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ORIGINAL ARTICLE

Prognostic significance of del 17p, ZAP-70 and CD38 as independent indicators for B-CLL: correlation to response to treatment and disease outcome

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Abstract Prognostic markers as CD38 and ZAP-70 and specific chromosomal abnormalities as del 17p have now been developed to refine the risk of progressive disease in chronic lymphocytic leukemia (CLL). This study analyzed 40 recently diagnosed, untreated B-CLL patients for CD38 and ZAP-70 expression by flow cytometry and for del 17p by conventional cytogenetics (CCG) and by fluorescence in situ hybridization (FISH) technique to evaluate their effect on the clinical course of CLL and as risk factors for disease progression in addition to their impact on response to treatment and disease outcome. Twenty healthy age- and sex-matched subjects were included as a control. The results revealed that CD38 and ZAP-70 expression were detected in 42.5% and 47.5% of cases, respectively. They were associated with an unfavorable clinical course. Higher levels were significantly associated with increased risk of unfavorable response to treatment ($P = 0.003$), with poor clinical outcome ($P = 0.0001$). Del 17p was detected in 35% of cases by FISH technique and in 7.5% by CCG. The deletion was significantly associated with progressive clinical course; poor response to treatment ($P = 0.007$) but not with disease outcome ($P = 0.103$).

Combined analysis of ZAP-70 and CD38 yielded concordantly negative results in 50% of patients and concordantly positive results in 40% of patients, while 10% were discordant. $CD38^+/ZAP\text{-}70^+$

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patients were significantly associated with progressive disease ($P < 0.05$) and with del 17p than $CD38^-/ZAP\text{-}70^-$ patients ($P = 0.008$).

Time to disease progression (TDP) was 6 months among $CD38^+/ZAP\text{-}70^+$ patients as compared to 16 months in $CD38^-/ZAP\text{-}70^-$ patients. In patients with discordant results, the TDP was 9 months. Over-representation of the three parameters (CD38, ZAP-70 and del 17p) was detected in 22.5% of cases, and pointed towards even shorter TDP (4.5 months), more aggressive disease; more resistance to chemotherapy and poor outcome thus providing a precise tool for identifying high-risk patients.

In conclusion, the combined expression of CD38 and ZAP-70 together with del 17p in CLL is a precise diagnostic tool for identifying high-risk patients and convey rapid progression; they are accurate predictors of clinical outcome thus could be used to indicate when more novel chemotherapeutic approaches are needed and provided help in guiding individual patient treatment.

1. Introduction

The clinical behavior of patients with chronic lymphocytic leukemia (CLL) is heterogeneous. Some patients have indolent disease without complications for many years others develop progressive disease requiring therapy within a short time after diagnosis. Early treatment of the former could lead to therapy-related complications that might compromise their quality of life and/or survival [1,2]. Defining markers that reliably can stratify patients into groups with good-risk or poor-risk disease could facilitate clinical trials evaluating the potential benefit of early treatment [3].

Gene expression analysis found that the expression of unmutated immunoglobulin heavy chain variable region genes (IGHV) predicts more aggressive CLL than the mutated IGHV [4] as they express a relatively small subset of genes, one of which encodes the zeta-chain associated protein of 70 kDa (ZAP-70). Measurements of this intracellular protein can be used as a surrogate marker for expression of U-IGHV [5].

ZAP-70 is a member of the syk family protein tyrosine kinases (PTKs); which plays a critical role in T-cell antigen receptor (TCR) signaling and T-cell development [6]; was found to be associated with the B-cell receptor (BCR) in CLL. CLL cells expressing high levels of ZAP-70 were found to be associated with enhanced signal transduction via the BCR complex, when compared to CLL cells with low expression of ZAP-70 [5].

CD38 ligation on mature B-cells protects it against apoptosis and upregulates the expression of Bcl-2 protooncogene [7]. It was observed that IgVH gene configuration and high expression of CD38 were associated with poor prognosis in CLL.

Other abnormalities associated with poor prognosis include deletions on chromosome 17p (p53 locus). This structural abnormality reflects the loss of genetic information at specific loci and consequently loss of its tumor suppression activity which may be involved in the pathogenesis of these tumors [8] and presumably account for much of the chemotherapy resistance of this disease [2,9].

Thus the aim of this study is to evaluate patients with CLL for CD38 and ZAP-70 expression by immunophenotyping; to detect the existence of 17p deletion by conventional karyotyping and FISH technique; to detect their relation to clinical progression; response to treatment and clinical outcome of the disease and to correlate between their presence and bad prognostic parameters.

2. Subjects and methods

2.1. Study population

This study included 40 recently diagnosed, untreated B-CLL patients who were presented to Hematology Oncology Clinic, Ain Shams University Hospitals in the period from June 2008 to May 2010. They were 24 males and 16 females, with a male to female ratio of 1.5:1. Their mean age was 58.7 ± 10.9 years. Twenty healthy age- and sex-matched subjects were considered as a control group.

