

Prevalence and prognostic impact of allelic imbalances associated with leukemic transformation of Philadelphia chromosome–negative myeloproliferative neoplasms

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Philadelphia chromosome–negative myeloproliferative neoplasms (MPNs) including polycythemia vera, essential thrombocythemia, and primary myelofibrosis show an inherent tendency for transformation into leukemia (MPN-blast phase), which is hypothesized to be accompanied by acquisition of additional genomic lesions. We, therefore, examined chromosomal abnormalities by high-resolution single nucleotide polymorphism (SNP) array in 88 MPN patients, as well as 71 cases with MPN-blast phase, and correlated

these findings with their clinical parameters. Frequent genomic alterations were found in MPN after leukemic transformation with up to 3-fold more genomic changes per sample compared with samples in chronic phase ($P < .001$). We identified commonly altered regions involved in disease progression including not only established targets (*ETV6*, *TP53*, and *RUNX1*) but also new candidate genes on 7q, 16q, 19p, and 21q. Moreover, trisomy 8 or amplification of 8q24 (*MYC*) was almost exclusively detected in

***JAK2V617F*⁻ cases with MPN-blast phase. Remarkably, copy number–neutral loss of heterozygosity (CNN-LOH) on either 7q or 9p including homozygous *JAK2V617F* was related to decreased survival after leukemic transformation ($P = .01$ and $P = .016$, respectively). Our high-density SNP-array analysis of MPN genomes in the chronic compared with leukemic stage identified novel target genes and provided prognostic insights associated with the evolution to leukemia. (*Blood*. 2010; 115(14):2882-2890)**

Introduction

Philadelphia chromosome–negative myeloproliferative neoplasms (MPNs) including polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF) are defined as clonal hematopoietic stem cell disorders and characterized by increased proliferation of terminally differentiated myeloid cells. The tyrosine kinase *JAK2* is directly linked to the pathogenesis of MPN with the identification of *JAK2V617F* as a recurring gain-of-function mutation.^{1,2} Almost all cases with PV, and roughly 50% of patients with ET and PMF, carry this specific mutation localized on chromosome 9p24.

The long-term outcome of patients with acute myeloid leukemia (AML) secondary to MPN, myelodysplastic syndrome (MDS), or treatment with cytotoxic agents is relatively poor compared with

patients with de novo AML. Patients with de novo and secondary AML have a similar spectrum of cytogenetic abnormalities, but the occurrence of cytogenetic changes associated with unfavorable risk such as 5q–, –7/7q–, trisomy 8, or complex karyotype is higher in secondary AML.^{3,4} However, so far only a small number of studies with limited number of cases have explored the chromosomal alterations and/or clinical markers associated with acceleration to blast phase of patients with MPN.

Previously, we developed the copy number analyzer for Affymetrix GeneChip (CNAG) program and the new algorithm allele-specific copy number analysis using anonymous references (AsCNAR).^{5,6} These techniques in combination with high-density single nucleotide polymorphism (SNP) array provide a robust and

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Table 1. Clinical features of MPN/MPN-blast phase cases (unmatched and matched)

	Unmatched MPN	Unmatched MPN-blast phase	Matched MPN	Matched MPN-blast phase
All cases, no. (%)	77 (56)	60 (44)	11 (50)	11 (50)
MPN diagnosis no. (%)				
PV samples	21 (27)	17 (28)	2 (18)	2 (18)
ET samples	31 (40)	18 (30)	1 (09)	1 (09)
PMF samples	25 (33)	25 (42)	8 (73)	8 (73)
Sex, M:F				
PV samples	1:2	1:1	1:1	1:1
ET samples	1:2	1:1	0:1	0:1
PMF samples	2:1	2:1	2:1	2:1
Mean age at diagnosis, y, ± SD*				
PV	57 ± 5	68 ± 5	—	—
ET	59 ± 6	69 ± 7	—	—
PMF	57 ± 6	65 ± 9	59 ± 9	65 ± 7
Mean blast count in bone marrow, ± SD, no. (%)*				
PV samples	< 5%	70 ± 20	—	—
ET samples	< 5%	66 ± 23	—	—
PMF samples	< 5%	70 ± 21	< 5%	66 ± 24
JAK2V617F (+) no. (%)				
PV samples	21/21 (100)	14/17 (82)‡	2/2 (100)	1/2 (50)
ET samples	18/31 (58)	6/18 (33)	0/1 (0)	0/1 (0)
PMF samples	16/25 (64)	12/25 (48)	5/8 (62.5)	4/8 (50)
c-MPL mutation positive, no. (%)				
PV samples	1/21 (5)†	0/17 (0)	0/2 (0)	0/2 (0)
ET samples	0/31 (0)	1/18 (6)	0/1 (0)	0/1 (0)
PMF samples	3/25 (12)	2/25 (8)	1/8 (12.5)	1/8 (12.5)

MPN indicates myeloproliferative neoplasm; PV, polycythemia vera; ET, essential thrombocytosis; M, male; F, female; and PMF, primary myelofibrosis.

*Data are available for 27 unmatched MPN (10 PV, 10 ET, and 7 PMF) and 54 unmatched MPN-blast phase (15 PV, 18 ET, and 21 PMF) cases, and 8 matched MPN (PMF) cases.

†This c-MPL mutation in a PV patient has already been validated and reported by Kawamata et al.⁸

‡Significantly fewer cases with JAK2V617F in blast phase vs chronic phase ($P = .045$).

detailed approach to detect large and small copy number changes, as well as copy number–neutral loss of heterozygosity (CNN-LOH). To obtain a comprehensive profile of genomic alterations associated with leukemic transformation in MPN, we applied this interrogational method and performed a systemic analysis of 159 samples obtained from patients either in chronic phase or blast phase of MPN.

