Frequent deletions of *JARID2* in leukemic transformation of chronic myeloid malignancies

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Chronic myeloproliferative neoplasms (MPN) and myelodysplastic syndromes (MDS) have an inherent tendency to progress to acute myeloid leukemia (AML). Using high-resolution SNP microarrays, we studied a total of 517 MPN and MDS patients in different disease stages, including 77 AML cases with previous history of MPN (*N* = 46) or MDS (*N* = 31). Frequent chromosomal deletions of variable sizes were detected, allowing the mapping of putative tumor suppressor genes involved in the leukemic transformation process. We detected frequent deletions on the short arm of chromosome 6 (del6p). The common deleted region on 6p mapped to a 1.1-Mb region and contained only the *JARID2* gene—member of the polycomb repressive complex 2 (PRC2). When we compared the frequency of del6p between chronic and leukemic phase, we observed a strong association of del6p with leukemic transformation (*P* = 0.0033). Subsequently, analysis of deletion profiles of other PRC2 members revealed frequent losses of genes such as *EZH2*, *AEBP2*, and *SUZ12*; however, the deletions targeting these genes were large. We also identified two patients with homozygous losses of *JARID2* and *AEBP2*. We observed frequent codeletion of *AEBP2* and *ETV6*, and similarly, *SUZ12* and *NF1*. Using next generation exome sequencing of 40 patients, we identified only one somatic mutation in the PRC2 complex member *SUZ12*. As the frequency of point mutations in PRC2 members was found to be low, deletions were the main type of lesions targeting PRC2 complex members. Our study suggests an essential role of the PRC2 complex in the leukemic transformation of chronic myeloid disorders. Am. J. Hematol. 87:245–250, 2012. © 2011 Wiley Periodicals, Inc.

Introduction

The hematopoietic stem cells maintain sufficient blood cell production that involves self-renewal and tight regulation of proliferation and differentiation. During the lifetime of an individual somatic mutations arise in hematopoietic stem cells that may cause dominance of one or more stem cell clones. Once a clone has been established, it undergoes further mutagenesis and the resulting impact on proliferation capacity and differentiation dynamics may give rise to hematological phenotypes with excessive or deficient production of terminally differentiated blood cells. These conditions in the myeloid compartment fall into two large phenotypic categories referred to as chronic myeloproliferative neoplasms (MPN) and myelodysplastic syndromes (MDS). MPN is characterized by excessive production of myeloid cells, whereas MDS often displays dysplasia and deficient myeloid cell production with or without the presence of blasts.

A proportion of BCR-ABL negative MPN cases, also named classical MPN, develop acute myeloid leukemia (AML; 5–7%). The average time from MPN diagnosis to transformation is ~6 years, with the transformation occurring faster in myelofibrosis then in polycythemia vera (PV) and essential thrombocythemia (ET) patients. Patients who develop AML have a poor prognosis with an average survival time after transformation less than 5 months [1]. Some patients experience disease progression characterized by either secondary myelofibrosis (post-PV or post-ET) or a so-called "accelerated phase" defined by pancytopenia and increased number of blasts in the bone marrow, but below 20% that is necessary to establish AML diagnosis. MDS patients are also prone to develop AML as a complication of the disease. Approximately 30% of MDS patients transform to AML within a few months to a few years [2]. Post-MPN and post-MDS AML share common phenotypic and molecular features, and have bad prognosis resulting in rapid progression of the terminal stage of the disease and death within only a few months after transformation. To date, no efficient therapy exists and the mechanism of transformation remains unclear.

During the clonal evolution of MPN and MDS, the acquisition of lesions that either abrogate differentiation or increase genome instability can transform patients from chronic phase of the disease to AML. In MPN, transformation to AML is often preceded by an accelerated phase characterized by variable degree of cytopenia and elevated

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Additional Supporting Information may be found in the online version of this article.

Conflict of interest: Nothing to report.

