the torrent suite variant called using the somatic settings. A mutation was called somatic when the mutant allele burden in buccal DNA was <25% of the value observed in granulocytes. In the great majority of somatic mutations (~90%), no signal was detected in the germline control DNA.

Sanger Sequencing and AS-PCR

For a minority of amplicons, no sequencing coverage was obtained with the Ion Torrent PGM. For these regions, Sanger sequencing for mutation validation was performed according to standard protocols. Allele-specific PCR (AS-PCR) of *CALR* exon 9 was performed as previously reported.¹³

Analyses of patient cell colonies

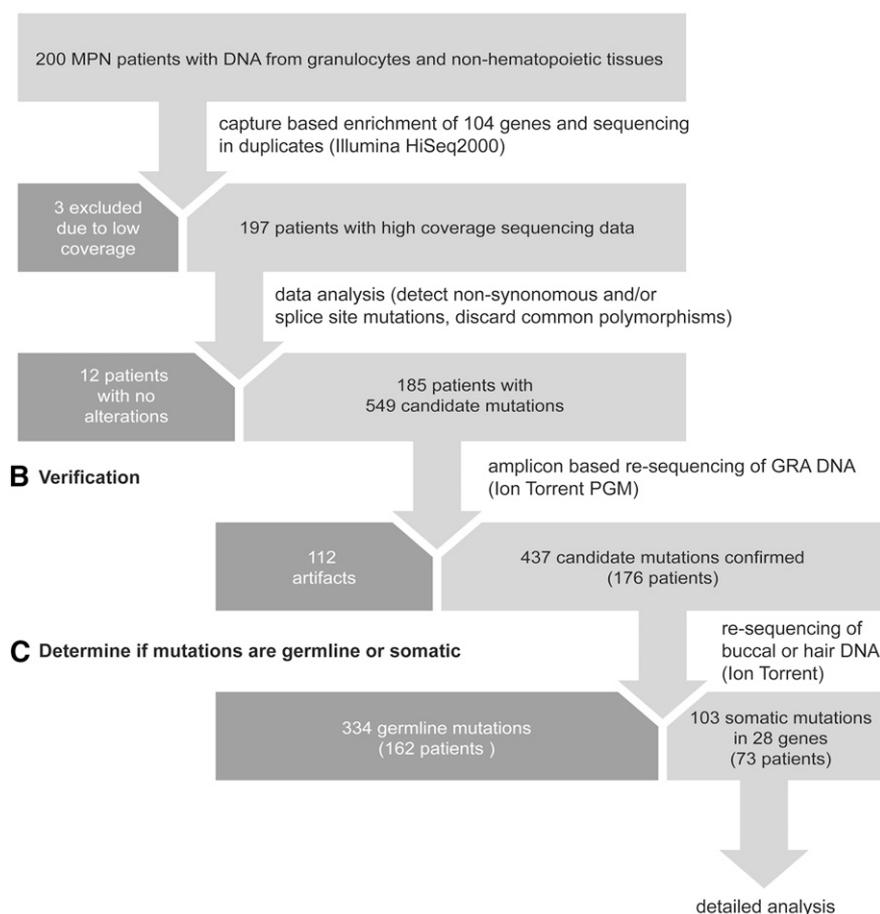
The colony assays were performed using peripheral blood mononuclear cells from patients as previously published.⁷ After 14 days, colonies were picked and analyzed individually for *JAK2* V617F using AS-PCR and for the presence of somatic mutations by Sanger sequencing, respectively. On average, 88 colonies per patient were analyzed. To determine the temporal order of mutation acquisition, at least 2 informative colonies were required. In total, we analyzed 33 patients.

Statistical analysis

Mutational status of genes mutated in ≥5 patients in the cohort was correlated with blood counts at diagnosis (hematocrit, platelets, leukocytes, and neutrophils) in generalized linear models adjusting for patient gender, disease (PV vs ET vs PMF), and age. Survival and transformation curves were estimated using the Kaplan-Meier univariate method and compared by the Mantel-Cox log-rank test. Primary end points were overall survival, defined as time between diagnosis and death by any cause, and transformation to acute myeloid leukemia (AML). Statistical analyses were performed using SPSS (version 20) and GraphPad Prism (version 6).

