RESEARCH ARTICLE

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Cytogenetic Subclone Burden: A New Biomarker Predicting Chronic Lymphocytic Leukemia Patients Outcome

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Abstract

Background: Chronic lymphocytic leukemia is the most prevalent adult leukemia that occurs in older patients and presents a variable course of the disease. Risk stratification of CLL is a matter of continuous improvement. Thus, this study aimed to assess the impact of the quantification of 17p del and 11q del cytogenetic subclones on the outcome of patients with chronic lymphocytic leukemia. **Patients and Methods:** This is a prospective study that involved 100 subjects with CLL. For all included patients; assessment of the cytogenetic subclones burden for 17p del and 11q del using the FISH technique was carried out. **Results:** CLL patients with a high 17p del (>33%) cytogenetic subclone burden showed significantly shorter lymphocyte doubling time (LDT), time to first treatment (TTFT), and progression free survival (PFS) compared to those with a lower burden. On contrary 11q del subclone(>30%) burden had an insignificant impact on LDT, TTFT and PFS. **Conclusion:** Quantification of 17pdel burden (>vs.≤33%) could be used for refining risk stratification of CLL patients.

Keywords: CLL- 17pdel- 11q del- Cytogenetic

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Introduction

Chronic lymphoproliferative disorders of B cells (BCLPD) are characterized by the accumulation of mature B lymphocytes in peripheral blood and bone marrow with progressive lymphadenopathy and splenomegaly. These disorders have been categorized according to their genetic changes, histopathological features and immunophenotype [1]. The most prevalent type of leukemia in adults among B-CLPD is chronic lymphocytic leukemia (CLL), with an estimated incidence of 4 to 6 occurrences per 100,000 persons each year in western countries [2, 3]. CLL is diagnosed by the presence of more than 5,000 monoclonal B cells per microliter with specific immune phenotypes [4]. Matutes Score was designed for CLL diagnosis [5]. It depends on the expression pattern of CD5, CD23, FMC7, SmIg, and CD22. Most CLL cases had a score of more than 3, whereas non-CLL cases had a score of less 3. Subsequently, it was demonstrated that CD79b may effectively substitute CD22 [6]. In 40–50% CLL cases, chromosomal abnormalities have been documented. The most frequent chromosomal changes are entire chromosome additions, like trisomy 12, and incomplete deletion of one chromosome as 6q, 11q, 17p and 13q. Certain cytogenetic abnormalities, including deletions of 11q or 17p, have poor prognosis [7-10] Many trials have been done to search for new biomarker for improvement risk stratification of CLL patients outcome [11-13]. In this context, Recent reports indicated that the degree of positivity of cytogenetic findings by FISH could have different impact on CLL patients outcome [14]. The purpose of this study was to assess the impact of quantification of 11qdel and 17pdel cytogenetic subclones burden on CLL patients outcome.

Materials and Methods

Patients

The present study is a prospective study conducted on 100 patients with CLL (61 men, 39 women); their mean age is 60.5 years, recruited from the inpatient and outpatient clinics of the Mansoura University Oncology Center for a two-year period from December 2019 to December 2021. This study was approved by the institutional review board of the Mansoura Faculty of Medicine and done according to declaration of Helsenki. All included patients gave their informed consent to participate in the current study. The sample size was calculated using an online sample size calculator (https://www.openepi.com/SampleSize/SSMean.htm) with anticipated prevalence of chronic

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lymphocytic leukemia and level of absolute precision of 2% with alpha error of 5% and study power of 90%. All patient data including age, complete hemogram, and course of the disease were collected from the patient's medical record. The diagnosis was based on the morphological examination of peripheral blood and bone marrow smears and was confirmed by immunophenotyping using panel of monoclonal antibodies for CLL (CD5, CD19, CD23, SIg, FMC7, CD79b, Kappa & lambda light chains, CD38, CD43).

Conventional prognostic parameters were identified at diagnosis for all included CLL patients: namely smudge cells count, Lymphocyte doubling time, CD38 expression, RAI staging. Cytogenetic studies were done using interphase FISH technique for detection of 17p del and 11q del.