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Contract grant sponsor: Austrian Science Fund; Contract grant number: P23257-B12; Contract grant sponsors: MPN Research Foundation, Fondazione Cariplo, Regione Lombardia; Contract grant sponsor: MPO; Contract grant number: FR-TI2/254; Contract grant sponsor: Associazione Italiana per la Ricerca sul Cancro (AIRC, Milano) "Special Program Molecular Clinical Oncology 5x1000" to AGIMM (AIRC-Gruppo Italiano Malattie Mieloproliferative - http://www-progettoagimm.it; studies in Pavia and Florence); Contract grant numbers: #1005, IG 9034;

Received for publication 15 November 2011; Accepted 15 November 2011

Am. J. Hematol. 87:245–250, 2012.

Published online 18 November 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/ajh.22257

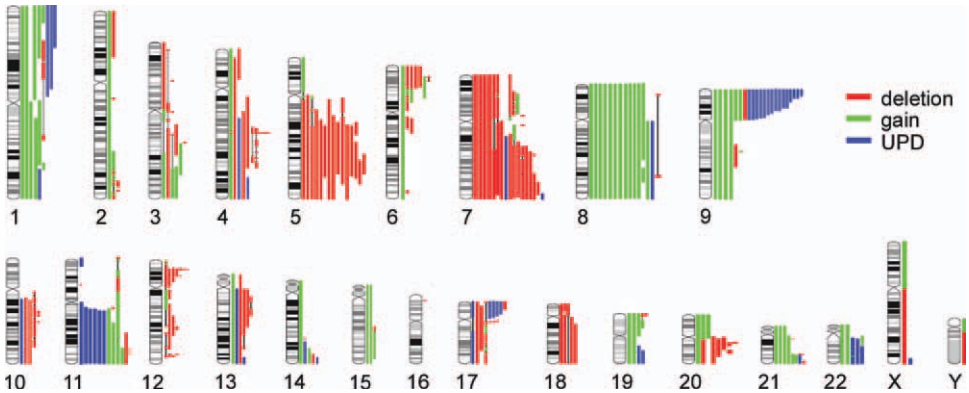

Figure 1. Karyoview of all chromosomal aberrations. Lesions were detected by Affymetrix SNP 6.0 arrays in 77 post-MPN (n = 46) and post-MDS (n = 31) AML patients. Physical positions and sizes of chromosomal aberrations are represented with green, red, and blue bars, indicating gains, deletions, and UPDs, respectively. The aberrations present in the same patient are depicted with thin black lines connecting the bars.

blasts in bone marrow (<20%) and in peripheral blood. As somatic deletions occur frequently in MPN and MDS, deletion mapping offers a powerful tool to identify tumor suppressor genes involved in the pathogenesis of these disorders. Recently, this approach allowed the identification of a number of novel myeloid tumor suppressors such as *IKZF1*, *CUX1*, and *FOXP1* [3–6]. In this study we applied deletion mapping in patients with post-MPN and post-MDS AML and identified frequent lesions of *JARID2*, a member of the polycomb repressive complex 2 (PRC2). Our study suggests that *JARID2*, and other PRC2 members, represent important tumor suppressors playing a crucial role in the leukemic transformation of chronic myeloid malignancies.

Patients and Methods

Patient samples. Peripheral blood samples from individuals diagnosed with MPN and MDS in different disease stages were obtained from several institutions in Italy, Austria, Serbia, and the Czech Republic. Written informed consent was obtained from all subjects in compliance with local ethics regulations. Genomic DNA was isolated according to standard procedures from granulocytes, mononuclear cell fractions, or the whole blood.

Microarray analysis. Microarray data were generated on a total of 517 samples. Of these samples, 77 were either post-MPN or post-MDS AML, 379 were chronic or accelerated phase MPN, and 61 chronic phase MDS. Genomic DNA from all patients was processed and hybridized to Affymetrix Genome-Wide Human SNP 6.0 arrays according to the manufacturer's instructions. Genotyping Console Version 3.0.2 software (Affymetrix) was used for the analysis of the raw data quality as well as identification of copy number alterations and losses of heterozygosity. The criteria used for definition of acquired (somatic) uniparental disomy (UPD) was telomeric position and the size of >1 Mb. In patients carrying numerous interstitial runs of homozygosity (>10 Mb) telomeric UPDs were excluded. Aberrations mapped to known copy number variation loci reported in the Database of Genomic Variants (DGV version 5, human reference genome assembly hg18) were not annotated.

