The prognostic implication of CD49d expression by flow cytometry and trisomy 12 detection by fluorescent in situ hybridization in chronic lymphocytic leukemia

Abstract:

Chronic lymphocytic leukemia (CLL) is the most common chronic lympho□proliferative disorder. This study done to detect the level of (CD)49d in C patients by flow cytometry and its correlation with the prognosis (survival) and with (trisomy12) detected by fluorescent in situ hybridization (FISH).

Methods: Clinico□hematological profiles done to 40 CLL patients. CD49d tested flow cytometry and trisomy12 was detected by FISH.

Results: CLL patients classified according to modified Rai staging system into: low risk 12.5%, intermediate risk 22.5% and high risk 65%. CD49d and trisomy12 positivity were detected in 29 patients (72.5%) and 22 patients (55%), respectively. There was a significant positive correlation between the percentage of trisomy12 and of CD49d cells in CLL patients (P =0.034). And also, between CD49d and CD38 (P =0.034). On the other hand, there was no significant relation between both CD49d and trisomy12 expression and modified Rai staging system.

As regard to overall survival (O.S) and disease free survival (DFS), both CD49d, trisomy12 positive cases were associated with shorter disease free, and overall survivals compared to the negative cases.

Regarding to the relation between the use of combination of fludarabine, cyclophosphamide, and rituximab (FCR) as a standard treatment in CLL and OS and DFS of patients in our study, we found that FCR account for the better outcome associated with its use.

Conclusion: CLL B-cell membrane expression of CD49d as measured by flow cytometry is a powerful prognostic parameter in patients with CLL. Its positive correlation with the trisomy12 and CD38 and the association of both CD49d and trisomy12 with short survival times indicate that they may have roles in the prognosis of CLL.

In addition, the use of FCR regimen in the treatment of CLL is associated with long survival of CLL patients.

Keywords:

Chronic lymphocytic leukemia, Prognosis, CD49d, trisomy12, FCR.

Introduction:

Chronic lymphocytic leukemia (CLL) defined as a lymphoproliferative disorder, composed by monomorphic round B-lymphocytes involving peripheral blood (PB), bone

marrow (BM) and lymphoid organs **(1)**. CLL is one of the most common types of leukemia in the Western world, however, infrequent in the Eastern. In Egypt, CLL was the most common subtype of leukemias, the National Cancer Registry reported over 80% of lymphoid leukemias are CLL **(2)**. It is the most common types of leukemia diagnosed in adult.

CD49d is a surface molecule that binds to the β-integrin CD29 to form very late antigen-4 (VLA-4), the expression of which promotes microenvironment-mediated proliferation of CLL leukemic cells and identifies a subgroup of patients characterized by progressive course and short survival **(3)**. It should be noted that the expression of CD49d correlates with some other prognostic factors. Specifically, with unmutated IGHV, CD38 and ZAP70 with the major cytogenetic lesions such as trisomy12. Moreover, trisomy12 CLL cases were characterized by the higher mean fluorescence intensity levels of CD49d compared with cases belonging to the other cytogenetic categories, probably facilitated through a NOTCH1 or methylation-mediated mechanism **(4)**.

The presence of cytogenetic abnormalities is a hallmark of CLL, and have historically been best studied by interphase fluorescence in situ hybridization (FISH) **(5)**. Trisomy12 is the third most common cytogenetic abnormality identified by fluorescence insitu hybridization (FISH). In some reports, trisomy 12 CLL carry an intermediate prognostic risk, while other reports suggest a certain degree of clinical heterogeneity, with a higher incidence of second malignant neoplasms and Richter transformation **(6)**.

The aim of the present study was to detect the level of expression of CD49d in CLL patients by flow cytometry and its correlation with the prognosis and with trisomy12detected by fluorescent in situ hybridization (FISH).

Patients and methods:

The study was included 40 CLL patients (22 men and18 women; age range 38-81 years). These patients were presented to South Egypt Cancer Institute Assiut University hospital in the period between December 2015 and July 2017. The study was approved by the Institutional Review Board of Faculty of Medicine, Assiut University. An informed written consent was taken from of all cases.