Methods

Samples

Bone marrow samples were collected at diagnosis by sterile procedure and distributed as follows. 1. Two milliliters were added to dipotassium ethylene-diamine-tetraacetate (EDTA) (1.2 mg of anhydrous salt per milliliter of blood) for performing a flow cytometric assay to detect antigens expression by lymphocytes 2. Two milliliters were delivered in heparinized tube for FISH (fluorescence in situ hybridization) studies.

Cytogenetic detection by FISH technique

The FISH technique includes 4 steps

Culture, Hybridization. Microscopy and Interpretation. The interphase FISH technique was performed on heparinized samples after optimization of the protocol using commercially available probe from Cytocell UK as follows: Heparinized samples were inoculated in the culture medium and kept overnight incubation, then harvesting was done to obtain a clear pellet. Slides were prepared from harvested specimens and viewed under a phase contrast microscope. Then, the following fluorescent-labeled probes were added: LSI p53 (17p13), LSI ATM l(11q22.3), and LSI D13S319 (13q14.3) (all probes were from Cytocell, UK). The slides were added to the Lecia ThermoBrite instrument (Thermobrite Leica, Technoscient, Germany) to allow the denaturation of the sample and probe simultaneously, and then hybridization and posthybridizatin washes were applied. Finally, the slides were counterstained by 10µl of DAPI and examined with the aid of an Olympus BX 61 fluorescent microscope (Optoscient company, Japan) that is fitted with filters suited for DAPI, rhodamine, and fluorescein. Using Cytovision Image Capture software and a monochrome digital camera, representative pictures were taken (Applied Imaging).

Interpretation of results

The signal screening was evaluated in 200 cells. The cut-off point for positivity for single deletion was 10% for del(17p), del(11q). In a normal cell, this probe will appear as discrete red and green spots, one for each homologue (2G, 2R). In cells with a single deletion, one red signal with two green signals (2G, 1R) will appear. The percentage of positivity was evaluated in positive cases for subclone classification (Figure 1, 2).

Statistical analysis

The information was examined, coded, and tabulated with the help of IBM's Statistical Software for the Social Sciences (2017 release) (version 25.0 of IBM SPSS Statistics for Windows, IBM Corp, Armonk, New York). Each parameter's data was collected, and analysis was performed as needed. To check if the data were normally distributed, the Kolmogorov-Smirnov test was carried out. We utilised the mean and standard deviation for parametric data. The median and range for non-parametric numerical data were adopted. We relied on frequency and percentage to represent non-numerical parameters. The means of the 2 groups were compared using the Student T test. The Mann–Whitney U test was used to determine whether or not there was a significant difference in the means of 2 groups when testing a nonparametric variable (U test). The chi-square test was used to analyse the link among two qualitative variables. When the projected count was less than 5 in more than twenty percent of the cells, we used Fisher's exact test to look for a link among the two qualitative variables. To evaluate the sensitivity and specificity of quantitative diagnostic tests that

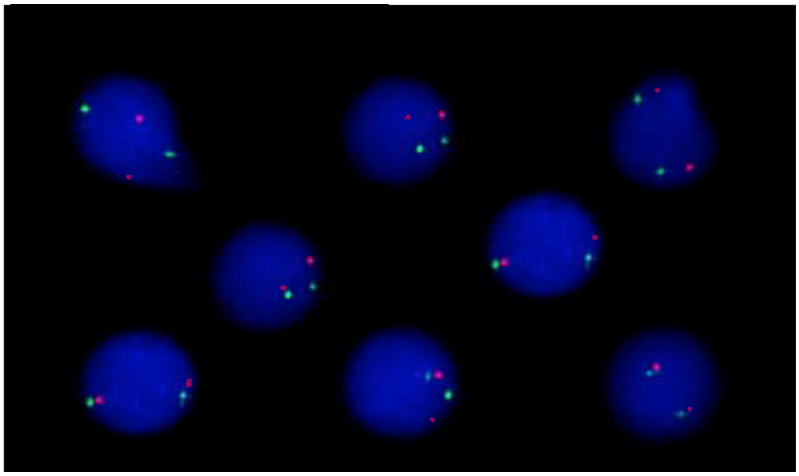

Figure 1(a). Representative CLL FISH Image of Case with Negative 17p del Shows Two Red Dots Represent LSI p53(17p13) Probe and Two Green Dots Represent D17Z1(CEP17) Probe for Control, One for each Homologue.

