GENOMIC PROFILING BY MULTIPLEX LIGATION - DEPENDENT PROBE AMPLIFICATION IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Abstract: The clinical management of severe pathological conditions, such as B-cell chronic lymphocytic leukemia (B-CLL), is subject to continuous optimization and re-evaluation. Patients may fully benefit from rapid, standardized laboratory tools designed to facilitate their early stratification according to disease risk, stage and prognosis. Such technologies may also aid the clinician in selecting the therapeutic option with the greatest chances of success. The presence of specific genetic abnormalities are frequently associated with the clinical outcome of oncologic patients in general, and B-CLL patients in particular. In the current study, a group of 58 B-CLL patients were evaluated for the detection of gene copy number alterations (deletions or duplication/ amplifications) within 45 distinct genetic targets, by means of a novel molecular methodology, Multiplex Ligation - Dependent Probe Amplification (MLPA). Simple or complex genetic defects were identified in 67% of cases, and the most common aberrations observed were: deletion of the short arm of chromosome 13 in 33% of cases, deletion of the long arm of chromosome 11 in 16% of cases, trisomy 12 in 16% of cases, and deletion of the short arm of chromosome 17 in 7% of cases. The main conclusion of the study presented here points towards MLPA as a potential key step of clinical management protocols in B-CLL, providing that it will be fully standardised for routine diagnosis.

INTRODUCTION

Unlike other hematologic malignancies, B cell chronic lymphocytic leukemia (B-CLL) is a very heterogeneous disease, in terms of genetic lesions detected [Chiorazzi et al, 2005; Dighiero and Hamblin, 2008]. A hierarchical association has been established between the presence of specific genetic abnormalities and the clinical outcome of B-CLL patients. The conclusion of the majority of studies in this area of research was that failure to maintain the genomic integrity associates with a particular disease status. In this respect, the deletion of the short arm (p) of chromosome 17 (del(17p)) and deletion of the long arm (q) of chromosome 11 (del(11q)) are associated with reduced survival, trisomy 12 (+12) and normal karyotype are associated with an intermediate prognosis, while del(q13), when detected as a single aberration, is associated with a favorable disease progression [Reddy, 2006; Seiler et al, 2006]. In recent years a number of studies have identified additional chromosomal phenotypes showing a high genomic complexity, including recurrent translocations (mostly unbalanced), complex aberrant karyotypes and single nucleotide polymorphisms correlated with the disease progression [Kujawski et al, 2008; Van Den Neste et al, 2007; Mayr et al, 2006; Grubor et al, 2009]. The technical accuracy in detection of chromosome deletions, duplications and gene amplifications has been proved of help for patient stratification in various other types of cancers and degenerative diseases [Buijs et al, 2006; Jeuken et al, 2006; Scarciolla et al, 2006]. The aim of the current study was to identify chromosome copy number variations (deletions or duplications) in a group of B-CLL patients, by Multiplex Ligation - Dependent Probe Amplification (MLPA), a rapid method that allows simultaneous processing of multiple samples, as it becomes increasingly necessary to early identify those B-CLL cases with a high-risk disease and hence poor prognosis, in order to allow for a thorough clinical management.

MATERIALS AND METHODS

Patient group. Fifty eight patients with B-CLL were included in the study group. Patients were hospitalized in the Hematology Clinic of Regional Institute of Oncology, Iasi, Romania and assigned, according to the Rai staging system, to Rai 0 (the least advanced), Rai 1/2, and Rai 3/4 (the most aggressive) clinical stages. The study was approved by the local Ethical Committee.

DNA extraction. After density gradient separation of peripheral blood cells, DNA was extracted from mononuclear cells. Promega Wizard Genomic DNA Purification kit was used to extract DNA from $5x10^6$ mononuclear cells. For each DNA sample the quantification was performedvaccording to principles described in detail elsewhere

[Mihasan et al., 2012], using aNanoDrop2000 spectrophotometer (ThermoScientific). Samples were diluted to 40ng/µL DNA concentration and stored at freezer until MLPA analysis was performed .