All patients were subjected to:

- History taking and clinical examination.
- Complete blood picture.
- Bone marrow examination
- Immunophenotyping: analysis was done by multicolor flow cytometry (FACS Caliber, BECTON DIKINSON, USA). Forward scatter and side scatter histogram were made to detect the lymphocyte population. Lymphocytes were then gated for

further analysis of different monoclonal antibodies as CD5, CD10, CD19, kappa, and lambda, FMC7, CD 23, CD 3, CD49d and CD38.

Immunophenotyping diagnosis of our CLL patients was done according to scoring system **(7)**.

- **Cytogenetic study:** the test aims to detect the presence and level of expression of trisomy 12 by fluorescence in situ hybridization (FISH) by using Alpha Satellite 12 plus probe (Cytocell, Catalogue No: REF LPH069), labelled in red, which recognized the centromeric repeat sequence D12Z3. FISH procedures were performed as usual, after Preparation of mitotic cells from short-term blood cultures, slide preparation, Pre-denaturation, denaturation, hybridization and final post-hybridization washes.

  Analysis was done by a fluorescent microscope (Carl Zeiss AxioSkop 2 Mot FL). The images were captured through a Leica CW 4000 camera assembled to a computer having FISH software (Carl Zeiss/Cytovision, Axiovision control 3.1). Slides showing more than 50% cells with fluorescent dots were selected for analysis. Then at least 200 cells were counted. In a normal cell, 2 red signals should be observed. While in a cell with trisomy12, there should be 3 red signals.

Statistical analysis:

Statistical calculation was performed with Statistical Package for Social Sciences (SPSS) software (version 16.0; SPSS Inc, Chicago, Ill).

Results:

CLL patients classified according to modified Rai staging system into: low risk 12.5%, intermediate risk 22.5% and high risk 65% as in Figure (1).

The expression of CD49d on malignant lymphocytes was detected in 29 patients (72.5%). However, the expression of CD38 on malignant lymphocytes was detected in 21 patients (52.5%) as in figure (2).

As regard to trisomy 12, 22 patients were trisomy12 positive (55 %).

As regard to correlation between trisomy12, CD49d and CD38; there was a significant relation between trisomy12 and CD49d expression on CLL group (P value = 0.001**) (r =0.49), a significant relation between CD38 and CD49d in the studied CLL group. (P value = 0.05*) (r =0.311). On the other hand, there was no significant relation between trisomy and CD38 in the studied CLL group as in table (1). In addition, there was no significant relation between modified Rai staging system and the presence of both CD49d and trisomy12 as in table (2).

Concerning the relation between the overall survival (OS) and the disease free survival (DFS) of the studied CLL group and the percentage of trisomy12 and CD49d expression, there was a significant relation between OS and both CD49d (P value= 0.0192) and trisomy12 (P value=0.0141) as in Table (3) & Figure (3, 4). As regard to DFS, there was a

significant relation between CD49d and DFS (P value=0.0190). However, there was no significant relation between trisomy12 and DFS (P value=0.1882) as in Table (3) & Figure (5, 6).

As regard to the relation between the overall survival (OS) and the disease free survival (DFS) and the percentage of patients receiving the standard treatment, There was a significant relation between the percentage of patients receiving the standard treatment (FCR) and OS (P value=0.0016) and DFS (P value=0.0168) as shown in Table (4) & Figure (7, 8).

Discussion:

In this study, CLL patients were classified according to modified Rai staging system into: low risk 12.5 %, intermediate risk 22.5% and high risk 65% in their first presentation.

In this study, 29 patients (72.5%) were CD49d positive and 11 patients (27.5%) were CD49d negative according to the use of cut off value of 30%. This result is approximately close to the results found by Hendy et al. who reported that, CD49d was positive in (75%) of all studied CLL cases **(8)**. Other studies done by Benedetti et al. and Wesam et al. found that, CD49d expression was positive in (37.7%) and (55.3 %) of cases respectively **(9,10)**. These variations in expression of CD49d in CLL patients between our results and previous studies may be due to the variability of the sample size and the possible ethnic variations between the studied groups.

Remarkably, the finding of differential CD49d expression in CLL is an older discovery than anticipated. In 1996, it had already been demonstrated that CD49d expression in CLL is variable, with higher expression of CLL samples of advanced (Rai III, IV) than early stages **(11)**.