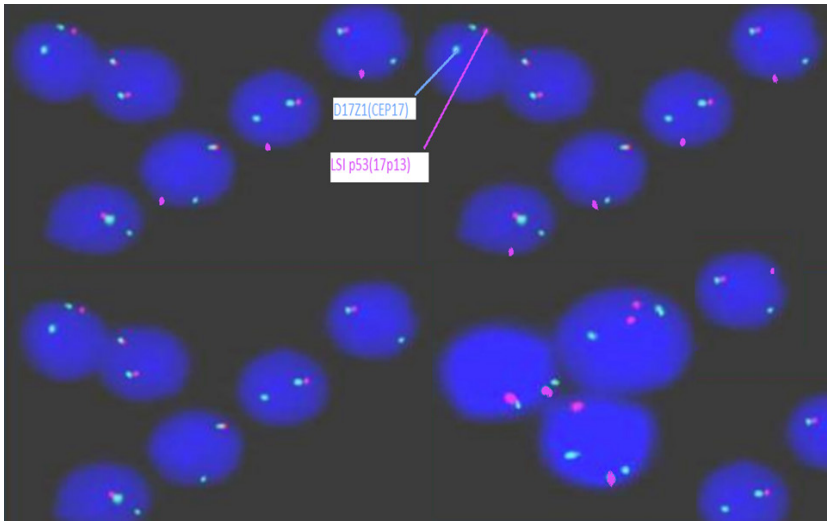

Figure 1(b). Representative CLL FISH Image of Case with 25% 17p del (50/200 cells), Red Dot Represents LSI p53(17p13) Probe and Green Dot Represents D17Z1(CEP17) Probe for Control

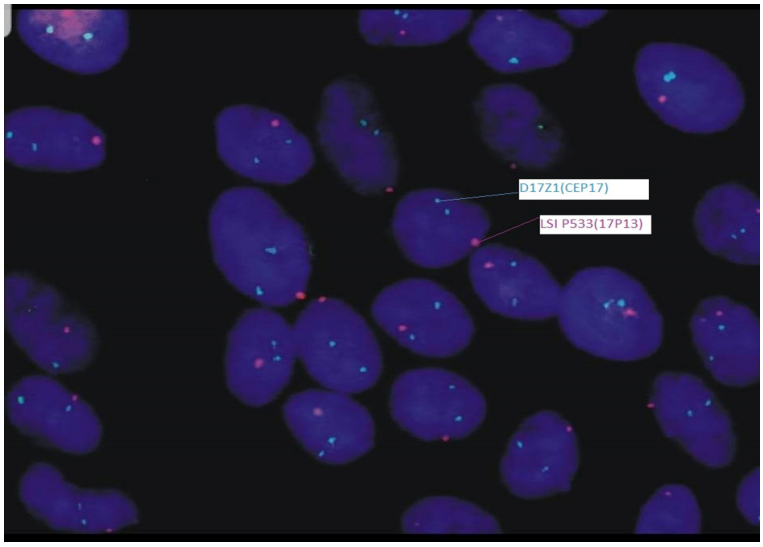

Figure 1(c). Representative CLL FISH Image of Case with 95% 17p del (190/200 cells), Red Dot Represents LSI p53(17p13) Probe and Green Dot Represents D17Z1(CEP17) Probe for Control

classify patients into two groups, we generated a receiver operating characteristic (ROC) curve. In the context of a 95% confidence interval, a p-value of less than 0.05 is considered statistically significant.

Results

The characteristics of CLL patients are shown in Table 1. Patients with CLL were stratified according to 17p del and 11q del into positive and negative ones. ROC curve was applied to identify the best cutoff that can sub-classify the positive ones into those with good or bad outcome (Figure 3). This analysis revealed that the best cut-off value of 17p del percent was (33%) (sensitivity was 83.3%, specificity was 85.7%). On the other hand, the best cut-off value of 11q del percent was (30%) (sensitivity was 72.7%, specificity was 75%). In this context, all studied CLL cases were stratified according to 17p del into 3 subgroups cases with negative 17p del (33%). Likewise, according to 11q del detection levels, CLL cases was classified into 3 subcategories which includes negative 11q del (30%) (Table 2, Figure 3).