Exome sequencing. DNA samples were processed and enriched for exons using Agilent SureSelect Human All Exon Kit (Agilent, Santa Clara, CA) and TruSeq DNA Sample Preparation and TruSeq Exome Enrichment Kit (Illumina, San Diego, CA) according to manufacturers' protocol. DNA libraries were sequenced using 51-bp paired end sequencing on HiSeq 2000 system (Illumina). Image analysis and base calling was performed using Real Time Analysis 1.12 software (Illumina). Resulting BCL files were converted to FASTQ format with the CASAVA 1.7 software (Illumina). Alignment of the reads and subsequent variant calling were performed in CLC Genomic Workbench 4.7 software (CLC bio, Aarhus, Denmark). For alignments, maximum of two mismatches or 2-bp insertions/deletions were allowed. Variants were called at coverage >10 and allelic frequency of a minimum of 15%. The variant lists were converted to MAQ format and GATK bed format and uploaded to SeattleSeq Annotation Server web tool (http://gvs.gs.washington.edu/ SeattleSeqAnnotation/). The annotated variants were filtered for those listed in dbSNP129 or 1,000 Genomes Project databases. Noncoding and synonymous variants were also removed. Finally, we applied Sanger sequencing for validating the variants in the genes encoding members of the PRC2 complex (*JARID2*, *AEBP2*, *EZH1*, *EZH2*, *SUZ12*, *PHF1*, *PHF19*, *EED*, *MTF2*, *RBBP4*, and *RBBP7*).

Single-gene mutational analysis and validation of exome sequencing. Exon sequencing was performed using BigDye Terminator version 3.1 cycle sequencing kit and the 3130xl Genetic Analyzer (Applied Biosystems). Sequence analysis was performed using Sequencher Version 4.9 software (Gene Codes). Primers were specifically designed for amplifying the regions of the variants.

All primer sequences and PCR conditions are available upon request.

Statistical analysis. Statistical significances of the distributions of frequencies of individual chromosomal aberrations between chronic phase and AML phase were determined using Fisher's exact test with the Bonfferoni correction for multiple testing.

Results

Identification of tumor suppressor genes by deletion mapping

To identify tumor suppressor genes that drive leukemic transformation in MPN and MDS, we aimed to map gene losses in transformed patients using high-resolution SNP array analysis (1.8 million probes per genome). We analyzed 77 AML cases with a previous history of either MPN (N = 46) or MDS (N = 31). In 16.9% of the patients, no chromosomal aberrations could be detected. We identified a number of recurrent deletions, gains, and acquired uniparental disomies (UPD) (Fig. 1). Deletions represented 59% of all cytogenetic lesions (total of 665 events). Because of the high frequency of recurrent deletion events in some genomic regions, it was possible to narrow down the common deleted regions (CDRs) to single genes. Among these recurrently deleted regions we confirmed known tumor suppressors such as *TET2*, *ETV6*, *IKZF1*, and *CUX1* [3–5,7–11].

In all analyzed samples, the vast majority of the deletions were hemizygous. The short arm of chromosome 6 acquired frequent hemizygous as well as a homozygous deletion, identifying a 1.1 Mb CDR. The CDR contained only a single gene *JARID2* (Fig. 2A). In addition to *JARID2*, we identified another homozygous loss as a result of two independent deletion events on the short arm of chromosome 12, forming a CDR that included the *AEBP2* gene (Fig. 2B). In both cases, the homozygous deletion arose by a two-step mechanism. In the first case, a 1.1-Mb deletion targeting *JARID2* was amplified through UPD of 36.5 Mb of the chromosome 6p (Fig. 2C). In the second case, the homozygosity was a consequence of two subsequent deletion events targeting *AEBP2*, the first one harboring a 22.7-