Methods

Patients and clinical samples

In total, samples from 148 patients were analyzed by SNP-array. One hundred fifty-nine samples were obtained, of which 88 (55%) were diagnosed with MPN in chronic phase (23 PV, 32 ET, 33 PMF) and 71 (45%), with MPN in blast phase (19 PV, 19 ET, 33 PMF). Diagnosis was based on the World Health Organization criteria,⁷ and an overview of patients, including clinical data, is given in Table 1. This study received institutional review board approval from the Cedars-Sinai Medical Center, and informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Given the relatively high incidence of homozygous JAK2V17F patients diagnosed with ET (3/18 in chronic phase, 2/6 in blast phase), which is usually lower for this disorder,⁹ we suggest that at least some cases diagnosed with ET may have been incorrect.

Samples were provided by (1) Department of Hematology, Mayo Clinic (n = 35); (2) Brigham and Women's Hospital, Harvard University, School of Medicine (n = 46); (3) Department of Hematology, Archet Hospital (n = 44); (4) MLL Munich Leukemia Laboratory, (n = 14); (5) Division of Hematology-Oncology, Chang Gung Memorial Hospital (n = 14); and (6) Division of Hematology, Sheba Medical Center and Sackler School of Medicine, Tel-Aviv University (n = 6).

SNP-Chip analysis

A total of 159 tumor specimens (MPN and/or MPN-blast phase) were analyzed on GeneChip SNP genotyping microarrays (GeneChip Mapping 50K and/or 250K arrays; Affymetrix) as described previously.^{5,6} After appropriate normalization of mean array intensities, signal ratios were calculated between tumors and anonymous normal references in an allele-specific manner. Genome-wide determination of allele-specific copy numbers (AsCNs) and detection of CNN-LOH at each SNP were inferred from the observed signal ratios based on the hidden Markov model using CNAG/AsCNAR algorithms (<http://www.genome.umin.jp>).^{5,6} For clustering of patient samples with regard to the status of copy number changes, as well as CNN-LOH, CNAG-Graph software (Tokyo University) was used. Size, position, and location of genes were identified with the University of California, Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu>)¹⁰ and Ensemble Genome Browser (<http://www.ensembl.org>).¹¹ Germline copy number changes previously described as copy number variants at Database of Genomic Variants (<http://projects.tcag.ca/variation>)¹² and UCSC Genome Browser were excluded. SNP-array data used in this study are available in the Gene Expression Omnibus (GEO) database under accession number GSE19647.¹³

Comparison of 50K versus 250K SNP-Chip analysis in MPN chronic phase

SNP-array analysis of 46 of our MPN samples (10 PV, 20 ET, 16 PMF; kindly provided by D.G.G. at Brigham and Women's Hospital, Harvard University) has already been reported by our group.⁸ At that time, only 50K arrays were available, whereas later in this study, the 250K arrays were accessible and used to analyze additionally 42 MPN and 71 MPN-blast phase samples. Because no significant differences in either number of deletions, duplications/amplifications, or CNN-LOH per case were found as analyzed by the 50K compared with 250K array (supplemental Table 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), we combined the analysis of both platforms in our

results. Supplemental Table 2 lists all individual samples and the array that was used.

Cytogenetics

Routine cytogenetic analysis with conventional banding techniques was performed in 35 of 88 MPN (10/23 PV, 10/32 ET, 15/33 PMF) and 63 of 71 MPN-blast phase (15/19 PV, 18/19 ET, 30/33 PMF) cases according to standard procedures as previously described.¹⁴ No routine fluorescent in situ hybridization (FISH) panel was applied, but in some cases, however, FISH analysis was performed to supplement conventional cytogenetic analysis (supplemental Table 2).

Allele-specific PCR for *JAK2V617F* mutation

For the detection of *JAK2V617F*, allele-specific polymerase chain reaction (PCR) was performed according to the previously reported method.¹⁵

Direct mutation screening

Primers were designed to amplify and sequence coding exons and splice junctions of the following genes: *TET2*, *c-CBL*, *TP53*, and *RUNX1*. We screened only the 11 matched samples that showed genomic changes in the particular gene regions. Primer details are available from the corresponding author (N.H.T.).

We evaluated all MPN and MPN-blast phase patients with 1pCNN-LOH for the *MPLW515* mutation (exon 10) by direct sequencing. If no mutation was detected in this cohort, we also screened the other coding exons of the *c-MPL* gene previously shown to be mutated in MPN.⁸

Validation of acquired genomic copy number changes including CNN-LOH

To confirm the somatic origin of genomic copy number changes, quantitative genomic real-time (QG RT)-PCR was performed on the genomic DNA from the hybridized MPN and matched MPN-blast phase samples according to the calculation method described by Weksberg et al.¹⁶ For example, we used primers for the *RUNX1* gene (21q22.12; supplemental Figure 1A) as well as *TET2* gene (4q24; data not shown) and a random region on chromosome 21q21.1 and 4p15.1, respectively, as a reference in patient 121.

Detection of acquired CNN-LOH was also validated by QG RT-PCR and subsequently by nucleotide sequencing. Three SNP sequences (rs919275, rs10854117, and rs10854117) on chromosome 19p in case 36 at diagnosis of PV, as well as at leukemic transformation, were determined (supplemental Figure 1B). The genomic region of each SNP site was amplified, and products were purified and sequenced (supplemental Figure 1C). In addition, we confirmed loss of CNN-LOH on 9p after leukemic transformation in matched case 120 using SNP sequences rs3858029, rs1360461, and rs10818814 on chromosome 9 (data not shown).

Homozygous deletions of *CUTL1* and *SH2B2* (case 138) as well as *PIG-A* (case 121) in both MPN and/or MPN-blast phase samples were also confirmed by QG RT-PCR (supplemental Figure 2). Primers for these experiments will be provided upon request.

Statistical analysis

Wilcoxon rank sum tests were used to assess differences in continuous variables, and categoric variables were assessed using chi-square tests, all with a significance level of $\alpha = .05$. The methods of estimations included the standard deviation (\pm SD) of the sampling distribution. Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control condition (* $P < .05$; ** $P < .001$). Survival analysis was performed using the Kaplan-Meier method, and survival curves were compared using the log-rank test.