Patients with high positive 17p del (> 33%) had significantly shorter LDT, shorter TTFT and shorter PFS than those with low positive 17p del (≤33%) as well as those with wild type. While patients with wild 17p del did not differ significantly from those with low positive 17p del (≤33%). Furthermore, high smudge cells count were significantly associated with wild and low positive 17p del (≤33%). On the other hand, no significant association was found regarding positive CD38, and RAI stages with 17p del among CLL group (Table 3).

Patients with high positive 11q del (> 30%) had significantly shorter LDT, shorter TTFT and shorter PFS than those with wild type, but did not differ significantly from those with low positive 11q del (≤30%). Also, more smudge cells were significantly associated with wild and low positive 11q del(≤30%). While no significant association was found regarding CD38, and Rai stages with 11q del among CLL group (Table 4).

Figures 1, 2 displayed FISH photos of CLL cases with different grades of 17p del and 11q del. Figure 1(a): Represent CLL FISH image of a case with negative 17p del. Figure 1(b): Represent CLL FISH image of a case with 25% 17p del. Figure 1(c): Representative CLL FISH image of a case with 95% 17p del. Figure 2(a): Represent CLL FISH image of a case with 22% 11q del. Figure 2(b): Represent CLL FISH image of a case with 80% 11q del.

Discussion

In chronic lymphocytic leukemia, deletion of the short arm of chromosome 17 is associated with rapid disease progression, as well as a poor response to treatment [15-17]. Previous studies stated that 17p del was positive in 5-10% at diagnosis [18, 19]. The present study revealed that 17p del was positive in 19%. The ROC curve of 17p del percent was performed to evaluate the validity of 17p del percent for the prediction of CLL patients outcome. High-accuracy AUC was found (AUC=0.923) at best cut-off value of 17pdel burden equal to ≥33% vs<33%.

We have found that patients with a lower percentage of cells with 17p del had significantly better outcome (better LDT, TTFT, PFS & OS) than those with a higher percentage of cells with 17p del, as well as those with wild type. Similarly, Strati et al. [20] showed that a higher rate complete remission (CR)/ non- complete remission(NCR) was associated with a "lower burden" of 17p del detected by FISH. Additionally, previous research showed that patients with 17p del as a low subclone burden had a survival advantage compared to patients with 17p del with a high burden [21, 22]. Yuan et al [14] demonstrated that the proportion of 17p del-positive cells and the size of 17p del subclones were significant indicators of prognosis in CLL. Furthermore, Li et al. [23] and Van Dyke et al. [24] found that CLL patients with a high proportion of cells

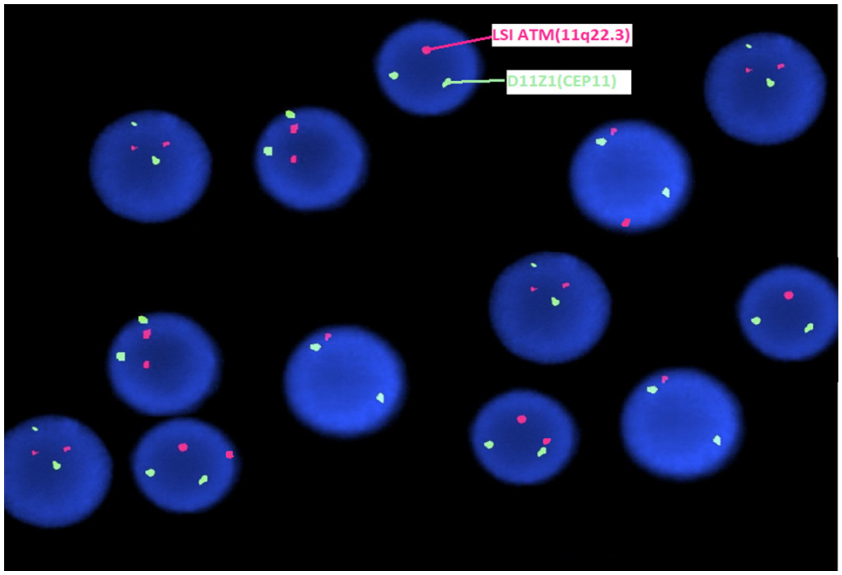

Figure 2(a). Representative CLL FISH Image of Case with 22% 11q del (44/200 cells), Red Dot Represents LSI ATM(11q22.3) Probe and Green Dot Represents D11Z1(CEP11) Probe for Control

