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HemaSphere EHA EUROPEAN HEMATOLOGY ASSOCIATION

ARTICLE

Comprehensive sequential genetic analysis delineating frequency, patterns, and prognostic impact of genomic dynamics in a real-world cohort of patients with lower-risk MDS

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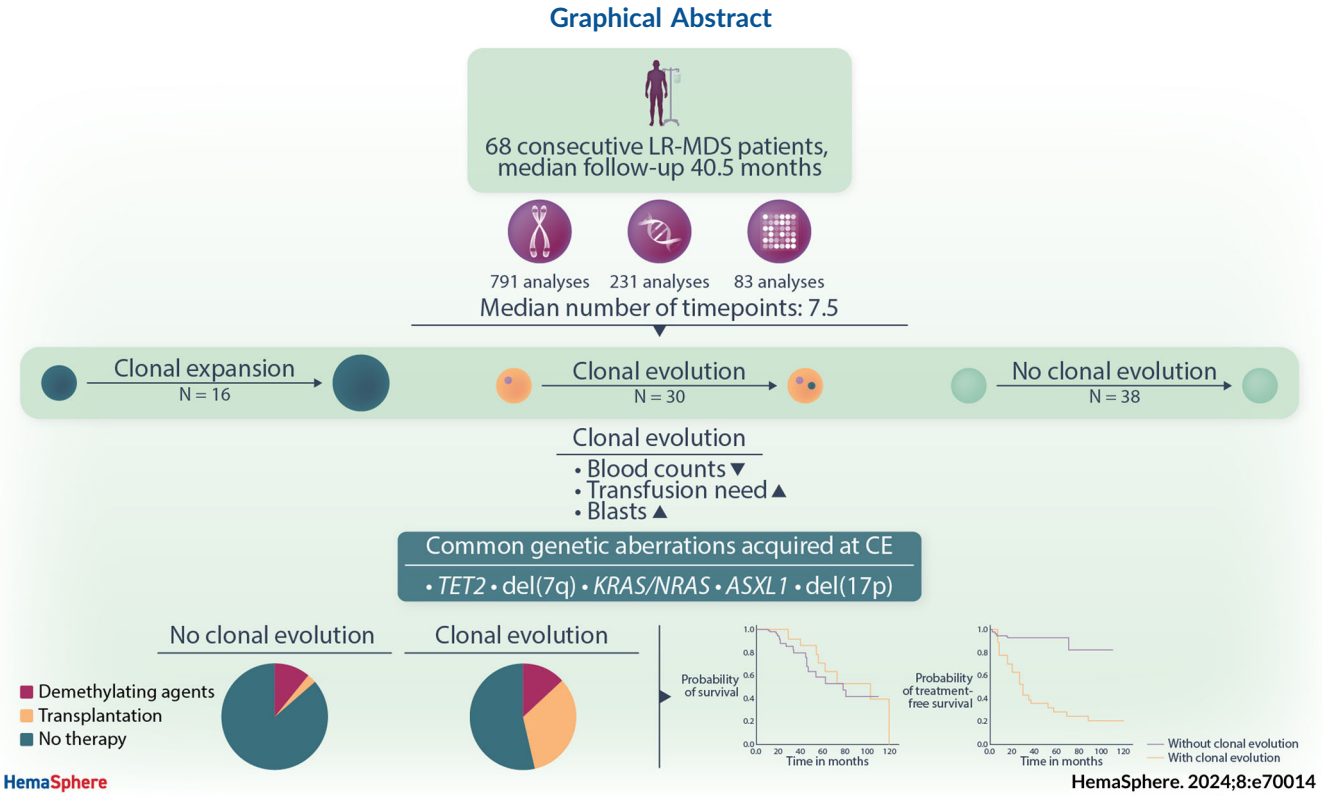
Graphical Abstract



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Abstract

The acquisition of subsequent genetic lesions (clonal evolution, CE) and/or the expansion of existing clones (CEXP) contributes to clonal dynamics (CD) in myelodysplastic syndromes (MDS). Although CD plays an important role in high-risk patients in disease progression and transformation into acute myeloid leukemia (AML), knowledge about CD in lower-risk MDS (LR-MDS) patients is limited due to lack of robust longitudinal data considering the long clinically stable courses of the disease. In this retrospective analysis, we delineate the frequency and the prognostic impact of CD in an unselected real-world cohort of LR-MDS patients. We screened 68 patients with a median follow-up of 40.5 months and a median of 7.5 (range: 2–22) timepoints for CE and CEXP detected by chromosomal banding analysis, fluorescence in situ hybridization, sequencing, and molecular karyotyping. In 30/68 patients, 47 CE events and a CD rate of 1 event per 4 years were documented. Of note, patients with at least 1 CE event had an increased probability for subsequent treatment. Unexpectedly, CE did not correlate with inferior outcomes, which could be reasonably explained by CD detection triggering the subsequent start of a disease-modifying therapy.

INTRODUCTION

Myelodysplastic syndromes (MDS) constitute a heterogeneous group of clonal hematopoietic stem cell diseases characterized by ineffective hematopoiesis, dysplasia of one or more hematopoietic cell lines and a 30% risk of progression to acute myeloid leukemia (AML).[1–3] The heterogeneity of the disease is reflected by a wide range of somatically acquired cytogenetic and/or molecular genetic aberrations. These aberrations may be present years before the clinical manifestation of MDS.[4] At diagnosis, cytogenetic aberrations are detected in about 50%[5,6] and mutated genes in up to 90% of cases.[7,8] The importance of genetic aberrations in the pathogenesis of MDS is

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reflected by their prognostic value.[9–11] Disease progression is closely related to genomic dynamics by sequential acquisition of novel genetic aberrations (clonal evolution, CE) and/or clonal expansion (CEXP). CE and CEXP both contribute to clonal dynamics (CD) and have been lumped together in most studies of clonal evolutionary events.[12,13] Additionally, previous studies could show that CE and CEXP are associated with transformation to AML and shorter overall survival (OS).[12,14–17]

Regarding the frequency of CE in MDS at diagnosis, karyotyping shows cytogenetic subclones originating from a primary clone in 13% of patients.[6] At diagnosis, in 58% of the patients multiple clones are inferred considering copy number alterations and gene mutations.[18] In longitudinal analyses, up to 29% of MDS patients are affected by CE detectable by karyotyping.[6,14,16,19] Considering cytogenetics and gene mutations, genetic evolution can be proved in up to 66% of MDS patients in longitudinal analyses.[20] Previous studies in MDS focused on CE at AML transformation, often based on a limited number of samples per patient and mostly comparing samples at MDS diagnosis and after leukemic transformation. Little is known about CE in lower-risk (LR) MDS patients[21] in most of whom the course is clinically stable over a long period. In this study, we analyzed the frequency, patterns, and prognostic impact of clonal dynamics by CE and CEXP in LR-MDS patients based on frequent comprehensive genetic analyses.

MATERIALS AND METHODS

Patient cohort

We enrolled 68 adult patients with LR-MDS in our study. All patients were defined as low-risk or intermediate-1 risk according to IPSS[22] and had up to 4.5 points according to the revised International Prognostic Scoring System (IPSS-R).[10] They were recruited at the University Medical Center Göttingen ($n = 64$), the Technical University Munich ($n = 3$), and the University Hospital Dresden ($n = 1$) between 2010 and 2021. Patients were included if at least two genetic analyses were available, with an interval of at least 6 months between them. The first genetic analysis had to be conducted within 12 months following the initial diagnosis of morphologically proven MDS. Three patients were included that did not meet the dysplasia criteria for MDS diagnosis and did not progress clinically or genetically to MDS during the observation time. One of these patients was diagnosed with clonal cytopenia of unknown significance (CCUS) and two with idiopathic cytopenia of unknown significance (ICUS). Patients with isolated 5q- were not included as their genetics under treatment was described by us before.[21] The study was approved by the local ethics committees (application number 02-02-14). Informed consent was obtained from all patients.