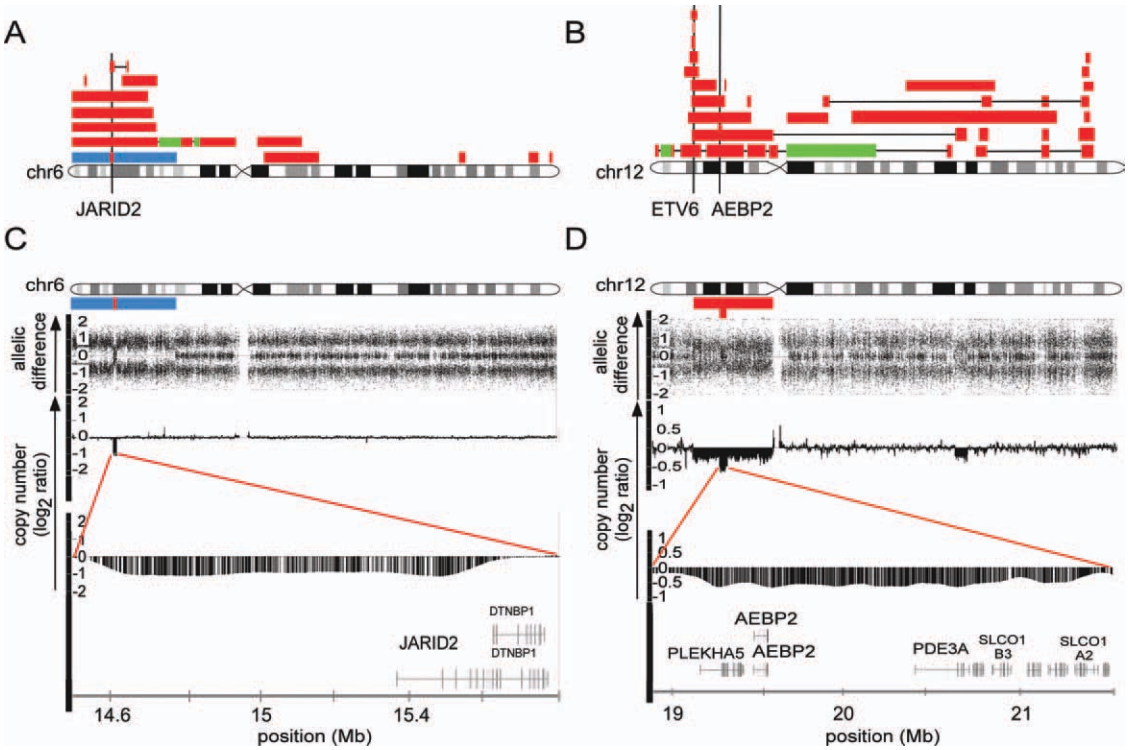

Figure 2. Deletions of *AEBP2* and *JARID2* genes. Positions and sizes of chromosomal aberrations are represented by horizontal bars; red indicating a deletion, green a gain, and blue an UPD. Bars connected by a black line indicate multiple aberrations present in the same patient. The output from the Genotyping Console Version 3.0.2. software represents the copy number data from Affymetrix SNP 6.0 arrays (log2 ratio to normal samples) as well as allelic difference. Chr, chromosome; Mb, megabase. (A) Chromosome 6 deletions detected in all analyzed patients. The commonly deleted region maps down to a single gene—*JARID2*. (B) Deletion profile of chromosome 12. The commonly deleted region harbors the *ETV6* gene; however, the homozygously deleted region in one patient identifies a secondary target of deletions—*AEBP2*, which is codeleted with *ETV6* in 40% of cases. (C) UPD of chromosome 6p with a homozygous deletion of ~1 Mb targeting a single gene *JARID2* in patient UPN_014 diagnosed with JAK2-V617F positive polycythemia vera in the accelerated phase. (D) Patient UPN_047 diagnosed with post-MDS AML carries a ~22.7-Mb deletion on chromosome 12p. Within this region lays a homozygous deletion ~1.8 Mb targeting *AEBP2*, suggesting a two-step deletion event.