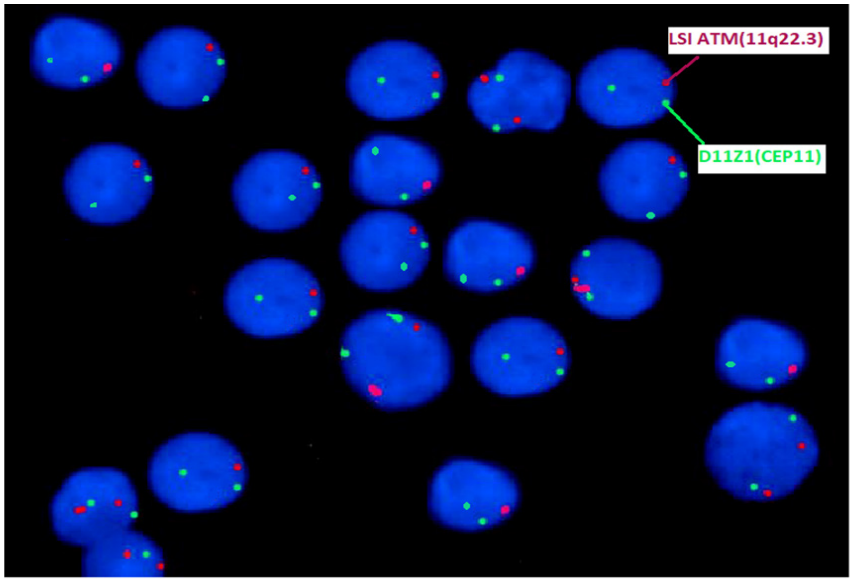

Figure 2(b). Representative CLL FISH Image of Case with 80% 11q del (160/200 cells), Red Dot Represents LSI ATM(11q22.3) Probe and Green Dot Represents D11Z1(CEP11) Probe for Control.

Table 1. Patients Characteristics of CLL Patients at Diagnosis

			CLL patients (n=100	
Age (years		mean±SD		60.5±10.8
Males		N (%)	61	61%
Females		N (%)	39	39%
Total leucocyte count (X10^9/L)		Median (min-max)	41.2	9.9-600
Absolute lymphocytic count (X10^9/L)		Median (min-max)	32.4	5.3-590
Hemoglobin level (g/dL)		Median (min-max)	11.1	5.6-15.8
Platelet count (X10^9/L)		Median (min-max)	147	13-473
Smudge cells	<30%	N, %	20	20%
	>30%	N, %	80	80%
Hepatomegaly		N, %	30	30%
Splenomegaly		N, %	76	76%
modified RAI staging	I	N, %	16	16.00%
	II	N, %	30	30.00%
	III	N, %	24	24.00%
	IV	N, %	30	30.00%
CD38 positive		N, %	46	46%
17pdel positive		N, %	19	19%
13qdel positive		N, %	19	19%
11pdel positive		N, %	15	15.00%
CLL patients response to therapy				
Complete remission (CR		N, %	11	18.3%
Non-complete remission (NCR		N, %	25	41.7%
Stationary course (SD		N, %	8	13.3%
Progressive course (PD		N, %	16	26.7%

with 17p del had unfavorable results, whereas those with a low proportion (<20,<25% respectively) had good results similar to those without 17p del.

