Transcription factor mutations in myelodysplastic/myeloproliferative neoplasms

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background
Aberrant activation of tyrosine kinases, caused by either mutation or gene fusion, is of major importance for the development of many hematologic malignancies, particularly myeloproliferative neoplasms. We hypothesized that hitherto unrecognized, cytogenetically cryptic tyrosine kinase fusions may be common in non-classical or atypical myeloproliferative neoplasms and related myelodysplastic/myeloproliferative neoplasms.

Design and Methods
To detect genomic copy number changes associated with such fusions, we performed a systematic search in 68 patients using custom designed, targeted, high-resolution array comparative genomic hybridization. Arrays contained 44,000 oligonucleotide probes that targeted 500 genes including all 90 tyrosine kinases plus downstream tyrosine kinase signaling components, other translocation targets, transcription factors, and other factors known to be important for myelopoiesis.

Results
No abnormalities involving tyrosine kinases were detected; however, nine cytogenetically cryptic copy number imbalances were detected in seven patients, including hemizygous deletions of *RUNX1* or *CEBPA* in two cases with atypical chronic myeloid leukemia. Mutation analysis of the remaining alleles revealed non-mutated *RUNX1* and a frameshift insertion within *CEBPA*. A further mutation screen of 187 patients with myelodysplastic/myeloproliferative neoplasms identified *RUNX1* mutations in 27 (14%) and *CEBPA* mutations in seven (4%) patients. Analysis of other transcription factors known to be frequently mutated in acute myeloid leukemia revealed *NPM1* mutations in six (3%) and *WT1* mutations in two (1%) patients with myelodysplastic/myeloproliferative neoplasms. Univariate analysis indicated that patients with mutations had a shorter overall survival (28 *versus* 44 months, P=0.019) compared with patients without mutations, with the prognosis for cases with *CEBPA*, *NPM1* or *WT1* mutations being particularly poor.

Conclusions
We conclude that mutations of transcription and other nuclear factors are frequent in myelodysplastic/myeloproliferative neoplasms and are generally mutually exclusive. *CEBPA*, *NPM1* or *WT1* mutations may be associated with a poor prognosis, an observation that will need to be confirmed by detailed prospective studies.

Key words: *RUNX1, AML1, CEBPA*, MDS, MPN, myeloproliferative.

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Introduction

Myeloproliferative neoplasms (MPN) are clonal hematopoietic stem cell disorders characterized by abnormal proliferation and survival of one or more myeloid cell types in the bone marrow and increased numbers of mature and immature cells in the peripheral blood.[1] In addition to the four classical MPN, polycythemia vera, essential thrombocythemia, primary myelofibrosis and chronic myeloid leukemia (CML), there are rarer subtypes referred to as non-classical or atypical MPN such as chronic eosinophilic leukemia (CEL), hypereosinophilic syndrome (HES), chronic neutrophilic leukemia or MPN unclassifiable (MPN-U).[2] These disorders may overlap with myelodysplastic/myeloproliferative neoplasms (MDS/MPN) including *BCR-ABL*-negative atypical CML, chronic myelomonocytic leukemia (CMML) and MDS/MPN unclassifiable (MDS/MPN-U), in which proliferation is accompanied by dysplastic features or ineffective hematopoiesis in other lineages.[3]

The molecular pathogenesis of atypical MPN and MDS/MPN is only partially understood. In many patients, aberrant activation of tyrosine kinase signaling has been found as a consequence of four principal mechanisms: (i) activating tyrosine kinase mutations, e.g. *FLT3* and *JAK2*,[4] (ii) mutations in downstream signaling components, e.g. *RAS*,[5,6] (iii) mutations in negative regulators, e.g. *CBL*[7-9] and (iv) constitutively active tyrosine kinase fusion genes arising as a consequence of genomic rearrangements.[10] Collectively, however, these abnormalities account for well under 50% of cases.

Of the tyrosine kinase fusions, *FIP1L1-PDGFRA* in CEL is unique in that it results from a cytogenetically invisible 800 kb interstitial deletion at chromosome band 4q12.[11] All other fusions in atypical MPN and MDS/MPN, most of which are extremely rare, are associated with visible karyotypic aberrations. However cytogenetically cryptic tyrosine kinase fusions have been described in acute lymphoblastic leukemia, either as a consequence of translocations involving regions that cannot be distinguished visually (*EML1-ABL*)[12] or, most remarkably, episomal amplification (*NUP214-ABL*).[13] The discovery of cryptic fusions relied on a fortuitous case with an incidental visible translocation (*FIP1L1-PDGFRA*) or the use of fluorescence *in situ* hybridization to screen for disruption of *ABL* (*EML1-ABL* and *NUP214-ABL*). It is, therefore, possible that many other similar abnormalities remain to be discovered.