Mb deletion, and the second one deleting 1.8 Mb of chromosome 12 (Fig. 2D). It is clear that, compared to single allele losses, deletion of both alleles could provide greater clonal advantage. Both loci also show frequent hemizygous deletion events in other patients in our cohort. In addition to the single case where *JARID2* was homozygously deleted in patient UPN_014, we found another five post-MPN AML cases where the deletion on chromosome 6p included *JARID2* (Fig. 2A). No other target gene on chromosome 6 has been reported in myeloid malignancies. Recurrent deletions are also present along the entire chromosome 12, mainly targeting *ETV6*, which has already been reported as a tumor suppressor frequently deleted in leukemia. We found that 40% of deletions targeting *ETV6* also codeleted *AEBP2*. Simultaneous loss of *ETV6* and *AEBP2* was present in a total of four patients, two diagnosed with post-MPN AML and two cases with post-MDS AML (Fig. 2B). We did not observe the deletion of both genes in any of the chronic phase samples. It is possible that the codeletion of *ETV6* and *AEBP2* provides stronger clonal advantage than the single gene deletions, accounting for the disease progression seen in patients.

Clinical phenotype associated with homozygous deletions of *AEBP2* and *JARID2*

The clinical data of patients UPN_047 and UPN_014, with homozygous losses of *AEBP2* and *JARID2*, respectively, are summarized in Table I. Patient UPN_047 with the homozygous *AEBP2* deletion had low levels of differentiated blood cells at diagnosis, accompanied with 6% blasts in peripheral blood and over 20% blasts in bone marrow. As the examination of blood and bone marrow indicated the existence of a previously unnoticed myelodysplastic phase, the patient was diagnosed as post-MDS AML. The patient had no history of prior cytotoxic therapy. Death occurred a few months after diagnosis.

TABLE I. Clinical Phenotype of Patients with Homozygous *JARID2* and *AEBP2* Deletions

	UPN_014	UPN_047
Diagnosis	PV	Post-MDS AML
Sex	M	F
Age at diagnosis (years)	68	70
Diagnosis at sample	Post-PV MDS (RAEB I)	Post-MDS AML
Age at sample (years)	75	70
Blood count at sample		
Leukocytes (10^9/l)	14.69	3.8
Hemoglobin (g/dl)	9.0	7.7
Hematocrit (%)	28.4	22.4
Platelets (10^9/l)	329	39
% blast in PB at diagnosis (%)	2	6
% blast in PB at sample (%)	5	6
Transfusion dependency	No	Yes
SPL at diagnosis	No	NA
SPL at sample	No	No
History of thrombosis	No	No
Secondary fibrosis	No	No
Therapy	Hydroxyurea Phlebotomy Anagrelide	Azacytidine
JAK2-V617F	Positive	NA
MPL-W515L	Negative	NA

UPN, unique patient number; PV, polycythemia vera; M, male; F, female; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; PB, peripheral blood; SPL, splenomegaly; NA, not applicable; RAEB I, refractory anemia with excess blasts.

TABLE II. Association of Chromosomal Deletions in PRC2 Loci, with Progression of Chronic Phase MPN/MDS to Acute Myeloid Leukemia

Gene name	Chromosome	Size of CDR	Number of genes in CDR	Chronic phase (MPN/MDS) (n = 440)	AML phase (n = 77)	P	P^*
JARID2	6p	1.1 Mb	1	1 (0.2%)	5 (6.5%)	0.0003	0.0033
AEBP2	12p	1.8 Mb	5	0	4 (5.2%)	0.0005	0.0055
SUZ12	17q	1.6 Mb	15	0	5 (6.5%)	0.0001	0.0011
EZH2	7q	15 Mb	>15	2 (0.5%)	17 (22%)	0.0001	0.0011

MPN, myeloproliferative neoplasm; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; CDR, commonly deleted region; Mb, megabase; P, P-value; P^*, P-value corrected for multiple testing using Bonferroni correction.

Patient UPN_014 was first diagnosed with JAK2-V617F positive PV and was treated with hydroxyurea. After 5 years of therapy, hematocrit levels were normalized and the patient continued on anagrelide for control of platelet count. Platelet count increased over time with a simultaneous decrease of hemoglobin levels. The patient was sampled at the "accelerated phase" of the disease characterized by anemia and 9% myeloblasts in the bone marrow.

