## Specific molecular mutation patterns delineate chronic neutrophilic leukemia, atypical chronic myeloid leukemia, and chronic myelomonocytic leukemia

Chronic neutrophilic leukemia (CNL) and atypical chronic myeloid leukemia (aCML) are rare entities grouped into the World Health Organisation (WHO) categories myeloproliferative and myelodysplastic/myeloproliferative neoplasms (MPN and MDS/MPN overlap), respectively. According to the WHO 2008 classification,[1] both entities are characterized by leukocytosis and a hypercellular bone marrow, predominantly consisting of granulocytic cells.

Chronic neutrophilic leukemia is diagnosed by the expansion of neutrophils in the peripheral blood, the exclusion of an elevated blast count, and hepatosplenomegaly. In contrast to other MPNs, before 2013, no molecular marker was known to prove clonality or could shed light on the molecular nature of the disease. Therefore, CNL had been diagnosed by a number of exclusion criteria eliminating evidence for other neoplasms or myelodysplastic syndromes.

Atypical CML is diagnosed by a similar approach, since diagnosis according to WHO has featured an increased number of neutrophil precursors, a defined threshold of blasts and monocytes, and dysplasia in the granulocytic lineage. A distinctive feature for differential diagnosis of CNL and aCML is the proportion of immature neutrophils (≥10% in aCML and <10% in CNL). As in CNL, other neoplasms and myelodysplastic syndromes should be excluded.

In addition, also chronic myelomonocytic leukemia (CMML) shares several of these characteristics and, therefore, needs to be discriminated from the other two entities, especially by the absolute number of monocytes for clinical decision making.

In the last three years, important markers have been identified for the diagnosis and differential diagnosis in these entities. *ASXL1, SRSF2*, and *TET2* were found to be frequently mutated in CMML.[2,3] *SETBP1* was identified to be frequently mutated in aCML, which was shown to co-occur frequently with mutations in *ASXL1* and CBL.[4,5] *CSF3R* mutations were found to associate with CNL and aCML.[6,7]

In Philadelphia negative MPNs, cytogenetic abnormalities occur, but the frequency differs and no specific abnormality has been defined in the different entities so far.[8] Therefore, the aim of our study was to determine the frequencies of the new armamentarium of genes, i.e. *ASXL1, CBL, CSF3R, SETBP1, SRSF2*, and *TET2* mutations in CNL, aCML, and CMML, to help guide the diagnosis and clinical decisions of these three, in part overlapping, entities. A total of 218 patients were diagnosed according to the WHO 2008 criteria, including 14 cases with CNL, 58 with aCML, and 146 with CMML (for more clinical details see *Online Supplementary Table S1*). Cytogenetics was available in 211 (97%) cases. In all cases, *BCR-ABL1* was excluded by RT-PCR and/or FISH, and *JAK2*V617F mutation was analyzed by melting curve analyses, as were *JAK2* exon 12 and MPL mutations in *JAK2*wild-type (wt) patients. *CALR* mutations were analyzed in *JAK2*wt CNL and aCML patients by Sanger sequencing. Presence of *PDGFR*-rearrangements was excluded in CNL by expression analyses of *PDGFRA* and *PDGFRB*. In all patients the mutational hot spot regions of *ASXL1, CBL, CSF3R, SETBP1*, and *SRSF2* were analyzed by Sanger sequencing. The complete coding region of *TET2* was analyzed by next generation sequencing in 217 of 218 cases. For more details see *Online Supplementary Appendix*.

Cytogenetic aberrations were detected in 54 of 211 cases (26%); the most frequent were trisomie 8 (n=14), deletion of the Y chromosome (n=7), del(20q) (n=3), and i(17)(q10) (n=3). However, there was no association to one of these entities.

Mutational analyses showed that *ASXL1* was frequently mutated in all three diseases, resulting in mutation frequencies of 57% in CNL (8 of 14), 66% in aCML (38 of 58), and 45% in CMML (66 of 146), respectively (Figures 1 and 2). A similar frequency of *ASXL1* mutations has previously been published in CMML.[9] However, the frequency in aCML was higher than the 23% reported by Piazza *et al.*[5] This finding of frequent appearance in CNL was surprising since little is known about clonality markers in addition to *CSF3R* in CNL. Furthermore, in CMML, MDS, and also PMF there is evidence that mutations in *ASXL1* provide prognostic information, with faster leukemic transformation.[9-12] *CBL* mutations clustered mostly in CMML patients (21%, 31 of 146), were less frequent in aCML (10%, 6 of 58), and not found in CNL (0 of 14), matching reported data.[13] In contrast, *CSF3R* was often mutated in CNL patients (43%, 6 of 14), but rarely in aCML and CMML cases, with only 2 patients each harboring a *CSF3R* mutation (3%, 2 of 58; 1%, 2 of 146). However, these 4 cases with *CSF3R* mutation in aCML and CMML showed neutrophil counts below 80% and increased monocyte numbers above 1000/μL in CMML cases and more than 10% neutrophilic precursors in aCML. Although a higher mutation frequency was reported for CNL patients, the rare occurrence of *CSF3R* mutations in aCML and CMML is in accordance with other reports.[6,7] Two mutation types have