Genetic analyses

All genetic abnormalities reported were somatic, MDS-associated, and nonconstitutional. During the observation time from 2010 to 2022, a total of 1105 genetic analyses were conducted, comprising 237 chromosome banding analyses, 554 fluorescence in situ hybridization analyses (FISH), 83 molecular karyotyping analyses, and 231 sequencing analyses. Among the 1105 analyses conducted, 63% were performed on peripheral blood samples, whereas 37% were conducted on bone marrow samples. Follow-up analyses were available for all patients, with a minimum of two consecutive analyses per individual, and a median of 7.5 analyses per patient (range: 2–22). Chromosome banding analysis of G-banded chromosomes from bone marrow cultures was performed as described elsewhere.[5,23,24] FISH analysis was performed using a panel containing 11 probes designed for MDS, commercially available (see Supporting Information S1: Table 1) following previous descriptions.[23,25,26] Next-generation sequencing (NGS) or Sanger sequencing was performed as previously described[27] using a targeted panel including up to 54 genes frequently mutated in myeloid neoplasms (see Supporting Information S1: Table 2). Molecular karyotyping was performed to detect copy-neutral losses of heterozygosity (cnLOH) that remain undetected by FISH using the Cyto Scan HD arrays (Thermo Fisher) as previously described.[28] FISH, sequencing, and molecular karyotyping were done either on bone marrow cells or on CD34+ peripheral blood cells after immunomagnetic enrichment.

Genetic evolution and expansion criteria

We differentiated between CE and CEXP in our study. CE was defined as new genetic aberrations, including somatic mutations or cytogenetic aberrations, emerging either during the natural course of the disease or under therapy. The "pattern indicative of CE at diagnosis" subcategory was defined in two ways: first, as different but depending clones with additional clonal chromosomal aberrations in parallel to the stem clone[6]; second, as a mutated gene located within a chromosomal region with loss of heterozygosity (LOH). CEXP was defined by two criteria: first, a 50% increase in clone size by FISH and/or chromosome banding analysis, by adapting the Cheson criteria[29]; and second, an increase of 10% or more in variant allele frequency (VAF), as detected through sequencing.[30] If CE and CEXP were observed simultaneously, the CE event was evaluated.

Statistics

Patient characteristics were compared using the Fisher's exact test for categorical variables, and the Wilcoxon rank-sum test for continuous variables. Survival outcomes associated with baseline characteristics were determined using standard Cox proportional hazards regression analysis. Survival analysis with genetic evolution as time-dependent covariate was performed using two approaches: in the first one, we employed Cox proportional hazards regression analysis and the Simon–Makuch plot with the Mantel–Byar test including genetic evolution as time-dependent covariate. OS was calculated from the first diagnosis to death from any cause, with censoring at the last follow-up and at allogeneic hematopoietic stem cell transplantation (HSCT). Treatment-free survival was calculated from first diagnosis to the start of disease-modifying therapy (DMT), including HSCT and demethylating agents (DMA; 5-azacitidine, decitabine), with censoring at last follow-up and at death. This first approach did not consider HSCT or death as a competing event.

The second approach was a novel approach called case-based sampling.[31,32] It allowed considering HSCT as a competing event and including the CE events as time-varying covariate. We used the implementation of the method as provided by the casebase package.[33,34] We tested several models, all of which used age at diagnosis, sex, and IPSS-R as constant predictors, as well as CE events as a time-varying predictor. Details about model selection can be found in the Supporting Information Methods.

The following R packages were applied: survival (version 3.5.7), RcmdrPlugin.EZR (version 1.63), and casebase (version 0.10.3) for survival analysis and swimplot (version 1.2.0), fishplot (version 0.5.1), survminer (version 0.4.9), ggplot2 (version 3.3.6), and ggpubr (version 0.4.0) for graphical representation.

RESULTS

Patient cohort

Patients in this study were diagnosed between 2010 and 2021. The cohort included MDS with single lineage dysplasia (MDS-SLD; 12%, 8/68), MDS with multilineage dysplasia (MDS-MLD; 53% 36/68), MDS with ring sideroblasts (MDS-RS; 6% 4/68), MDS with excess blasts (MDS-EB1, -EB2; 10%, 7/68), chronic myelomonocytic leukemia (CMML; 3%, 2/68), MDS unclassifiable (MDS-U; 4%, 3/68), and other low-risk myeloid diseases (MDS/MPN, 4%, 3/68; hypoplastic MDS, 3%, 2/68; ICUS, 3%, 2/68; CCUS, 1%, 1/68) according to the WHO classification of 2016.[35] The median age of the study population was 71 years (range: 26–87 years). The patients were stratified as follows: very low (34%, 23/68), low (43%, 29/68), or intermediate risk (24%, 16/68) according to IPSS-R.[10] The median observation time for the study cohort was 40.5 months (range: 7–117 months). The median blood counts at initial diagnosis were as follows: hemoglobin 10.2 g/dL (range: 6.3–14.3), platelets 115×10^9/L (16.5–483.0), white blood counts 3.8×10^9/L (0.1–50.8), absolute neutrophil count (ANC) 1.9×10^9/L (0.1–20.9). Ferritin at initial diagnosis was 223 µg/L (6–2933). The median bone marrow blast count at initial diagnosis was 2% (range: 0%–10%). Among all patients, 11 (16%) transformed to AML. At the time of transformation, four patients showed CE and four patients CEXP. A total of 11 out of 68 patients (16%) underwent HSCT. Of those, 4/11 (36%) were categorized at initial diagnosis as very low, 2/11 (18%) low, and 5/11 (45%) as intermediate risk according to IPSS-R. Among the 11 transplanted patients, 4 (33%) had developed secondary AML (sAML) at the time of HSCT. Twenty-six percent (18/68) of all patients received DMT (including DMA, such as 5-azacitidine and decitabine). All of them experienced deterioration in blood counts/increasing blasts as a precondition to start in-label DMT. Among the patients who underwent HSCT, only one out of 11 (9%) received induction therapy without prior DMA administration during the observation period; 7/11 (64%) received DMA exclusively; 2/11 (18%) received DMA followed by induction therapy due to leukemic progression; while 1/11 (9%) did not receive DMA or induction before HSCT, primarily because the blast count was below 5% at the time of HSCT. Details of the study population are shown in Table 1.

Cytogenetics (including chromosome banding analysis and FISH) and molecular karyotyping were available for all patients. According to IPSS-R, the karyotypes were classified as follows: very good risk in 9/68 (13%) patients (-Y), 52/68 (76%) as good risk (with 49 of them having a normal karyotype and the remaining showing isolated 12p- or 20q-), intermediate risk in 6/68 (9%), and one patient (1%) had -7, indicating a poor karyotype. Notably, -Y was never acquired during CE. Sequencing was performed in 60 patients (88%). In 43/60 patients (72%), somatic mutations were identified in the analyzed genes. The most common genes mutated at initial diagnosis were *TET2*, detected in 19 patients, *ASXL1* in 15 patients, *SRSF2* in 13 patients, *SF3B1* in eight patients, and *RUNX1* in seven patients. All genetic aberrations acquired during clonal evolution are shown in Figure 1. Further details regarding genetic aberrations at the time of diagnosis and during the follow-up can be found in Table 2 and Supporting Information S1: Table 3.