Deletions of PRC2 loci in leukemic transformation

As *AEBP2* and *JARID2* encode proteins that are members of the PRC2 complex, we closely examined the aberration profile of the other PRC2 members (*EZH1/2*, *EED*, *SUZ12*, *RBBP4*, *RBBP7*, *MTF2*, *PHF1*, *PHF19*) in all 77 leukemic phase cases. Members of the complex are scattered across the genome and no focal deletions of individual members other than *JARID2* and *AEBP2* were found. Instead, the genes were often a part of larger deletions. Deletions covering the region of at least one member of the PRC2 complex were present in 35.6% of postchronic phase AML patients. Among the most frequently deleted were chromosomal regions containing *EZH2*, *JARID2*, *SUZ12*, and *AEBP2* (in 22, 6.5, 6.5, and 5.2% of patients, respectively; Table II). To investigate the presence of such deletions in the chronic phase, we included 440 chronic phase patient samples in this study. The results obtained for 61 MDS chronic phase patient samples were combined with previously reported MPN chronic phase cytogenetic aberration profiles for 379 patients (321 chronic phase MPN and 58 accelerated phase MPN or secondary myelofibrosis) [4]. Regions of *EZH2*, *JARID2*, and *SUZ12* that were deleted in the leukemic phase showed statistically significant differences compared to the frequencies found in chronic phase (Table II). Loss of the region including *EZH2* was detected in 0.5% of chronic phase samples (P = 0.0011), *JARID2* in only 0.2% (P = 0.0033) and loss of the chromosomal region covering *SUZ12* (P = 0.0011) was not found in any chronic phase case. Overall, deletions covering regions of PRC2 members were present in only 1.4% of all chronic phase cases and in 33.7% of the post-MPN and post-MDS AML cases (P = 0.0001). We did not observe mutual exclusivity of deletions of different PRC2 members. When we closely examined the *SUZ12* locus, a number of deletions were found in the region that targeted both *NF1* and *SUZ12* (Fig. 3A). Interestingly, in one patient with post-PV AML with a monosomy of chromosome 17, a homozygous deletion targeting only *NF1* was detected (Fig. 3B).

Point mutation frequency in PRC2 complex members

Next generation exome sequencing was used for sequencing 40 patient samples (31 chronic phase MPN, 6 post-MPN AML, and 3 post-MDS AML). We identified five variants in the PRC2 members *SUZ12*, *EED*, *EZH1*, *EZH2*, and *PHF19*. Variants were present in three MPN patients who had secondary myelofibrosis and one patient with post-MPN AML. The control tissue (T-cells) was available only from the patient with the *SUZ12* mutation in which we managed to show that the mutation is of somatic origin (Fig. 3C). This patient carried a somatic A to C substitution at the first position of codon ACA and the mutation was amplified to homozygosity by an acquired UPD of the long arm of chromosome 17 (Fig. 3C,D). The T598P mutation found in this patient is localized in the VEFS domain of Suz12 and is predicted to be possibly damaging with a high score (0.797) according to the PolyPhen-2 prediction tool. Unfortunately, patients with *EED*, *EZH1*, *EZH2*, and *PHF19* single nucleotide variants had no control tissue available; therefore, their somatic or germline origin could not be determined. The variant found in *EZH2* was predicted to be damaging based on the PolyPhen2 score and targets an amino acid residue that lies within the domain II of the protein, which is frequently targeted by somatic mutations in myeloid disorders [12]. We also performed sequencing of the entire coding region of PRC2 complex members affected by UPDs (Supporting Information Table I), as well as *JARID2* in all patients with a deletion of chromosome 6p, but could not identify any mutations. The overall frequency of mutations in PRC2 members was found to be low, suggesting that deletions are the main type of defects in *JARID2* and *AEBP2* in myeloid malignancies, and the same seems to be also true for other members of the PRC2 complex.

Discussion

PRC2 is a multimeric protein complex found in mammals that negatively regulates gene expression by trimethylating lysine 27 of histone H3 through the enzymatic activity of Ezh2 [13–15]. The core of the complex is formed by Ezh2, Suz12, and Eed, which are essential for its integrity and enzymatic function [16–18]. Other proteins identified to be part of the PRC2 complex are Aebp2, Jarid2, Ezh1, Rbbp4/7, Phf1, Mtf2, and Phf19 [19–27]. Both Jarid2 and Aebp2 contain DNA binding domains and have a role in the recruitment and binding of PRC2 to target genes. In addition, Aebp2 enhances the enzymatic activity of PRC2 by interacting with some of its components. Recent findings implicate that the PRC2 complex plays a role in hematopoiesis through epigenetic regulation of proliferative and self-renewal capacities of hematopoietic stem cells [28–30]. *EZH2* and *SUZ12* were found to be targeted by deletions or inactivating mutations in myeloid malignancies [31–33].

