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Clonal evolution in chronic lymphocytic leukemia studied by interphase fluorescence in-situ hybridization

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The results of repeated interphase fluorescence in-situ hybridization (I-FISH, FISH) examination of 97 CLL patients and correlation of these findings with IgVH hypermutation status, ZAP-70 and CD38 expression are presented. The appearance of new, FISH-detectable, genomic aberrations during disease course, described as clonal evolution (CE), was observed in 26% of patients. The most frequent newly acquired cytogenetic abnormality was 13q deletion in 64% (16/25). In contrast to earlier studies, there was no correlation found between CE and either one of single negative prognostic factors (unmutated IgVH; CD38 positivity; ZAP-70 positivity). However, the combination of all three negative factors correlated with CE highly significantly (p=0.005) and moreover, also with a shift from lower to higher FISH risk category (p=0.010). As the prognostic data were known in all patients, this study represents the complete insight on the association of CE and other risk parameters in CLL.

Key words: *CLL; FISH; clonal evolution; prognostic markers; high-risk deletions*

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the Western world and it follows an extremely variable clinical course [1]. As most patients with CLL are diagnosed in early disease stages, the identification of markers having prognostic power has been in the focus of intensive investigation. It is widely accepted that the hypermutation status of the immunoglobulin heavy-chain variable-region (IgVH) genes and specific genomic aberrations have the major impact on long-term survival as well as on treatment effectiveness. While the IgVH status has been reported to remain stable [2], genomic aberrations may be acquired during the disease course. Such clonal evolution (CE), when observed by interphase fluorescence in situ hybridization (I-FISH, FISH), is more common than originally believed [3]. This phenomenon becomes critically important especially when the subsequent FISH analysis reveals one of the high-risk aberrations (17p or 11q deletion) as it adversely affects treatment efficacy [4]. This raised a question whether it is possible to predict – taking into consideration the risk factor pattern – which patient will undergo CE and the risk shift assessed by FISH, respectively

Recent studies on CE detected by FISH in CLL and its relation to other prognostic markers such as CD38, ZAP-70 and IgVH gene mutation status have inconsistent results. Shanafelt et al. [3] reported the increasing occurrence of CE after more than 5 years of observation and concluded that ZAP-70 positive patients may be more likely to experience CE (n=159; CE in 13/31 ZAP-70 positive v 3/29 ZAP-70 negative, p=0.008; statistically relevant association with CD38 positivity or unmutated IgVH gene not found). Stilgenbauer et al. [5] observed CE only in CLL patients with unmutated IgVH genes (n=64, p=0.002; ZAP-70 and CD38 had not been examined) and presented CE as an independent adverse prognostic factor.

Our aim was to determine the association and statistical relevance between CE and the other markers in the series of CLL patients, sequentially examined at our institution.

Patients and methods

Ninety-seven patients with CLL diagnosed according to NCI-WG criteria [6] were enrolled into current study, based

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on two or more sequential samples available with a minimal time period of 5 months following the baseline FISH analysis. The median age at diagnosis was 58 years (range 35-81). The male to female ratio was 1.26 (54 men and 43 women). Median time between diagnosis and first FISH analysis was 1 month (range 0-98 months). The estimated median observation time since the first genetic examination was 66 months (range 22-304 months). The time to CE was defined as the time from the baseline FISH analysis (including initially no aberration found in the extent of examination) to the first follow-up FISH analysis with at least one newly detected genetic abnormality and its median value was 33 months (range 5-87 months). In all samples, CLL infiltration was immunophenotypically confirmed by flow cytometry (median infiltration 64%, range 43-92).

Genomic aberrations with known prognostic relevance were analyzed at various time points during the disease course with a probe set allowing the detection of trisomy 12, del(13)(q14.3), del(11)(q22.3) - ATM gene deletion, and del(17)(p13.1) - deletion of p53 gene (Abbott Laboratories, IL, USA). ZAP-70 and CD38 expression analyses were performed by flow cytometry (FACSCalibur, Becton-Dickinson, La Jolla, CA, USA) and IgVH mutation analyses by sequencing (ABI Prism 310 Genetic Analyzer, Applied Biosystems, CA, USA) as previously described [7, 8]. The estimated cut-off levels were as follows: for I-FISH 5%, for ZAP-70 20% and for CD38 30% of positive cells. For IgVH mutation analyses, cut-off value of 2% (i.e. 98% of homology with germ-line genes) was set to discriminate between the mutated and unmutated genes [8]. All of these analyses were successfully performed in all patients, thus the data were complete for all 97 patients. Statistical considerations were performed using SPSS software version 15. Differences in nominal variables between baseline FISH groups were evaluated using Fisher's exact test. Patients' characteristics is listed in Table 1.