Results

We characterized a cohort of 200 MPN patients from whom paired granulocyte and nonhematopoietic DNA samples were available (Figure 1). Clinical characteristics of the patients at diagnosis of MPN are summarized in Table 1. Serial blood samples were available

A Initial mutational screening**Figure 1. Targeted NGS in MPN: study design and workflow.**

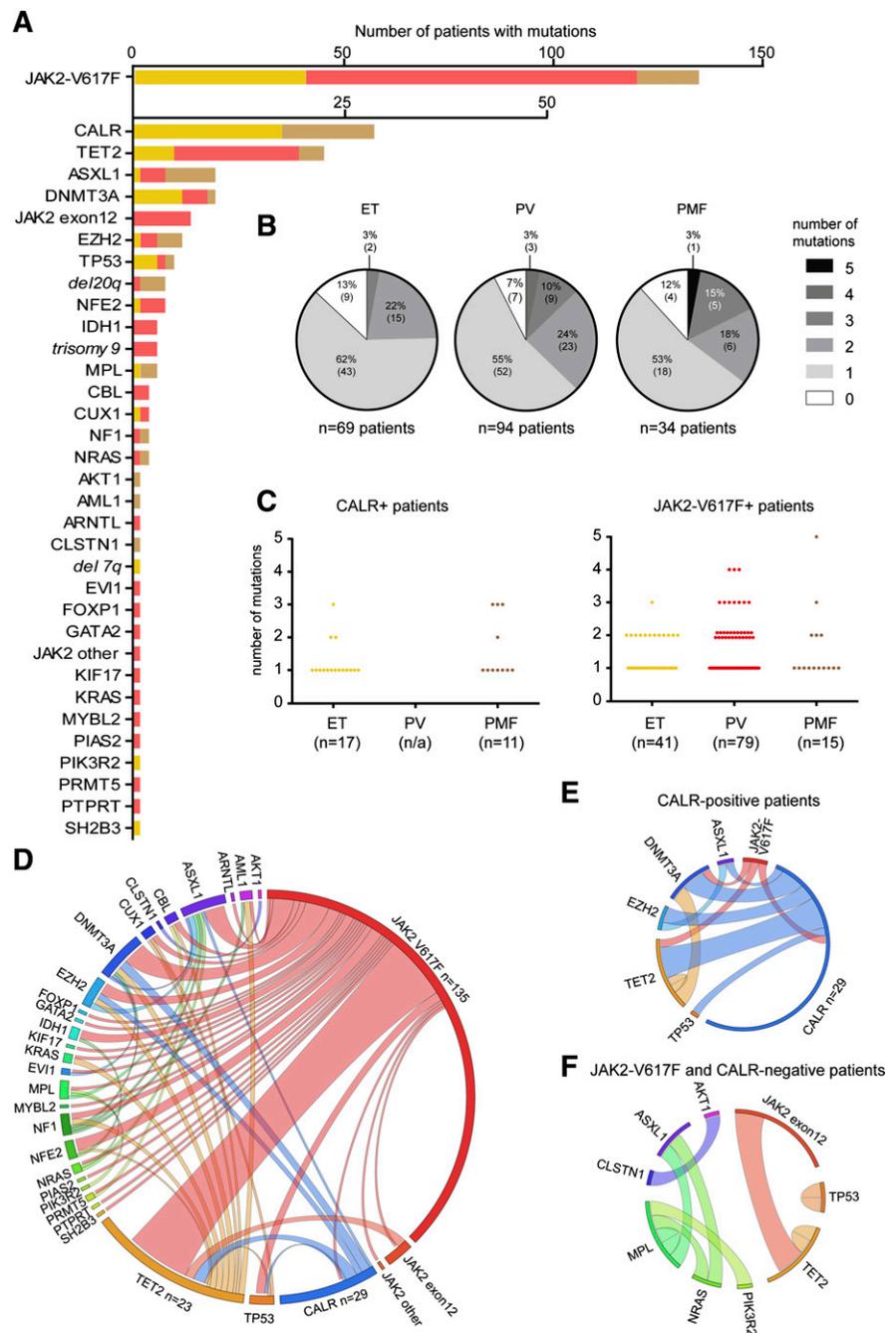
for 143 of 200 (72%) patients. To detect the maximal number of candidate mutations, granulocyte DNA from the most recent patient sample was used for initial sequencing. The workflow is summarized in Figure 1. We used the Agilent SureSelect method to capture exons and flanking regions of 104 selected genes with known or possible role in MPN (supplemental Figure 1A). To reduce PCR and sequencing artifacts, all DNA samples were processed and sequenced in duplicates and only sequence alterations that were present in both duplicate samples and displayed a mutant allele burden of >5% were further analyzed. The average exon coverage of Illumina sequencing per patient was 370-fold (supplemental Figure 2A), and only 3 patients had to be excluded due to insufficient coverage (Figure 1A). Resequencing of granulocyte DNA confirmed 437 of the 549 candidate mutations (80%) that were detected in the original screening (Figure 1B). Using DNA derived from buccal mucosa or hair follicles, we found that 334 of the 437 mutations were germline (76%) and 103 were somatic (24%) (Figure 1C). Furthermore, we screened our cohort also for mutation in the *CALR* gene using AS-PCR.¹³ Overall, 41 of 94 PV patients (44%), 20 of 69 ET patients (29%), and 12 of 34 PMF patients (35%) carried somatic mutations other than *JAK2* V617F or *CALR*.