MLPA. MLPA technique is based on polymerase chain reaction, used for the simultaneous quantification of up to 45 different genomic targets in a single experiment, by means of amplification of specifically hybridized probes. Five µL of stock DNA were used for each MLPA reaction. For each sample run at least one control DNA from normal subjects (non-B-CLL) was tested in parallel. MLPA was performed using the commercial kits CLL MRC Holland SALSA MLPA P037 and P038, as previously described [Fabris et al, 2011]. Hybridization, ligation and PCR amplification of samples were performed using a thermocycler Labcycler SensoQuest (Biomedical Electronics) and for the electrophoretic migration CEQ 8000 (Beckman Coulter) was used. MLPA data interpretation was performed using the Coffalyser.Net software, version 2013.

RESULTS AND DISCUSSIONS

Nineteen (33%) out of the 58 patients investigated did not show any genetic defect based on the screening performed using the P037 and P038 MLPA kits (as exemplified in Figure 1A). A total of 49 patients (67%) were identified with simple or complex genetic aberrations (Table 1). Of these, the most common aberrations identified were: del(13q) in 33% of cases, del(11q) in 16%, +12 in 16% (as exemplified in Figure 1B), and del(17p) in 7% of cases. Our results are in line with previous studies [Döhner et al, 2000; Šindelářová et al, 2005; Durak et al, 2009; Bullinger et al, 2001; Dewald et al, 2003; Glassman and Hayes, 2005; Giertlova et al, 2011] performed on larger groups of patients (79-325 patients), also reporting an increased frequency of del(13q), a similar incidence of del(11q) and +12, and a reduced frequency of del(17p).

The similar results obtained in our study may be explained by the homogeneous distribution of disease stages within our group of patients (Table 2). Our study included patients with B-CLL in various Rai disease stages. Nine out of all patients investigated were in Rai0 disease stage, 33 in Rai1/2 stage and 16 in Rai3/4. Patients identified with a genetically normal background were distributed on clinical stages as follows: four patients in Rai0 stage, 12 in Rai1/2, and 3 in Rai3/4 (Table 2). All five patients in Rai0 bearing genetic anomalies were identified having simple changes, while only six out of those 13 patients in Rai3/4 identified had simple genetic lesions, the other seven bearing complex aberrations. Twelve patients in Rai1/2 had simple molecular aberrations, and 9 patients had complex and heterogeneous aberrations (Figure 1C). Overall, a progressive accumulation of genetic aberrations was observed within the group of B-CLL patients evaluated.

13q14 deletion, involving genes located on the long arm of chromosome 13, is the most common genetic abnormality, reported to be detected in approximately 50% B-CLL patients [Peterson et al, 1992; Bullrich et al, 1996; Wolf et al, 2001]. Although, when present as simple alteration, it is generally associated with a favorable clinical course, patients with an aggressive disease may also share the defect. This is probably due to the different points of rupture, affecting distinct genes located on the region 14 of chromosome 13, leading to an increased instability of the genome [Cimmino et al, 2005; Calin et al, 2002; Ouillette et al, 2008; Palamarchuk et al, 2010; Hammarsund et al, 2004]. Ouillette [Ouillette et al, 2008] proposed the classification of chromosome 13q14 deletions in two distinct categories: type I, with no defect of RB1 gene and type II, in which RB1 gene has been deleted. RB1 gene is a critical regulator of cell cycle progression (by blocking transcription and by cell cycle arrest) and genomic stability (by stabilizing histone methylation).