Within the same concept, our study also revealed that, higher expression of CD49d was associated with advanced disease stage but these results were statistically insignificant, this may be due to low number of our studied cases. This also matched to the results of Wesam E Elderiny, who found that higher levels of CD49d in advanced stages **(10)**.

As regard to correlation between CD49d and CD38 Zucchetto and colleagues were the first in 2006 that reported the strong association of CD38 and CD49d expression on CLL cells using both parameters as categorical variables **(12)**. These findings were found to be in harmony with our study results where we found that, there was a significant positive correlation between CD49d and CD38 expression in CLL patients. In addition, this is in agreement with Kamel et al. and Buggins et al. **(13, 14)**.

By using Kaplan-Meier curves, patients with CD49d positivity had shorter survival and disease free survival than those negative for CD49d (using 30% as cut off level for

CD49d positivity). This is in agreement with Gattei et al., who reported that, when analyzed retrospectively, CLL patients with ≥30% CD49d-positive tumor cells revealed significantly shorter treatment-free and overall survival than patients with <30 % CD49d positivity **(15)**. A prospective analysis indicated that an alternative cut-off level of 45 % CD49d expression might be superior to the 30 % level **(16)**. Following these first reports, the prognostic relevance of increased CD49d expression was rapidly and unequivocally confirmed by several groups, using the 30 % cut-off level. All of them found that, CD49d expression consistently identifies a subgroup of CLL characterized by poor outcome in their study **(3, 17, 18, 19)**.

Concerning trisomy 12 in our CLL patients, there was a higher incidence of trisomy 12 than that previously thought, (20%) according to WHO 2008 **(20)**. 55% of our study group were found to be positive for trisomy 12. Other studies including the incidence of trisomy 12 in CLL patients revealed under estimation of these results in comparison to our results. Dal Bo et al., Bulian et al. and Alp. reported that, trisomy 12 was found in 13.2%, 15-20% and between 16% and 35% respectively **(21, 22, 23)**. A much lower frequency of trisomy12 (13.2%) reported by Dal Bo et al. On reviewing their study, we found that the majority (50%) of CLL patients who were included in that study had early-stage disease. In contrast, only 12.5% (5/40) of the patients included in our study were at low risk group in the modified Rai staging system, with the majority (65%) were at high risk groups.

As regard to the relation between the staging of the disease and the incidence of the trisomy 12 finding in CLL patients we found that, there was no significant relation between trisomy 12 positivity and staging system. This is in agreement with Alp. **(23)**. On the other side Witzig et al. stated that, there was an increased frequency of trisomy 12 in patients with more advanced Rai stages **(24)**.

In contrast, there was a significant relation between trisomy 12 positivity and CD49d in our study. This is in agreement with Gooden et al., Baumann et al .and Riches et al. they found that a higher CD49d expression was frequently linked to trisomy 12 positive cases in their study **(4, 25, 26)**. On the other hand more recently Bullian et al. stated that, despite the high frequency of NOTCH1 and BIRC3mutations and of CD49d and CD38 overexpression, these markers failed to convey a prognostic risk in trisomy12 CLL, while there is a peculiar clinical relevance of IGHV mutations in tris12 CLL patients **(22)**.

In this study, patient's group with trisomy12 positivity had shorter survival times than those without trisomy12. This is in agreement with Juliusson et al., Bulian et al. and González-Gascón y Marín et al., they found that overall survival was shorter in patients with high trisomy12 expression in comparison to those with low trisomy12 expression **(22, 27, 28)**. These results found to be contradirectory to results reported by Döhner et al. who their prospective trials suggests that overall survival in trisomy12 positive cases was favorable despite progression-free survival may be shorter **(29)**.

As regard to the treatment, our study patients were classified according to type of treatment into 2 subgroups: 35% were recieved the standard treatment (FCR) and 65% were received other lines of treatment (CVP and CHOP).

In our study, we found that the use of the standard treatment was associated with prolonged disease free and overall survivals in comparison to the other lines of treatment. This is in agreement with Strati et al. and Herishanu et al., they found that FCR regimen was the first regimen achieves long remissions and prolonged overall survival in first-line treatment CLL patients **(30, 31)**.

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fludarabine-cyclophosphamide-rituximab regimen for chronic lymphocytic leukemia outside clinical trials: the Israeli CLL Study Group experience. Haematologica, 100, 662-9.