Regarding baseline clinical parameters and outcome, gender and age were significantly associated with OS in the univariate analysis (female vs. male: hazard ratio [HR]: 0.32, 95% confidence interval [CI]: 0.17–0.86, $p = 0.024$; age (years): HR: 1.09, CI: 1.03–1.16, $p = 0.003$) in our cohort. However, blood counts, bone marrow blasts, and IPSS-R at diagnosis did not show a significant association with OS. Genetics at initial diagnosis showed no association with OS, neither with the presence of a normal karyotype nor the total number of genetic aberrations (Table 3). Additionally, we investigated whether the year of initial diagnosis did influence survival because of the retrospective nature of our study and the long observation time. As a result, the year of initial diagnosis was not associated with OS.

Clonal evolution

CE was detected in 30 out of 68 patients (44%). Figure 2 gives an overview of each patient's clinical course. In total, 47 CE events were observed in a total observation time over all patients of 3193 months resulting in a CE rate of 1 event every 5.7 years: 7 events (15%) at diagnosis (pattern indicative of CE at diagnosis) and 40 events (85%) during the follow-up. Considering the CD rate (combining both CE and CEXP events), we calculated a rate of one event every 4.0 years.

Six patients showed a pattern indicative of CE at initial diagnosis but no further CE events. Among the 24 patients with at least one CE event during the follow-up, the median time from diagnosis to the first CE event was 22 months (range: 1–85 months). From the 47 CE events observed in total, 41 were observed under the natural course of the disease and 6 events under DMT. These six events under DMT were observed in four different patients. In total, 20 patients showed morphologic progression (transformation from <5% blasts or from 5% to 20% blasts to a higher blast count, hence 5%–20% blasts or ≥20% blasts/AML transformation). Four patients who progressed directly to AML without prior evolution to MDS with increased blasts underwent regular monitoring, rendering it unlikely that any phase of increased blasts was missed. In total, 13/20 (65%) patients with morphologic progression also showed CE. Furthermore, morphologic progression was more frequently observed in the group of patients with CE compared to the group of patients without CE ($n = 13$ vs. $n = 7$, $p = 0.048$) (Table 1).

DMT was started because of deterioration of blood counts/morphologic progression in all patients (in-label treatment). While just 5/38 patients (13%) without CE were treated with DMT, 13 patients (43%) with CE were treated with DMT. Of these, 10/18 (56%) received DMT within 3 months of detection of CE. The median time from detecting the first CE event to the start of DMT was 2 months, with a range from 0 to 16 months.

CEXP was detected in 16 out of 68 patients (24%). Of those, 12 also had CE events during the observation time. The most frequently mutated genes and cytogenetic abnormalities involved in CEXP were *EZH2*, *IDH1*, and *SRSF2* (3 events), *CBL* and *TET2* (2 events) followed by *ASXL1*, *BCORL1*, *KRAS*, *SF3B1*, monosomy 7, 7q-, trisomy 8, 13q-, 20q-, and -Y (1 event). The median time from diagnosis to the first CEXP was 23 months (4–108 months). Among the 16 patients with CEXP, seven received therapy. One patient had CEXP during therapy, another one had CEXP within one month before the therapy started and the third one within one month after the therapy ended.

Comparing patients with and patients without CE, we point out that they were balanced in terms of baseline clinical data (Tables 1 and 4): no differences regarding the gender ratio, age at initial diagnosis, cytomorphologic subtypes, AML transformation rate, BM blasts, ANC count, Hb value, death ratio, proportion of patients with normal karyotype, ferritin levels at diagnosis. Significant differences were observed for the platelet counts (CE: 105 (35–384; $\times 10^9$/L) vs. no CE: 138 (16.5–483; $\times 10^9$/L), $p < 0.001$), the IPSS-R stratification, the proportion of patients receiving therapy (CE: 43% vs. no CE: 13%, $p = 0.002$) or underwent HSCT (CE: 33% vs. no CE: 3%, $p < 0.001$), observation time (CE: 49 (10–117) vs. no CE: 32.5 (7–107) months, $p < 0.001$), transfusion dependency (Table 4, CE: 64% vs. no CE: 42%, $p = 0.003$), and deterioration of blood counts during the observation

TABLE 1 Baseline characteristics of 68 MDS patients with and without clonal evolution.

Parameter	All patients, *N* = 68		Patients with CE, *N* = 30		Patients without CE, *N* = 38		*p* Value
Baseline characteristics at diagnosis							
Gender (M/F), *n* (ratio)	46/22	2.1	21/9	2.3	25/13	1.9	0.797
Median age, years (range)	71	26–87	68	50–83	72	26–87	0.693
Cytomorphologic subtypes (WHO 2016)							
MDS with single lineage dysplasia (MDS-SLD)	8	12%	4	13%	4	11%	0.409
MDS with ring sideroblasts (MDS-RS)	4	6%	3	10%	1	3%	
MDS with multilineage dysplasia (MDS-MLD)	36	53%	15	50%	21	55%	
MDS with excess blasts (MDS-EB1, -EB2)	7	10%	4	13%	3	8%	
MDS, unclassifiable (MDS-U)	3	4%	2	7%	1	3%	
CMML	2	3%	1	3%	1	3%	
Other[a]	8	12%	1	3%	7	18%	
IPSS-R (*n* = 68)							
Very low	23	34%	15	50%	8	21%	0.007
Low	29	43%	6	20%	23	61%	
Intermediate	16	24%	9	30%	7	18%	
Blood counts, median/range							
Hemoglobin (g/dL)	10.2	6.3–14.3	10.8	6.3–13.5	10.1	7.3–14.3	0.406
Platelets (10^9/L)	115	16.5–483	105	35–384	138	16.5–483	<0.001
ANC (10^9/L)	1.9	0.1–20.9	1.5	0.3–20.9	2.1	0.1–17.4	0.285
Ferritin (µg/L), median/range	223	6–2,933	255	57–922	217	6–2933	0.252
Bone marrow blasts (%), median/range	2	0–10	2	0–8	1	0–10	0.641
Year of initial diagnosis	2010–2021		2010–2020		2010–2021		0.131
Genetics at diagnosis							
Normal karyotype	49	72%	19	63%	30	79%	0.272
Number of aberrations per patient (median)							
Number of chromosomal aberrations (*n*)[b]	0	0–3	1	0–3	0	0–3	0.021
Number of molecular aberrations (*n*)[c]	1	0–5	2	0–5	1	0–5	0.007
Total number of aberrations (chrom. + molecular)	2	0–6	3	0–6	1	0–5	0.002
Events during the observation time							
Observation time (median, months)	40.5	7–117	49	10–117	32.5	7–107	<0.001
Disease-modifying therapy (DMT)	18	26%	13	43%	5	13%	0.002
Hematopoietic stem cell transplantation (HSCT)	11	16%	10	33%	1	3%	<0.001
Morphologic progression (incl. AML transformation)	20	29%	13	43%	7	18%	0.048
AML transformation	11	16%	5	17%	6	16%	1.000
Death	25	37%	8	27%	17	45%	0.139
Rate of genetic analysis timepoints (median rate/year)	2.6 (0.5–13.1)		2.5 (0.7–8.4)		2.0 (0.5–13.1)		0.725

Note: The values shown are median and range for continuous variables and the absolute number and percentage for categorical variables. CE, clonal evolution; CMML, chronic myelomonocytic leukemia; F, female; M, male; MDS, myelodysplastic syndrome; MDS-EB1/MDS-EB2, MDS with excess blasts 1/2; WHO, World Health Organization.[35]

[a]Other: MDS/MPN, hypoplastic MDS, idiopathic cytopenia of unknown significance (ICUS), clonal cytopenia of unknown significance (CCUS); IPSS-R, International Prognostic Scoring System-revised.[10] AML, acute myeloid leukemia; ANC, absolute neutrophil count; dL, deciliter; g, gram; L, liter; *N*, number; µg, micrograms.

