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

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SUPPLEMENTARY INFORMATION

1. Cohort information

The total cohort consisted of 218 patients. The male to female ratio was 2.0 (145/73). The median age was 73.0 years, ranging from 21.9 to 91.0 years. Further clinical data for each entity are given in Table S1.

CMML cases were already included in previous studies by Meggendorfer *et al.*,[1] and Itzykon *et al.*[2] Also aCML cases were already included in Meggendorfer *et al.*[3]

The study design adhered to the tenets of the Declaration of Helsinki and was approved by our institutional review board before its initiation.

	CNL (n=14)	**aCML (n=58)**	**CMML (n=146)**
Clinical characteristics			
Male/female (ratio)	7/7 (1.0)	38/20 (1.9)	100/46 (2.2)
Median age (years)	73.7 (31.0-91.0)	74.6 (44.9-88.7)	72.4 (21.9-90.5)
Median WBC ($x10^9$/L)	38.7 (25.2-90.5)	33.9 (11.3-206.5)	13.8 (1.6-160.0)
Median PLTs ($x10^9$/L)	190.0 (100.0-984.0)	148.0 (6.0-2,300.0)	88.0 (3.0-1,385.0)
Median Hb (g/dL)	12.0 (10.0-16.0)	10.2 (6.0-15.0)	11.0 (4.0-17.0)
Median Neutrophils (% of WBC)	89 (79-98)	66 (26-81)	n.a.
Median Monocytes (absolut/µL)	n.a.	n.a.	5,382 (1,089-57,582)
Cytogenetics (n=211/218)			
Normal karyotype	11	40	107
Aberrant karyotype	1	18	34

Table S1. Clinical chracteristics of the cohort. Median values and ranges are given. Abbreviations: aCML, atypical chronic myeloid leukemia; CNL, chronic neutrophilic leukemia; CMML, chronic myelomonocytic leukemia; Hb, hemoglobin; PLT, platelets; WBC, white blood cell; n.a., not addressed.

2. Methodical information

2.2 Cytomorphology

In all cases bone marrow and peripheral blood smears underwent May Giemsa Gruenwald staining. For cytomorphology, 100 nucleated cells were counted in the peripheral blood, 200 in the bone marrow. Cytochemistry was performed for myeloperoxidase (MPO) and non-specific esterase (NSE), and iron staining was done for detection of ring sideroblasts in cases with increased erythropoiesis or anemia. Classification of the disease entities and dysplasia was rated according to WHO criteria.[4]

2.3 Cytogenetics and fluorescence in situ hybridization (FISH)

Chromosome banding analysis was performed in 211/218 cases after short-term culture. Karyotypes were analyzed after G-banding and described according to the International System for Human Cytogenetic Nomenclature.[5] Interphase FISH was applied with probes for *BCR-ABL1* (MetaSystems, Altlussheim, Germany).

2.4 Isolation of nucleic acid

DNA or RNA from fresh bone marrow or peripheral blood cells was isolated after Ficoll separation of mononucleated cells. DNA was isolated using the DSP DNA Midi Kit and the QIAsymphony instrument (Qiagen, Hilden, Germany). RNA was isolated using the MagNa Pure LC system with the corresponding mRNA HS Kit (Roche Applied Science, Mannheim, Germany). RNA was reverse transcribed with 500 U SuperScript II Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA) in a 50 µl reaction using random hexamers as primers.

2.5 Melting curve and expression analyses

Melting curve analyses for *JAK2*V617F, *JAK2* exon 12 and *MPL*W515 were performed like previously described in Schnittger *et al*.[6-8] Expression levels of *PDGFRA* and *PDGFRB* were analyzed by quantitative real time PCR, as described in Erben *et al*.[9]