Results

Lower frequency of *JAK2V617F* and 9p alterations after leukemic transformation

In the present study, we examined 159 samples (88 MPN and 71 MPN-blast phase) from a total of 148 patients. An overview of the clinical

features of matched and unmatched cases including sex, age, leukemic blast infiltration, and mutational status (*JAK2V617F*, *c-MPL*) is provided in Table 1. The sex ratio of male and female patients in chronic phase was 1:2 for PV and ET, whereas after transformation, the ratio was balanced with 1:1. For PMF patients, the male-to-female ratio was 2:1 in both MPN chronic and blast phase.

Overall, the incidence of *JAK2V617F* was almost 20% less in the blast phase compared with the chronic phase for both the matched and unmatched MPN cases (unmatched cases: $P < .05$; Figure 1A). Cases that were negative for *JAK2V617F* were also exclusively negative for 9p duplication, trisomy 9, or 9pCNN-LOH in the chronic as well as leukemic stage of MPN. 9pCNN-LOH was noted approximately 3 to 4 times more often than 9p duplication and/or trisomy 9 in *JAK2V617F*⁺ MPN cases during either the chronic or blast phase (Figure 1A), but the frequency of 9pCNN-LOH was significantly less in the blast crisis compared with the chronic phase of unmatched PMF and PV patients (supplemental Table 3). In contrast, unmatched ET cases had about the same frequency of 9pCNN-LOH in the chronic phase versus the blast phase of the disease. Furthermore, in the analysis of the 11 matched MPN cases, 7 were positive for *JAK2V617F* (64%), 4 had 9p CNN-LOH (37%), and 1 had 9p duplication (9%) at first diagnosis (Figure 1A). In comparison, 2 of these patients were *JAK2V617F*⁺ with either trisomy 9 or 9pCNN-LOH during their chronic phase (1 PV, 1 PMF), but no longer had detectable *JAK2V617F* with a normal chromosome 9 after leukemic evolution (Figure 1B).

JAK2V617F mutational status had no impact on time to transformation or survival

In the evaluation of clinical data for MPN-blast phase patients, no significant correlation was noted between the prevalence of *JAK2V617F* at transformation and either age, percentage of leukemic blast cells in the marrow, or pretreatment with alkylating agents and/or hydroxyurea (data not shown). Moreover, we found no statistical association between either time to leukemic transformation or overall survival and the *JAK2V617F* status at transformation in PV, ET, or PMF patients. The overall survival of MPN-blast phase patients with *JAK2V617F* versus blast phase patients without this mutation is provided in Figure 2A ($P = .6$). In addition, with respect to the comparably low frequency of *MPLW515*-positive MPN-blast phase patients (6%), we noted no impact of the *c-MPL* mutational status on either time to transformation (data not shown) or the overall survival in MPN patients who underwent leukemic transformation ($P = .5$; Figure 2B).

However, regardless of the mutational status of MPN-blast phase patients, we noted that the time from diagnosis of MPN to leukemic transformation was significantly shorter in those with pre-existing PMF (median, 58 months) compared with patients with either prior PV (median, 98 months) or ET (median, 110 months; $P = .01$). This earlier transformation resulted in a decreased overall survival from the time of diagnosis of the underlying MPN in leukemic patients with preceding PMF patients compared with preceding PV or ET ($P = .02$; Figure 2C), which is congruent with previously published results.¹⁷

Increased number of additional genomic changes after leukemic transformation

Altogether, a relatively low number of genomic alterations was found by SNP-array analysis in the chronic phase of the MPN samples (Figure 3A). In contrast, 2 to 3 times more abnormalities per sample were detected after leukemic evolution in both matched and unmatched cases with MPN (both $P < .001$; Figure 3A). We

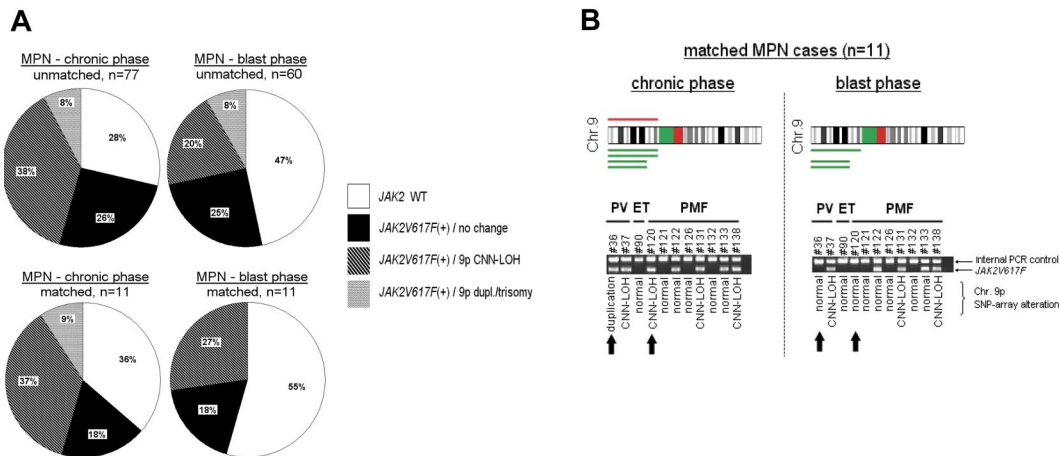


Figure 1. Frequency of *JAK2V617F* and associated alterations on chromosome 9. (A) Diagrams represent matched and unmatched MPN cases in chronic versus blast phase. Indicated are frequencies of *JAK2V617F* and association to 9p duplication (dupl)/trisomy 9 or 9pCNN-LOH. Data and statistical evaluation for underlying MPN subgroups are shown in supplemental Table 3. (B) CNAG software represents duplication (red) and CNN-LOH (green) on 9p detected in 11 patients with matched samples (chronic MPN vs MPN-blast phase). In addition, allele-specific PCR for the detection of *JAK2V617F* was performed in these samples. Arrows indicate 2 MPN patients who were initially positive for *JAK2V617F* in association with 9p imbalances; leukemic transformation was accompanied with loss of *JAK2V617F* and a normal chromosome 9.

