Clonal evolution in chronic lymphocytic leukemia: acquisition of high-risk genomic aberrations associated with unmutated VH, resistance to therapy, and short survival

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ABSTRACT

In chronic lymphocytic leukemia (CLL), the acquisition of new genomic aberrations during the disease course (*clonal evolution*) is thought to be an infrequent phenomenon but comprehensive analyses are limited. Genomic aberrations were analyzed by fluorescence *in situ* hybridization (FISH) at various time points during the disease course of 64 CLL patients. Results were correlated with the mutation status of the immunoglobulin heavy-chain variableregion genes (VH) and clinical characteristics. Following a median observation time of 42.3 months (range 23.2-73) after first genetic study, 11 out of the 64 (17%) patients showed clonal evolution with the following newly acquired aberrations: del(17p13) (n=4), del(6q21) (n=3), del(11q23) (n=2), +(8q24) (n=1), and evolution from monoallelic to biallelic del(13q14) (n=3). Interestingly, clonal evolution only occurred among cases with unmutated VH status. The group with clonal evolution showed a higher rate of progression in Binet stage (82% vs. 28%), a possibly greater need for treatment (91% vs. 62% previously untreated patients received their first therapy), and a higher hazard rosk of death (HR = 2.97, 95% CI 1.40-6.27, p=0.004) in multivariable analysis. The estimated median survival time after the occurrence of clonal evolution was 21.7 months. Expansion of the clone with del(17p13) was observed in all patients during treatment, indicating *in vivo* resistance to therapy. In multivariable Andersen-Gill regression analysis, clonal evolution was identified as an independent prognostic factor for overall survival. Clonal evolution only occurred in CLL with unmutated VH indicating to karyotypic instability as a pathomechanism. Acquisition of genomic aberrations was associated with poor outcome based on multivariable analysis. *In vivo* resistance to chemotherapy of CLL clones with del(17p13) emphasizes the need for alternative treatment approaches in these patients.

Key words: CLL, clonal evolution, genetic instability, p53.

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Chronic lymphocytic leukemia (CLL) is characterized by a variable clinical course and the mutation status of the immunoglobulin heavy-chain variable-region genes (VH) together with genomic aberrations have emerged as prognostic factors.[1-7] While the VH status remains stable, genomic aberrations may be acquired over time and it has been suggested that this may partly account for the more aggressive clinical course of CLL with unmutated VH genes.[2] Sequential chromosome banding analyses indicated the acquisition of genomic aberrations over time (*clonal evolution*) as an infrequent phenomenon in CLL.[8-11] However, only limited data are available from FISH studies of interphase cells, a more sensitive method for the detection of genomic aberrations in CLL.[12-20] Auer *et al.* and Hjalmar *et al.* found the acquisition of trisomy 12 in none of 41 and 2 of 77 CLL cases.[15,16] Chevalier et al. observed additional genomic aberrations with an extended probe set, in 13/31 (42%) CLL cases after a median time of 83 months.[17] There was no association between clonal evolution and CD38 expression or disease progression but the acquisition of del(17p13) was associated with death in 7/11 cases. The VH status was not reported and sequential samples during treatment were not available. Shanafelt et al. recently reported the increasing occurrence of clonal evolution over time (after 5+ years: 27% incidence, n=63) and the association with ZAP-70 positive CLL.[19]

Study design

Sixty-four patients from a single institution diagnosed according to standard criteria[22] were enrolled based on the availability of two or more sequential samples after a minimum follow-up time of 24 months after the first genetic investigation. Clinical characteristics

Table 1. Patient characteristics at initial evaluation.