[b]Median of chromosomal aberrations including copy number neutral loss of heterozygosity (cnLOH) detected by FISH, chromosome banding analysis, or molecular karyotyping at diagnosis.

[c]Median of molecular aberrations detected by sequencing at diagnosis.

time (Table 4, CE: 63% vs. no CE: 40%, $p = 0.002$). In terms of ESA therapy, chelation therapy, or other supportive therapies there was no statically significant difference between patients with CE and those without CE (Table 4). Notably, statistically significant differences became evident looking at the median number of aberrations per patient at the time of initial diagnosis: considering both, chromosomal as well as molecular alterations, the median was 3 (0–6) for patients that acquired CE during the observation time and 1 (0–5) for patients without CE ($p = 0.002$). Considering the chromosomal aberrations only, the median was 1 (0–3) for the CE group and 0 (0–3)

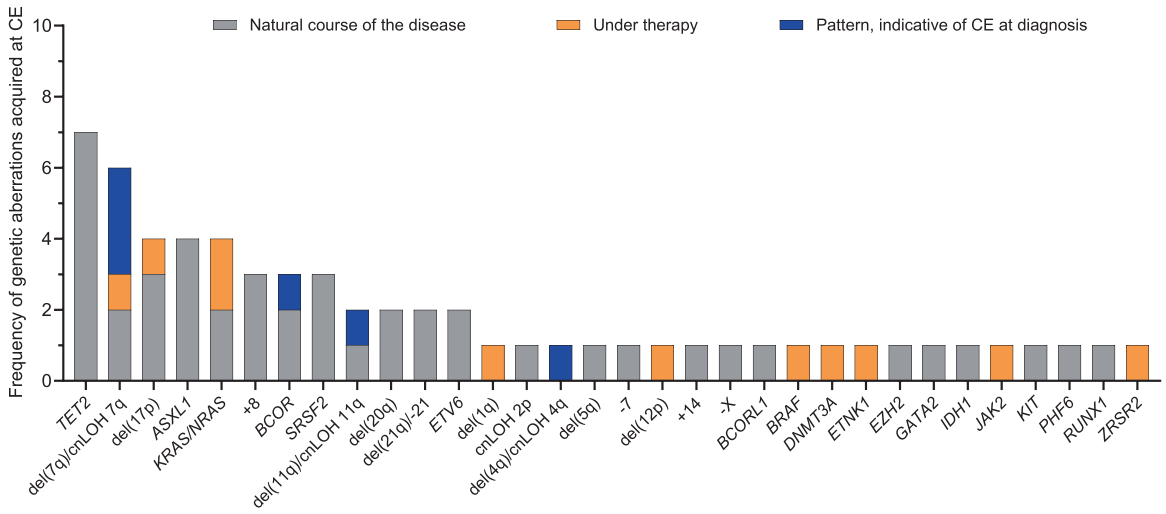

FIGURE 1 **Genetic aberrations acquired during CE**. The *Y*-axis displays the frequency of the involvement of a genetic aberration in CE. CE, clonal evolution; cnLOH, copy-neutral loss of heterozygosity.

for the no CE group ($p = 0.021$). Considering the molecular aberrations only, the median was 2 (0–5) for the group with CE and 1 (0–5) for the group without CE ($p = 0.007$). The 590 timepoints with genetic analyses performed in these 68 patients (median 7.5, range: 2–22) resulted in a median rate of 2.5 timepoints every year for the CE group and 2.0 timepoints for the group without CE ($p = 0.725$, statistically insignificant).

To answer the question whether clonal evolution was associated with shortened survival, we performed several Cox models with CE or the number of aberrations as a time-dependent covariate (Table 3). In the univariate analysis for OS, the HR for the acquisition of CE or an increasing number of aberrations was not significantly increased. Furthermore, there was no statistically significant effect in the multivariate models including the IPSS-R points at diagnosis. For treatment-free survival, the HR was significantly increased for clonal evolution (HR: 3.12, 95% CI: 1.74–5.57, $p < 0.001$) and the number of genetic aberrations (HR: 1.40, 95% CI: 1.20–1.64, $p < 0.001$) in the univariate models. They were also significantly increased in the multivariate models including the IPSS-R points at initial diagnosis (Table 3). Similar effects were shown in the Simon–Makuch plots. Stratifying patients at the occurrence of their CE event, we delineated distinct timeframes—those devoid of CE and those encompassing CE. Subsequently, no significant differences were observed regarding survival for patients who acquired CE compared to those who did not (Figure 3A). However, treatment-free survival was significantly shorter for patients who acquired CE compared to patients who did not (Figure 3B).

As the models described above do not consider competing risks, we also performed a novel case-based sampling approach. The best model that resulted from this approach was the Weibull model (description of the model selection in the Supporting Information Materials). Just like in the above-described models, CE count was not predictive of death after accounting for transplantation as a competing event (Supporting Information S1: Table 4).

Patient with clonal dynamics

Figure 4 exemplarily shows details of patient 54 who was initially diagnosed with MDS-MLD, <5% of bone marrow blasts, and an IPSS-R score of 1.5 points (very low-risk). Blast counts and blood counts were stable during the observation time. The patient received supportive care and chelation therapy starting at the initial diagnosis. Initially, loss of the Y chromosome and mutations in *CBL*, *EZH2*, *TET2* (2x), *ZRSR2*, and cnLOHs in 11q and 21q were detected. Genetic analysis was done at initial diagnosis and every 3–6 months.

During the natural course of the disease, the patient acquired a mutation in *BCORL1* and a cnLOH in 7q, 21q- (blue and green clone, respectively), and finally 17p- and 20q- (orange clone). Thus, the patient showed an ancestral clone (gray clone) and several subclones with an increasing number of genetic aberrations through a branching evolution (red and yellow clone) and then a linear evolution pattern (blue and orange clone). Finally, the subclone with further linear evolution outcompeted the other subclones and populated the whole compartment.

DISCUSSION

Here, we report on frequent longitudinal genetic analyses of LR-MDS patients to delineate CD encompassing CE and CEXP in detail and to dissect the clinical consequences of CD in an unselected real-world cohort of patients and different treatment modalities. We believe that this can be an important step in understanding CD, given the limited literature available up to now. Our data show that CD is a rather frequent event in LR-MDS, although being even more frequent in high-risk cases, with a CD rate of just one event in 4 years in our cohort. This reflects the long period of a clinically stable course in these low-risk patients. However, the long median observation time in our study of 40.5 months finally resulted in 53% of patients with CD and 44% with CE. Comparing treatment modalities in patients with and without CE, we found that 13/30 (43%) patients with CE and 5/38 (13%) without CE received DMT; 10/30 (33%) of CE patients and 1/38 (3%) of patients without CE underwent HSCT, and 5/30 (17%) of CE patients and 6/38 (16%) of patients without CE transformed to AML. Although there is an association between CE and treatment, there is also an obvious and significant interdependency between CE and worsening of blood counts, providing the basis for in-label therapeutic intervention. However, the design of our study did not allow us to clearly separate the impact of CE, deterioration of blood counts, and morphologic progression on treatment decisions.