Figures and legends:

Figure (1):

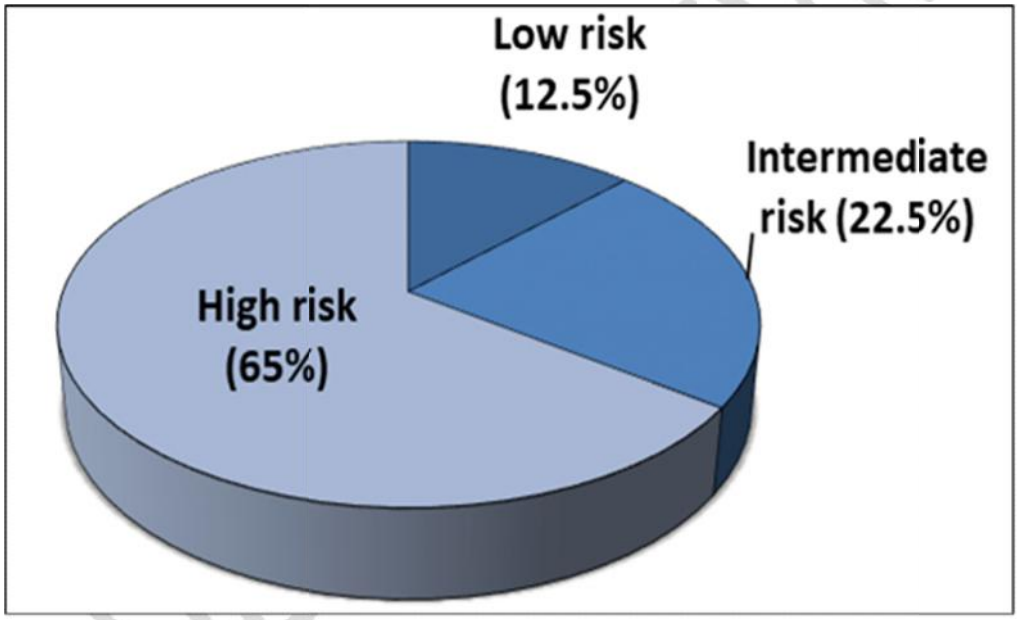

Figure (1): The distribution of CLL patients, according to modified Rai staging system

Figure (2):

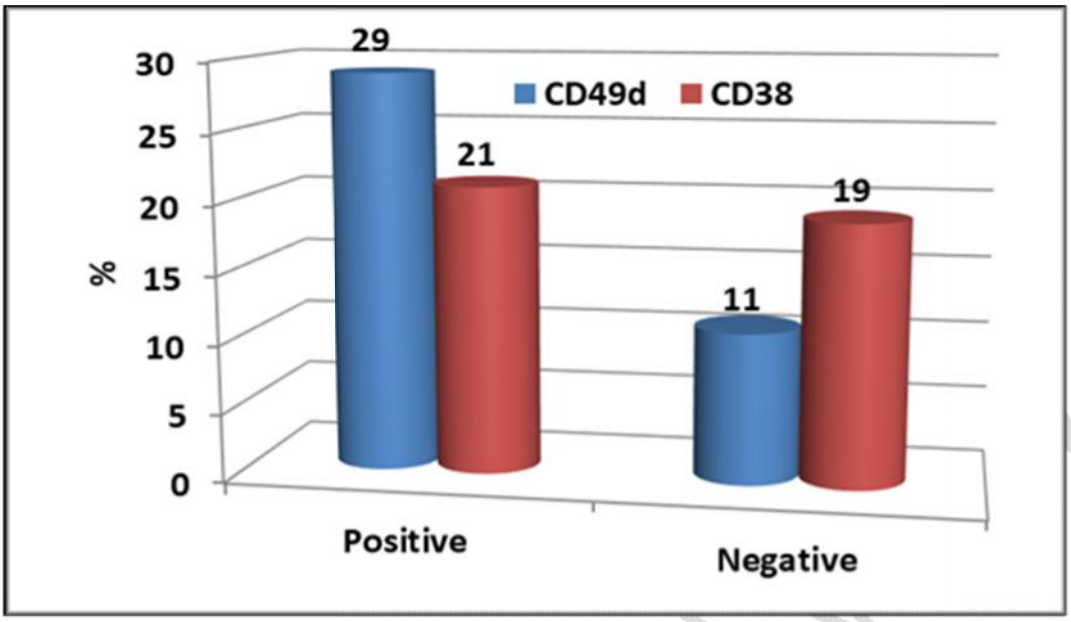

Figure (2): Expression of CD49d and CD38 on malignant lymphocytes in CLL patients

