Detection and characterization of homozygosity of mutated *CALR* by copy neutral loss of heterozygosity in myeloproliferative neoplasms among cases with high *CALR* mutation loads or with progressive disease

Myeloproliferative disorders (MPN) are a heterogeneous group of diseases characterized by aberrant proliferation of one or more of the myeloid lineages.[1] *BCR-ABL1* negative MPN include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), which have overlapping clinical features and a common molecular basis.[1] Molecular mutations frequently involve genes encoding for specific kinases, leading to constitutively activated signal transduction pathways that cause the abnormal proliferation. The most common acquired somatic mutation in *BCR-ABL1* negative MPN affects the kinase *JAK2* (V617F), and is present in approximately 95% of cases with PV and in approximately 60% of patients with ET or PMF.[2] Further frequent mutations in MPN involve MPL (leading to activation of the JAK-STAT pathway similar to *JAK2* mutations) and CALR (calreticulin), which is mutated in approximately 20-30% of patients with ET and in 25-35% of PMF cases.[3,4] Thus, the vast majority of MPN patients (97%) carries mutations (mut) in one of these three genes in a nearly mutually exclusive manner.[4,5] The

Table 1. Description of selected cases with CALR mutations.

Case number	CN-LOH (showing chromosome bands involved and affected region in bp)	Partial trisomy 19	Type of *CALR* mutation	Mutation load >60%	Progressive disease	Mutations in *ASXL1*, *SRSF2*, *EZH2* and/or *IDH1/2*
1	19p13.12p12(15,866,918-22,183,880)	–	1	–	acceleration (ET)	yes
2	19p13.3p12(0-21,772,847)	–	2	yes	acceleration (ET)	–
3	19p13.3p13.12(0-15,739,462)	–	2	yes	acceleration (PMF)	–
4	19p13.3p11(0-24,616,235)	–	2	yes	–	–
5	19p13.3q13.33(0-48,706,919)	–	2	yes	blast crisis (ET)	–
6	19p13.3p11(0-24,616,235)	–	2	yes	–	–
7	19p13.3p13.3(0-6,541,488)	–	other	yes	–	–
8	19p13.3p13.2(0-7,229,566)	–	other	yes	–	–
9	19p13.3p13.12(0-16,022,738)	–	other	yes	–	–
10	19p13.3p12(0-22,158,389)	–	other	yes	acceleration (ET)	–
11	19p13.3p13.2(0-13,822,430)	–	other	yes	acceleration (PMF)	–
12	–	–	1	–	blast crisis (ET)	–
13	–	–	1	–	acceleration (PMF)	yes
14	–	–	1	–	–	yes
15	–	–	1	–	–	yes
16	–	–	1	–	acceleration (PMF)	yes
17	–	–	1	–	acceleration (PMF)	–
18	–	–	1	–	acceleration (PMF)	–
19	–	–	1	–	–	yes
20	–	–	1	–	–	yes
21	–	–	1	–	–	yes
22	–	–	1	–	–	yes
23	–	–	1	–	acceleration (PMF)	–
24	–	–	1	–	–	yes
25	–	–	1	–	–	yes
26	–	–	1	yes	–	–
27	–	–	1	yes	–	–
28	–	–	1	yes	–	–
29	–	–	1	yes	–	–
30	–	–	1	yes	–	–
31	–	–	1	yes	–	–
32	–	–	1	yes	–	–
33	–	yes	1	yes	–	–
34	–	yes	1	yes	–	–
35	–	–	1	yes	–	–

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36	–	–	1	–	–	yes
37	–	–	2	–	blast crisis (ET)	–
38	–	–	2	–	–	yes
39	–	–	2	–	blast crisis (ET)	–
40	–	–	2	–	acceleration (ET)	–
41	–	–	2	–	blast crisis (ET)	yes
42	–	–	2	yes	–	–
43	–	–	2	–	–	yes
44	–	–	mixed	–	–	yes
45	–	–	other	–	–	yes
46	–	–	other	–	–	yes
47	–	–	other	–	blast crisis (PV)	–
48	–	–	other	–	–	yes
49	–	–	other	yes	–	–
50	–	–	other	yes	–	–

CN-LOH: copy neutral loss of heterozygosity; ET: essential thrombocythemia; PMF: primary myelofibrosis; PV: polycythemia vera.