Results and discussion

Sixty three out of 97 patients (65%) showed specific genomic aberrations at baseline FISH analysis. The most frequent aberration was del(13)(q14.3) (46 cases, 47%; in 29 cases, 30% as a sole genomic abnormality). ATM gene deletion – del(11)(q22.3) – was found in 21 (22%), chromosome 12 trisomy in 12 (12%) and p53 gene deletion – del(17)(p13.1) – in 5 patients (5%). Genomic aberrations at study entry are shown in Table 2.

Clonal evolution (CE), defined as newly acquired aberration during the disease course, was observed in twenty-five patients (26%). Most commonly newly detected genomic aberration was del(13)(q14.3) (monoallelic and/or biallelic) in 16 patients. 4 patients acquired del(11)(q22.3) – ATM deletion, 4 patients del(17)(p13.1) – p53 deletion and 1 patient trisomy 12. In patient no.5, subsequent FISH analysis revealed ATM gene deletion and also the loss of the second 13q14.3 allele, whereas patient no.9 newly acquired gene p53 deletion and del(13)(q14.3). Detailed characterization of patients with CE - initial I-FISH finding, the acquired aberration(s), time to CE, therapy before the initial I-FISH (prior therapy - initial), therapy after initial but before follow-up I-FISH (therapy

Table 2. Genomic aberrations at study entry.

Prevalence; n (%)	63 (64.9)
Aberrations per case; median (range)	1 (0-3)
Specific aberrations; n (%)	
del(13)(q14.3)	46 (47.4)
sole del(13)(q14.3)	29 (29.9)
trisomy 12	12 (12.4)
del(11)(q22.3)	21 (21.6)
del(17)(p13.1)	5 (5.2)

Table 1. Cohort definition with regard to CE.

	Total (n=97)	**With CE** (n=25)	**Without CE** (n=72)	**P-value**
Median age at dg in years (range)	58 (35-81)	56 (35-75)	59 (35-81)	
Male; n (%)	54 (55.7)	11 (44.0)	43 (59.7)	
Rai stage at dg; n (%)				
0	45 (46.4)	11 (44.0)	34 (47.2)	
I	30 (30.9)	9 (36.0)	21 (29.2)	
II	15 (15.5)	3 (12.0)	12 (16.7)	
III	3 (3.1)	2 (8.0)	1 (1.4)	
IV	4 (4.1)	0 (0.0)	4 (5.6)	
Prior therapy; n (%)	15 (15.5)	7 (28.0)	8 (11.1)	
Unmutated IgVH; n (%)	53 (54.6)	17 (68.0)	36 (50.0)	**p=0.162**
ZAP-70 positive; n (%)	48 (49.5)	16 (64.0)	32 (44.4)	**p=0.108**
CD38 positive; n (%)	45 (46.4)	15 (60.0)	30 (41.7)	**p=0.162**
Died; n (%)	19 (19.6)	6 (24.0)	13 (18.1)	**p=0.563**
Unmutated IgVH + ZAP-70 positive + CD38 positive; n (%)	30 (30.9)	14 (56.0)	16 (22.2)	**p=0.005**

Table 3. Patients with clonal evolution.

No.	Gender/ age at dg	Initial I-FISH finding	Follow-up I-FISH finding	Time to CE (mths)	IgVH hypermutation status	ZAP-70	CD38	Prior therapy - initial	Therapy during follow-up
1	M/61	N	***del(13)(q14.3)***	18	unmutated	+	+	yes	yes
2	M/62	N	***bial. del(13)(q14.3)***	9	unmutated	+	+	no	no
3	F/65	del(13)(q14.3)	del(13)(q14.3), ***del(11)(q22.3)***	33	unmutated	+	+	no	yes
4	F/54	N	***del(13)(q14.3)***	76	unmutated	-	-	no	no
5	M/52	del(13)(q14.3)	monoal.+***bial***.del(13)(q14.3), ***del(11)(q22.3)***	56	unmutated	+	+	yes	yes
6	F/57	N	***del(13)(q14.3)***	24	unmutated	+	+	no	no
7	M/54	del(13)(q14.3), del(11)(q22.3)	monoal.+***bial.***del(13)(q14.3), del(11)(q22.3)	48	unmutated	+	+	no	yes
8	F/55	trisomy 12	trisomy 12, ***del(17)(p13.1)***	47	unmutated	+	+	yes	yes
9	M/50	N	***del(13)(q14.3), del(17)(p13.1)***	25	unmutated	+	+	no	yes
10	F/70	N	***del(17)(p13.1)***	5	unmutated	+	+	no	yes
11	F/52	N	***del(13)(q14.3)***	29	unmutated	+	+	yes	yes
12	F/63	N	***trisomy 12***	29	unmutated	+	+	yes	yes
13	M/51	del(11)(q22.3)	***del(13)(q14.3),*** del(11)(q22.3)	64	unmutated	+	+	no	yes
14	M/42	trisomy 12	trisomy 12, ***del(13)(q14.3)***	6	unmutated	+	+	no	yes
15	M/58	N	***del(17)(p13.1)***	13	unmutated	-	-	no	no
16	F/39	N	***del(13)(q14.3)***	5	unmutated	+	-	no	no
17	M/49	N	***del(11)(q22.3)***	60	unmutated	+	+	yes	yes
18	M/69	N	***del(13)(q14.3)***	87	mutated	-	-	no	no
19	F/58	N	***monoal.+bial.del(13)(q14.3)***	17	mutated	-	-	no	no
20	M/56	N	***del(13)(q14.3)***	57	mutated	-	-	no	no
21	F/55	N	***del(11)(q22.3)***	52	mutated	-	+	no	no
22	F/75	del(13)(q14.3)	monoal.+***bial***.del(13)(q14.3)	70	mutated	-	-	no	no
23	F/56	N	***monoal.+bial.del(13)(q14.3)***	31	mutated	-	-	yes	yes
24	F/35	N	***del(13)(q14.3)***	84	mutated	+	-	no	no
25	F/58	del(13)(q14.3)	monoal.+***bial.***del(13)(q14.3)	40	mutated	-	-	no	no