Frequency and distribution of mutations in patients with MPN

By NGS, we found that 28 of 104 (27%) of genes analyzed were mutated in at least 1 of the 197 MPN patients (supplemental Table 1). By AS-PCR, in addition, 17 of 69 (25%) ET patients and 11 of

34 (32%) PMF patients carried mutations in *CALR*. After *JAK2* V617F and *CALR*, the most frequently observed mutations affected genes implicated in epigenetic regulation (*TET2*, *ASXL1*, *DNMT3A*, *EZH2*, and *IDH1*) (Figure 2A). We also identified 2 novel somatic mutations in the tumor suppressor *NFI*. Furthermore, we found mutations in *NFE2*, which had only been described in one recent report,²² and *CUX1*, uncovered previously in an MPN patient transforming to AML.²³ Recurrent somatic mutations were also observed in the genes *TP53*, *CBL*, *MPL*, and *NRAS*. Nonrecurrent mutations were detected in 16 other genes (Figure 2A). By measuring the relative read abundance of targeted regions in patients and normal controls, the NGS approach also detected copy number alterations, for example, deletions on chromosome 20q (Figure 2A and supplemental Figure 3). The distribution of mutations per patient is summarized in Figure 2B and supplemental Figure 2G. Overall, 20 of 197 patients (10%) had no detectable somatic mutation in any of the genes analyzed (9 ET, 7 PV, and 4 PMF). Two or more somatic mutations were found in 65 of 197 (33%) patients. The frequencies of somatic mutations in patients positive for either *CALR* or *JAK2* V617F are depicted in Figure 2C. Circos diagrams show the cooccurrence of all somatic mutations (Figure 2D) and the cooccurrence of events in *CALR*-positive patients (Figure 2E) and patients negative for mutation in both *JAK2* V617F and *CALR* (Figure 2F). In contrast to the recently published studies that reported *JAK2* and *CALR* mutations to be mutually exclusive,^{13,14} we observed coexistence of *JAK2* V617F and *CALR* mutations in 1 ET patient (Figure 2E). This coexistence was confirmed in granulocytes from 3 independent time points 1.5 years apart.

Figure 2. Frequency and distribution of mutations in patients with MPN. (A) Number of patients with mutations in the genes is indicated. ET patients are depicted in yellow, PV patients in red, and PMF patients in light brown. Numeric chromosomal aberrations are marked in italic font. (B) Distribution of somatic mutations among the 197 MPN patients according to phenotype. The shades of gray indicate the number of somatic mutations per patient. (C) Average number of somatic mutations per patient in *CALR*-positive (left panel) and *JAK2-V617F*-positive individuals (left panel) observed in ET, PV, and PMF patients, respectively. (D) Circos plot illustrating cooccurrence of somatic mutations in the same individual. The length of the arc corresponds to the frequency of the mutation, whereas the width of the ribbon corresponds to the relative frequency of co-occurrence of 2 mutations in the same patient. (E) Circos plot showing cooccurrence of somatic mutations in *CALR*-positive patients. (F) Circos plot showing co-occurrence of somatic mutations in patients negative for *JAK2 V617F* and mutations in *CALR*.