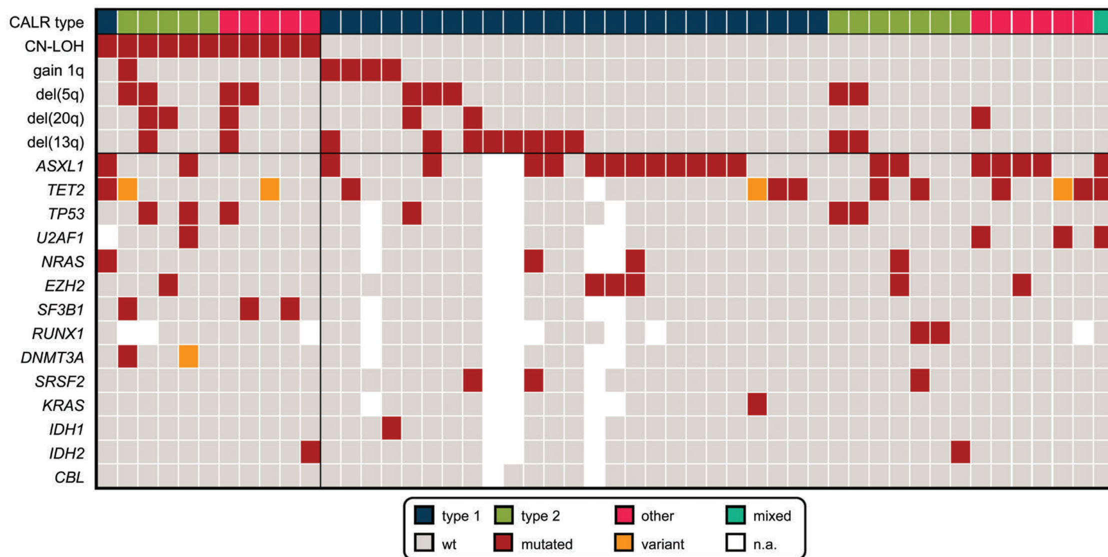

Figure 1. Summary of accompanying cytogenetic and molecular genetic aberrations. The result of each analysis is depicted for the total cohort of 50 cases; each column represents one patient. Type 1, type 2, other, and mixed (type 1 and type 2) denote the respective *CALR* types. wt: wild-type; n.a.: not analyzed.

CALR gene codes for a developmentally highly conserved and multifunctional protein playing a role as a Ca^{2+} binding chaperone in the endoplasmic reticulum lumen ensuring proper (glyco-) protein folding, thus preventing protein aggregation. One of its targets is represented by major histocompatibility complex (MHC) class I molecules that mediates its assembly and cell surface expression.[6-8] For *JAK2*, copy neutral loss of heterozygosity (CN-LOH) of 9p24 leading to homozygosity of *JAK2*V617F has been described in a number of studies and was associated with a distinct phenotype and with disease progression.[2,9] By contrast, high mutational loads (≥60%) are seen less frequently in *CALR* mutated cases, and mutated *CALR* homozygosity by CN-LOH has so far only rarely been described in MPN,[3,10] although it was found that homozygous mutations are associated with acquired myeloperoxidase deficiency.[11] Thus, the aim of the present study was to identify cases with *CALR* CN-LOH in a cohort of MPN cases with the help of the parameters high *CALR*mut loads and/or progressive disease, as well as cytogenetic and molecular genetic characterization of these cases in comparison to *CALR*mut patients without CN-LOH.

Overall, 50 cases with a CALRmut were included in this study. Bone marrow and/or peripheral blood samples had been sent for diagnosis to the MLL Munich Leukemia Laboratory in the period January 2007 and February 2016. Patients agreed to the use of laboratory data for research studies. The study was carried out in accordance with the Declaration of Helsinki. To detect cases with a *CALR* CN-LOH, cases were selected according to two different criteria. First, cases with progressive disease, pos-