In this study, we hypothesized that hitherto unrecognized, cytogenetically cryptic tyrosine kinase fusions might be common in patients with atypical MPN or MDS/MPN. Since cryptic fusions are frequently associated with DNA copy number changes, we performed a targeted screen of all tyrosine kinases as well as tyrosine kinase signaling components, known translocation targets and transcription factors using custom designed, high-resolution targeted array comparative genomic hybridization (CGH). We sought to determine whether copy number changes characterize atypical MPN and MDS/MPN of unknown molecular etiology and whether they could be used as a tool to help identify novel fusion genes or other driver mutations.

Design and Methods

Patients and clinical samples

Pretreatment leukocyte genomic DNA from 68 patients was studied by targeted array CGH. Patients had MDS/MPN (CMML, n=9; atypical CML, n=8; MDS/MPN-U, n=14); CEL/HES, n=17; MPN-U, n=16; chronic neutrophilic leukemia, n=3, or acute basophilic leukemia, n=1. All samples tested negative for *BCR-ABL*, *FIP1L1-PDGFRA*, *JAK2*^{V617F} and none had karyotypic abnormalities suggestive of other known tyrosine kinase fusions. Nine patients with CEL/HES showed a significant response to imatinib treatment in the absence of any known imatinib-sensitive abnormality. A further 187 patients with MDS/MPN (CMML, n=97; atypical CML, n=68; MDS/MPN-U, n=22) were analyzed for sequence variants by direct sequencing. The study was approved by the Internal Review Boards from participating institutions and informed consent was provided according to the Declaration of Helsinki.

Targeted array comparative genomic hybridization

We designed Agilent HD-CGH Microarrays (Agilent Technologies, Palo Alto, CA, USA) using the 4x44K format. Slides consisted of four individual arrays, each containing 44,000 60-mer oligonucleotide probes. Individual oligonucleotides were selected from the Agilent eArray online database (*https://earray.chem.agilent.com/erray*). Each array targeted 500 genes including all 90 tyrosine kinases plus downstream tyrosine kinase signaling components, other translocation targets, transcription factors, and other factors known to be important for myelopoiesis (for a full list of targeted genes see *Online Supplementary Table S1*). For each target gene, 50-100 probes were selected that spanned the gene plus flanking sequences of up to 200 kb, providing a resolution of up to 5-10 kb. Samples were processed according to the Oligonucleotide Array-based CGH for Genomic DNA Analysis Protocol (version 4.0, Agilent Technologies). Briefly, 1.5 μg of the patients' and reference DNA were labeled with either Cy3 or Cy5 by random priming after restriction enzyme digestion. Differentially labeled patients' and control DNA was mixed, denatured and hybridized to array slides for 24 h under stringent conditions. Slides were then washed, dried and scanned using an Agilent G2505B microarray scanner and Agilent Scan Control software (version A.7.0.1). Microarray images were analyzed by Agilent Feature Extraction software (version 9.5.3.1) and the data were subsequently imported into CGH Analytics software (version 3.4.40, Agilent Technologies) for downstream analysis.

Since the proportion of background normal cells in our samples was unknown and likely to be variable between cases, it was important to determine that our targeted arrays could detect heterozygous deletions in a background of normal cells. We found that *FIP1L1-PDGFRA* was readily detectable when EOL1 DNA was diluted 1:2.5 with normal DNA (Figure 1A and B). Since EOL1 cells have two copies of the del(4) and one normal 4, this is equivalent to detection of a heterozygous deletion when normal cells constitute 50% of the sample.

Human genome-wide array comparative genomic hybridization

In some cases in which targeted array CGH revealed copy number changes that extended beyond the boundaries of the probes targeting a gene of interest, commercially available Agilent Human Genome CGH Microarrays in 244K format (containing 244,000 coding and non-coding human sequences with a genome-wide resolution of approximately 7-9 kb) were used to identify the full extent of the copy number abnormali-