	All cases (n=64)	*With evolution (n=11)*	*Without evolution (n=53)*	*P-value*
Age in years; median (range)	59 (30-77)	61 (44-71)	59 (30-77)	0.82
Female gender; n (%)	18 (28)	4 (36)	14 (26)	0.48
Binet stage; n (%)				0.39
A	32 (50)	4 (36)	28 (53)	
B	27 (42)	6 (55)	21 (40)	
C	5 (8)	1 (9)	4 (7)	
Prior therapy; n (%)	8 (13)	1 (9)	7 (13)	1.00
Unmutated VH; n (%)	35/58 (60)	11 (100)	24/47 (51)	0.002
Genomic aberrations				
Prevalence; n (%)	59 (92)	10 (91)	49 (92)	1.00
Aberrations per case; median (range)	1 (0-3)	1 (0-2)	1 (0-3)	1.00
Specific aberrations; n (%)				
del(13q14)	39 (61)	7 (63)	32 (60)	
del(13q14) single	23 (36)	2 (18)	21 (40)	
del(11q23)	14 (22)	4 (36)	10 (19)	
+(12q21)	14 (22)	1 (9)	13 (25)	
del(6q21)	6 (9)	2 (18)	4 (8)	
del(17p13)	1 (2)	0 (0)	1 (2)	

and prior therapy are shown in Table 1. The estimated median observation time since first genetic investigation was 61 months (range 27-93): 68 months (range 46-68) in the group with and 60 months (range 27-93) in the group without clonal evolution (log rank test; p=0.23). The median time interval between diagnosis and first investigation by FISH was 2.5 months (range 0-122). This was not significantly different when compared with the group with (median 3.5 months, range 0-17.5) and the group without (median 1.5 months, range 0-122) clonal evolution (Mann-Whitney test; p=0.87). The time to clonal evolution was defined as time from initial genetic investigation to the first follow-up investigation showing at least one newly acquired aberration (*clonal evolution*). VH sequencing and FISH were performed as previously described with a probe set allowing the detection of the following trisomies (+) and deletions (del) and translocations (t): +(3q26), del(6q21), +(8q24), del(11q23), +(12q21), del(13q14), t(14q32), and del(17p13).[5,6] All statistical computations were performed using the R software environment, version 2.4.1.[25] Pairwise comparisons of categorical variables were made by Fisher's exact test. The Mann-Whitney test was used to analyze quantitative variables. The distributions of survival times and times to clonal evolution were estimated using the method of Kaplan-Meier.[23] Comparisons of observation times were made by log-rank statistics. A penalized Andersen- Gill regression model was used to identify prognostic factors for overall survival.[24] Regularization was needed to account for the dependence of VH mutation status and clonal evolution. Age, Binet stage, cytogenetic risk status as well as VH mutation status at time of first genetic investigation and the detection of clonal evolution as time-dependent covariate were included in the model as possible prognostic factors. The genomic aberration risk status was defined as *low risk* for single del(13q14) (n=23) and *high risk* for occurrence of del(11q23) or del(17p13) (n=15). All other cases were defined as *intermediate risk* (n=26).

Results and Discussion

At initial analysis, 59 out of the 64 cases (92%) had genomic aberrations: 36 (56%) had 1, 19 (30%) had 2 and 4 (6%) had 3 aberrations. The most common abnormalities were del(13q14) (20% biallelic, 36% as single abnormality), del(11q23), +(12q21), del(6q21), +(3q26), +(8q24), and del(17p13) (Table 1). After a median observation time of 42 months (range 23-73), 11 out of the 64 cases (17%) showed newly acquired aberrations (i.e. *clonal evolution*) during the disease course (*Figure 1A, online supplement*). The limited overall case number of the current study allows only an estimate of the rate of clonal evolution in CLL in general which is also influenced by the observation time. Four patients acquired a del(17p13), 3 a del(6q21), 2 a del(11q23), and 1 a +(8q24). Three patients initially had a monoallelic del(13q14) but in the follow-up samples a biallelic del(13q14), i.e. they acquired a new del(13q14) in the second chromosome 13. Two of them already had other aberrations at initial analysis (Figure 1a, No. 63 and 64) and 1 (Figure 1a, No. 62) also acquired del(17p13) and del(6q21). Therefore, no patient acquired the favorable del(13q14) as single aberration during clonal evolution. Ten patients acquired 1 new aberration while a single patient (Figure 1a, No. 62) acquired 3 aberrations (del(6q21), del(17p13) and biallelic del(13q14)). Therefore, overall 13 new aberrations were observed in the 11 patients with clonal evolution. The acquisition of biallelic del(13q14)), i.e. loss of the second allele, during the evolution of CLL after initially monoallelic del(13q14), is compatible with combined genetic and epigenetic pathomechanisms in 13q14 as recently reported.[26]