2.6 PCR and Sanger sequencing

Existence of *BCR-ABL1* fusion transcript was analyzed by PCR using the Qiagen Taq PCR Master Mix (Qiagen), as described in Cross *et al*.[10] The PCR fragments, containing the respective mutational hotspot regions for the genes *ASXL1* (exon 13), *CALR* (exon 9), *CBL* (exon 7 and 8), *CSF3R* (exon 14 and 17), *SETBP1* (amino acid 800 to 935), and *SRSF2* (exon 1, covering amino acid Pro95) were amplified with the Qiagen Taq PCR Master Mix (Qiagen) or with AccuPrime GC-Rich DNA Polymerase (Invitrogen) in cases of *ASXL1* amplicon 4 (ASXL1 exon 13 PCR4) and *SRSF2* from either genomic DNA or cDNA

templates, using the primers described previously[1;3;11;12] or given in Table S2. The amplicons were analyzed by Sanger sequencing using BigDye Terminator v1.1 cycle sequencing chemistry (Applied Biosystems, Carlsbad, CA) and ABI3130xl or ABI3730xl Genetic Analyzer instruments (Applied Biosystems).

Primer	Nucleotide sequence (5'-3')
CALR-E09-F	GACCTCTGGCAGGTCAAGTC
CALR-E09-R	TTCTCGAGTCTCACAGAGACATT
CSF3R-E14-F	GAGGCAGCTTTACCATCCAG
CSF3R-E14-R	GTACCCTCCAAACAGCCATC
CSF3R-E17-F	CTGGGGCAGACATCTGAAAT
CSF3R-E17-R	CCAGCTAGCTCAGGCCTTTA

Table S2. Primer sequences for amplification of *CALR* exon 9 and *CSF3R* exons 14 and 17.

2.7 Next-generation sequencing (NGS)

Next-generation deep-sequencing of the complete coding region of the *TET2* gene was performed using the 454 GS FLX amplicon chemistry (Roche Applied Science) as previously described.[13]

2.8 Statistical analyses

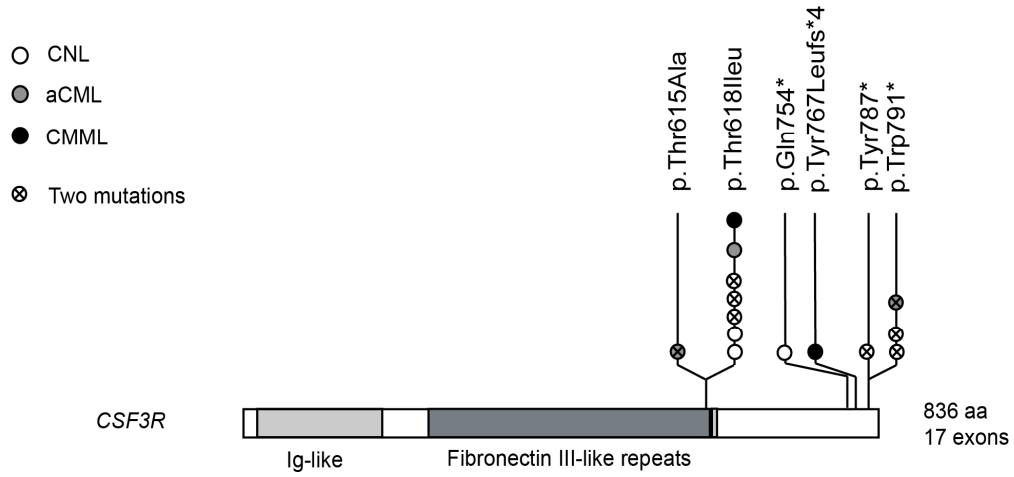
Dichotomous variables were compared between different groups using the χ^2-test or Fisher's exact test and continuous variables by Student's T-test. Results were considered significant at $p<0.05$; the reported p-values are two-sided. Adjustment for multiple testing was not done. Statistical analyses were performed using SPSS version 19.0 (IBM Corporation, Armonk, NY).

3. Mutational and corresponding clinical information

3.1 Analyses of CSF3R mutations in CNL, aCML, and CMML patients

All *CSF3R* mutated patients were further analyzed regarding the two different mutation types: membrane proximal and truncation mutations. There was no significant distribution of these two mutation types within the three addressed entities. The different mutations, distribution in the entities, as well as co-occurrence with a other mutation in *CSF3R* are given in Figure S1.