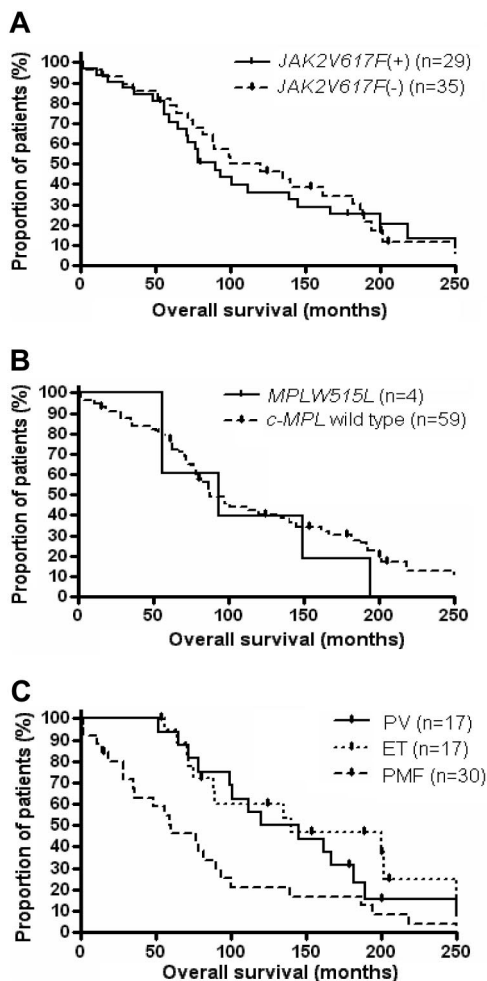


Figure 2. Overall survival of MPN patients with subsequent transformation to blast crisis. Kaplan-Meier plots of all MPN-blast phase patients from the diagnosis of pre-existing MPN were stratified for (A) the presence or absence of a *JAK2V617F* mutation at transformation, (B) the presence or absence of a *MPLW515L* mutation at transformation, and (C) the underlying type of MPN.

found no statistical relationship between the *JAK2V617F* status and the number of genomic changes in matched as well as unmatched samples (data not shown). However, samples from ET patients had fewer copy number changes than those from either PV or PMF patients in the chronic phase, which was highly significant in the unmatched cases ($P < .001$; Figure 3A, supplemental Figure 3A). After leukemic transformation, a similar number of SNP-array changes occurred in cases with prior ET compared with those with pre-existing PV and PMF (unmatched cases: $P = .59$). Statistical evaluation of the matched samples divided into each subentity was not possible because of the small number of cases (Figure 3Aii and supplemental Figure 3B). A subanalysis of the number of either deletions, duplications/amplifications, or CNN-LOH per case, matched and unmatched, is shown in supplemental Figure 3.

Compared with the cytogenetic data, SNP-array analysis detected more than 2-fold of additional chromosomal changes in the MPN samples of either chronic or blast phase, whereas SNP-array practically captured all cytogenetic abnormalities (Figure 3B).

Candidate genes involved in leukemic transformation of MPN patients

SNP-chip analysis detected several additionally altered regions in patients after leukemic evolution compared with the MPN chronic phase in both unmatched (Figure 4; supplemental Figure 4) and matched (Figure 5A) cases. The altered regions included chromosome 8q (*MYC*), 12p (*ETV6*), 17p (*TP53*), and 21q (*RUNX1*), which are already known to be involved in leukemogenesis.¹⁸⁻²² Trisomy 8 was detected in 12% of unmatched and 9% of matched cases in MPN-blast phase; interestingly, almost all these samples were negative for *JAK2V617F*. PMF patient 148, who was also *JAK2V617F*⁻, showed amplification of 8q24.21 in blast crisis involving the *MYC* gene. MPN-blast phase patients with trisomy 8 did not show an inferior outcome compared with cases without this abnormality ($P = .11$; data not shown).

In 20% of unmatched cases in MPN-blast phase, deletions (12%) or CNN-LOH (8%) occurred on **chromosome 17 including *TP53* at p13.1**. Deletions on the short arm of chromosome 17 were detected significantly often in MPN-blast phase patients who received prior treatment with hydroxyurea with or without the addition of alkylating agents ($P = .035$, Table 2). Supplemental

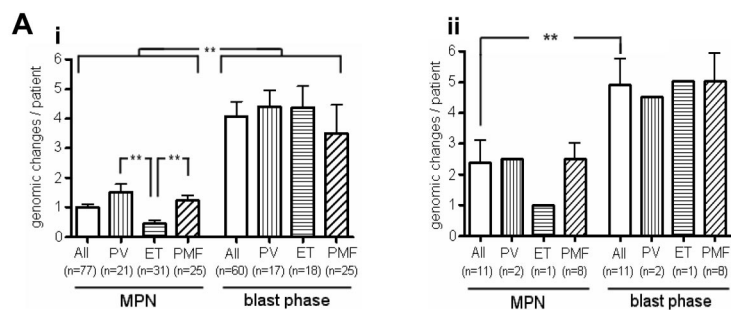


Figure 3. Genomic alterations per MPN patient in chronic versus blast phase. (A) Mean of SNP-array alterations per patient in MPN versus MPN-blast phase with (i) unmatched samples and (ii) matched samples (\pm SD); $**P < .001$. (B) Mean of SNP-array aberrations compared with cytogenetic alterations per patient in chronic versus blast phase with (i) unmatched samples and (ii) matched samples (\pm SD).