Figure (3):

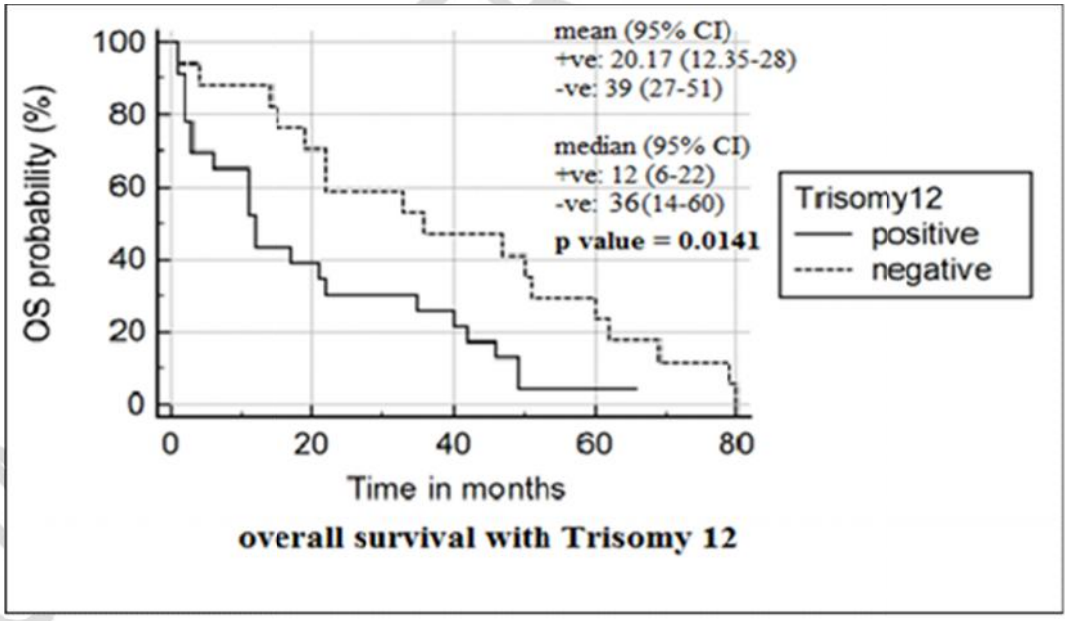

Figure (3): Kaplan –Meier curves show the relation between overall survival (OS) and patients with and without trisomy12.

Figure (4):