Table 1. The incidence of genetic defects in patients with B-CLL compared with existing data in the literature

Group of patients (N)	del(13q)	del(11q)	+12	del(17p)	No alterations detected	All alterations detected	Study
58	33%	16%	16%	7%	33%	67%	Present study
325	55%	18%	16%	7%	18%	82%	Dohner et al, 2000
217	54%	12%	16%	16%	30%	70%	Sindelarova et al, 2005
79	33%	5%	15%	8%	49%	51%	Durak et al 2009
258	62%	11%	10%	6%	22%	78%	Bullinger et al, 2001
139	51%	25%	12%	3%	19%	81%	Stilgenbauer et al, 1996
113	64%	15%	25%	8%	23%	77%	Dewald et al, 2003
100	40%	23%	11%	12%	36%	64%	Glassman and Hayes, 2005
86	31%	17%	19%	6%	55%	45%	Giertlova et al, 2011

Table 2 The frequency of identified genetic abnormalities in B-CLL patients according Rai clinical staging

Genetic defect	**Rai clinical stage**
	Rai0 (N=9)
Normal genotype	4
+12	3
del(13q) 14 RB1+	1
del(13q) 14 RB1-	1
	Rai1/2 (N=33)
Normal genotype	12
+12	4
del(13q) 14 RB1+	2
del(13q) 14 RB1-	4
del(13q) 14 RB1+ and SF3B1 mutated	1
del(13q) 14 RB1+ and NOTCH1 mutated	2
SF3B1 mutated	2
del(6q)21-q26	1
del(11q)	1
+12 and del(13q)14RB1+	1
del(11q) and del(13q) 14 RB1+	1
del(11q) and del(13q) 14 RB1-	1
del(13q) 14 RB1- and +8q24 and SF3B1 mutated	1
	Rai3/4 (N=16)
Normal genotype	3
del(13q) 14 RB1-	2
del(11q)	3
del(17p)	1
del(11q) and +8q24	1
del(13q) 14 RB1- and del(17p)	1
del(11q) and del(17p) and + 8q24 and SF3B1 mutated	1
del(13q) 14 RB1+ and +2p24	1
del(11q) and del(13q) 14 RB1-	1
+12 and del(17p)	1
del(13q) 14RB1+ and +8q24 and NOTCH1 mutated	1