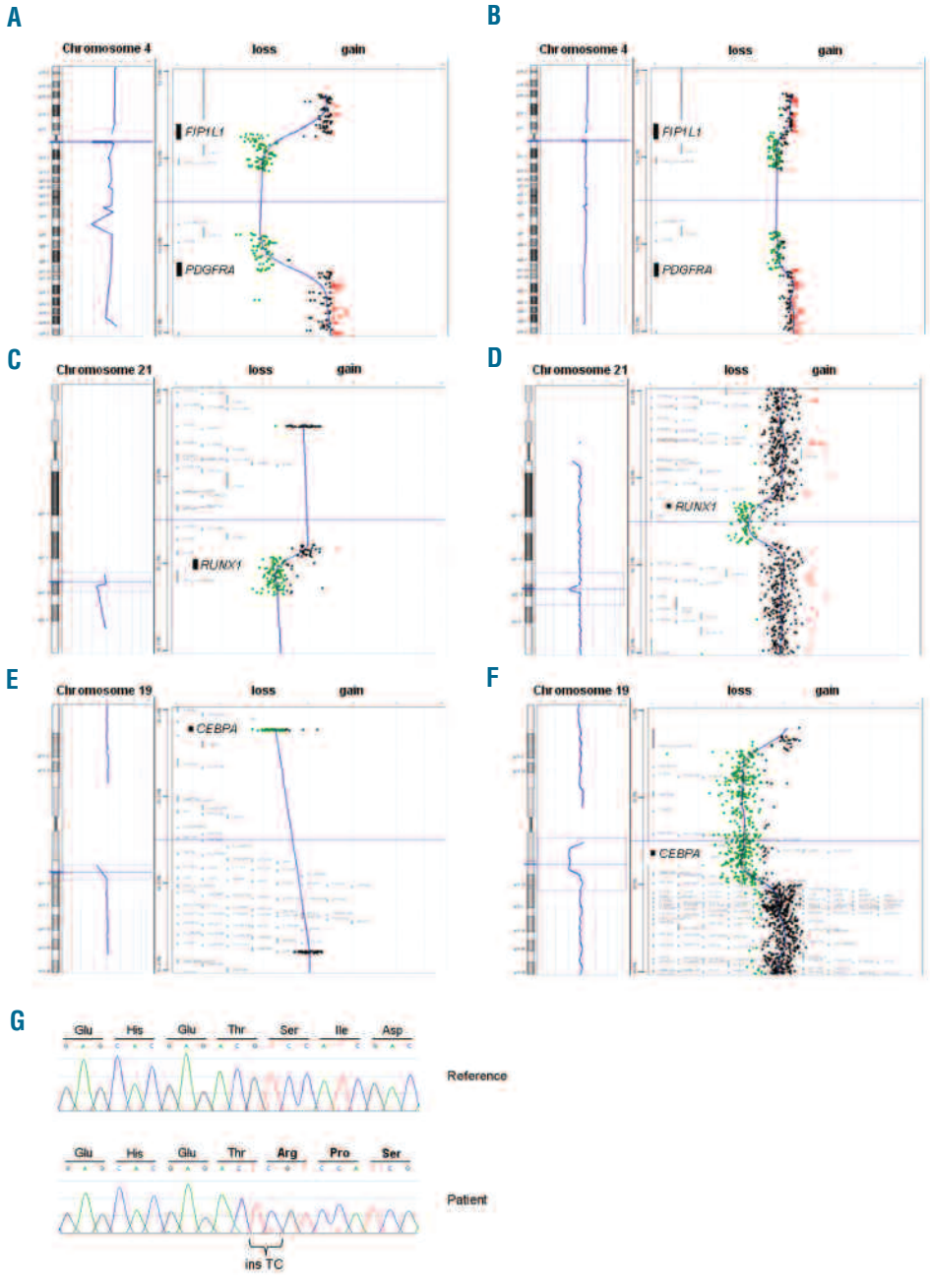

Figure 1. Targeted array CGH and sequencing profiles. (A) Control experiments demonstrating that targeted arrays were readily able to identify the 800kb *FIP1L1-PDGFRA* deletion in EOL1 cells in a background of normal cells. EOL1 cells harboring two copies of the del(4) and one normal chromosome 4. (B) 1:2.5 mixtures of EOL1 DNA with normal DNA, simulating a heterozygous deletion with 50% background normal cells. (C) *RUNX1* deletion in a patient with atypical CML. Targeted array CGH profile of chromosome 21 (left) with zoom of the respective region (right) showing a hemizygous deletion of *RUNX1*. (D) Whole-genome 244K array CGH results confirm this observation and further characterize the deletion as a 841kb deletion including *RUNX1*. (E) *CEBPA* deletion in a patient with atypical CML. Targeted array CGH profile of chromosome 19 (left) with zoom of the respective region (right) showing a hemizygous deletion of *CEBPA*. (F) Whole-genome 244K array CGH results further characterize the deletion as a 6.3Mb deletion including *CEBPA* and several other genes. (G) Sequencing result of the atypical CML patient with a *CEBPA* deletion. A homozygous 2 bp insertion was observed resulting in a frameshift at amino acid threonine 60 (bottom panel). The *CEBPA* reference sequence is shown in the top panel.