Our data show that *JARID2* and *AEBP2* are homozygously or hemizygously deleted in post-MPN and post-MDS progression stage patients, mainly AML. Patient UPN_014 is not a true chronic phase case, since the clinical data show clear progression of the disease with an increased number of blasts in the bone marrow. Other members of the PRC2 complex are also found affected by larger deletions and if shown to be targets of these deletions, could be novel tumor suppressors with a role in the disease progression from chronic phase to AML. PRC2 members could also be second targets of deletions, with an additive affect to the deletion of other tumor suppressors. Most of the patients acquire only hemizygous deletions covering the PRC2 complex members, suggesting that haploinsufficiency could already be sufficient to manifest a phenotypic effect. In the patient carrying the *JARID2*

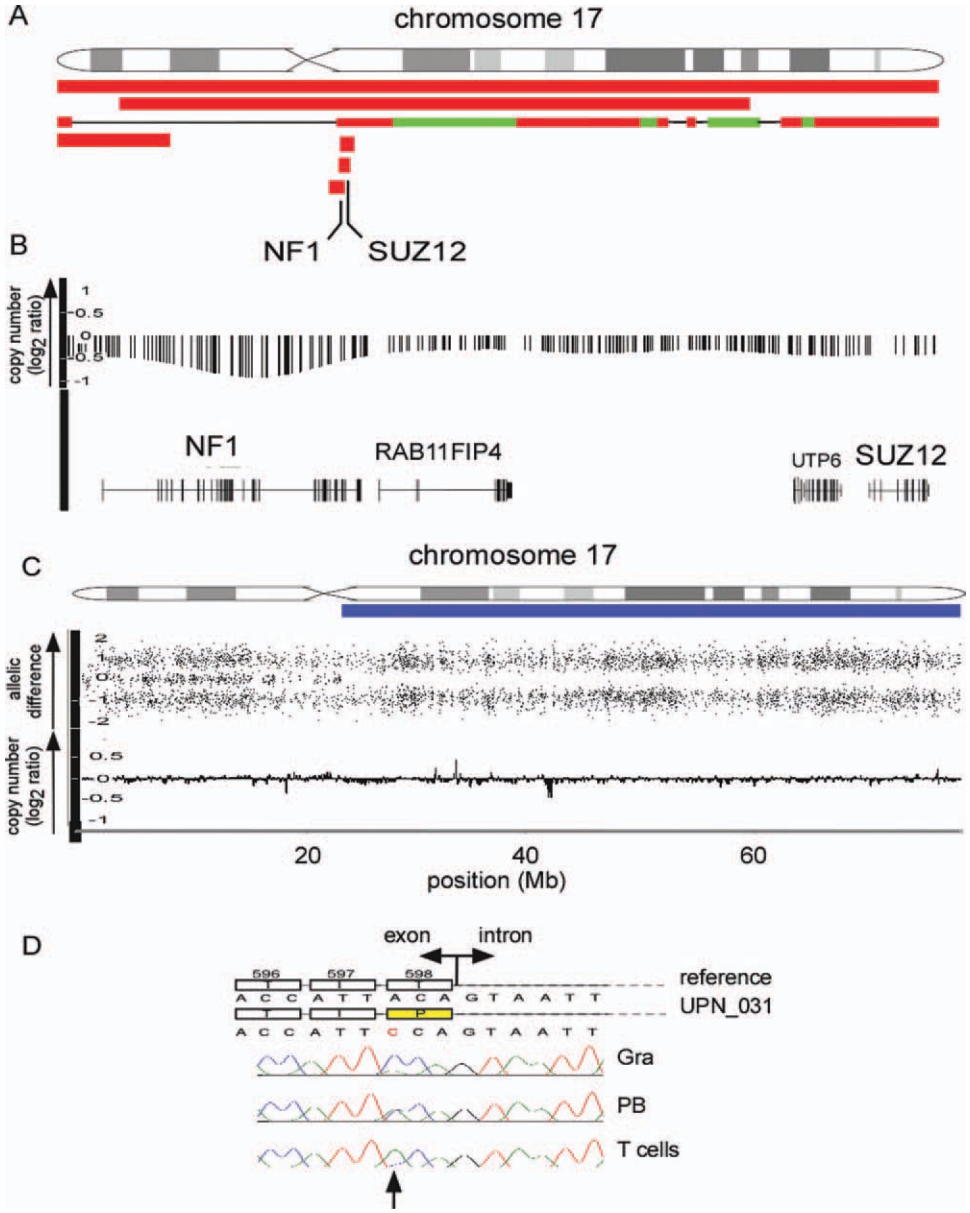

Figure 3. Summary of chromosome 17 defects. Chromosomal aberrations' sizes and positions are depicted as horizontal bars; green indicating gains, red deletions, and blue UPD. The outputs from the Genotyping Console Version 3.0.2. software represent the copy number data from Affymetrix SNP 6.0 arrays (log2 ratio to normal samples) as well as allelic difference. (A) Deletions of chromosome 17 in 77 analyzed post-MPN and post-MDS AMLs. Common deleted region maps to *NF1* locus. (B) Homozygous deletion targeting a single gene *NF1* on chromosome 17q in a post-MPN AML patient UPN_068. (C) UPD of chromosome 17q in patient UPN_031 diagnosed with secondary myelofibrosis. (D) Somatic mutation of SUZ12-T598P in patient UPN_031 carrying a UPD of chromosome 17q. Both mutated and wt alleles are present in the peripheral blood DNA sample. However, only the granulocytes carry the mutated allele, which is absent from the T-cells used as the control.

homozygous deletion, the *JAK2* mutation causing MPN accounted for the PV diagnosis, while the two subsequent aberrations resulting in the homozygous deletion of *JARID2* could have led to the progression of the disease, causing a phenotype switch from MPN to RAEB (refractory anemia with excess blasts), with a likeliness to further develop AML. Interestingly, the Jumonji mutant mice embryos (analog of human *JARID2*) die as a result of anemia due to the reduction of common myeloid and erythroid progenitor levels [34].