Remarkably, clonal evolution was only observed in cases with unmutated VH (Figure 1B, online supplement). Due to the limited case number studied, it cannot be excluded that clonal evolution may also occur in CLL with mutated VH genes, but this seems to be less likely compared with unmutated VH. This observation is in line with a recently proposed model of CLL pathogenesis in which repetitive interaction with antigen promotes clonal growth of VH unmutated CLL cells allowing the acquisition of additional genetic changes and transformation to a more aggressive phenotype.[2] Most but not all of the patients with clonal evolution had received chemotherapy. This suggests a contributory immunodeficiency effect of chemotherapy. In 53 out of the 64 patients, there was no acquisition of new aberrations (i.e. no clonal evolution). In 10 of these 53 cases, significant (>15%) changes were observed in the proportion of cells with specific aberrations. In 4 of these 10 cases, all with del(13q14), the size of the clone grew over time. By contrast, there were 6 cases in whom the clone with the initial aberration decreased or completely disappeared. The individual aberrations of these cases were: +(12q21) (n=4), +(3q27) (n=2), del(11q23) (n=1), and del(13q14) (n=1). One case each with +(12q21) had coexistence of +(3q27) or del(13q14) respectively. All of these patients had received therapy before follow-up analysis

Table 2. Patients' follow-up.

	All cases (n=64)	*With evolution (n=11)*	*Without evolution (n=53) VH mutated and unmutated cases (n=53)*	*Without evolution VH unmutated cases (n=24)*
Treatment; n (%)	50/63 (79)	11 (100)	39/52 (75)	21 (88)
Lines of therapy; median (range)	2 (0-10)	4 (1-9)	2 (0-10)	3 (0-10)
New treatment of previously untreated patients; n (%)	42/63 (67)	10 (91)	32/52 (62)	18 (75)
Events:				
Progression in stage; n (%	21/54 (39)	9/11 (82)	12/43 (28)	7/21 (33)
Deaths; n (%)	22 (34)	7 (64)	15 (28)	9 (38)
Survival in months: median (95% confidence interval)				
since initial investigation	69.2 (68.4 - Inf)	68.4 (53.2 - Inf)	76.2 (69.1 - Inf)	69.2 (48.0 - Inf)
since clonal evolution	–	21.7 (5.9 - Inf)	–	–