Table 1. Copy number abnormalities found by targeted array CGH.

Patient	Disease	Chromosome	Band	Gene	CNA	CNA start position	CNA end position	CNA size (kb)	N. of probes
1	Atypical CML	5	5q21.3	*PJA2*	Gain	108662279	108714138	51.9	22
2	Atypical CML	5	5p12	*FGF10*	Loss	44387421	44399741	12.3	14
		15	15q21.1	*FGF7*	Loss	47498995	47525965	27.0	28
3	MPN-U	17	17q23.1	*CLTC*	Loss	55117687	55118542	0.9	3
4	HES	19	19p13.11	*JUND*	Loss	18251575	18251854	0.3	5
5	Atypical CML	19	19q13.11	*CEBPA*	Loss	38473374	38495178	21.8	50
6	Atypical CML	19	19p13.11	*JUND*	Loss	18251575	18251854	0.3	5
		21	21q22.12	*RUNX1*	Loss	35044781	35543405	498.6	200
7	MPN-U	22	22q13.2	*L3MBTL2*	Gain	39916010	39959748	43.7	14

CNA: copy number abnormality; CML: chronic myeloid leukemia; MPN-U: myeloproliferative neoplasm unclassifiable; HES: hypereosinophilic syndrome. Genomic positions are according to NCBI build 35 (hg17) and indicate the minimum size of the abnormality.

ties. The sample processing was performed as described for the targeted arrays except that 2 μg of patients' and reference DNA were used.

Scoring criteria

Copy number changes less than 10 Mb were regarded as cytogenetically cryptic. Abnormalities in three consecutive oligonucleotide probes were required to call a copy number imbalance by array CGH. Copy number changes were compared to the Database of Genomic Variants[14] to exclude known constitutional copy number variants.

Mutation analysis

Mutation analysis was performed on whole-genome amplified DNA material using the Illustra™ GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Buckinghamshire, UK). The entire coding regions of *RUNX1* (exons 3-8) (ENST00000344691) and *CEBPA* (exon 1) (ENST00000328368) and selected exons of *NPM1* (exon 12) (ENST00000296930) and *WT1* (exons 7 and 9) (ENST00000332351) were analyzed by direct sequencing of polymerase chain reaction products using published primer sequences[15-18] and standard techniques on an ABI 3130 Genetic Analyzer (Applied Biosystems, Warrington, UK) with Mutation Surveyor software (SoftGenetics, State College, PA, USA). A dropping factor (relative intensity drop of the wild-type allele peak relative to that seen in a concurrently run normal sample) of 60% or more was considered indicative of a biallelic mutation whereas a dropping factor of less than 60% was considered as monoallelic. This is a conservative definition and since we did not know the relative proportion of malignant and non-malignant cells in our samples the true incidence of homozygosity may be underestimated. All mutations were confirmed on unamplified genomic DNA by sequencing in both directions.

Results

Detection of copy number imbalances in the majority of patients

Sixty-six of 68 patients (97%) harbored detectable genomic imbalances with a median of five (range, 1-19) copy number abnormalities per patient. Of the 424 copy number imbalances detected, 252 (59%) were amplifications and 172 (41%) were deletions involving 41 of the 500 targeted genes. The median size of the copy number changes was 3.0 kb (range, 0.1 – 498.6 kb) involving a median of six oligonucleotide probes (range, 3-200). Forty patients showed either gain or loss of DNA material and 26 patients had both. One patient with CMML and one patient with MPN-U did not show any copy number abnormality within the targeted genes. Of the 41 regions showing copy number imbalance, 20 were within regions of known copy number variants. Thirteen additional regions with a median size of 1.2 kb (range, 0.1 - 18.2 kb) were identified that showed copy number loss in some individuals and copy number gain in others strongly suggesting that they are copy number variants, despite not being listed in the Database of Genomic Variants (*Online Supplementary Table S2*).