TABLE 2 Chromosomal aberration(s) and gene(s) mutated at initial analysis and at clonal evolution (CE) (continued on next page). (continued on next page)

ID	Chromosomal aberration(s) at first diagnosis	Gene(s) mutated at first diagnosis	Genetic aberrations acquired at clonal evolution
1	Normal	NP	
2	Normal	Negative	
3	Normal	*ASXL1 (x2)*, *IDH2*, *SRSF2*, *TET2*, *U2AF1*	
4	-Y	Negative	
5	Cryptic 4q-	NP	
6	20q-	*ASXL1*, *ETV6*, *U2AF1*	1st: *ETV6*, 2nd: 12p-[a], 3rd: 7q-[a]
7	-Y	*KRAS*	
8	cnLOH in 1p and 4q	*IDH2*, *SF3B1*	
9	Normal	*ASXL1*, *IDH2*, *SF3B1*	
10	cnLOH in 7q	*ASXL1*, *CEBPA*, *EZH2*, *SETBP1*	1st: pattern indicative of CE at diagnosis: cnLOH 7q/*EZH2*, 2nd: *ETV6*
11	cnLOH in 7q	*ASXL1*, *CUX1*, *SETBP1*, *SRSF2*	7q-
12	Normal	*TET2 (x2)*, *SRSF2*	*ASXL1*
13	Normal	*ASXL1*, *U2AF1*	
14	Cryptic 5q-, cnLOH in 7q	*EZH2*, *RUNX1*	Pattern indicative of CE at diagnosis: cnLOH 7q/*EZH2*
15	cnLOH in 14q, del(20q), del(20q)x2	NP	1st: pattern indicative of CE at diagnosis: del(20q) x 2
16	Normal	*SF3B1*, *TET2*	1st: *TET2*, 2nd: *TET2*
17	+14, -Y	Negative	Pattern indicative of CE at diagnosis: +14
18	cnLOH in 4q, -Y	*EZH2*, *PHF6*, *TET2*, *ZRSR2*	Pattern indicative of CE at diagnosis: cnLOH 4q/*TET2*
19	Normal	Negative	*IDH1*
20	+8	*SRSF2*	
21	12p-	*ASXL1*, *RUNX1*, *SRSF2*	
22	Normal	NP	
23	Normal	*KIT*, *SRSF2*, *TET2 (x2)*	
24	Cryptic 6q+, cnLOH in 22q, -Y	*ZRSR2*	1st: *TET2*, 2nd: *EZH2*
25	Normal	Negative	1st: *ASXL1*, 2nd: *+8*, 3rd: *ZRSR2*[a]
26	Cryptic 21q-	*ASXL1*, *SRSF2*, *TET2*	+8
27	Normal	Negative	
28	Normal	*TET2* (x2), *ZRSR2*	
29	Normal	Negative	
30	Normal	*ASXL1*, *TET2*	*SRSF2*
31	-Y	*TET2*	1st: *TET2*, 2nd: *PHF6*
32	Normal	*DNMT3A*	
33	Normal	Negative	
34	13q-	Negative	
35	Normal	*EZH2*	
36	Normal	NP	
37	Normal	*MPL*, *SRSF2*	
38	Cryptic 2p-	*TET2*, *U2AF1*	1st: cnLOH 2p, 2nd: *+8*
39	-7, 7q-, r(7)	NP	
40	Normal	Negative	
41	Normal	*SRSF2*, *TET2*	*KRAS*
42	cnLOH in 7q	*BCOR*, *CEBPA*, *EZH2*, *TET2* (x2), *ZRSR2*	Pattern indicative of CE at diagnosis: cnLOH 7q/*EZH2*
43	Normal	*RUNX1*, *SRSF2*, *STAG2*	1st: *ASXL1*, *BCOR*, *KRAS*, 2nd: *GATA2*
44	-Y	Negative	
45	Normal	*EZH2*, *SF3B1*, *TET2* (x2)	*17p-*, *-X*

TABLE 2 (Continued)

ID	Chromosomal aberration(s) at first diagnosis	Gene(s) mutated at first diagnosis	Genetic aberrations acquired at clonal evolution
46	Normal	Negative	*ASXL1*
47	Normal	*RUNX1*	
48	cnLOH in 11q, -Y	*CBL (x2)*, *SF3B1*, *TET2* (x2), *ZRSR2*	Pattern indicative of CE at diagnosis: cnLOH 11q/*CBL*
49	Normal	*ASXL1*, *RUNX1*	
50	+8	*SF3B1*	1st: *TET2 (x2)*, *-7*, 2nd: *DNMT3A* (2x)[a], 3rd: 17p-[a]
51	Normal	NP	-21
52	5q-, 11q-, -Y	NP	
53	Normal	*ASXL1*, *PHF6*	
54	-Y, cnLOH 11q, cnLOH 21q	*CBL*, *EZH2*, *TET2* (x2), *ZRSR2*	1st: *BCORL1*, cnLOH 7q, 2nd: 21q-, 3rd: 17p-, 20q-
55	Normal	Negative	
56	Normal	*ASXL1*, *EZH2* (x2), *RUNX1*	1st: *KIT*, 5q-, 11q-, 17p-, 20q-, 2nd: *BRAF*[a], *ETNK1*[a], *JAK2*[a], *KRAS*[a], *NRAS*[a], 1q-[a]
57	Normal	Negative	
58	Normal	Negative	
59	Normal	*ASXL1*, *SRSF2*, *TET2*	
60	Normal	*IDH1*	*SRSF2*
61	Normal	*SF3B1*, *TET2*, *ZRSR2(x2)*	
62	cnLOH in 7q	*ASXL1*, *RUNX1*, *SRSF2*	
63	20q-	*ASXL1*, *NRAS*, *SRSF2*, *TET2*	*TET2*
64	Cryptic 20q-	*IDH1*, *U2AF1*	
65	Normal	*IDH2*	
66	cnLOH in 19q	Negative	1st: *SRSF2*, 2nd: *BCOR*, 3rd: *RUNX1*
67	Normal	Negative	
68	-Y	*SF3B1*, *TET2*	*TET2*

Abbreviations: CE, clonal evolution; NP, not performed.

[a]Acquired under disease-modifying therapy. 1st, 2nd, and 3rd refer to the consecutive CE events.

In previous studies focusing on either unselected MDS cohorts or high-risk MDS only, the phenomenon of CD was found to be significantly associated with transformation to AML and shorter OS.[12,14–17,19,21] This was also observed in our previous study of MDS patients with isolated 5q-, where CE was detected in 21%–34% of patients (by chromosome banding analysis and FISH on CD34+ peripheral blood cells), and CE was associated with a significantly shorter AML-free survival.[21] However, our current analysis of a cohort restricted to LR-MDS could not confirm these findings. Counterintuitive to our genetic data as well as to the published data mentioned above, the acquisition of CD did not worsen outcomes in our patients. Since the future acquisition of CD is unknown at the start of the study, evaluating its impact on outcome presents a challenge.[36] To properly account for CD as covariate, it was included in the Cox proportional hazard regression model as time-dependent variable. In addition, our patients were exposed to multiple potential events and the occurrence of one event precluded the occurrence of the others (death, HSCT, DMT). Traditional Cox proportional hazard regression models do not consider these competing risks. There may be a bias in the results of our first approach that just considered time-dependent covariates but not competing risks. Therefore, we additionally performed a novel statistical approach accounting for both. Both approaches resulted in no significant influence of CD on survival. A further possible bias in survival analysis could be the impact of comorbidities that were not considered in our study.

