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Clonal evolution in chronic lymphocytic leukemia detected by fluorescence *in situ* hybridization and conventional cytogenetics after stimulation with CpG oligonucleotides and interleukin-2: A prospective analysis

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ABSTRACT

Chronic lymphocytic leukemia (CLL) patients may acquire new chromosome abnormalities during the course of their disease. Clonal evolution (CE) has been detected by conventional chromosome banding (CBA), several groups also confirmed CE with fluorescence *in situ* hybridization (FISH). At present, there are minimal prospective data on CE frequency determined using a combination of both methods. Therefore, the aim of our study was to prospectively assess CE frequency using a combination of FISH and CBA after stimulation with CpG oligonucleotides and interleukin-2.

Between 2008 and 2012, we enrolled 140 patients with previously untreated CLL in a prospective trial evaluating CE using FISH and CBA after stimulation. Patients provided baseline and regular follow-up peripheral blood samples for testing.

There was a median of 3 cytogenetic examinations (using both methods) per patient. CE was detected in 15.7% (22/140) of patients using FISH, in 28.6% (40/140) using CBA, and in 34.3% (48/140) of patients by combining both methods. Poor-prognosis CE (new deletion 17p, new deletion 11q or new complex karyotype) was detected in 15% (21/140) of patients and was significantly associated with previous CLL treatment ($p = 0.013$).

CBA provides more complex information about cytogenetic abnormalities in CLL patients than FISH and confirms that many patients can acquire new abnormalities during the course of their disease in a relatively short time period.

1. Introduction

The clinical course of chronic lymphocytic leukemia (CLL) is remarkably heterogeneous. Approximately half of CLL patients never require treatment; however, there is a group of patients with a very poor prognosis and a median overall survival (OS) of 1–2 years regardless of repeated therapies [1–4]. A significant prognostic factor correlating with the clinical course of CLL and OS is the presence of some chromosomal aberrations [5,6]. At present, fluorescent *in situ* hybridization (FISH) is the standard method used to detect genomic aberrations in CLL [6]; however, this method is not able to detect all chromosomal changes which can also play an important role in CLL pathogenesis. Conventional chromosome banding analysis (CBA) has not been used in CLL as malignant B-lymphocytes did not proliferate *in vitro* [5]. Nevertheless, after introducing the new CLL cell stimulation methodology with CpG oligonucleotides and interleukin-2 (IL-2) [7], detecting chromosomal aberrations using CBA has become possible in up to 80% of CLL cases [8,9]. It is of importance that additional aberrations modifying the CLL patients' prognostic stratification (prognosis defined according to FISH) can be detected using CBA [10]. Chromosomal translocations are found in up to 34% of CLL cases [10]. Although the original concept considered CLL as a genetically stable disease [11], clonal evolution (CE) with the acquisition of new cytogenetic aberrations occurs in 17–26% of patients during the course of the disease

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Table 1
Baseline patients' characteristics and clinical data at the start of the study.

	All patients (n = 140)	Patients with poor prognosis CE (n = 21)	Patients without poor prognosis CE (n = 119)	p-Value (poor prognosis CE *vs.* no poor prognosis CE)
Median age (years)	64 (37–75)	64 (38–75)	63 (37–73)	NS
Male/female ratio	86/54	15/6	71/48	NS
Rai stage				
0	36 (25.7%)	5	31	NS
I	50 (35.7%)	6	44	
II	8 (5.7%)	1	7	
III	19 (13.6%)	4	15	
IV	27 (19.3%)	5	22	
IGHV mutational status (n = 135)				
Mutated	60 (42.9%)	8	52	NS
Unmutated	75 (53.6%)	13	62	
CD38				
Negative	107 (76.4%)	15	92	NS
Positive	33 (23.6%)	6	27	
ZAP-70 (n = 129)				
Negative	60 (42.9%)	6	54	0.157
Positive	69 (49.3%)	13	56	
Therapy before CE				
No	54	3	51	0.013
Yes	86	18	68	

CE, clonal evolution; poor prognosis CE, clonal evolution with new poor prognosis abnormalities detected during the follow-up (new 17p deletion, new 11q deletion, or new complex karyotype); NS, not significant.