Serial samples were available from 28 of the 73 patients (38%) carrying somatic mutations other than *JAK2 V617F* or *CALR*. To estimate the mutation rate, we determined whether 38 somatic mutations found in the most recent sample were already present in the first patient sample that was available (Figure 3A and supplemental Figure 4). We found that the vast majority of mutations (36/38, 95%) were already detectable in the first sample and only 2 somatic mutations were acquired in a total of 133 patient years of follow-up (supplemental Figure 4). Thus, a patient would have to live ~66 years to acquire 1 mutation in the targeted region.

Clinical correlations and risk stratification

We analyzed the impact of the number of mutations other than *JAK2 V617F* on survival and transformation into AML using the log-rank

test for trend. We observed that increased number of somatic mutations lead to a significantly reduced overall survival and increased the risk of transformation into AML (Figure 3B). Patients with mutations in *TP53*, *TET2*, or other genes involved in epigenetic regulation (*ASXL1*, *DNMT3A*, *EZH2*, and *IDH1*) were analyzed separately (Figure 3C-E). We had serial samples from 4 out of 5 patients carrying *TP53* mutations. In these 4 patients, the *TP53* mutations were detected at a low allelic burden in the first available sample and remained low for several years (Figure 3C). After loss of the WT allele through mitotic recombination or deletion, the hemi- or homozygous *TP53* clone expanded rapidly in 3 out of 4 patients, and these 3 patients transformed to AML (Figure 3C), whereas 1 patient remained stable at a low allelic burden. The fifth patient with *TP53* mutation (from whom serial samples were not available) also