Regarding 11q del, this is the first study to investigate the value of 11q del subclone identification. The present study showed that 11q del was positive in 15% of the cases. The ROC curve of 11q del percent was performed for the assessment of the validity of 11q del percent for

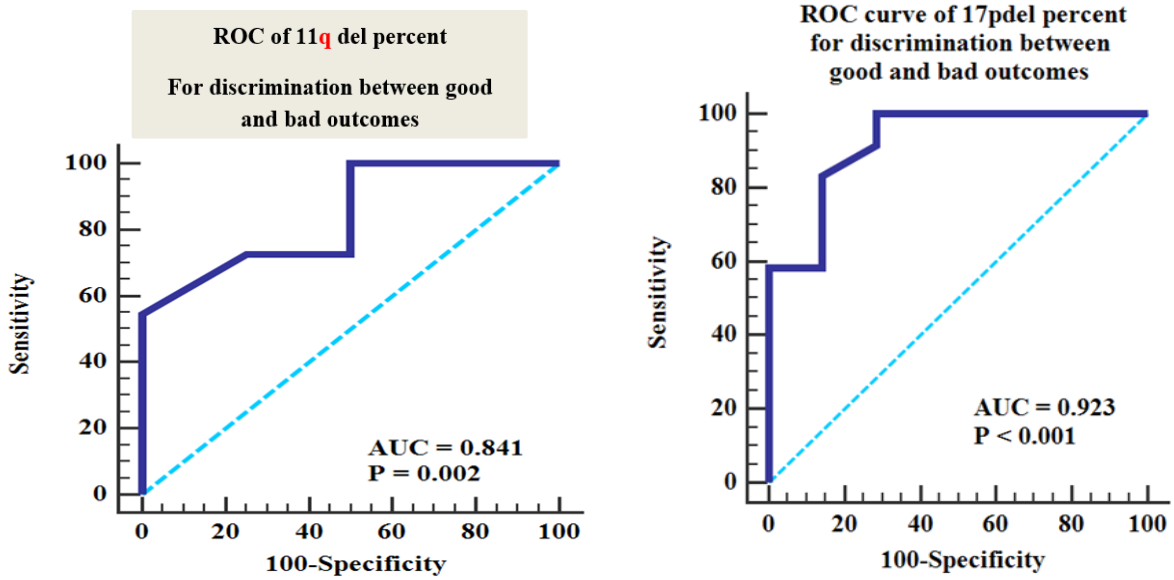

Figure 3. ROC Curve to Determine the Best Cut off Value that Discriminate the Cytogenetic Burden Better Classify CLL Patients into Good and Bad Outcomes Regarding 17pdel and 11q del., The high accuracy AUC was found (AUC=0923) at best cut of value of 17p del percent (=33%), sensitivity was 83.3%, while specificity 85.7%. Regarding 11q del moderate accuracy AUC was found (AUC=0.841) at best cut of value of 11p del percent (=30%), sensitivity was 72.7%, while specificity 75%.

Table 2. Classification of Positive CLL Case Regarding 17pdel and 11qdel into High Positive Cases and Low Positive Cases according to the Finding Addressed by the ROC Curve.

	CLL patients with positive 17pdel (n=19)		CLL patients with positive 11qdel (n=15)	
Percentage of positivity	10-33%	>33%	10-30%	>30%
Number of CLL cases	11	8	6	9

Table 3. Comparison of LDT, TTFT, PFS, CD38, Smudge Cells and RAI Stages According to 17p del among CLL Group

		17p del						
		Wild (n=81)	Positive<33% (n=10)	Positive>33% (n=9)	*P1*	*P2*	*P3*	*P4*
LDT (months)	Cumulative 1y-LDT (%)	81.7	100	25	<0.001	0.259	<0.001	0.007
	Mean LDT	45.171	23	10.375				
	95% CI	36.451 to 53.891	23.000 to 23.000	4.750 to 16.000				
TTFT (months)	Cumulative 1y-TTFT (%)	49.9	35.7	22.2	0.045	0.885	0.023	0.001
	Mean TTFT	13.529	13.24	5.378				
	95% CI	10.411 to 16.648	4.163 to 22.317	-0.403 to 11.159				
PFS (months)	Cumulative 5 y-PFS %	70.5	75	22.5	<0.001	0.655	<0.001	0.002
	Mean PFS	54.1	44.75	12.25				
	95% CI	44.171 to 64.075	30.746 to 58.754	4.770 to 19.730				
CD38%	Negative	44 (54.3%)	4 (40.0%)	6 (66.7)	0.566	0.508	0.726	0.37
	Positive	37 (45.7%)	6 (60.0%)	3 (33.3%)				
Smudge cells%	Less than 30%	11 (13.6%)	3 (30.0%)	6 (66.7%)	0.002	0.18	0.001	0.179
	More than 30%	70 (86.4%)	7 (70.0)	3 (33.3%)				
RAI stages	0-II	37 (45.7%)	3 (30.0%)	6 (66.7%)	0.309	0.503	0.301	0.179
	III-IV	44 (54.3%)	7 (70.0%)	3 (33.3%)				