The results of this study show that *SUZ12* is targeted by both deletions and mutations. It has previously been shown that the loss of Suz12, the core component of PRC2 complex, enhances hematopoietic stem cell activity. The *SUZ12* locus is in a close proximity of the *NF1* tumor suppressor gene reported to be mutated in MPN [35]. Larger deletions may target both *SUZ12* and *NF1* simultaneously, likely resulting in a stronger effect on clonal progression or leukemic transformation. Same may be true for *EZH2* and *CUX1*, and *AEBP2* and *ETV6* codeletions, indicating that these PRC2 complex members could be secondary targets of deletions rather than drivers of the pathogenesis.

Our hypothesis is that disruption of these genes inactivates the function of PRC2 resulting in de-repression of its target genes, which leads to decreased differentiation potential of the myeloid progenitors that eventually can lead to leukemic transformation. This hypothesis corresponds to our finding that deletions of regions harboring the PRC2 complex members are present in one-third of post-MPN or post-MDS AML patients that we analyzed, and that their presence is significantly associated with leukemic transformation.

The leukemic transformation of chronic myeloid neoplasms is poorly understood. Recent studies implicated transcription factors (*ASLX1*, *CUX1*, *IKZF1*, *RUNX1*, *ETV6*), epigenetic regulators (*TET2*, *DNMT3A*, *IDH1/2*), *CBL*, and the p53 pathway in the leukemic transformation of chronic phase MPN and MDS [4,36–45]. In this study, we identified yet other tumor suppressors affecting transcriptional regulation. The diversity of lesions associated with the leukemogenesis in MPN and MDS suggests that many pathways are involved in this process. Identifying the relevant gene targets of the leukemia-associated transcription factors and early detection of these lesions are the prerequisites for novel therapeutic strategies capable of tackling the aggressive nature of leukemia arising from MPN and MDS.

Acknowledgments

The authors thank Martin Schalling for technical assistance.

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