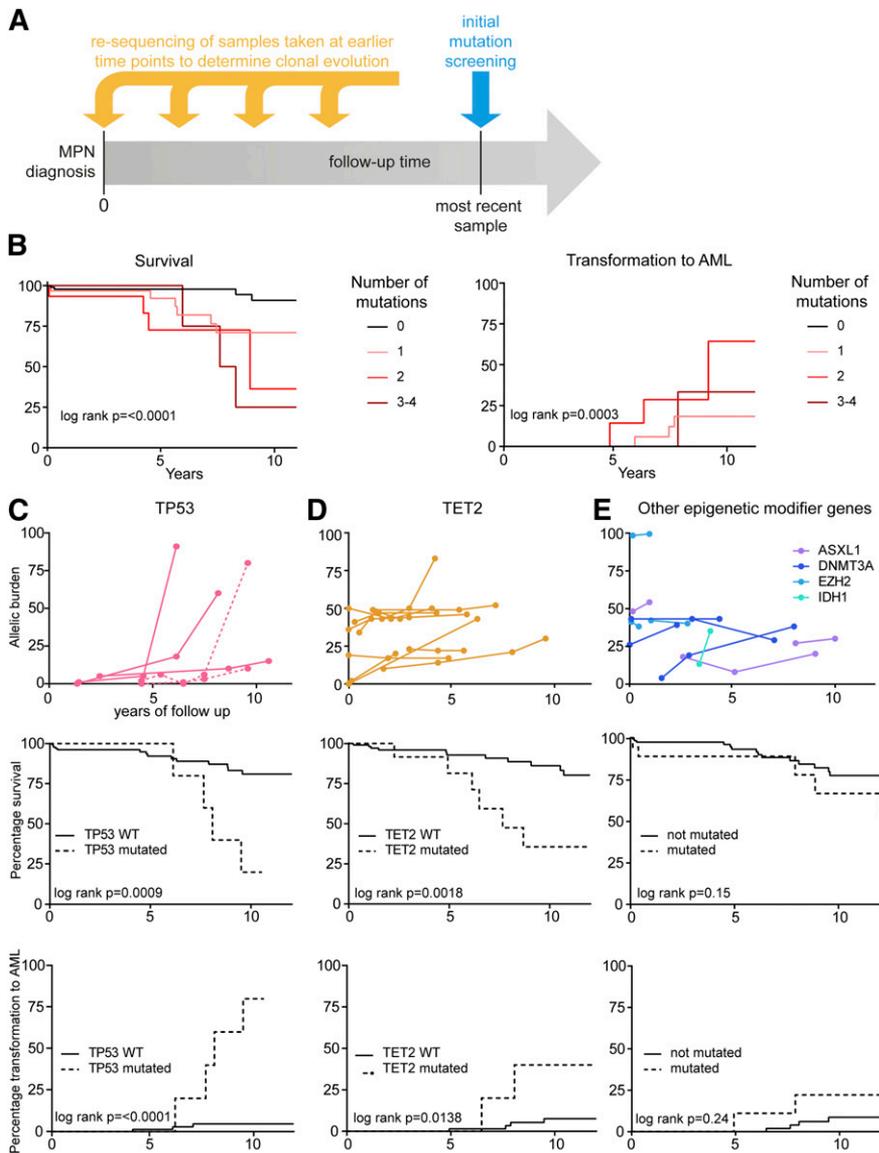


Figure 3. Analysis of sequential samples: clinical correlations and risk stratification. (A) Scheme of resequencing of mutations in serial samples to determine the time of acquisition and clonal evolution. (B) Kaplan-Meier curves for the probabilities of survival (left panel) and transformation into AML (right panel). Numbers indicate the number of somatic mutations per patient omitting *JAK2* V617F and *CALR* mutations. (C) Time course of the TP53 mutant allele burden in serial follow-up samples of 4 MPN patients with available follow-up samples (upper panel). One patient harbored 2 distinct TP53 mutations (dotted lines), only one of which displayed loss of heterozygosity. Survival (middle panel) and transformation to AML (lower panel) is shown below for 5 patients with mutations in TP53. (D) Time course of the TET2 mutant allele burden in serial follow-up samples of 12 MPN patients (upper panel). Survival (middle panel) and transformation to AML (lower panel) is shown below for 23 patients with mutations in TET2. (E) Time course of the mutant allele burden of epigenetic modifiers (*ASXL1*, *DNMT3A*, *EZH2*, and *IDH1*) in serial follow-up samples of 11 MPN patients (upper panel). Survival (middle panel) and transformation to AML (lower panel) is shown below for 29 patients with mutations in *ASXL1*, *DNMT3A*, *EZH2*, or *IDH1*.

transformed to AML. Serial blood samples were also available in 12 out of 23 patients with mutations in *TET2*, and in 11 of these patients, the *TET2* mutation was already present in the initial sample. Patients carrying *TET2* mutations had significantly reduced overall survival and an increased risk of leukemic transformation (Figure 3D). The number of individual patients with mutations in *DNMT3A*, *ASXL1*, *EZH2*, or *IDH1* was low, and when combined as a group, these patients showed no significant differences in the clinical course (Figure 3E). Thus, only 2 patients acquired a mutation during follow-up. One of these patients was treated with hydroxyurea (TP53 mutation), whereas the second patient was treated with aspirin only (TET2 mutation).

In addition, we observed correlations between mutation status and blood counts at diagnosis. Patients with an increased number of somatic mutations had a significantly higher leukocyte count (supplemental Figure 5A). Moreover, individuals with *ASXL1* mutations had significantly lower hemoglobin levels than their WT counterparts (supplemental Figure 5B), whereas patients carrying *EZH2* mutations had a significantly increased leukocyte count (supplemental Figure 5C).

Clonal evolution

For clonal analyses, we focused on patients carrying mutations in epigenetic modifier genes. To address the temporal order of acquisition, we genotyped DNA from single colonies grown in methylcellulose and plotted the results for each of the colonies analyzed (Figure 4A). The mutations could be classified as occurring before, after, or in a clone separate from *JAK2* V617F, and 1 example for each of these patterns is shown in Figure 4A. The results from all patients analyzed are summarized in Figure 4B. We found that mutations in *TET2* and *DNMT3A* were predominantly acquired before *JAK2* V617F or coexisted as separate clones (biclinal disease). Mutations in *ASXL1* and *EZH2* occurred before, after, or separate from *JAK2* V617F, whereas in 3 patients, *IDH1* mutation occurred exclusively after *JAK2* V617F (Figure 4B).