P1, comparison between wild, low positive, high positive 17p del; *P2*, comparison between wild, low positive 17p del; *P3,* comparison between wild, high positive 17p del; *P4*, comparison between low positive, high positive 17p del

the prediction of good patient outcome (CR, NCR) and bad patient outcome (SD and PD). A moderate accuracy AUC was found (AUC=0.841) at the best cut-off value of 11q del percent (30%).

Table 4. Comparison of LDT, TTFT, PFS, CD38, Smudge Cells and RAI Stages According to 11q del among CLL Group.

		11q del						
		Wild (n=85)	Positive <30% (n=6)	Positive ≥30% (n=9)	*P1*	*P2*	*P3*	*P4*
LDT (months)	Cumulative 1y-LDT (%)	83.8	83.3	33.3	0.001	0.823	<0.001	0.116
	Mean LDT	46.355	17.75	11.778				
	95% CI	37.624 to 55.085	12.114 to 23.386	6.106 to 17.450				
TTFT (months)	Cumulative 1y-TTFT (%)	51.1	25	11.1	0.008	0.493	0.002	0.135
	Mean TTFT	14.082	5.758	3.322				
	95% CI	11.006 to 17.158	2.413 to 9.103	-1.011 to 7.655				
PFS (months)	Cumulative PFS %	92.1	83.3	64.8	<0.001	0.975	<0.001	0.262
	Mean 5y-PFS	54.342	44.5	15.126				
	95% CI	44.411 to 64.273	29.292 to 59.708	8.453 to 21.798				
CD38	Negative	47 (55.3%)	3 (50.0%)	4 (44.4%)	0.838	0.801	0.727	0.833
	Positive	38 (44.7%)	3 (50.0%)	5 (55.6%)				
Smudge cells	Less than 30%	13 (15.3%)	1 (16.7%)	6 (66.7%)	0.005	0.928	0.002	0.119
	More than 30%	72 (84.7%)	5 (83.3%)	3 (33.3%)				
RAI stages	0-II	39 (45.9%)	2 (33.3%)	5 (55.6%)	0.703	0.687	0.73	0.608
	III-IV47	46 (54.1%)	4 (66.7%)	4 (44.4%)				

P1, comparison between wild, low positive, high positive 11q del; *P2*, comparison between wild, low positive 11q del; *P3*, comparison between wild, high positive 11q del; *P4*, comparison between low positive, high positive 11q del

In the current study, it is evident that patients with a higher percentage of cells with 11q del had a significantly worse outcome (worse LDT, PFS and TTFT) than those with wild type. On the contrary, those with a lower proportion of cells with 11q del showed no significant difference from the wild type. This finding could be explained on the basis of the results in a recent study done by Álamo et al. [10] who, demonstrates that biallelic BIRC3 deletion through 11q del and mutation triggers non-canonical NF-κB signaling, driving BCL2 overexpression and conferring clonal advantage, which could account for the negative predictive impact of BIRC3 biallelic inactivation in CLL.

In conclusion, quantification 17pdel burden (>vs.≤33) could be used for refining risk stratification of CLL patients .

Author Contribution Statement

Salah Aref: Conception and supervision; Sherin Abdel-Aziz : Interpretation and analysis of data; Mohamed Sabry: Interpretation and analysis of data; Mohamed Aref: Preparation of the manuscript and revision for important intellectual; Mona Mansour and Wesam El Dosoky: Laboraory work, intrepretation of data and prepration of the manuscript.

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Ethical Statement

The study was approved by Mansoura Faculty of Medicine IRB.

Informed consent

All subjects included in this study gave informed consent.

Conflict of interest

The authors declare no competing interests

Availability of data

The data of the present study is available upon request to the corresponding author.

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