To exclude possible confounders in our models, we reviewed further clinical and genetic data. Visits with genetic analyses could have been scheduled more often for patients with higher risk, resulting in a higher probability to detect CD. However, the rates of genetic analyses performed were evenly distributed between patients with and without CE acquired. During the long observation period, standards for therapy and genetic tests changed. We could exclude a statistically significant difference by the time point of initial diagnosis between patients who acquired CD and those who did not.

The probability of treatment with DMT or HSCT was significantly higher in patients with CE compared to patients without CE. Through our close genetic monitoring performed on peripheral blood and different to the recent publication of Neukirchen et al.,[14] there was no bias regarding analysis timing and frequency between patients that acquired CD and those that did not Therefore, we hypothesize that patients who experienced CE are more likely to experience a deterioration of blood counts/increase in blast cells that significantly influenced the decision to timely start a DMT in-label and/or HSCT and that our lower-risk patients benefited from the detection of CE as a trigger for the onset of DMT and/or HSCT.

It is obvious that especially in LR-MDS with long follow-up the number of patients with therapeutic needs will increase over time as it is the case in our cohort.

TABLE 3 COX regression modeling for overall survival and treatment-free survival.

	HR (95% CI)	*p* Value
Overall survival (standard model considering baseline characteristics at diagnosis, univariate analysis)		
Gender F vs. M	0.32 (0.17–0.86)	0.024
Age (years)	1.09 (1.03–1.16)	0.003
Blood counts		
Hemoglobin (g/dL)	0.93 (0.77–1.12)	0.425
Platelets (10^9/L)	1.00 (0.99–1.00)	0.606
ANC (10^9/L)	1.10 (0.98–1.23)	0.098
Bone marrow blasts (%)	0.95 (0.75–1.19)	0.641
IPSS-R (0–4.5 points)	0.98 (0.69–1.41)	0.923
Normal karyotype (yes vs. no)	1.48 (0.59–3.74)	0.406
Total number of aberrations (chromosomal + molecular)	0.94 (0.76–1.17)	0.591
Year of initial diagnosis	0.97 (0.81–1.16)	0.723
Overall survival (model with CE or the number of aberrations as time-dependent covariate, univariate analysis)		
Clonal evolution (1st event: 1 point, 2nd event: 2 points, 3rd event: 3 points)	1.18 (0.68–2.04)	0.544
Total number of aberrations (chromosomal + molecular)	1.14 (0.95–1.36)	0.155
Overall survival (model with CE as time-dependent covariate, multivariate analysis)		
Clonal evolution (1st event: 1 point, 2nd event: 2 points, 3rd event: 3 points)	1.19 (0.69–2.07)	0.549
IPSS-R (0–4.5 points) at initial diagnosis	1.00 (0.70–1.45)	0.984
Overall survival (model with the number of aberrations as time-dependent covariate, multivariate analysis)		
Total number of aberrations (chromosomal + molecular)	1.17 (90.96–1.42)	0.125
IPSS-R (0–4.5 points) at initial diagnosis	1.12 (0.75–1. 68)	0.576
Treatment-free survival (model with CE or the number of aberrations as time-dependent covariate, univariate analysis)		
Clonal evolution (1st event: 1 point, 2nd event: 2 points, 3rd event: 3 points)	3.12 (1.74–5.57)	<0.001
Total number of aberrations (chromosomal + molecular)	1.40 (1.20–1.64)	<0.001
Treatment-free survival (model with CE as time-dependent covariate, multivariate analysis)		
Clonal evolution (1st event: 1 point, 2nd event: 2 points, 3rd event: 3 points)	3.87 (2007–7.23)	<0.001
IPSS-R (0–4.5 points) at initial diagnosis	1.66 (1.06–2.61)	0.028
Treatment-free survival (model with the number of aberrations as time-dependent covariate, multivariate analysis)		
Total number of aberrations (chromosomal + molecular)	1.66 (1.36–2.02)	<0.001
IPSS-R (0–4.5 points) at initial diagnosis	2.32 (1.37–3.91)	0.002

Abbreviations: AML, acute myeloid leukemia; ANC, absolute neutrophile count; CE, clonal evolution; CI, confidence interval; dL, deciliter; F, female; g, gram; HR, hazard ratio; HSCT, hematopoietic stem cell transplantation; IPSS-R, International Prognostic Scoring System-revised;[10] M, male.

Our patients may also have benefited from further supportive care such as chelation therapy or erythropoiesis-stimulating agents. Iron chelation improves the outcome of MDS patients.[37] Erythropoiesis-stimulating agents also improve outcomes and reduce transfusion needs and therefore iron overload.[38–42] Besides other negative effects, iron overload may increase oxidative stress and consequently—among others—further genetic damage and CD, even in LR-MDS. However, the number of patients who received iron chelation or erythropoiesis-stimulating agents was too low in our cohort to test our hypothesis of a positive effect of supportive therapy on patients with CD.

Regarding the effect of the number of aberrations at diagnosis on further CD, it was reported that patients with cytogenetic aberrations at initial diagnosis are more likely to acquire additional genetic aberrations over time.[6,15] The initial number of genetic aberrations in our cohort is in the range of previously described unselected MDS cohorts,[6] albeit our analysis is restricted to lower-risk patients. At initial diagnosis, nine of our patients showed loss of the Y-chromosome which is associated with a very good prognosis according to IPSS-R.[10] If a loss of the Y-chromosome was not associated with CE, this could be a bias in this calculation. Due to the limited number of patients with genetic aberrations at diagnosis and subsequent CD in our lower-risk cohort, we could not confirm the hypothesis that a low initial number of genetic aberrations is associated with a lower probability of further CE.[6]

Usually, genetic analyses in MDS require bone marrow aspirates. Due to ethical reasons, it is not possible to perform frequent bone marrow aspirates in LR-MDS. Most of these patients are in stable disease. The results of frequent bone marrow examinations would not affect treatment decisions and thus would not be justified. Alternatively to bone marrow, using peripheral blood for genetic analyses is much less of a burden for the patient and the physician. As these patients may suffer from cytopenia and therefore may need supportive therapy, blood counts are regularly monitored in these patients. We could previously show that genetic analyses of immunomagnetically enriched CD34+ peripheral blood provide comparable results as those based on bone marrow blood.[23–26] This approach allows serial and frequent genetic analyses even in lower-risk patients.[21] In our current study, 63% of genetic analyses were performed on peripheral blood, which underlines the applicability of our approach in clinical routine. However, since we adopted a defined FISH probes panel and targeted sequencing of commonly mutated genes, we cannot rule out the possibility of mutations not covered by our methods. Therefore, the extent of clonal expansion might be even greater than reported in our study.

There are fluctuating clones (e.g., a clone with 21q- in patient 54, see Figure 4) that would have been missed if the genetic analysis was just done at initial diagnosis and progression or leukemic transformation. Our approach allows the observation of clones that do not prevail and therefore, may be more benign. For the management of MDS, it would be helpful to know which clones do not require immediate therapy because they may not contribute to the further progression of the disease.