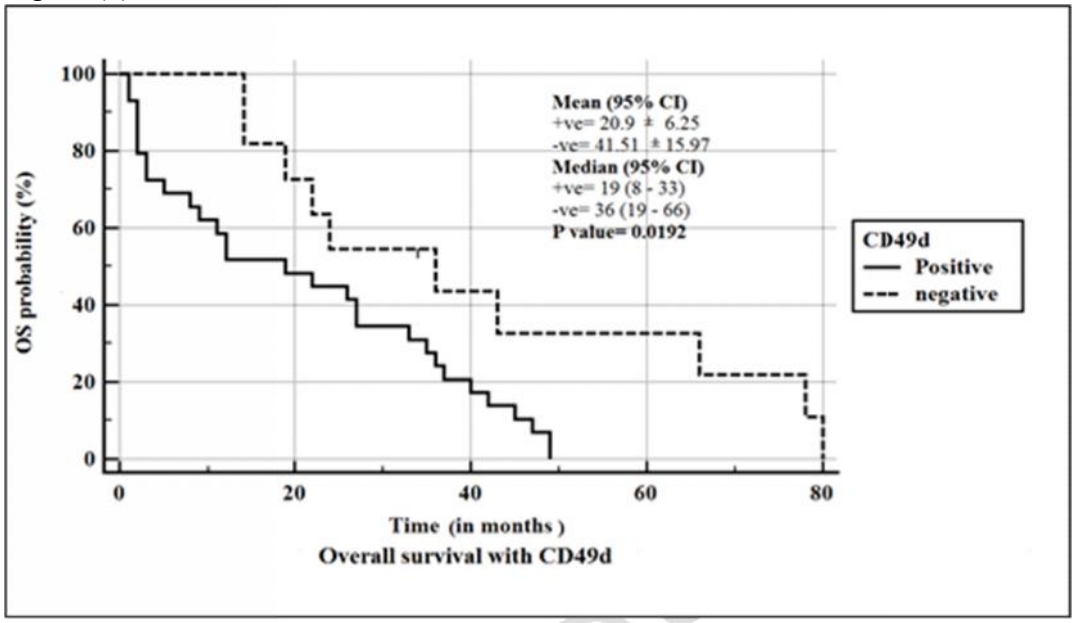

Figure (4): Kaplan-Meier curves show the relation between overall survival (OS) and patients with and without CD49d.

Figure (5):

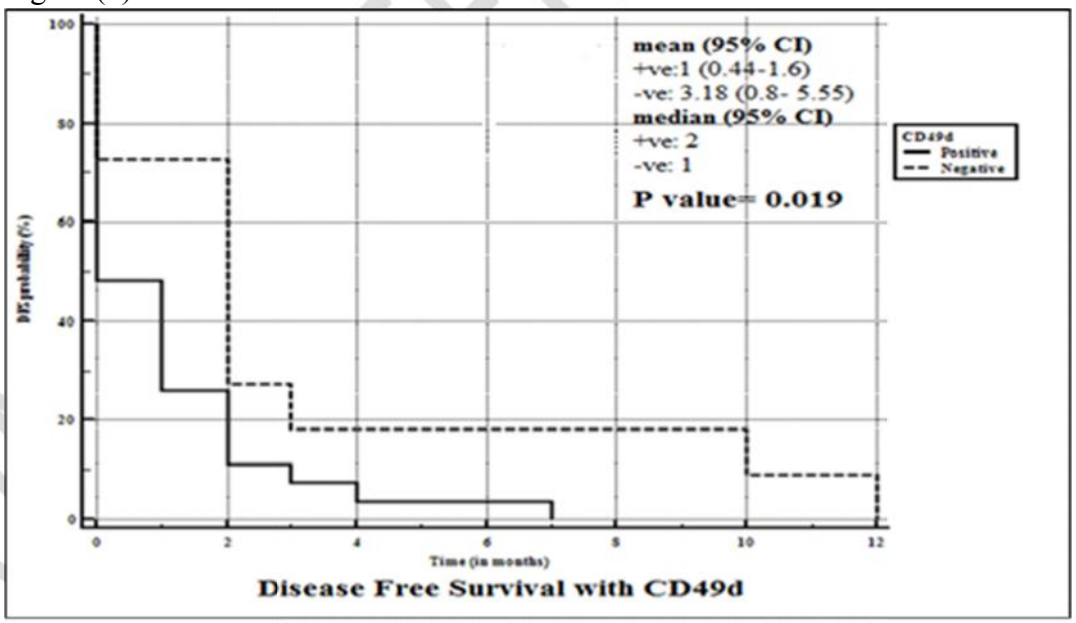

Figure (5): Kaplan-Meier curves show the relation between the disease free survival (DFS) and patients with or without CD49d expression.

Figure (6):