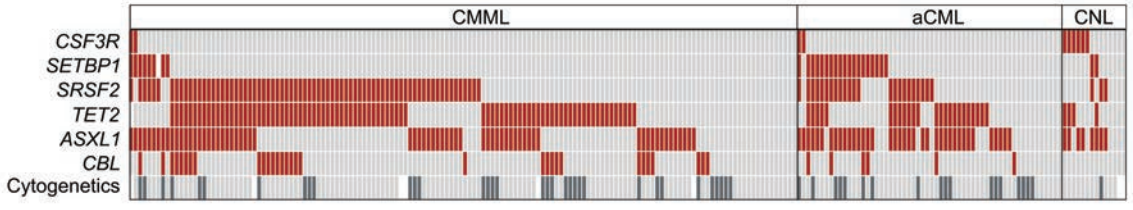


Figure 1. Mutational screening of CNL, aCML, and CMML. Alignment of gene mutations, cytogenetics, and entity information. Each column represents one of the 218 patients analyzed for *CSF3R, SETBP1, SRSF2, TET2, ASXL1, CBL*, and karyotype (shown in rows). Upper rows: red: mutated gene, light gray: non-mutated gene. Cytogenetics: dark gray: aberrant karyotype (n=54), light gray: normal karyotype (n=157). White: no data available.

**A**

| | CNL | aCML | CMML | CNL *vs* aCML | CNL *vs* CMML | aCML *vs* CMML |
|---|---|---|---|---|---|---|
| *ASXL1* | 8/14 (57%) | 38/58 (66%) | 66/146 (45%) | | | .013 |
| *CBL* | 0/14 | 6/58 (10%) | 31/146 (21%) | | | |
| *CSF3R* | 6/14 (43%) | 2/58 (3%) | 2/146 (1%) | <.001 | <.001 | |
| *SETBP1* | 2/14 (14%) | 19/58 (33%) | 9/146 (6%) | | | <.001 |
| *SRSF2* | 3/14 (21%) | 23/58 (40%) | 74/146 (51%) | | .049 | |
| *TET2* | 4/14 (29%) | 24/58 (41%) | 86/146 (59%) | | .045 | .029 |
| *TET2 + SRSF2* | 0/14 | 12/58 (21%) | 52/145 (36%) | | .005 | .044 |
| | | | | | | |
| *BCR-ABL1* | 0/14 | 0/58 | 0/146 | | | |
| *JAK2* | 0/14 | 2/58 (3%) | 10/146 (7%) | | | |
| *CALR* | 0/14 | 2/56 (4%) | n.a. | | | |
| *MPL* | 0/14 | 1/56 (2%) | 1/136 (1%) | | | |
| | | | | | | |
| *PDGFRA/B* | 0/14 | n.a. | n.a. | | | |

**B**

| CNL | *ASXL1* | *CBL* | *CSF3R* | *SETBP1* | *SRSF2* | *TET2* |
|---|---|---|---|---|---|---|
| *TET2* | | | | | | X |
| *SRSF2* | | | | | X | |
| *SETBP1* | | | n.s. | X | | |
| *CSF3R* | | | X | | | |
| *CBL* | | X | | | | |
| *ASXL1* | X | | | | | |

| aCML | *ASXL1* | *CBL* | *CSF3R* | *SETBP1* | *SRSF2* | *TET2* |
|---|---|---|---|---|---|---|
| *TET2* | | | | | | X |
| *SRSF2* | .010 | | | .004 | X | |
| *SETBP1* | | | | X | | |
| *CSF3R* | | | X | | | |
| *CBL* | | X | | | | |
| *ASXL1* | X | | | | | |

| CMML | *ASXL1* | *CBL* | *CSF3R* | *SETBP1* | *SRSF2* | *TET2* |
|---|---|---|---|---|---|---|
| *TET2* | .029 | | | .001 | .004 | X |
| *SRSF2* | | | | | X | |
| *SETBP1* | .001 | | .003 | X | | |
| *CSF3R* | | | X | | | |
| *CBL* | | X | | | | |
| *ASXL1* | X | | | | | |

**Legend**

| | | |
|---|---|---|
| A | | Association of gene mutation and entity |
| | n.a. | Not addressed |
| B | | Mutations concomitant |
| | | Mutations mutually exclusive |
| | n.s. | Not significant |

**Figure 2.** Association of gene mutations. (A) Table showing the mutation frequencies of the analyzed genes for each entity separately. Significant associations are filled in gray. (B) Scheme showing association of gene mutations within each entity. Positive association (concomitant): light gray, negative association (mutually exclusive): dark gray. *P*-values for significant associations are given; $P<0.05$ was considered significant.