Molecular karyotyping allowed us to analyze the effect of cryptic cytogenetic aberrations (small deletions and cnLOH) that cannot be detected by chromosome banding analysis. Cryptic cytogenetic aberrations, as well as other cytogenetic aberrations, were rarely observed within CD events. However, 10% of our patients (7/68) showed a pattern indicative of CE at diagnosis based on a mutated gene in a region of a cnLOH. A potential mechanism of cnLOH resulting from mitotic recombination in somatic cells of patients with mutations in the region of the cnLOH has been proposed before. It was suggested that

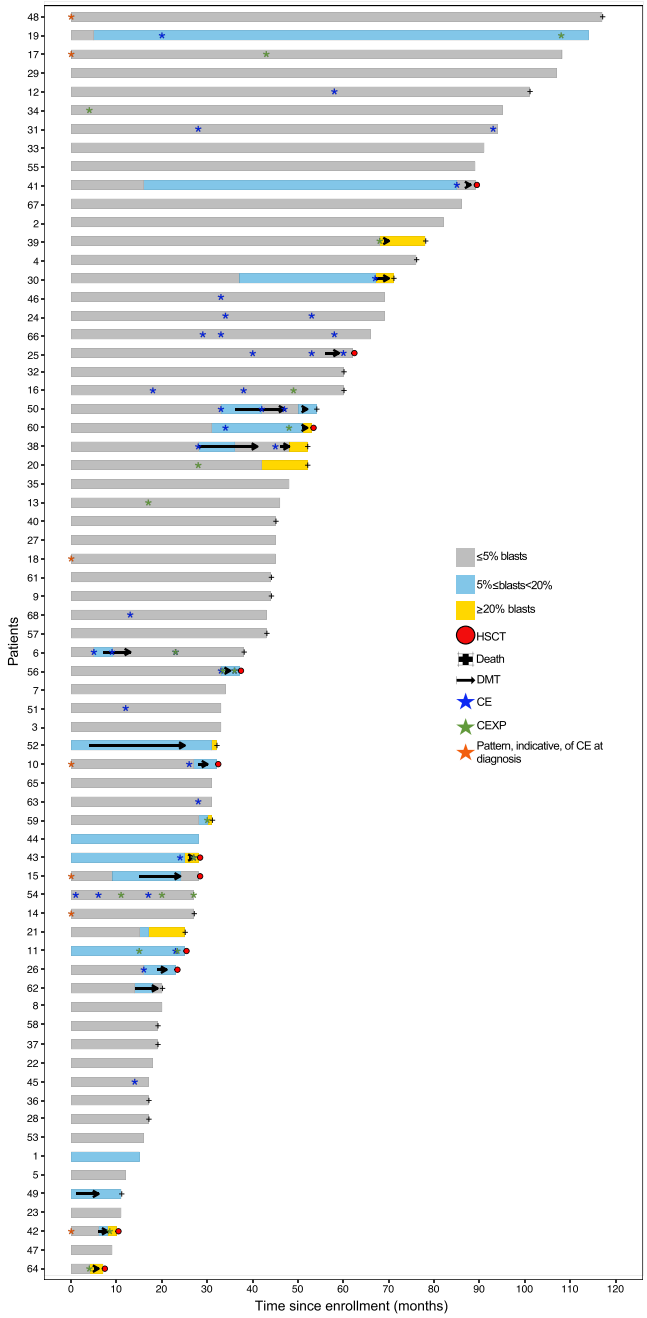

FIGURE 2 **Clinical course of the 68 patients.** The color of the bars corresponds to the medullary blasts, less than 5% in gray; in light blue blasts ≥5% and ≥20% in yellow. The red spots show the HSCT. The black "+" shows when patients died and the black arrow the time when therapy was applied. The orange "*" shows patterns, indicative of CE, at diagnosis, the blue one CE, and the green one CEXP. CE, clonal evolution; CEXP, clonal expansion; DMT, disease-modifying therapy; HSCT, hematopoietic stem cell transplantation.

daughter cells with cnLOH and a homozygous mutation have a selective growth advantage.[43,44] Notably, a mutated gene within a region of a cnLOH was observed much more frequently at first diagnosis compared to being newly acquired during the course of the disease. Therefore, this pattern seems to be involved at the onset of the disease. Generally, cnLOHs are associated with poor survival. This was observed in ICUS[45] as well as in AML.[46,47] In the previous study about

TABLE 4 Supportive therapies and change of blood counts over time among our cohort of patients with and without clonal evolution.

Parameter (%)	Patients with CE	Patients without CE	*p* Value
Supportive therapies			
Transfusion dependency	18/28 (64%)	15/36 (42%)	0.003
ESA therapy	5/28 (18%)	11/37 (30%)	0.069
Chelation therapy	5/28 (18%)	7/37 (19%)	1.000
Other supportive therapies	10/28 (36%)	12/37 (32%)	0.654
Blood counts			
Deterioration of blood counts	15/24 (63%)	8/20 (40%)	0.002

Note: Blood counts were calculated based on any deterioration in hemoglobin, platelets, leukocytes, and neutrophils, as adapted from disease progression criteria according to Cheson[29]: at least a 50% decrease from diagnosis in leukocytes, neutrophils, or platelets up to the last follow-up, along with a reduction in hemoglobin by ≥2 g/dL between diagnosis and the last follow-up, and transfusion dependency.

Abbreviations: CE, clonal evolution; ESA, erythropoiesis-stimulating agents; other supportive therapies: ATG, anti-thymocyte globulin; G-CSF, granulocyte-colony stimulating factor, hydroxyurea, and romiplostim.

ICUS, the poor impact of a copy number aberrations or a cnLOH on survival was higher than that of gene mutations.[45]

In contrast to individuals with clonal hematopoiesis,[48] CEXP without CE was a rare event in our cohort of LR-MDS patients. Using the example of *TET2*, in our cohort, 19 patients showed a *TET2* mutation at initial diagnosis, but only one patient showed an expansion of the initial *TET2* mutated clone. However, six patients acquired *TET2*-mutations during CE and *TET2* was the most frequent aberration detected at CE in our cohort. Recently, based on data collected from 285 individuals tracked up to 13 years, distinct patterns of lifelong clonal behavior that can negatively impact clonal hematopoiesis have been described.[48] In particular, mutations in *DNMT3A* were associated with slower clonal expansion, but earlier expansion, than *TET2*-mutant clones.

Finally, we aimed to check if previously described patterns of CE associated with prognosis could also be observed in LR-MDS. Due to the low number of patients with more than one aberration in banding analysis, we could not verify the effect of the clonal subtypes defined by Schanz et al.[15] on the outcome. We also could not verify previous reports about mutated genes that may be associated with progression. The acquisition of mutations in signaling genes (e.g., RAS pathway), recently described as associated with CD and AML-transformation,[49,50] was rare in our low-risk patient cohort. Three patients acquired a mutation in *KRAS* (2x during the natural course of the disease, 1x under DMT), one further patient showed a mutation in *NRAS* (under DMT), and a mutation in *KIT* in another patient (during the natural course of the disease). One patient with an initial mutation in *KRAS* (patient 7) and one with an initial mutation of *KIT* (patient 23) did not show CD. Further patients with initial mutations in signaling genes with subsequent CE were one patient with an initial mutation in *CBL* and one with an initial mutation in *NRAS*. Mutations in transcription factors, suggested to modulate signaling genes,[50] were also rarely acquired in our lower-risk patients: 1x *RUNX1* and 1x *ETV6* (all under the natural course of the disease). The most frequently affected genes involved in DNA methylation were *TET2* (7x) and *IDH1* (1x); in RNA splicing: *SRSF2* (3x) and in chromatin modification: *ASXL1* (4x) and *EZH2* (1x). Although patients with founding mutations in DNA methylation genes were reported to transform to AML, these mutations were recently not described to be associated with CD.[49] We hypothesize that, although CE was observed