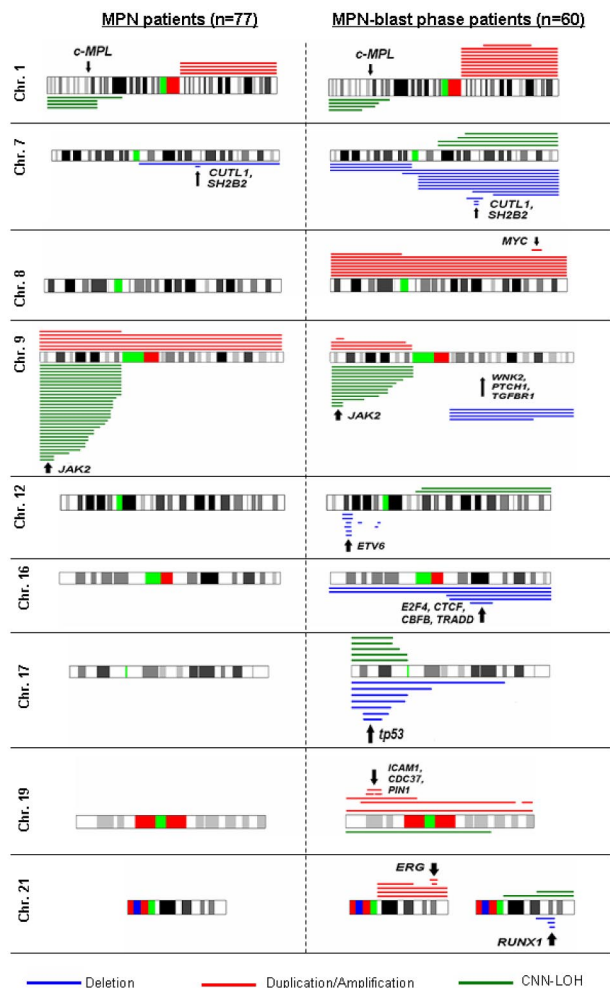
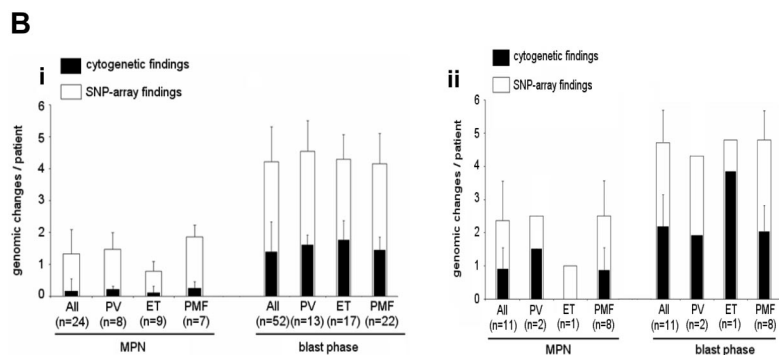


Figure 4. Overview of gains and losses detected by CNAG software. Indicated are the most common altered regions in unmatched MPN-blast phase patients ($n = 60$; right-sided cytobands) compared with unmatched MPN patients ($n = 77$; left-sided cytobands). Each line represents 1 sample with either deletion (blue), duplication/amplification (red), or CNN-LOH (green). Candidate genes of the minimal altered regions are highlighted by arrows.

Table 4 indicates pretreatment (hydroxyurea and/or alkylating agents) of 47 MPN-blast phase patients and their individual SNP-array findings. Deletion or CNN-LOH on 17p in unmatched blast phase cases was associated with either complex karyotype or isochromosome 17 ($P = .01$), and significantly decreased survival (with 17p deletion: $P = .012$; with 17p CNN-LOH: $P = .018$). One of the 11 matched MPN samples (case 120) acquired a 17p deletion at diagnosis of blast phase (Figure 5A), resulting in a hemizygous mutant *TP53* (M133K; Figure 5Bi).

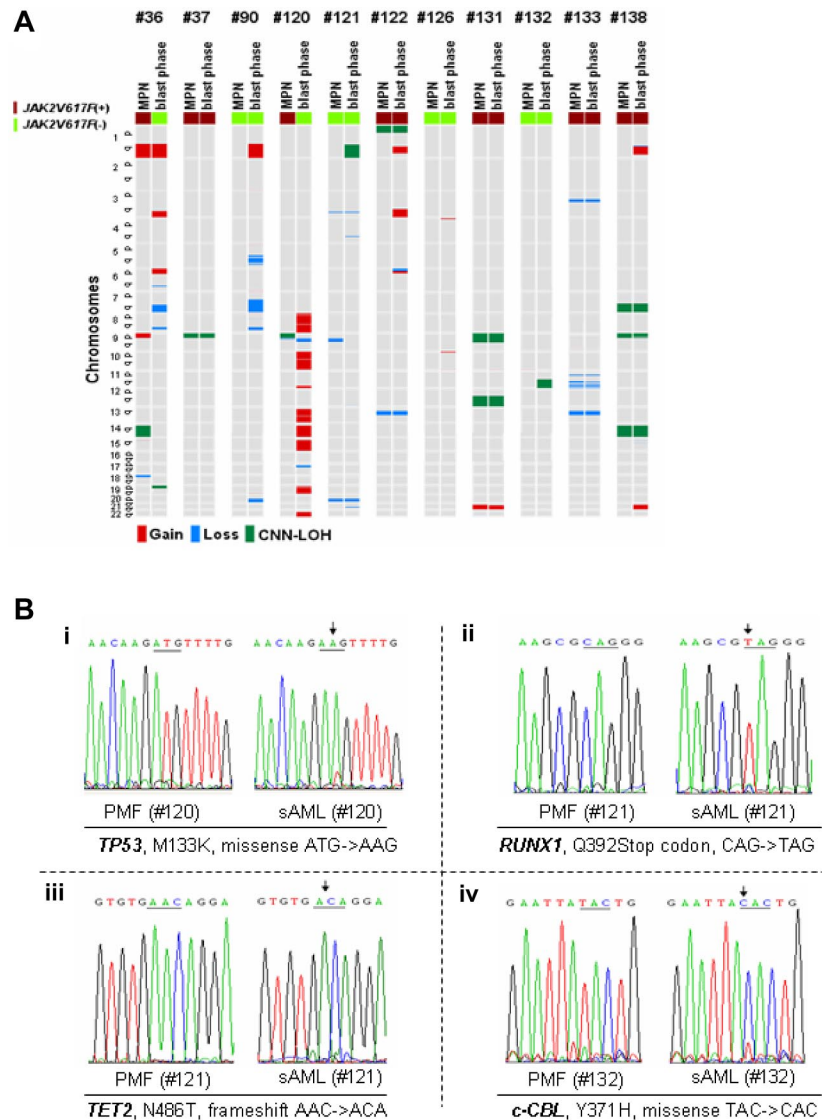
On chromosome 21, SNP-chip analysis revealed either deletions or CNN-LOH in 8% of unmatched cases in MPN-blast phase involving the transcription factor *RUNX1* at q22.12. Patient 121 acquired a small deletion of that locus in the leukemic sample (Figure 5A) associated with a mutation of the Runt domain of the *RUNX1* gene on the remaining allele (Q392Stop codon; Figure 5Bii).