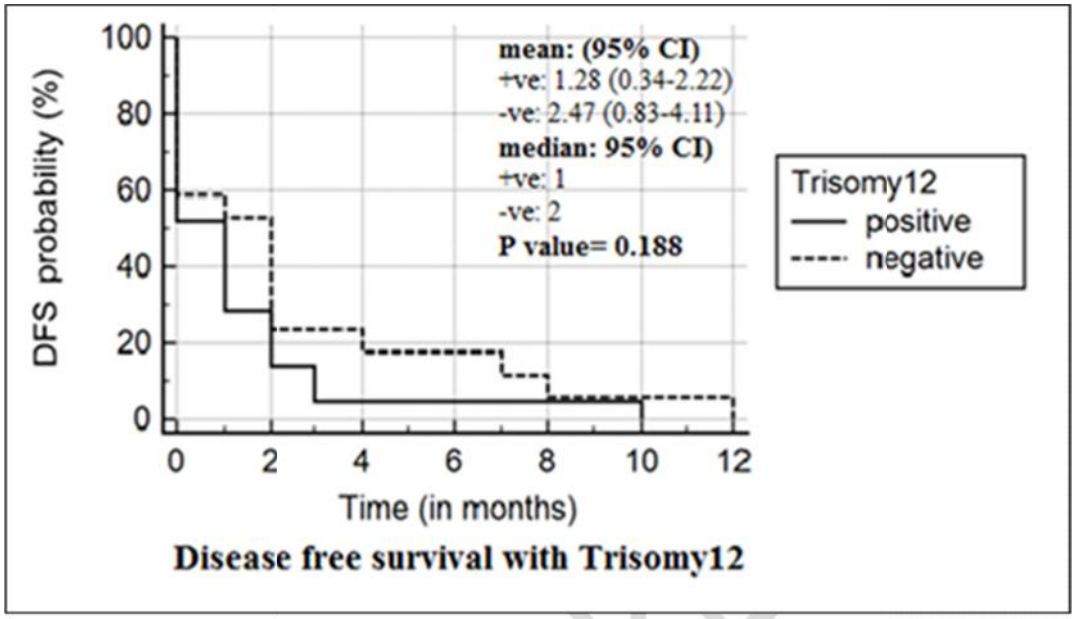

Figure (6): Kaplan-Meier curves show the relation between disease free survival (DFS) and patients with or without trisomy12.

Figure (7):

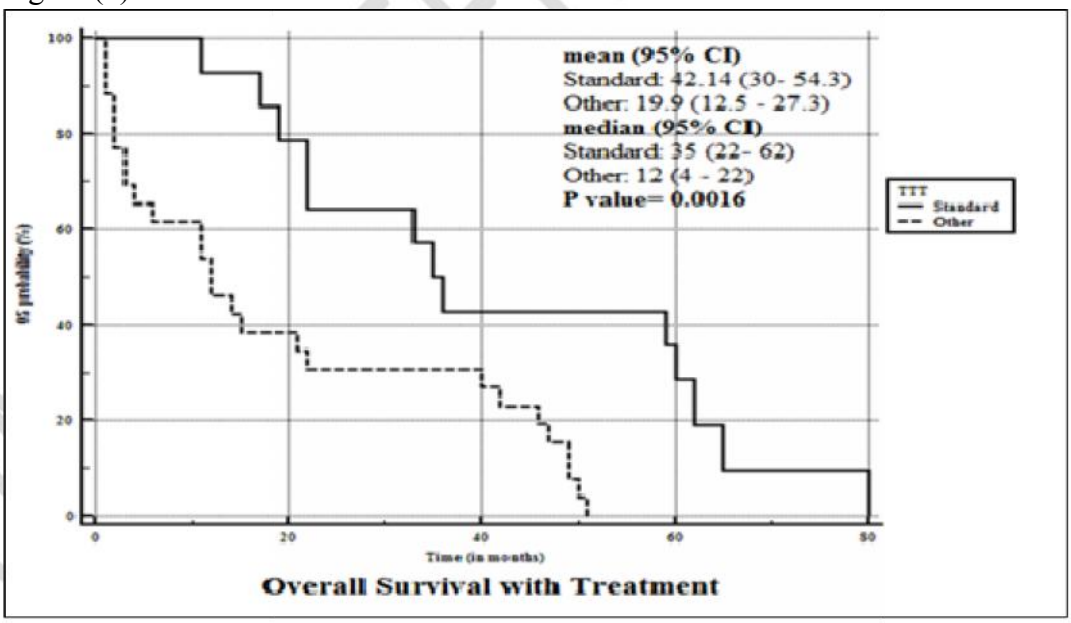

Figure (7): Kaplan-Meier curves show the relation between the overall survival (OS) and patients with and without the standard treatment (FCR).