Identifications of novel copy number abnormalities

After eliminating copy number variants, nine cytogenetically cryptic imbalances at eight loci remained in seven patients (Table 1). Their median size was 21.8 kb (range, 0.3 - 498.6kb) involving a median of 14 oligonucleotide probes (range, 3-200). Seven copy number changes represented loss of DNA material including a 499 kb deletion of *RUNX1* at 21q22.12 (Figure 1C) and a 22 kb deletion of *CEBPA* at 19q13.11 (Figure 1E) in two patients with atypical CML. Two copy number imbalances represented gain of DNA material (*PJA2* and *L3MBT2L*). None of the imbalances affected tyrosine kinases.

Breakpoint analysis of RUNX1 and CEBPA deletions

For this initial study we focused on the *RUNX1* and *CEBPA* deletions because of their known prognostic significance in AML; the other abnormalities are still under investigation. In patients with *RUNX1* or *CEBPA* deletions the copy number abnormalities extended beyond the limits of the probe set targeting these genes. Whole-genome array CGH was, therefore, performed to characterize the genomic boundaries of these abnormalities (Table 2). An 841 kb-deleted region including only *RUNX1* was identified in the patient with *RUNX1* deletion (Figure 1D). In the patient with the *CEBPA* deletion, a region of 6.3 Mb was identified with involvement of adjacent genes which were not represented on the initial targeted array (Figure 1F).

Mutation analysis of candidate genes reveals frequent mutations of transcription factors

We focused on *RUNX1* and *CEBPA* because of their known involvement in AML. Mutation analysis of the remaining alleles in the two patients with deletions revealed non-mutated *RUNX1* and a 2 bp insertion within *CEBPA* leading to a frameshift at amino acid threonine 60 (Figure 1G). A mutation screen of a further 187 patients with MDS/MPN identified *RUNX1* mutations in 27 (14%; CMML, n=18; atypical CML, n=4; MDS/MPN-U, n=5) and *CEBPA* mutations in 7 (4%; CMML, n=4; atypical CML, n=3) patients. Three patients had two *RUNX1* mutations leading to a total of 30 *RUNX1* mutations (frameshift ins/del, n=13; missense, n=12; nonsense, n=5). Four mutations (13%) were homozygous. Eighteen *RUNX1* mutations (60%) were located within the RUNT domain (amino acids 50-177) and four (13%) were within the transactivation domain (amino acids 291-371) (Figure 2A). One patient had two *CEBPA* mutations leading to a total of eight *CEBPA* mutations (frameshift ins/del, n=5; missense, n=2; nonsense, n=1). Three mutations (38%) were homozygous and mutations were spread throughout the coding sequence (Figure 2B). A recurrent in-frame insertion of 6 bp (H196_P197dup) within the TAD2 domain of *CEBPA* was identified in 13 cases but has previously been identified as a polymorphism.[19,20]

Mutation analysis of other transcription factors known to be frequently mutated in AML revealed *NPM1* mutations in 6/187 (3%) and *WT1* mutations in 2/187 (1%) MDS/MPN patients. Taken together, mutations in transcription factors were identified in 41/187 (22%) MDS/MPN patients. Of these, only one patient had mutations in two different genes (*CEBPA* and *WT1*) indicating that transcription factor mutations are generally mutually exclusive (Table 3 and *Online Supplementary Table S3*).

Prognostic impact of transcription factor mutations

Transcription factor mutations have prognostic significance in AML and we, therefore, assessed the impact of mutational status on survival in the 127 MDS/MPN

patients for whom data were available. On univariate analysis patients with mutations of transcription factors had a shorter overall survival and progression-free survival compared with mutation-negative cases (overall survival: 28 *versus* 44 months, *P*=0.019, log-rank test; progression-free survival: 21 *versus* 30 months, P=n.s). (Figure 3A and B). Patients with *CEBPA*, *NPM1*, or *WT1* mutations had a particularly poor prognosis (*P*=0.0047, *P*=0.0024 and *P*=0.058, respectively, with regard to overall survival). In contrast, there was no significant difference between *RUNX1*-mutated cases and those without transcription factor mutations (Figure 3C and D). It should be emphasized, however, that our cohort of patients was derived from different centers, their treatment was heterogeneous, the numbers of *CEBPA*, *WT1* and *NPM1* mutated cases were small and the analysis was done retrospectively. Clearly, therefore, prospective studies that take into account all relevant prognostic variables will be necessary to confirm the prognostic significance of transcription factor mutations.