Deletion or CNN-LOH on 4q24 spanning the *TET2* gene was detected in 6% of unmatched blast phase cases and 1% in chronic phase. One *TET2* mutation was found by nucleotide sequencing in the matched MPN samples. *JAK2V617F*⁻ case 121 had no genomic imbalances on 4q at diagnosis of PMF, but acquired a microdeletion (1 Mbp) on 4q24 (*TET2*) after leukemic evolution 1 year later (Figure 5A). The remaining allele had a *TET2* frameshift mutation (N486T; Figure 5Biii), and the mutation was absent in the matched PMF sample.

CNN-LOH involving 11q23.3, which has been shown to be strongly associated with *c-CBL* mutations,²³ had an even lower frequency, with only 2% of unmatched MPN cases in either chronic phase or blast crisis. The *JAK2V617F*⁻ patient 132 had 11q CNN-LOH with a homozygous *c-CBL* missense mutation (Y371H) in the MPN-blast phase sample. Both the CNN-LOH and the mutation were absent in the corresponding chronic phase, 2 years before disease progression (Figure 5A-Biv).

Besides these already well-known targets, SNP-array analysis detected commonly altered regions on chromosomes 1, 7, 16, 19, and 21 encompassing potentially new candidate genes involved in MPN transformation. These imbalances were either absent or at least very infrequent in the chronic phase of the disease (Figures

Figure 5. Gains and losses in matched MPN samples and mutational analysis. (A) Most commonly altered genomic regions in MPN samples (left sample column) compared with matched blast phase samples (right sample column) evolved from 11 patients (2 PV, 1 ET, 8 PMF). Each line represents 1 sample with either deletion (blue), duplication/amplification (red), or CNN-LOH (green). (B) Hemizygous *TP53* mutation detected in MPN-blast phase sample of case 120 associated with acquired 17q deletion, which was not present in the MPN phase of case 120. (ii) Hemizygous *RUNX1* mutation detected in MPN-blast phase sample (case 121) associated with acquired deletion at 22q22.1, which was not present in the MPN phase of case 121. (iii) Hemizygous *TET2* mutation detected in MPN-blast phase sample of case 121 associated with acquired cryptic deletion on 4q24, which was not present in the MPN phase of case 121. (iv) Homozygous *c-CBL* mutation detected in MPN-blast phase sample of case 132 associated with acquired 11q CNN-LOH, which was not present in the MPN phase of case 132.



4 and 5A, supplemental Figure 4). Ten percent of unmatched and 18% of matched MPN-blast phase cases had either duplication/amplification or CNN-LOH on 19p. The commonly involved region spanned a small locus (2 Mbp) at 19p13.2, where, among others, the genes *PINI*, *ICAMI*, and *CDC37*, which have been associated with carcinogenesis, are located.²⁴⁻²⁶ In addition, the minimal region (1.8 Mbp) of amplifications/duplications/trisomy on chromosome 21 detected in 8% of unmatched and 9% matched

MPN-blast phase samples harbored the oncogenic transcription regulator *ERG* (q22.2).

Table 2. Pretreatment in 47 MPN-blast phase cases and frequency of 17p and 7q deletions

Pretreatment				
Hydroxyurea	-	+	-	+
Alkylating agents	-	-	+	+
SNP-array alteration (17p vs 7q)				
No <i>del</i> (17)(p), no <i>del</i> (7)(q)	17	16	0	1
<i>del</i> (7)(q)	2	2	2	2
<i>del</i> (17)(p)	0	4*	0	1*
<i>del</i> (17)(p) and <i>del</i> (7)(q)	0	0	0	0

Numbers of blast-phase patients are presented.

MPN indicates myeloproliferative neoplasm; and SNP, single nucleotide polymorphism.

*A total of 5 cases with *del*(17)(p) pretreated with hydroxyurea ($P = .035$).

Complete or partial deletion ($-7/7q-$), as well as CNN-LOH of the long arm of chromosome 7, was one of the most common abnormalities detected by SNP-array analysis in up to 25% of unmatched and 27% matched samples evolved in the blast phase. SNP-array also revealed 3 unmatched cases (32, 87, and 116) with a heterozygous microdeletion encompassing the 7q22.1 locus, which was not detectable by cytogenetic analysis. Moreover, case 138 with 7qCNN-LOH had a homozygous deletion on 7q22.1 in both the matched MPN and MPN-blast phase samples (supplemental Figure 2A). The minimally deleted region spanned a small region of 0.88 Mbp at 7q22.1 covering only 2 target genes, *CUTL1* and *SH2B2*. The homozygous deletion of these genes in patient 138 was confirmed by QG RT-PCR (supplemental Figure 2B). Deletions of the long arm of chromosome 7 were found more often in MPN-blast phase patients pretreated with hydroxyurea and/or alkylating agents, but the findings were not statistically significant ($P = .2$; Table 2).