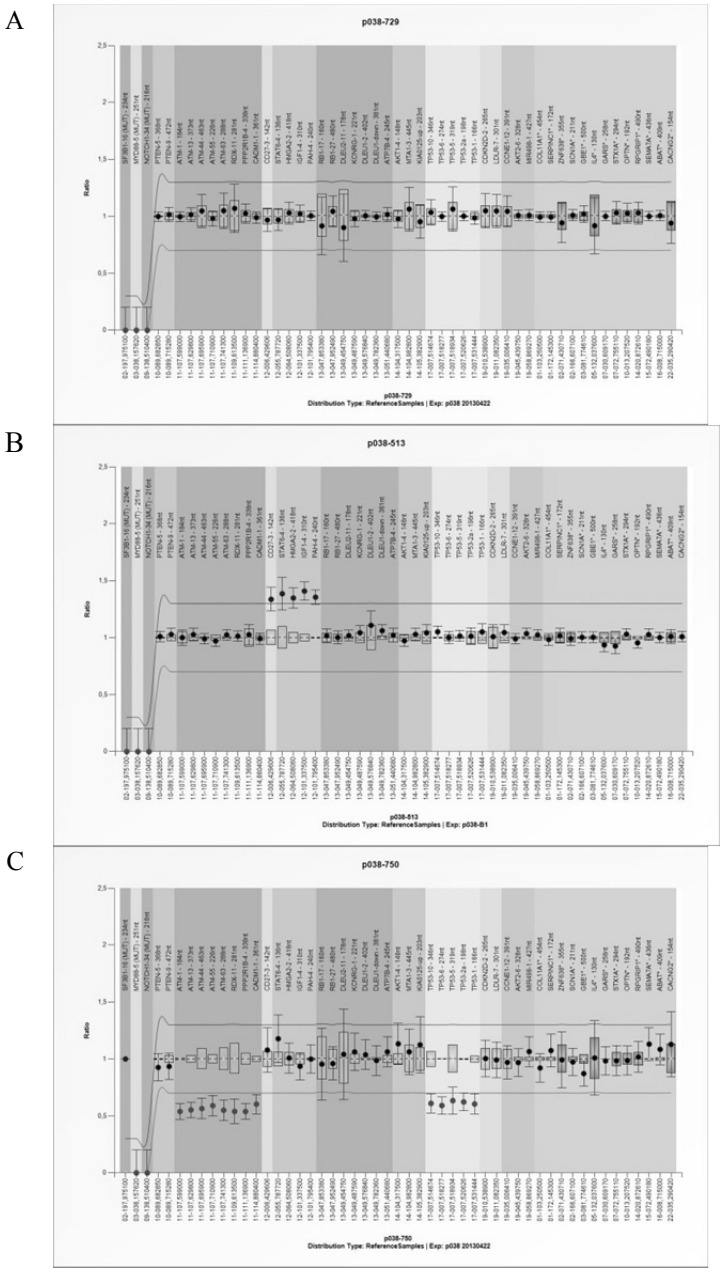

Figure 1. Examples of three distinct genetic profiles within the B-CLL patient group evaluated. A. Patient (patient code 729, Rai0 clinical stage) with no identifiable genetic aberrations based on the P038 MLPA kit. B. Patient (code 513, Rai1/2 clinical stage) with +12. C. Patient (code 750, Rai3/4 clinical stage) with multiple aberrations: del(11q) and del(17p) and + 8q24 and SF3B1 mutated.

As a result, loss of one or both RB1 alleles may differentially affect the progression of B cell chronic lymphocytic leukemia [Pickering and Kowalik, 2005; Hernando et al, 2004]. Molecular regions within the 13q14 targeted in our study were: RB1, FNDC3A, DLEU2, KCG, MIR15A, DLEU1, DLEU7 and ATP7B. In our study group, 20 patients (34.5% of the total) were identified as having deletions within 13q14. Thirteen patients had del(13q14) as a unique genomic aberration (65% of all patients presenting del(13q14)), while seven patients had del(13q14) accompanied by other changes in gene copy number, classified as complex mutations (35% of all patients with del(13q14)). Among patients presenting del(13q14) as single aberration, there were patients also presenting NOTCH1 mutations (two patients) or SF3B1 (one patient), and another patient had deletions within both 13q14 regions. Type I and type II del(13q14) classified according to the involvement of RB1 gene were found in equal proportions in the group investigated in this study (10 patients in each category).

Trisomy of chromosome 12 is the third most frequent chromosomal aberration in B-CLL patients and frequently appears as a single abnormality. According to the genetic subclassification used in clinical practice, +12, when present as a unique aberration, is associated with an intermediate prognosis, with a median time to progression of 33 months and a median survival of 114 months [Döhner et al, 2000]. In our group of 58 B-CLL patients, nine patients (15.5%) had +12. As a unique anomaly, +12 was found in seven patients, while two patients presented this defect accompanied by other anomalies. When the Rai staging system was assessed, three patients with +12 (as a unique anomaly) were in Rai0, five patients were in Rai1/2 (one with multiple genetic defects) and only one patient in Rai3/4 stage, concomitantly presenting del(17p).

Deletion 11q22 - 23q occurs in approximately 20% of B-CLL cases [Döhner et al, 2000] and represents the second most frequent genetic defect associated with B-CLL. This genetic abnormality defines a subgroup of patients with progressive disease and poor prognosis [Fegan et al, 1995]. In fact, leukemic cells present a high survival rate, possibly through inhibition of apoptosis or alteration of genes involved in cell cycle control [Korz et al, 2002].

ATM gene (ataxia - telangiectasia mutated) is included within the minimum deleted region of chromosome 11q22-23 in B-CLL patients [Stilgenbauer et al, 1996; Schaffner et al, 1999]. ATM is known to regulate the genomic integrity by controlling the cell cycle, preventing DNA damage, activating DNA repair, and triggering apoptosis when the DNA repair fails [Kastan and Lim, 2000]. The group of B-CLL patients enrolled in the current study showed a frequency of 15.5% for del(11q) (nine out of 58 patients). Four cases have been identified presenting del(11q) as a simple aberration. One may note that none of the patients in Rai0 stage enrolled in the study presented del(11q). Within Rai1/2 stage group, three patients carrying the aberration were identified (one case as a simple aberration and two cases associated with other abnormalities). Del(11q) positive B-CLL patients in Rai3/4 stage were identified in six cases, three as a single defect and three with other abnormalities.