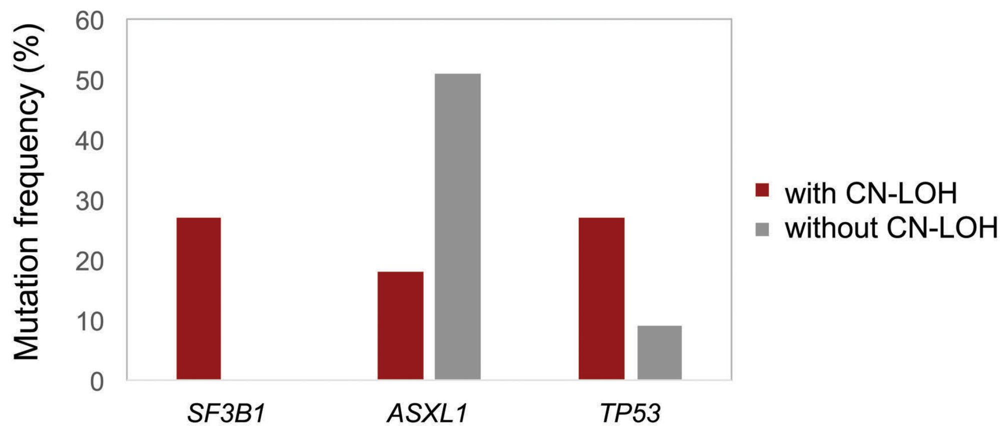

Figure 2. Frequency of accompanying molecular mutations in cases with and without *CALR* copy neutral loss of heterozygosity (CN-LOH). Mutation frequencies of genes that show differences between the two subgroups (with CN-LOH: red; without CN-LOH: gray) are depicted.

tulating a similar correlation as for *JAK2*, as homozygosity of *JAK2*V617F is known to be associated with disease progression.[2,9] Cases with progressive disease were defined by: i) accelerated phase; ii) blast crisis; or iii) the presence of at least one mutation in *ASXL1, SRSF2, EZH2* and/or *IDH1/2* as markers for progression. Second, cases with a high *CALR*mut load, which could potentially be caused by: i) a *CALR* CN-LOH; ii) a deletion of the *CALR* wild-type (wt) allele with a concomitant *CALR*mut in the other allele; or iii) at least a partial trisomy 19 including the *CALR* gene. For some cases, more than one criterion was present (Table 1). It should be noted that, for large *CALR* deletions, the mutation load might be over-estimated due to preferential amplification. The selected cases included: 1) n=23 (46% of cases) with a mutation load ≥60% determined by gene scan analysis; 2) n=17 (34%) cases with progressive disease (accelerated phase: n=11, 22%; blast crisis: n=6, 12%) according to cytomorphology; and/or 3) n=19 (38%) cases displaying mutations in *ASXL1, SRSF2, EZH2* and/or *IDH1/2*. All cases were investigated by genomic arrays (SurePrint G3 ISCA CGH+SNP Microarray, Agilent, Waldbronn, Germany). Images were analyzed using the DEVA Software v.1.2.1 (Roche Nimblegen) and Nexus Copy Number 6.1 (Biodiscovery Inc., El Segundo, CA, USA). Aberrations were evaluated in each sample using BioDiscovery's Fast Adaptive States Segmentation Technique (FASST2) algorithm. In addition, chromosome preparations and banding analysis were performed in all 50 cases according to standard methods, as previously described.[12] Amplicon next-generation sequencing was performed to detect mutations in *ASXL1, CBL, DNMT3A, EZH2, IDH1/2, KRAS, NRAS, RUNX1, SF3B1, SRSF2, TET2, TP53*, and *U2AF1* for all cases. The template library was generated with the TruSeq Custom Amplicon Low Input Kit and sequenced with the NextSeq (Illumina, San Diego, CA, USA; sensitivity: 3%). Next-generation sequencing (NGS) data were analyzed using the Sequence Pilot (v.4.1.1 Build 510 for the Illumina platform, JSI Medicalsystems, Kippenheim, Germany). SPSS (v.19.0.0) software (IBM Corporation, Armonk, NY, USA) was used for statistical analysis. All reported *P*-values are two-sided; *P*≤0.05 was considered statistically significant. Variants of unknown significance were excluded from statistical analysis.