Discussion

In this study, we performed a systematic search for cytogenetically cryptic abnormalities in patients with atypical MPN and MDS/MPN using custom designed, high-resolution targeted array CGH. Array CGH has the potential to pick up small genomic imbalances that are below the resolution of conventional cytogenetics. Targeted arrays were designed to focus on all 90 tyrosine kinases plus a range of other genes known to be involved in leukemia and myelopoiesis, e.g. known translocation genes, transcription factors as well as selected cytokines and receptors. Our aim was to detect small DNA copy number changes that might indicate the presence of novel fusion genes or other driver mutations.

After excluding all known or likely copy number variants, nine cytogenetically cryptic copy number imbalances were detected in seven patients, including hemizygous deletions of *RUNX1* or *CEBPA* genes. A further mutation screen of 187 MDS/MPN patients revealed frequent *RUNX1* and *CEBPA* mutations. *RUNX1* (also named *AML1* or *CBFA2*) is located on chromosome band 21q22.12 and encodes the alpha subunit of the core-binding factor (CBF) complex.[21] This complex activates and represses transcription of key regulators of growth, survival and differentiation pathways. *RUNX1* is one of the most frequent targets of chromosome translocations in leukemia and somatic mutations have also been identified, especially in AML M0 subtype,[22,23] *de novo* high-risk MDS,[24] and therapy-related MDS/AML.[15,25] Until recently only a small number of patients with CMML had been examined for *RUNX1* mutations and this gene has not been examined at all in atypical CML or other related diseases. In CMML, Harada *et al.* did not find *RUNX1* mutations in four patients,[24] nor did Preudhomme *et al.* in 27 patients.[23] Here, we report *RUNX1* mutations in 27 of 187 MDS/MPN patients (14%) with a high mutation frequency seen in CMML (19%). Three patients had two *RUNX1* mutations leading to a total of 30 *RUNX1* mutations. Eighteen mutations were likely causative changes predicted to result in premature chain termination and 12 were missense substitutions that have not been reported as single nucleotide polymorphisms. Similar to our results, two recent studies reported *RUNX1* mutations in 9 of 30 (33%) and 30 of 81 (37%) CMML patients, respectively.[26,27] In accordance with these studies, we also detected the majority (60%) of *RUNX1* mutations within the N-terminal RUNT domain, which is the most conserved region of RUNX family members and is directly involved in DNA binding and interactions with CBFβ.[28] Kuo *et al.* described a higher risk of AML progression in CMML patients with *RUNX1* mutations at the C-terminal compared to the N-terminal region.[27] Although we observed a similar trend in our cohort (*data not shown*), the number of patients with C-terminal *RUNX1* mutations was small in our study (n=6) as well as in that by Kuo *et al.* (n=9). Overall we saw no difference in outcome between *RUNX1*-mutated cases and those without transcription factor mutations. In contrast, *RUNX1* mutations have

Table 2. Copy number boundaries obtained using human genome-wide array CGH.

Patient	Disease	Chromosome	Band	Gene	CNA	44K targeted array CGH			244K whole-genome array CGH		
						CNA start position	CNA end position	CNA size (kb)	CNA start position	CNA end position	CNA size (kb)
5	Atypical CML	19	19q13.11	*CEBPA*	Loss	38473374	38495178	21.8	33611665	39919594	6307.9
6	Atypical CML	21	21q22.12	*RUNX1*	Loss	35044781	35543405	498.6	35047874	35889005	841.1

CNA: copy number abnormality; CML: chronic myeloid leukemia. Genomic positions, according to NCBI build 35 (hg17), determined by the locations of internal end probes within the CNA.

Table 3. Total number of patients with mutations of transcription factors.

Diagnosis	Number of analyzed patients	Number (%) of patients with mutations							
		RUNX1		*CEBPA*		*NPM1*		*WT1*	
CMML	97	18	(19)	4	(4)	6	(6)	1	(1)
Atypical CML	68	4	(6)	3	(4)	0		1	(1)
MDS/MPN-U	22	5	(23)	0		0		0	
Total	187	27	(14)	7	(4)	6	(3)	2	(1)

CMML: chronic myelomonocytic leukemia; CML: chronic myeloid leukemia; MDS/MPN-U: myelodysplastic/myeloproliferative neoplasm unclassifiable. Polymorphisms are not included.

been shown to be associated with a poor prognosis in *de novo* and therapy-related MDS.[15,24] A recent study on 470 adult AML patients found that *RUNX1* mutations were a marker for a significantly lower complete remission rate and shorter disease-free and overall survival.[29]