[12–18]. CE is associated with shorter OS and a new finding of deletion 17p is important to determine a treatment strategy [19–21]. In the majority of published analyses to date, CE has been monitored using FISH [13–18]. Therefore, the aim of the present study was to prospectively assess CE frequency using the combination of both FISH and CBA after stimulating with CpG oligonucleotides and IL-2 in CLL patients. We compared these methods and evaluated how frequently new adverse cytogenetic findings occurred when correlated with treatment and other prognostic factors (therapy, variable part of the immunoglobulin heavy chain [*IGHV*] gene mutation status, CD38 and ZAP-70 expression).

2. Patients and methods

2.1. Patients

Between 2008 and 2012, we enrolled 140 patients with previously untreated CLL in a prospective trial evaluating CE by FISH and CBA. In cases enrolled more than 1 year after CLL diagnosis, FISH data from previous tests were available. The patients provided baseline and follow-up (every 12 months in untreated patients with the stable disease or earlier, before each therapy, in treated patients) peripheral blood samples for testing. All blood samples were processed with written informed consent in accordance with the Declaration of Helsinki under protocols approved by the local ethical committee.

2.2. FISH

FISH analyses were performed on the interphase nuclei of cultured peripheral blood cells using a panel of DNA probes to detect prognostically significant aberrations: deletion 13q and trisomy 12 (LSI D13S319/LSI 13q34/CEP 12, Vysis, Downers Grove, IL, USA; or XL DLEU/LAMP/12cen and XL DLEU/LAMP/12cen MetaSystems, Altlussheim, Germany), deletion 11q and deletion 17p (LSI p53/LSI ATM, Vysis, Downers Grove, IL, USA; or XL ATM/p53 probe, MetaSystems, Altlussheim, Germany), rearrangement 14q32 (LSI IgH DC Break Apart Probe; Vysis, Downers Grove, IL, USA). Two hundred interphase nuclei were evaluated. The cut-off level for each individual probe was determined based on a negative sample analysis and calculated as the mean +3SD. Chromosomal aberrations were categorized according to Döhner's hierarchical model [6].

2.3. CBA

Peripheral blood (100–1000 μL) was cultivated in complete RPMI 1640 medium with 20% fetal bovine serum and with CpG DSP 30 oligonucleotides (2 μM; TIB Molbiol, Berlin, Germany) and interleukin IL-2 (200 U/mL; PeproTech, Rocky Hill, NJ, USA). Colcemid (0.15 μg/mL; Sigma, St. Louis, MO, USA) was added to the culture for the last 5 or 24 h. A minimum of five metaphases were karyotyped and chromosomal aberrations were reported according to the International System for Human Cytogenetic Nomenclature (ISCN 2009). Complex aberrations were assessed using M-FISH and M-BAND methods (MetaSystems, Altlussheim, Germany).

2.4. Mutation status of IGHV

The *IGHV* gene's mutation status was examined using polymerase chain reaction (PCR) followed by PCR amplicon sequencing. The sequences obtained were analyzed using an IMGT/V-QUEST tool and database. The unmutated *IGHV* gene was defined using sequence identity to germ-line sequence ≥98%; the mutated *IGHV* gene was defined by identity <98%.

2.5. Flow cytometry

Flow cytometry was used to analyze CD38 (cut-off expression 30%) and ZAP-70 (cut-off expression 20%). Flow cytometric data acquisition was performed on a FACSCantoII flow cytometer (Becton Dickinson, NJ, USA) after previously eliminating red cells using FACS Lysing Solution (Becton Dickinson, NJ, USA).

2.6. Statistical analysis

Pearson's Chi-square test was used to test the correlation between CE and selected risk factors. p-Values of <0.05 were considered significant.

3. Results

From 140 enrolled patients, 76 had newly diagnosed CLL; the remaining 64 patients were diagnosed more than 1 year before the start of our study (median 19 [range 12–204] months). All patients were previously untreated. At baseline, 50% of patients had a stable disease without therapy and 50% of patients exhibited a progressive disease requiring treatment. During the study follow-up, another 16 patients developed disease progression. The median age of patients was 64 (range 37–75) years at baseline. The cohort included 86 men and 54 women. The median number of cytogenetic examinations (using both methods) was 3 (range 2–5) per patient. Baseline patients' characteristics are summarized in Table 1.

3.1. Cytogenetics

At baseline, chromosomal aberrations were detected using FISH in 72.6% (102/140) of patients. The most frequent aberration was deletion 13q in 39.3% (55/140) of patients. Deletion 11q was detected in 17.1% (24/140), trisomy 12 in 8.6% (12/140) and deletion 17p in 7.9% (11/140) of patients.