Fig. S1



Mutation type	Case number
Membrane proximal (p.Thr618Ile) sole	4
Truncation mutation sole	2
Membrane proximal + truncation mutation	4

Figure S1. Schematic overview of *CSF3R* mutations within the different entities. Location, mutations and mutation numbers are given. One dot represents one mutation: white dot: CNL, grey dot: aCML, black dot: CMML, crossed dot represents a mutation appearing together with a second mutation in *CSF3R*.

3.2 Correlation of gene mutations in the total cohort

The co-occurrence of gene mutations was analyzed in the total cohort (n=218) and resulted in the following correlations: *TET2* and *SRSF2*, *ASXL1* and *SRSF2*, *SETBP1* and *SRSF2*, as well as *ASXL1* and *SETBP1* mutations occurred more frequently together. In contrast, *TET2* and *SETBP1* were nearly mutually exclusive. The mutation frequencies as well as the correlations and corresponding *p*-values are given in Figure S2.

Fig. S2

Total cohort	*ASXL1*	*CBL*	*CSF3R*	*SETBP1*	*SRSF2*	*TET2*
TET2 **(52%)**				<.001	.002	x
SRSF2 **(46%)**	.021			.018	x	
SETBP1 **(14%)**	<.001			x		
CSF3R **(5%)**			x			
CBL **(17%)**		x				
ASXL1 **(51%)**	x					

Figure S2. Scheme showing associations of gene mutations. Mutation frequencies within the total cohort are given in parenthesis. Positive association (concomitant): light grey, negative association (mutually exclusive): dark grey. Significant *p*-values are given; $p<0.05$ was considered as significant.

3.3 Clinical characteristics of the three different entities corresponding to mutations within the marker genes

The cohort was analyzed regarding the clinical data age, sex, leucocytes, haemoglobin levels, platelet counts, neutrophil and monocyte counts, and cytogenetic information. Clinical median values of *CSF3R* wild type and mutated CNL cases, *SETBP1* wild type and mutated aCML cases, as well as *TET2* and *SRSF2* wild type or single mutated and *TET2* and *SRSF2* mutated CMML cases are given in Table S3.

	CNL (n=14)			aCML (n=58)			CMML (n=146)		
	*CSF3R*wt	*CSF3R*mut	*p*	*SETBP1*wt	*SETBP1*mut	*p*	*SRSF2*wt or *TET2*wt or single mutated	*SRSF2*mut + *TET2*mut	*p*
Clinical characteristics									
Case number	8	6		39	19		93	52	
Male/female (ratio)	2/6 (0.3)	5/1 (5.0)	n.s.	25/14 (1.8)	13/6 (2.2)		58/35 (1.7)	41/11 (3.7)	.043
Median age (years)	73.7 (64.3-91.0)	71.9 (31.0-79.7)		74.7 (44.9-88.7)	73.6 (59.4-83.3)		72.2 (21.9-90.5)	72.9 (56.3-85.8)	
Median WBC ($x10^9$/L)	31.9 (27.6-90.5)	41.4 (25.2-69.8)		33.1 (11.3-140.0)	44.0 (14.9-206.5)		13.0 (1.6-160.0)	16.2 (2.2-74.5)	.013
Median PLTs ($x10^9$/L)	352.0 (100.0-984.0)	171.0 (101.0-283.0)		144.0 (6.0-2,300.0)	216.5 (24.0-607.0)		98.5 (16.0-1,385.0)	79.5 (3.0-711.0)	
Median Hb (g/dL)	11.1 (10.0-15.0)	14.2 (10.0-16.0)		9.9 (8.0-15.0)	12.0 (6.0-14.0)	.016	10.6 (4.0-17.0)	11.8 (7.0-15.0)	
Median Neutrophils (% of WBC)	88.5 (80-98)	89 (79-91)		66 (26-81)	31		n.a.	n.a.	
Median Monocytes (absolut/µL)	n.a.	n.a.		n.a.	n.a.		5,382 (1,089-5,782)	4,750 (256-17,135)	.008
Cytogenetics (n=211/218)									
Normal karyotype	5	6		28	12		64	43	
Aberrant karyotype	1	0		11	7		27	7	