For patients with 3 or more somatic mutations, the results from single-colony analyses are shown in Figure 4C. In patients carrying mutations in *JAK2* V617F and epigenetic modifier genes or mutations in *TET2* or *DNMT3A* were predominately acquired as the first event. In 2 patients, *CALR* mutations were acquired first

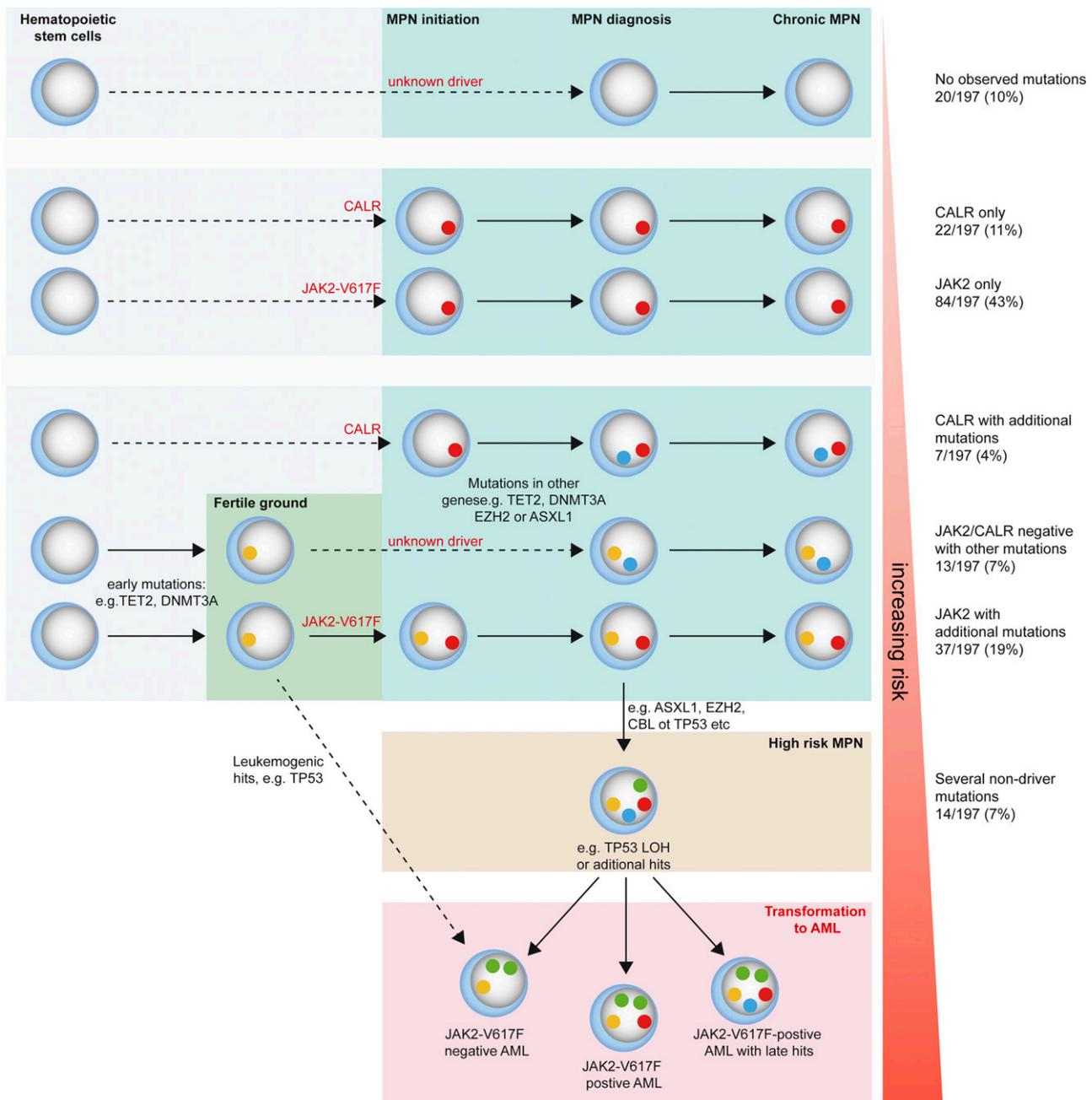


Figure 5. Model of MPN disease evolution and risk stratification in correlation to mutational events.

study detected ~ 0.2 somatic mutations per Mb in 151 MPN patients, showing that MPN has a low frequency of somatic mutations compared with other malignancies (eg, 0.37 mutations per Mb for AML and ~ 1 mutation per Mb for multiple myeloma).^{14,29}