In total, we were able to identify 11 out of 50 cases (22%) with CN-LOH by array CGH, suggesting that CN-LOH of 19p13 is quite a common event in cases with high *CALR* mutation loads and/or progressive disease. While the type 1 (c.1099_1150del52) *CALR* mutation was the most frequent mutation type in cases without CN-LOH (25 out of 39, 64%), it was rare in *CALR* CN-LOH cases (1 out of 11, 9%; *P*=0.009). Thus, in cases with CN-LOH, it was mainly the type 2 (c.1154_1155insTTGTC) mutation (5 out 11, 45%) and rare mutation types (5 out of 11, 45%) that were detected (Table 1). It can be hypothesized that the observed association of CN-LOH with type 2 *CALR*mut causes a higher potency of activation of the JAK-STAT signaling pathway. Of note, the only case with *CALR* CN-LOH showing the type 1 mutation was found to harbor a *TET2*mut and CN-LOH encompassing 4q23q28 including the *TET2* gene. As the *CALR*mut load was clearly lower compared to the *TET2*mut load (49% *vs.* 100%), in this case, the *CALR*mut might only belong to a subclone. The remaining 10 cases (91%) with CN-LOH showed a *CALR*mut load of >60% (median: 81%; range 49-100%). In 13 out of 39 cases (33%) without *CALR* CN-LOH a mutation load >60% was found (median: 48%; range: 3-100%). The reasons for the high mutation load of the majority of these 13 cases without CN-LOH are unclear. A partial trisomy 19 involving the *CALR* locus was only detected by genomic arrays in two of these 13 cases; in the remaining 11 cases no aberration involving chromosome 19 that could, therefore, potentially explain the high mutation loads was found (Table 1). The cases with *CALR* CN-LOH presented more frequently with MPN in acceleration compared to the cases without CN-LOH (5 out of 11, 45% *vs.* 6 out of 39, 15%; *P*=0.048). Almost all of the patients in accelerated phase with CN-LOH (4 out of 5, 80%) showed a mutation load ≥60% (81%, 78%, 94% and 100%, respectively); the remaining case was the patient with concomitant *TET2* and *CALR* CN-LOH. The chromosomal aberrations detected as recurrent abnormalities by array CGH analyses in the total cohort regarded: del(13q) (12 out of 50, 24%), del(5q) (9 out of 50, 18%), del(20q) (6 out of 50, 12%), and gain of 1q (5 out of 50, 10%) (Figure 1). Del(5q) and del(20q) showed a trend for greater frequency in cases with CN-LOH [del(5q): 4 out of 11, 36% *vs.* 5 out of 39, 13%; *P*=0.093;

del(20q): 3 out of 11, 27% *vs.* 3 out of 39, 8%; *P*=0.111]. No differences were observed in the other aberrations between cases with and without CN-LOH: del(13q): 2 out of 11 cases, 18% *vs.* 10 out of 39 cases, 26%; gain of 1q: 1/11=9% *vs.* 4 out of 39=10%. With regard to the advanced disease state of the selected cohort, mutation analyses revealed a high frequency of *ASXL1*mut (44%), followed by mutations in *TET2* (19%), *EZH2* (13%), *TP53* (13%), *U2AF1* (9%), *NRAS* (9%), and *SF3B1* (7%) in the total cohort. Interestingly, *SF3B1*mut were exclusively detected in cases with *CALR* CN-LOH (3 out of 11, 27% *vs.* 0 out of 34, 0%; *P*=0.012), whereas *ASXL1*mut tended to be more frequent in cases without CN-LOH (19 out of 37, 51% *vs.* 2 out of 11, 18%; *P*=0.083); however, this was not statistically significant (Figure 2). TP53mut were detected more often in the subgroup of cases with *CALR* CN-LOH, although, once again, this was not statistically significant (3 out of 11, 27% *vs.* 3 out of 35, 9%). In addition, *TP53*mut showed a significant correlation to del(5q) [*TP53*mut in cases with del(5q): 5 out of 9, 56% *vs. TP53*mut in cases without del(5q): 1 out of 37, 3%; *P*=0.001], an association that is well-known in MDS (myelodysplastic syndrome) cases, where *TP53*mut are proposed to play a role in disease progression and contribute to transition to acute myeloid leukemia.[13,14] However, a correlation of *TP53*mut with del(5q) has also been described for patients with MPN and MDS/MPN, thus corroborating the present results.[15] To analyze if the CALRmut was present in the main clone or in a subclone of the respective patient, its mutation load was compared to the mutation load of accompanying mutations. CALRmut was considered to constitute the main clone if the difference between the loads was >10%: in the vast majority of cases *CALR*mut is found in the main clone (48 out of 51; 94%). In summary, we can conclude that, in cases with high CALRmut loads and/or progressive disease, *CALR* CN-LOH is quite common (22%). While cases without CN-LOH frequently show the type 1 *CALR*mut, while mainly the type 2 mutation and rare mutation types were detected in CALR CN-LOH cases. Moreover, we found that CN-LOH cases show a distinct pattern of chromosomal aberrations and additional molecular mutations, as they are associated with del(5q) and del(20q) and mutations in *SF3B1* and *TP53*, whereas *ASXL1*mut are less frequent.

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