Table S3. Clinical characteristics of the three entities CNL, aCML, and CMML differentiated by mutations within the marker genes. Median value, ranges, and *p*-values are given. Abbreviations: aCML, atypical chronic myeloid leukemia; CNL, chronic neutrophilic leukemia; CMML, chronic myelomonocytic leukemia; Hb, hemoglobin; PLT, platelets; WBC, white blood cell; n.a., not addressed; n.s., not significant. $P<0.05$ was considered as significant.

4. Reference list

1. Meggendorfer M, Roller A, Haferlach T, Eder C, Dicker F, Grossmann V, et al. SRSF2 mutations in 275 cases with chronic myelomonocytic leukemia (CMML). Blood 2012;120:3080-8.

2. Itzykson R, Kosmider O, Renneville A, Gelsi-Boyer V, Meggendorfer M, Morabito M, et al. Prognostic score including gene mutations in chronic myelomonocytic leukemia. J Clin Oncol 2013;31:2428-36.

3. Meggendorfer M, Bacher U, Alpermann T, Haferlach C, Kern W, Gambacorti-Passerini C, et al. SETBP1 mutations occur in 9% of MDS/MPN and in 4% of MPN cases and are strongly associated with atypical CML, monosomy 7, isochromosome i(17)(q10), ASXL1 and CBL mutations. Leukemia 2013;27:1852-60.

4. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, 4th ed. Lyon: International Agency for Research on Cancer (IARC), 2008.

5. Shaffer LG, McGowan-Jordan J, Schmid M. ISCN 2013: an international system for human cytogenetic nomenclature. Basel, New York: Karger, 2013.

6. Schnittger S, Bacher U, Kern W, Schroder M, Haferlach T, Schoch C. Report on two novel nucleotide exchanges in the JAK2 pseudokinase domain: D620E and E627E. Leukemia 2006;20:2195-7.

7. Schnittger S, Bacher U, Haferlach C, Geer T, Muller P, Mittermuller J, et al. Detection of JAK2 exon 12 mutations in 15 patients with JAK2V617F negative polycythemia vera. Haematologica 2009;94:414-8.

8. Schnittger S, Bacher U, Haferlach C, Dengler R, Krober A, Kern W, et al. Detection of an MPLW515 mutation in a case with features of both essential thrombocythemia and refractory anemia with ringed sideroblasts and thrombocytosis. Leukemia 2008;22:453-5.

9. Erben P, Gosenca D, Muller MC, Reinhard J, Score J, del Valle F, et al. Screening for diverse PDGFRA or PDGFRB fusion genes is facilitated by generic quantitative reverse transcriptase polymerase chain reaction analysis. Haematologica 2010;95:738-44.

10. Cross NC, Melo JV, Feng L, Goldman JM. An optimized multiplex polymerase chain reaction (PCR) for detection of BCR-ABL fusion mRNAs in haematological disorders. Leukemia 1994;8:186-9.

11. Gelsi-Boyer V, Trouplin V, Adelaide J, Bonansea J, Cervera N, Carbuccia N, et al. Mutations of polycomb-associated gene ASXL1 in myelodysplastic syndromes and chronic myelomonocytic leukaemia. Br J Haematol 2009;145:788-800.

12. Grand FH, Hidalgo-Curtis CE, Ernst T, Zoi K, Zoi C, McGuire C, et al. Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. Blood 2009;113:6182-92.

13. Kohlmann A, Klein HU, Weissmann S, Bresolin S, Chaplin T, Cuppens H, et al. The Interlaboratory RObustness of Next-generation sequencing (IRON) study: a deep sequencing investigation of TET2, CBL and KRAS mutations by an international consortium involving 10 laboratories. Leukemia 2011;25:1840-8.