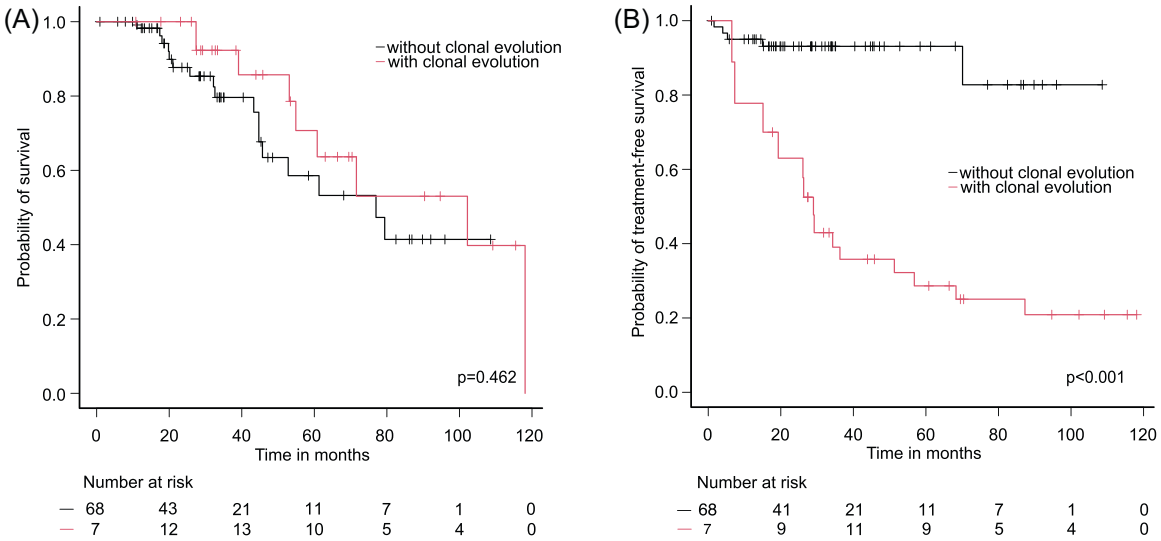

FIGURE 3 **Survival of LR-MDS patients based on CE. (A)** No significant differences regarding survival for patients who acquired CE compared to those who did not. **(B)** Treatment-free survival was significantly shorter for patients who acquired CE compared to patients who did not; Simon–Makuch plot; *p*-value from the Mantel–Byer test. CE, clonal evolution.

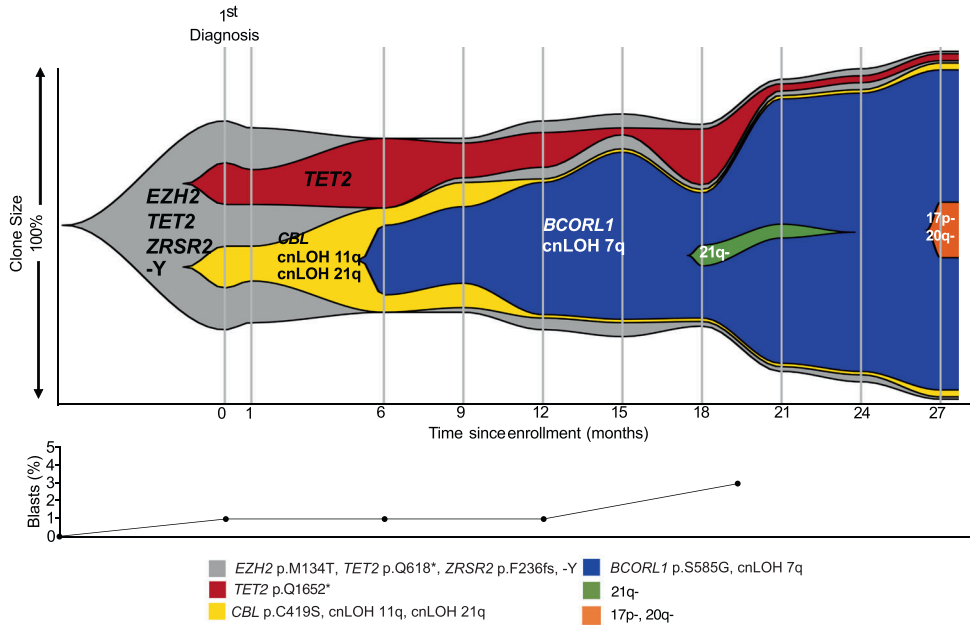

FIGURE 4 **Clonal dynamics were observed in patient 54 during the spontaneous course of the disease.** Gray lines depict the timepoints when the genetic analyses were performed. Below the fishplot, a corresponding graph recapitulates the blast counts.

in our lower-risk patients, genetic aberrations associated with rapid progression were rare and therefore, CE did not result in rapid progression. Additionally, our results and statistical calculations imply that patients with CD may have benefited from DMT.

The purpose of this study was to elucidate the clonal dynamics in LR-MDS patients since published data on clonal dynamics in MDS is mainly based on high-risk MDS with leukemic transformation thus not covering the important early phases of MDS. The unexpected missing adverse effect of CD on outcome reflects the unselected real-world character of our patient cohort with a therapeutic need evolving during the observation period. The proof of CD was closely associated with the timely implementation of a DMT in our study, thus, possibly turning a seemingly disadvantage (genetic progression and deterioration of blood counts) into a clinical benefit for the patients.

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AUTHOR CONTRIBUTIONS

Paolo Mazzeo, **Christina Ganster**, and **Detlef Haase**: Conceptualization. **Paolo Mazzeo**, **Christina Ganster**, **Doris Steinemann**, **Maximilian**

Schieck, **Catharina Müller-Thomas**, **Hannes Treiber**, **Friederike Braulke**, **Ulrich Germing**, **Katja Sockel**, **Ekaterina Balaian**, **Julie Schanz**, **Uwe Platzbecker**, **Katharina Götze**, and **Detlef Haase**: Data curation. **Christina Ganster**: Formal analysis. **Paolo Mazzeo** and **Christina Ganster**: Investigation. **Paolo Mazzeo**, **Christina Ganster**, **Katayoon Shirneshan**, **Katharina Rittscher**, **Elzbieta Brzuszkiewicz**, **Doris Steinemann**, **Maximilian Schieck**, and **Detlef Haase**: Methodology. **Paolo Mazzeo**, **John Wiedenhöft**, and **Christina Ganster**: Software. **Christina Ganster** and **Detlef Haase**: Supervision. **Paolo Mazzeo**, **Christina Ganster**, **Katayoon Shirneshan**, **Katharina Rittscher**, **Elzbieta Brzuszkiewicz**, and **Detlef Haase**: Validation. **Paolo Mazzeo**, **Christina Ganster**, and **Detlef Haase**: Writing—original draft. **Paolo Mazzeo**, **Christina Ganster**, **John Wiedenhöft**, **Katayoon Shirneshan**, **Elzbieta Brzuszkiewicz**, **Hannes Treiber**, and **Detlef Haase**: Writing—review and editing. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

Detlef Haase is a member of the Advisory board and received research support, as well as honoraria from Bristol Myers Squibb and Celgene. All other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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ETHICS STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the local Ethics Committee of the University Medical Center Göttingen (application number 02-02-14). Informed consent was obtained from all patients.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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