been identified in *CSF3R*, the membrane proximal mutations and truncating mutations.[6,7] While the membrane proximal mutations are mostly missense mutations in exon 14, with p.Thr618Ile as most prominent representative, the truncating mutations are mostly frameshift and nonsense mutation in exon 17. Maxson *et al*. demonstrated in cell culture experiments that membrane proximal mutations are sensitive to JAK inhibitors while truncating mutations are more sensitive to SRC kinase inhibitors.[6] This might indicate a potential therapy for these patients.[14] Surveying the two mutation types of *CSF3R* showed that, in our cohort, 4 cases carried a membrane proximal and 2 cases a truncation mutation, while 4 cases were affected by both types. There was no relation of any type to any morphological entity (*Online Supplementary Figure S1*). *CSF3R* mutations were thus significantly associated with CNL ($P<0.001$). On the other hand, mutations in *SETBP1* were also differentially distributed within the three entities ($P<0.001$) entities and correlated to aCML, where 33% (19 of 58) patients were *SETBP1* mutated, while in CNL and CMML the mutation frequencies were lower with 14% (2 of 14) and 6% (9 of 146), respectively, in line with other published cohorts.[6,7,14] *SRSF2* mutations were detected at a high frequency within the CMML group (51%, 74 of 146), as described previously.[2,3] Here we could show that a nearly as high proportion of *SRSF2* mutations was observed in aCML patients (40%, 23 of 58), and also a notable number of CNL patients was *SRSF2* mutated (21%, 4 of 14). Although *SRSF2* was mutated in all three entities, this molecular marker was distributed differentially and associated mostly with CMML ($P=0.06$). This was even more prominent in combination with mutations in *TET2*. *TET2* was most frequently mutated in CMML cases (59%, 86 of 146), followed by aCML cases (41%, 24 of 58) and also in CNL (29%, 4 of 14), fitting the already reported frequencies for CMML or aCML.[15] Both mutated *TET2* alone and con-

comitant mutations with *SRSF2* associated significantly with CMML (*P*=0.012 and *P*=0.004, respectively). Remarkably, in CNL, a concomitant detection of mutated *SRSF2* and *TET2* was not observed in any case; however, this relationship was not statistically significant.

Focusing on co-occurrence of gene mutations showed in CNL patients an equal distribution of mutated genes, without any significant co-occurrence of gene mutations (Figures 1 and 2). However, although not significant, *SETBP1* and *CSF3R* mutations were mutually exclusive in CNL patients (0 of 6), while 3 of the 4 *CSF3R* mutated cases (75%) within the two other entities showed an additional *SETBP1* mutation. In aCML patients, this was different. In this entity *SETBP1* mutations were more often associated with *SRSF2* mutations (*P*=0.004). Additionally, *SRSF2* mutations also often co-occurred with mutated *ASXL1* (*P*=0.010). Even more associations were found in CMML patients, where mutated *TET2* and mutated *SRSF2*, as well as mutated *SETBP1* and *ASXL1*, occurred more frequently together (*P*=0.004 and *P*=0.001, respectively). In contrast, *TET2* and *ASXL1* as well as *TET2* and *SETBP1* rarely showed co-occurring mutations in CMML (*P*=0.029 and *P*=0.001, respectively). All these correlations were also analyzed and confirmed in the total cohort (n=218) (*Online Supplementary Figure S2*). Looking at clinical data in these three entities regarding differences in cases with wild-type or mutated marker genes showed that CNL patients carrying a *CSF3R* mutation were more often male (5 of 6 *vs.* 2 of 8 *CSF3R*wt). In aCML, *SETBP1* mutated patients showed a higher hemoglobin level compared to *SETBP1*wt patients (12.0 *vs.* 9.9 g/dL; *P*=0.016). Comparing CMML patients with combined *TET2* and SRSF2 mutation (*TET2*mut/*SRSF2*mut) with patients having either no mutation (*TET2*wt/*SRSF2*wt) or only one mutation in these two genes (*TET2*mut/*SRSF2*wt or *TET2*wt/*SRSF2*mut) showed that patients with *TET2* and *SRSF2* mutations had higher white blood cell counts (16.2 *vs.* 13.0 x$10^9$/L; *P*=0.013), a less pronounced monocytosis (4750 *vs.* 5382/µL; *P*=0.008), and were more often male (*P*=0.043) (*Online Supplementary Table S3*).

In conclusion, the mutational landscape of *ASXL1, CBL, CSF3R, SETBP1, SRSF2*, and *TET2* shows some common features, but also indicates characteristic and individual molecular patterns in CNL, aCML, and CMML. Taking the new mutations into account, mutational analyses of *JAK2, CALR*, and *MPL* should be considered when there is an increase in polymorphonuclear leukocytes, to strengthen the diagnosis of MPN. Alterations in *CSF3R* could suggest a diagnosis of CNL, while in *SETBP1* mutations could suggest a diagnosis of aCML. On the other hand, an ongoing monocytosis would direct the mutational analyses to *SRSF2* and *CBL* that would rather indicate CMML in mutated cases. Independently, the investigation of *ASXL1* and *TET2* as frequently mutated genes in all entities could help to prove clonality and to distinguish malignant diseases from reactive changes. Furthermore, mutation in *ASXL1* is a negative prognostic marker in AML, MDS, and PMF and should, therefore, be tested for its prognostic impact in MPN.

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