Also worth mentioning, 1 microdeletion encompassing the chromosome X-linked *PIG-A* gene occurred in male patient 121 at

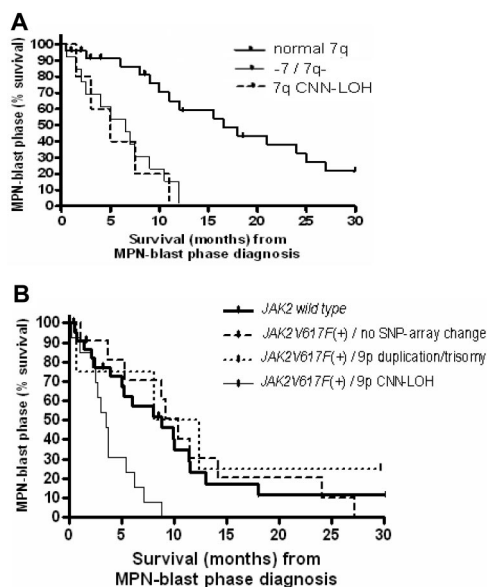


Figure 6. Survival analysis in MPN-blast phase. (A) Survival from the time of diagnosis of blast phase in transformed MPN patients with normal chromosome 7 (normal 7q) compared with either monosomy 7 ($-7/7q-$) or 7qCNN-LOH. (B) Survival from the time of diagnosis of blast phase in transformed MPN patients with homozygous *JAK2V617F*⁺ associated with 9pCNN-LOH compared with either heterozygous *JAK2V617F*⁺ with 9p duplication/trisomy 9 or no abnormality, or patients without the mutation (*JAK2 wild type*). Median survival (months) and the case numbers for each group (transformed PV, ET, or PMF) are listed in supplemental Table 5.

leukemic transformation (supplemental Figure 2C). This patient had a normal chromosome X in his chronic phase of PMF.

CNN-LOH is a marker of poor survival in MPN patients after leukemic evolution

SNP-array technology provides efficient and effective detection of segmental CNN-LOH. In the present study, the most prominent regions for CNN-LOH besides chromosome 9p (*JAK2*) were on 7q and 17p (*TP53*) in patients with MPN-blast phase. In marked contrast to CNN-LOH on 9p, CNN-LOH on 7q or 17p almost never occurred in the chronic phase of the disorder in matched and unmatched samples. As mentioned previously, cases with CNN-LOH and/or deletion of 17p were associated with either complex karyotype or isochromosome 17 and decreased survival.

As also expected, survival in the MPN-blast phase was significantly decreased in patients with $-7/7q-$ (median, 3.75 months) compared with those without chromosome 7 alterations (median, 9 months; $P = .008$). In addition, the unbalanced translocation, *der(1;7)(q10;p10)*, a nonrandom chromosomal abnormality rarely found in AML, was detected by SNP-chip and FISH in 7% of unmatched samples after leukemic evolution and was also associated with an inferior outcome compared with patients without chromosome 7 imbalances ($P = .014$). Strikingly, survival continued to be significantly decreased in MPN-blast phase, when cases with only 7qCNN-LOH were compared with those with a normal 7q ($P = .01$; Figure 6A; supplemental Table 5).

The *JAK2V617F* mutational status in terms of heterozygosity or homozygosity appeared to have no influence on the duration to leukemic evolution. Regardless of the underlying MPN subgroup, no statistical difference in the time to leukemic transformation was found comparing *JAK2V617F*⁺ patients with normal chromosome 9 to mutant positive blast phase patients with either 9p duplication/trisomy 9 ($P = .28$) or 9pCNN-LOH ($P = .21$). In-

stead, we found that homozygous *JAK2V617F* had an impact on survival after MPN transformation. Blast phase patients with 9pCNN-LOH resulting in a homozygous *JAK2* mutation had a worse outcome (median, 4 months) compared with *JAK2V617F*⁺ MPN-blast phase patients with either 9p duplication/trisomy 9 (median, 7.5 months) or no abnormality on 9p (median, 9 months), as well as patients without *JAK2V617F* (median, 7 months, $P = .016$; Figure 6B; supplemental Table 5). Homozygous *JAK2V617F* in association with CNN-LOH diagnosed at leukemic transformation was independent of known risk factors such as 5q⁻, $-7/7q-$, or complex karyotype ($P > .05$).

Discussion

Oncogenic *JAK2* signaling is an important event in MPN.^{1,2} Recently, we and others showed that homozygosity for *JAK2V617F* is closely related to chromosome 9pCNN-LOH in MPN patients.^{1,6,8,9} However, the transformation process of MPN to MPN-blast phase is not well understood.

Recent findings suggested that transition from heterozygosity to homozygosity for *JAK2V617F* is associated with a hyperproliferative disease profile and may be important for disease progression, at least from PV to secondary myelofibrosis.²⁷ Moreover, Barosi et al showed in a longitudinal prospective study that the presence of a *JAK2V617F* hematopoietic clone was significantly associated with leukemic transformation in PMF.²⁸ This is in contrast to our present findings showing that not only the mutational status of *JAK2V617F*, but also 9pCNN-LOH with homozygous *JAK2V617F*, had no impact on the time to leukemic transformation in patients with MPN-blast phase. In addition, 2 of the 11 matched MPN samples, initially positive for *JAK2V617F* with either trisomy 9 or 9pCNN-LOH, became negative for these abnormalities after leukemic transformation. Although only tested in unpaired samples, PMF and PV samples also showed a significantly smaller number of both *JAK2V617F*⁺ and 9pCNN-LOH in the blast phase compared with the chronic phase. Interestingly, and also contrary to the previously cited studies, Tefferi et al noted a significant association between a low *JAK2V617F* allelic burden and evolution to blast phase in a large cohort of PMF patients.²⁹ Even though these data are not completely congruent with our findings, the results of Tefferi et al and our results point to the coexistence of a more dominant *JAK2V617F*-negative clone with a higher propensity to undergo clonal evolution. This is congruent with recent studies indicating that *JAK2V617F*⁺ MPN can result in *JAK2V617F*⁻ MPN-blast phase.^{30,31} But still, some of our matched cases with *JAK2V617F*⁺ had no change in abnormalities including *JAK2* mutational status as well as 9pCNN-LOH, allowing the existence of a common pre-*JAK2V617F* clone. Taken together, the presence of *JAK2V617F* appears not to be a prerequisite for leukemic transformation of MPN, suggesting that additional genetic events are required for full transformation.