The *CEBPA* gene, located on chromosome band 19q13.11, encodes the transcription factor CCAAT/enhancer-binding protein-alpha which is essential for normal differentiation of granulocytes.[30] The involvement of *CEBPA* in leukemogenesis has been confirmed in many studies, with inactivating mutations reported predominately in AML M0, M1 and M2.[16,31-34] Mutations are usually acquired but can occasionally be inherited.[35] Only a small number of CMML patients have been investigated for *CEBPA* mutations to date. Kaeferstein *et al.* found no *CEBPA* mutations in five CMML patients,[36] whereas Shih *et al.* reported *CEBPA* mutations in three of 15 CMML patients (20%).[37] We identified eight different *CEBPA* mutations in seven of 187 MDS/MPN patients (4%). Six mutations were likely causative changes predicted to result in premature chain termination and two were missense substitutions that have not been reported as single nucleotide polymorphisms.

Following our finding of *RUNX1* and *CEBPA* mutations in MDS/MPN we screened for mutations in two other transcription factors known to be mutated frequently in AML. We detected *NPM1* mutations in six (3%) and *WT1* mutations in two (1%) MDS/MPN patients. Only one patient had mutations in two different genes (*CEBPA* and *WT1*) indicating that transcription factor mutations are generally mutually exclusive in MDS/MPN. This is similar to findings from a study on AML in which only 17/312 patients (5%) had more than one mutation in hypothetical class II genes (*NPM1*, *CEBPA*, and *MLL*) and 12/241 patients (5%) had more than one class I mutation

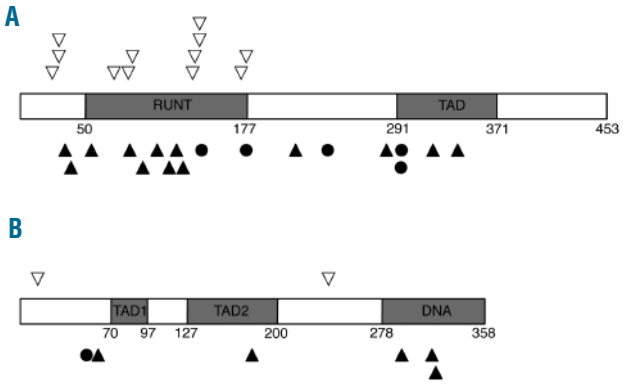

Figure 2. Schematic presentation of (A) RUNX1 protein and (B) *CEBPA* protein with locations of mutations. The majority (60%) of *RUNX1* mutations were found within the RUNT domain. *CEBPA* mutations were spread throughout the coding sequence. △ Missense mutation; ▲ frameshift insertion/deletion mutation; ● nonsense mutation; TAD, transactivation domain; DNA, DNA binding domain.