Figure (8):

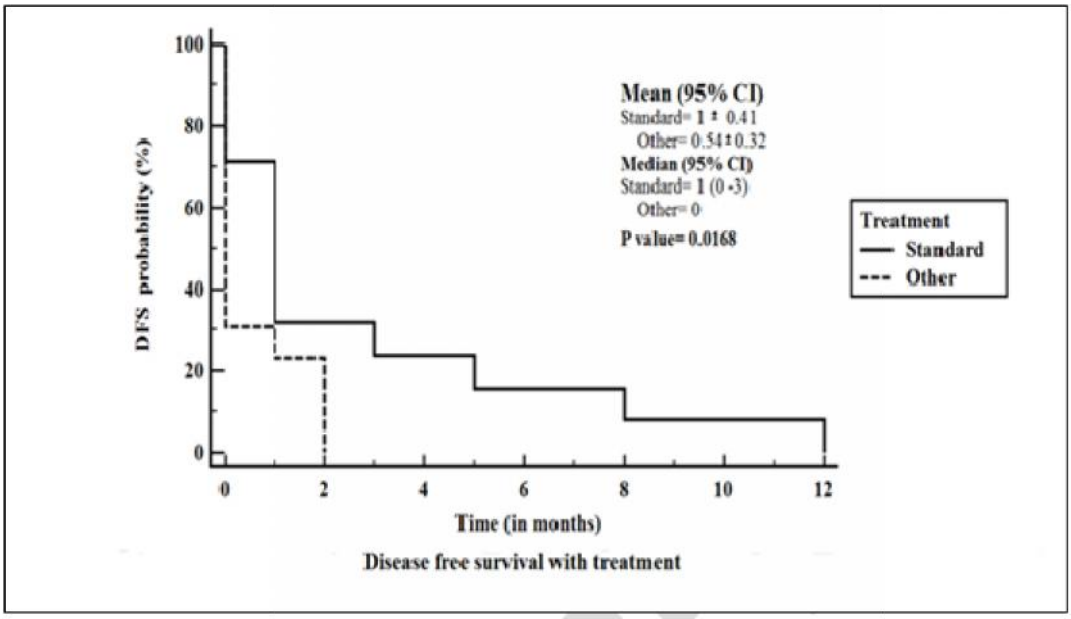

Figure (8): Kaplan-Meier curves show the relation between the disease free survival (DFS) and patients with and without the standard treatment (FCR).

Tables and legends

Table (1):

Percentage	**CD49d**		**CD38**	
	R	**P**	**R**	**P**
CD38	0.311	0.049*		
Trisomy12	0.49	0.001**	0.195	0.228

Table (1): The relation between CD49d, CD38 and Trisomy12.

Significant P value < 0.05

Table (2):

Percentage	**Grade**			**P. value**
	Low (N = 5)	**Intermediate (N = 9)**	**High (N =26)**	
CD49d	38.94 ±35.27	41.46 ±31.72	60.52 ±32.62	0.20
Trisomy12	6.02 ± 4.42	8.87 ± 5.71	6.95 ± 4.37	0.48

Table (2): The relation between the Modified Rai staging system and CD49d and Trisomy12.

Significant P value < 0.05

Table (3):

	Trisomy12		p. value	CD49d		P. value
	Positive trisomy12 NO (22)	**Negative trisomy12 NO (18)**		**Positive CD49d NO (29)**	**Negative CD49d NO (11)**	
OS (months)	20.17± 7.82	39 ± 12	0.0141*	20.9 ± 6.25	41.51±15.97	0.0192*
DFS (months)	1.28 ± 0.92	2.47 ± 1.64	0.1882	1.0 ± 0.56	3.18 ± 2.38	0.0190*

Table (3): The relation between the overall survival (OS) and the disease free survival (DFS) of the studied CLL patient's group and the percentage of trisomy12 and CD49d expression.

Significant P value < 0.05 **OS:** Overall survival

DFS: Disease free survival

Table (4):

	Standard treatment (FCR)	Other (CVP,CHOP)	P. value
OS (months)	42.14 ± 12.14	19.9 ± 7.4	0.0016**
DFS (months)	1 ± 0.41	0.54 ± 0.32	0.0168*

Table (4): The relation between the overall survival (OS) and disease free survival (DFS) and the percentage of patients receiving the standard treatment.

Significant P value < 0.05

FCR: Fludarabine, Cyclophosphamide, and Rituximab

CVP: Cyclophosphamide, Vincristine, and Prednisone

CHOP: Cyclophosphamide, Hydroxydaunorubicin, Oncovin and prednisone