and had a normal WBC count with a low proportion (20-38%) of lymphocytes indicative of remission. Interestingly, 4 out of 5 patients in whom the initial aberration was undetectable after therapy initially had a trisomy 12, indicating this clone is paticulary sensitive to treatment. Among the 11 cases with clonal evolution, the type and distribution of the aberrations are shown in Figure 1a. In 9 of the 11 cases (82%), a median number of 3 lines of therapy (range 1-5) had been administered before clonal evolution was detectable, while one patient each acquired a del(17p13) and a biallelic del(13q14) after initially monoallelic del(13q14) without prior therapy. These data indicate that a CLL clone with del(17p13) may be acquired with or without prior therapy. In all 3 patients with acquisition of del(17p13), for whom several samples were available over time, the proportion of cells with a del(17p13) grew from the time point of their first identification (37%, 26% and 46%) until the last follow-up (76%, 64% and 73%) after 2 to 14 months (*Figure 2, online supplement*). All of these patients received chemotherapy with various regimens (chlorambucil (n=4), fludarabine (n=3), FC (n=2), rituximab (n=1)) during this period. This observation is in line with treatment failure associated with del(17p13) in 7 CLL,[18-20, 27-29] and provides direct evidence for *in vivo* resistance of the clone with del(17p13) towards alkylators and purine analogs. Alemtuzumab was not used in these patients. A baseline clinical characteristics of the patients with and without clonal evolution were not significantly different regarding age, gender, stage, and prior therapy (Table 1). The comparison of the groups with and without clonal evolution at follow-up is given in Table 2. In the group of patients without clonal evolution, a progression in Binet stage was observed in 12/43 (28%) cases, treatment with a median of 2 lines of therapy was administered in 39/52 (75%) patients, and death occurred in 15 (28%) cases (10 due to disease progression, and one each due to infection, treatment, second malignancy, and unknown cause). In the group with clonal evolution, a progression in Binet stage was observed in 9/11 cases (82%) treatment with a median of 4 lines of therapy was administered in all 11 patients (100%) and death occurred in 7 cases (64%) (6 due to CLL, and 1 due to second malignancy). A comparison of the group with evolution and the subgroup of patients with unmutated VH among the cases without evolution is given in Table 2. The estimated median overall survival time from the date of the first genetic investigation was 68.4 months in the group with and 76.2 months in the group without clonal evolution. The median time to clonal evolution was 42.3 months (range 23.2-73) and the estimated median survival time after occurrence of clonal evolution was 21.7 months (95% confidence interval 5.9 - Inf). These observations point to a similarity between the two groups at baseline but poor outcome once clonal evolution has occurred. To further evaluate this, multivariable analysis of overall survival was performed using a penalized Andersen-Gill model including the variables of age, Binet stage, genomic aberration risk status, VH status as baseline covariates and detection of clonal evolution as time-dependent covariate. Only the occurrence of clonal evolution and high risk genomic aberrations at presentation (del(11q23), del(17p13)) versus low risk aberrations (del(13q14) single) were identified as significant prognostic factors: age: HR (1 year increase) 0.99, 95% CI 0.96-1.02, p=0.47; stage: HR (B:A) 1.10, 95% CI 0.67-1.80, p=0.71; HR (C:A) 1.28, 95% CI 0.56-2.89, p=0.56; genomic aberrations at baseline: HR (intermediate:low) 1.18, 95% CI 0.74-1.86, p=0.49; HR (high:low) 1.66, 95% CI 1.01-2.73, p=0.04; VH: HR (unmutated:mutated) 1.31, 95% CI 0.81-2.12, p=0.28; clonal evolution: HR (yes:no) 2.97, 95% CI 1.40-6.27, p=0.004. This indicated clonal evolution as an independent adverse prognostic factor by itself despite occurring only among cases with unmutated VH. The survival times of patients with clonal evolution in this study may be the result of different mechanisms which are inter-related partly such as the presence of unfavorable genomic aberrations, the VH mutation status, karyotypic instability, and treatment sensitivity. The limited case number and retrospective nature of this study mean results must be interpreted

with caution. Clinical trials are needed for a systematic evaluation of clonal evolution in relation to other risk markers before the consequences for patient management can be determined.

Authors' contributions

SSt: designed research, performed research, contributed vital analytical tools, collected data, analyzed data, drafting and final approval of manuscript; SSa: performed research, collected data, analyzed data, drafting approval of manuscript; LB: performed research, collected data, analyzed data, drafting and final of manuscript; AB: performed research, collected data, analyzed data, drafting and final manuscript; EL: designed research, performed research, collected data, analyzed data, and final approval of manuscript; DW: performed research, collected data, drafting and final approval of manuscript; AK: performed research, collected data, analyzed data, drafting approval of manuscript; DK: performed research, collected data, analyzed data, drafting and final manuscript; PL: designed research, contributed analytical tools, drafting and final manuscript: HD: designed research, performed research, contributed vital analytical tools, analyzed data, drafting and final approval of manuscrip.

Conflict of interest

The authors reported no potential conflicts of interest.

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