NOTCH1 gene mutations have been described as being associated with a poor prognosis, present in about 10% of B-CLL cases, and having a prevalence proportionally increased with disease progression [Fabbri et al, 2011; Puente et al, 2011; Rossi et al, 2012]. NOTCH1 gene is of major importance in the control of hematopoiesis [Milner and Bigas, 1999]. In our group, out of 58 patients, only 3 (5.17%) had mutations of NOTCH1. In two cases the mutation was accompanied by del(13q14), both patients being assigned to Rai1/2 clinical stage. In the third case, in stage Rai3/4, the mutation was accompanied by del(13q14) and +8q24.

Mutations of the gene coding for the splicing factor 3b, subunit 1 (**SF3B1**), are not very well understood in terms of their functional consequences [Cazzola et al, 2013; Wahl et al, 2009]. To our knowledge, splicing defects have not been reported to be involved in the pathogenesis of B-CLL. A study by Wang et al in 2011 described that 14 of 91 B-CLL patients presented mutations in the SF3B1, 7 of mutations being at the K700. K700 mutation within the transcription factor SF3B1 was recorded in our group in 5 cases (8.6%). A number of 2 patients in stage Rai1/2 presented mutated SF3B1 as a simple defect, without other changes in the number of gene copies studied. Mutated SF3B1 was also detected within a complex genetic background (accompanied by del(13q), +8q24, del(11q), and del(17p) (see Table 2)) in three B-CLL patients, two in Rai1/2 and one in Rai3/4.

Deletion of 17p. Deletion of the TP53 tumor suppressor gene, located in the 17p13 region, has been associated with drug resistance and represents an independent prognostic factor in B-CLL [Döhner et al, 2000]. Poor response to treatment with fludarabine or alkylating agents in these patients is attributed to the inactivation by deletion / mutation in the TP53, a key element required for DNA repair mechanisms [Wang et al, 2011]. Within the group of B-CLL patients studied here, four patients (7%) had a deletion of the short arm of chromosome 17, including the TP53 gene. All patients were classified in Rai3/4 clinical stage and only one patient presented del(17p) as a unique anomaly.

Deletion of q6 (21, 23, 25, 26) is rarely reported in B-CLL patients, while its correlation with prognosis or response to treatment is still being evaluated [Laurenti et al, 2011; Philipp et al, 2011]. A number of genes involved in various signaling pathways (such as the constitutive activation of the transcription factor NF-kB) have been located within the long arm of chromosome 6. In our study group only one B-CLL patient presented deletion in the long arm of chromosome 6, within the region 6q25-26, including three genes: ESR1, IGF2R and PARK2. All these genes are involved in growth, differentiation and functioning of the reproductive system, the skeletal growth, normal functioning of the cardiovascular and nervous systems, transport within the Golgi apparatus, and targeting of protein substrates for degradation in the proteasome [Philipp et al, 2011].

CONCLUSIONS

The majority (67%) of patients within the B-CLL group enrolled in the current study were identified as bearing simple or complex genetic aberrations. 2. The distribution of the most common genetic defects (del(13q), del(11q), +12, del(17p)) found in the current study among B-CLL patients is similar to that previously reported in the literature. 3. MLPA, although not yet fully standardised for routine diagnosis, may become soon a reliable tool within the optimized clinical management protocols, as it is suited for a rapid, early identification of those B-CLL cases with high-risk and poor prognosis.

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Abreviations:

+12	trisomy 12
+8q24	duplication of the long arm of chromosome 8, region 24
B-CLL	B cell chronic lymphocytic leukemia
del(11q))	deletion of the long arm (q) of chromosome 11
del(13q)	deletion of the short arm (p) of chromosome 17
del(17p)	deletion of the short arm (p) of chromosome 17
MLPA	Multiplex Ligation - Dependent Probe Amplification