SNP-array analysis was able to capture practically all cytogenetic abnormalities and to uncover additional lesions with potentially important clinical implications. The number of genomic alterations was more than 2 to 3 times greater in the blast phase as in the chronic phase of matched and unmatched cases with MPN. Noticeably, ET patients had fewer alterations in their chronic phase samples compared with the PV and PMF cases, whereas the number was comparable in all 3 MPN subgroups after their transformation. Being aware of the increased number of new

genomic changes enables investigators to focus on the identification of causative genes associated with the evolution of MPN to leukemia.

Commonly altered regions in blast crisis samples were detected on chromosomes 8, 12, 17, and 21 encompassing *MYC*, *ETV6*, *TP53*, and *RUNX1*, respectively, which are already known to be involved in the development of de novo and secondary AML.¹⁸⁻²² Gain of chromosomal material at 8q24.21 was almost exclusively found in *JAK2V617F*⁻ samples, suggesting that increased activity of *MYC* might allow selection of clones that do not require the *JAK2* gain-of-function mutation. Furthermore, deletion of 17p (*TP53*) was significantly associated with prior exposure to hydroxyurea as well as a complex karyotype in samples with MPN-blast crisis, which is in accordance with recent results.^{32,33} Interestingly, not only deletion, but also 17pCNN-LOH, was associated with a complex karyotype, a poor prognostic marker in myeloid malignancies.

In addition, regions on chromosomes 1q, 7q, 16q, 19p, and 21q were frequently altered in the evolution to the leukemic phase and may harbor promising new candidate genes. Abnormalities involving chromosome 7 are frequently detectable in de novo and secondary AML,³⁴⁻³⁷ and preceding studies have found a critical breakpoint region involving a locus at centromeric band 7q22, whereas the telomeric breakpoint varies from q32 to q36. Interestingly, the minimal deleted region in our cohort was located at 7q22.1 encompassing only 2 promising target genes, *SH2B2* (previously named *APS*) and *CUTL1*. *SH2B2* regulates and enhances *JAK2*-mediated cellular responses,³⁸ and the *CUTL1* gene encodes for a *CUT* family member of the homeodomain proteins that can repress the expression of developmentally regulated myeloid genes.³⁹ Moreover, genome-wide inspection for minimal regions of duplications/amplifications and CNN-LOH revealed several interesting genes, such as *PINI*, *ICAM1*, and *CDC37* on 19p as well as *ERG* on 21q. Whereas the latter 3 targets have been shown to possess potential progrowth activity in de novo AML and/or MDS,^{25,26,40} *PINI* is known to be overexpressed in a variety of cancers and may act as an oncogene via promotion of cell cycle progression and proliferation.²⁴

Mutations of the *c-CBL* gene are tightly associated with 11qCNN-LOH and are commonly diagnosed in patients with chronic myelomonocytic leukemia.^{23,41,42} Although MPN shares clinical as well as hematologic features with chronic myelomonocytic leukemia, we detected 11qCNN-LOH only in a minority of our study population, suggesting that *c-CBL* mutations are rare events leading to transformation of chronic MPN to leukemic blast phase.

In contrast to recent findings showing frequent LOH on 4q associated with *TET2* mutations in patients diagnosed with MDS/MPN,⁴³ we detected CNN-LOH or deletions at 4q24 (*TET2*) only in a minority of our patients in the chronic as well as blast phase of MPN. Nevertheless, our study was not sufficient to explore these findings in more detail and make conclusions on tumor suppressor *TET2* and its potential role in leukemic transformation.

However, with regard to the variety of detected allelic imbalances, we suggest that no single candidate gene or molecular pathway is sufficient and necessary to cause transformation of chronic MPN to blast phase. Like de novo AML, MPN-blast phase appears to be a heterogeneous disease prone to have evolved multiple mechanisms to provide a proliferative advantage to the abnormal leukemic clone.

CNN-LOH involving chromosomal regions that are also frequently affected by deletions may have prognostic implications similar to the deletions visible by karyotyping. In our study,

prognostic evaluation was based mainly on SNP-array data from blast phase samples without the incorporation of SNP-array results from the matched chronic phase. Moreover, we implied the survival and clinical outcome only of MPN patients who underwent leukemic transformation, without comparison with survival and outcome in untransformed chronic phase. However, as expected, blast phase patients with loss of chromosomal material on 7q showed poor survival, because this is known to be predictive for rapid progression and poor response in AML therapy.³⁵⁻³⁷ MPN-blast phase patients with cytogenetically undetectable 7qCNN-LOH had comparable survival rates to those with -7/7q- in their leukemic cells, which is in accordance with previously published data.⁴⁴

In addition, 9pCNN-LOH with homozygous *JAK2* mutation was also linked to an inferior outcome in MPN-blast crisis in comparison with patients with either heterozygous *JAK2V617F* or wild-type *JAK2*. In contrast to LOH on 17p, the prognostic impact of 9pCNN-LOH was independent of established risk factors such as -7/7q-, 5q-, or complex karyotype. Although *JAK2V617F* in association with 9pCNN-LOH appeared to have no impact on the time to MPN transformation, we suggest that the homozygous driver mutation in combination with additional newly acquired aberrations in terms of a second hit may have an implication on the clinical course of MPN-blast phase patients.

In conclusion, high-density SNP-array technology allowed precise identification of chromosomal aberrations, including CNN-LOH, and complemented conventional cytogenetic techniques in patients with chronic and transformed MPN. Our analysis provided prognostic details to further improve clinical prognosis, as well as novel interesting candidate genes potentially involved in the transformation of MPN.

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Authorship

Contribution: N.H.T and U.O.K. performed the research, analyzed the data, and wrote the paper; D.H.T.L., N.K., G.B.I., T.L., T.W., D.N., M.K.-M., M.K., M.S., L.-Y.S., A.N., and S.D.R. assisted with the research; C.M.-T., R.M., T.H., D.G.G., and A.T. designed and performed the research; and S.O. and H.P.K. directed the overall study.

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