Our analyses illustrate that one of the strongest predictors of outcome is the number of somatic mutations that occur in addition to *JAK2*, *CALR*, or *MPL* (Figure 3B). Interestingly, in our cohort, the group of patients carrying either no detectable somatic mutation or a mutation in *JAK2*, *CALR*, or *MPL* only had a particularly favorable prognosis. None of these patients showed leukemic transformation, suggesting that most genes with prognostic relevance are part of the gene set that we analyzed. In a study with a similar design in MDS patients, an association of time to AML transformation and number of mutations was found.³⁰ We observed that mutations in *TP53* and

TET2 were associated with particularly poor outcome (Figure 3C and 3D). *TET2* mutations were recently reported as negative prognostic markers in patients with intermediate-risk AML,³¹ and although a previous study in MPN showed no correlation between *TET2* mutational status and survival,³² other studies found an increased incidence of *TET2* mutation in blasts from patients with leukemic transformation.¹⁶ For *TP53*, we observed that mutations were present in a heterozygous state for an extended period of time during the chronic MPN phase without clonal expansion. However, after loss of the WT allele by either chromosomal deletion or uniparental disomy, the hemi- or homozygous *TP53* clone rapidly expanded, ultimately leading to leukemic transformation (Figure 3C). Thus, patients with *TP53* mutations represent a high-risk group, and screening for *TP53* mutations in MPN patients should be considered.

Because in the chronic phase of MPN the allelic burden of *TP53* mutation was <15%, sensitive methods such as NGS are needed for reliable detection. The prediction of disease progression by *TP53* mutations corresponding to our observations was described previously for patients with low-risk MDS³³ and chronic lymphocytic leukemia.³⁴ One recent study reported *TP53* mutations to be frequent in leukemic blasts of transformed MPN patients,¹⁷ whereas in chronic MPN from the same cohort, 2 out of 65 patients carried monoallelic *TP53* mutations.

By dissecting the clonal architecture of patients carrying ≥ 3 distinct somatic mutations, we found diverse patterns, some compatible with a linear acquisition of mutations, but also several cases with an apparent biclonal structure (Figure 4). Overall, such a biclonal pattern was found in 7 of 33 patients (21%), which illustrates that in many patients, the clonal architecture cannot be imputed using allele burden of mutations alone. In general, mutations in *TET2* and *DNMT3A* were early genetic events acquired before *JAK2* V617F, whereas mutations in *ASXL1*, *EZH2*, or *IDH1* were often acquired after *JAK2* V617F. In contrast, mutations in *CALR* appeared to be an early event in the limited number of patients analyzed, consistent with previous reports.^{13,14} One patient (p101) showed a complex 3 clonal pattern, and the *CALR* clone was present only in a minority of the colonies (Figure 4C).

Based on our data and previous studies, a model is presented in Figure 5. With the current methodologies, 10% of MPN patients show no detectable somatic mutations in the 105 genes analyzed (top row). In an additional 54% of patients, *JAK2* V617F or *CALR* were the only detected mutations. These 64% of MPN patients in our cohort displayed the most favorable prognosis and the lowest risk of disease progression. In the remaining 36% of MPN patients, we detected combinations of >1 somatic mutation, and in some patients, we can define the stage and order of acquisition. In the case of *JAK2*-V617F-positive MPN, often a somatic mutation occurred before the acquisition of *JAK2* V617F, compatible with providing a “fertile ground” for MPN disease initiation.³⁵ In contrast, *CALR* mutations appear to be the initiating event that could be followed by mutations in same set of genes as observed in *JAK2*-positive MPN. Patients

with multiple mutations formed a high-risk category, with increased risk of transformation and reduced survival. Although based on a limited number of patients, the acquisition of *TP53* appears to be a particularly unfavorable event, and loss of heterozygosity was invariably associated with progression to AML.

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Authorship

Contribution: P.L. designed and performed research, analyzed data, and wrote the paper; A.K. and R.N. performed research, analyzed data, and wrote the paper; R.L., H.H.S., I.N., and C.B. performed research; S.G., T.L., J.P., and M.S. provided clinical data and analyzed results; R.K. designed research and analyzed data; and R.C.S. designed research, analyzed data, and wrote the paper.

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The current affiliation for T.L. is Kantonsspital St. Gallen, St. Gallen, Switzerland.

Correspondence: Radek C. Skoda, Department of Biomedicine, Experimental Hematology, University Hospital Basel, Hebelstrasse 20, 4031 Basel, Switzerland; e-mail: radek.skoda@unibas.ch.

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