N – no aberration detected; CE – clonal evolution; monoal. – monoallelic; bial. – biallelic; italics – newly acquired aberration(s)

during follow-up) and prognostic markers – is summarized in Table 3.

As some of the patients received chemotherapy in various regimens before the baseline FISH testing and also before clonal evolution occurred, we have not statistically evaluated the association of the antileukemic treatment and CE. Nonetheless, it is obvious from Table 3 that new genomic aberrations may be acquired with or without prior therapy.

We have not proved any statistically significant relationship of clonal evolution either with IgVH mutation status, ZAP-70 or CD38 positivity. However, in patients displaying combination of all three negative prognostic factors (unmutated IgVH, ZAP-70 positive and CD38 positive), the statistical association with CE was found (p=0.005; see Table 1). Except one, all patients with CE and mutated IgVH acquired a favourable monoallelic and/or biallelic del(13)(q14.3).

In eight patients, the newly detected aberrations changed their classification from a lower (13q deletion/ normal/ trisomy 12) to a higher (11q or 17p deletion) FISH risk category using the hierarchical Döhner system [9]. Statistical evaluation of the risk features focused on these 8 cases (patient no. 3, 5, 8, 9, 10, 15, 17 and 21) is listed in Table 4, and shows significant relation to CD38 positivity and borderline relation to unmutated IgVH gene (p=0.015 and p=0.051, respectively). Nevertheless, the patients positive for all three risk factors (unmutated IgVH, ZAP-70 positive and CD38 positive) were significantly more likely to acquire high-risk genomic aberration within CE (p=0.010; see Table 4).

Table 4. Patients with shift from lower to higher risk FISH category; n=8.

	Total	With shift	Other finding	P-value
Unmutated IgVH; n (%)	53/97 (54.6)	7/8 (87.5)	1/8 (12.5)	**p=0.051**
ZAP-70 positive; n (%)	48/97 (49.5)	6/8 (75.0)	2/8 (25.0)	**p=0.132**
CD38 positive; n (%)	45/97 (46.4)	7/8 (87.5)	1/8 (12.5)	**p=0.015**
Unmutated IgVH + ZAP-70 positive + CD38 positive; n (%)	30/97 (30.9)	6/8 (75.0)	2/8 (25.0)	**p=0.010**

In accordance with Shanafelt et al.[3], once we classified the time of observation into two categories – less and more than 50 months, the subsequent FISH analyses revealed new genomic aberrations more often in patients followed-up longer than 50 months - on a borderline statistical significance (27/31 vs. 45/66; p=0.051). Worth mentioning, this was also caused by the sample availability (maximum time interval between FISH analyses was 87 months). Short overall survival was associated with unmutated VH gene status (p=0.001) as well as with the presence of high-risk deletions (p=0.001) (data not shown). The Kaplan-Meier curves evaluating overall survival were presented in our previous study [10].

The acquisition of p53 or ATM deletions is not rare and occurrence of these high-risk deletions, revealed at any time point of the disease course, adversely affects the patients' overall survival. Even though this study proved that patients displaying risk prognostic profile unmutated IgVH + ZAP-70 positive + CD38 positive are more likely to undergo clonal evolution, both sensitivity and specificity of this relation is limited as well as CE cohort size. Therefore, we recommend examining patients with CLL sequentially by FISH for the early detection of high-risk deletions and consequently, early decision on risk-adapted treatment.

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