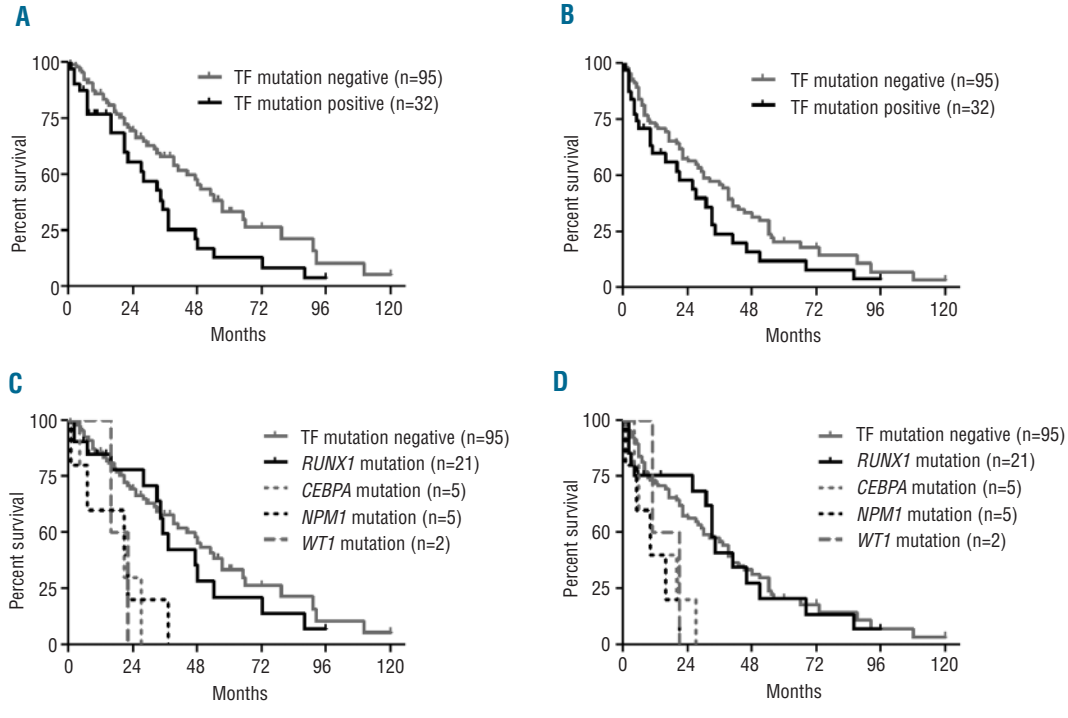

Figure 3. Clinical significance of transcription factor (TF) mutations in MDS/MPN. Kaplan-Meier estimates of (A) overall survival and (B) progression-free survival for 127 MDS/MPN patients with or without mutations of transcription factors. Overall survival was significantly lower in mutation-positive compared to mutation-negative cases (P=0.0190, log-rank test). Kaplan-Meier estimates of (C) overall survival and (D) progression-free survival for each specific mutation. Patients with *CEBPA*, *NPM1* or *WT1* mutations collectively showed a highly significant shorter overall survival (P<0.001) and progression-free survival (P<0.001) compared to cases without transcription factor mutations. Considering each gene individually, the P values for *CEBPA*, *NPM1* and *WT1* were 0.0047, 0.0024 and 0.0583, respectively, for overall survival and 0.0096, 0.0018 and 0.174, respectively for progression-free survival.

(*FLT3-ITD*, *FLT3-TKD*, and *NRAS*).[38] All *NPM1* mutations we found were identical and correspond to mutation type A according to Falini *et al.*[17] Type A mutations are the most frequent *NPM1* mutations in AML and the resulting shift in the reading frame alters the C-terminal portion of the NPM protein.

In a preliminary analysis we observed a shorter overall survival in patients with mutations of transcription factors compared with mutation-negative cases. This effect was accounted for principally by cases with *CEBPA*, *NPM1*, or *WT1* mutations. None of these patients had a concomitant *FLT3*-ITD. In contrast to our results, *CEBPA* and *NPM1* mutations (without *FLT3*-ITD) are linked to a favorable outcome in AML[16,33,38] whereas the prognostic impact of *WT1* mutations in AML is controversial.[18,39,40] Three recent studies found that only *CEBPA* double mutations but not single mutations (as predominately observed in our study) are associated with a favorable prognosis in AML.[34,41,42]

We did not find any cytogenetically cryptic abnormality involving tyrosine kinases in our cohort, which included nine patients with *FIP1L1-PDGFRA* negative CEL/HES who responded to imatinib.[43] Due to our focus on tyrosine kinases and the high resolution of our arrays, it seems unlikely that we missed any tyrosine kinase fusions, unless they were perfectly or nearly perfectly balanced. As reported elsewhere, we did find an unusual constitutional polymorphic *TFG-GPR128* fusion using our targeted arrays but this was not associated with any obvious phenotype.[44]

Since our approach was hypothesis-driven and focused on selected genes we only covered less than 2% of human genes on our targeted arrays and thus other important abnormalities may have been missed. Human genome-wide array CGH or single nucleotide polymorphism array analysis are useful tools for finding alterations that can point to novel candidate genes and pathways and new abnormalities have been detected using these approaches. Gelsi-Boyer *et al.* identified novel mutations of the polycomb-associated gene *ASXL1* in 17 out of 39 patients with CMML (43%).[45] Delhommeau *et al.* found frequent *TET2* mutations in about 15% of patients with various myeloid cancers including two of nine patients (22%) with CMML.[46] A high incidence of *TET2* mutations in CMML patients was confirmed in three other recent studies.[47-49] It is likely that mutations of these genes will also be found in atypical CML and related atypical MPN.

We conclude that mutations of transcription and other nuclear factors (particularly *RUNX1*) are frequent in MDS/MPN patients and thereby give molecular-based support for the grouping of those diseases into the same World Health Organization category (MDS/MPN).[2] In contrast, cryptic tyrosine kinase fusion genes are rare. Transcription factors are promising targets for future developments of novel targeted therapies.

Authorship and Disclosures

TE, AC and NCPC designed the study and performed experiments; KW, CH-C, JS, AJ and FG performed or assisted with the laboratory work and data analysis; KZ, AR and AH provided samples and clinical data; TE and NCPC wrote the paper; all authors contributed to the final version.

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