Patients were selected and diagnosed as CLL according to the International CLL Workshop Criteria and staged according to Binet and Rai system [10]: Peripheral blood (PB) persistent absolute lymphocytosis ($> 5 \times 10^9$/L) for at least 3 months period and a characteristic immunophenotype: $CD5^+$, $CD19^+$, $CD23^+$, restricted K or λ light chain expression and CD5/CD19 co-expression. Patients showing negative CD23 and CD5 expression were excluded.

During the follow-up period, patients were *treated* by one of the following lines depending on age, performance status and stage of the disease: Chlorambucil orally (0.2 mg/kg/day) and prednisone (20 mg/m^2/day); CVP regimen: Cyclophosphamide (400 mg/m^2/IV on days 1–3), Vincristine (1.4 mg/m^2 IV on day 1) and oral prednisone (100 mg/m^2 on days 1–5); Fludarabine (25 mg/m^2 IV on days 1–3) and cyclophosphamide (250 mg/m^2 IV on days 1–3).

Follow-up of patients to evaluate response to initial chemotherapy after 3–6 cycles. According to symptoms, organomegaly, lymphadenopathy, hemoglobin (Hb) levels, platelet counts, PB and bone marrow (BM) lymphocytes, patients were classified into complete remission (CR), partial remission (PR), progressive disease (PD), or stable disease (SD).

The patients were followed up over a period of 18–24 months to detect time to disease progression (TDP) (duration from date of initial response until disease relapse or progression) and to evaluate the disease outcome.

2.2. Methods

2.2.1. Routine investigations

All patients and controls were subjected to the following:

- Thorough clinical history and examination particularly for pallor, purpuric eruptions, hepatomegaly, splenomegaly and lymphadenopathy.

– Chest X-ray, abdomino-pelvic ultrasound, CT scan for clinical staging.
– Laboratory investigation, including liver and kidney function tests and serum lactic dehydrogenase (LDH). Complete blood count (CBC), using Coulter (GenS) (Coulter electronics, Bechman, USA) with examination of Leishman-stained peripheral blood (PB) smears

2.2.2. Bone marrow aspiration and biopsy
This was done for patients only for morphological examination and to detect the type of infiltration.

2.2.3. Immunophenotyping (IPT)
IPT of lymphocytes from lysed whole peripheral blood using Coulter Epics XL Flow cytometer (Hialeah, FL, USA)

2.2.3.1. Confirming the diagnosis of CLL using the following monoclonals (Dako). Fluorescein isothiocyanate conjugated CD45, CD3, CD4, CD20, FMC7, HLADR kappa light chains; Phycoerythrine (PE) conjugated CD5, CD23, CD10, CD22, CD56, CD79b, CD8, lambda light chain; Phycoerythrine-Cyanine 5 (PECya5) conjugated CD19.

2.2.3.2. Estimation of surface CD38 and cytoplasmic ZAP-70 expression [11] using monoclonal antibodies. PE conjugated CD38 (Dako) and FITC conjugated ZAP-70 (Becton Dickinson, San Jose, CA, Bioscience) with an intrastain kit (Dako) provided with a permeabilizing agent. Specific isotypic control for FITC, PE and PC-5 (negative control) conjugated monoclonal antibody was used.

Lymphocytes were selected in the forward scatter versus side scatter dot blot and additionally gated as CD19/CD5 positive cells. Results were expressed as a percentage of gated cells showing positive expression over the corresponding isotypic control with cutoff $>20\%$ for ZAP-70 and $>30\%$ for CD38 (Fig. 1).

2.2.4. Cytogenetic analysis of 17p (p53) deletion
2.2.4.1. Conventional cytogenetic analysis (CCA) (G-banding) [8]. Principle: It depends on the examination of spontaneously dividing cell population by blocking cell division at metaphase stage with an inhibitor of spindle formation (Colcemid). The procedure was done according to classic technique. Setup of four cultures was done under complete aseptic conditions (direct, short term, over night exposure to colcimide and 72 h cultures) followed by harvesting, slide preparation, banding and slide analysis on chromoscan image system. At least 230 banded metaphase spreads were counted and analyzed. Metaphase were karyotyped and interpreted according to the International System for Human Cytogenetic Nomenclature

2.2.4.2. Fluorescence in situ hybridization (FISH) of 17p (p53) deletion using labeled LSI probe [12] (Kreatech Biotechnology Amsterdam, Netherlands). Principle: Target DNA was denatured and hybridized to chemically modified single stranded nucleic acid (fluorophore-labeled) probe, homologous to targeted genomic DNA sequence (17p13.1) using locus specific identifier (LSI) Tp53 probe (Vysis). Reaction conditions were adjusted so that hybridization only occurs between probe and target DNA sequence of high hemology followed by counter staining by a DNA specific florescent dye (DAPI) that emits in different range.

Results were interpreted by scanning most viable metaphase and at least 100 interphase (for clear signals, no overlapping or splitting) in every case under the chromoscan.

Hybridization of LSI Tp53 probe to normal cells revealed two red signals, while abnormal cells revealed single red signal (Fig. 2). A case was considered positive for 17p deletion if $>6\%$ of nuclei showed single red signal.

2.3. Sampling

For CBC and IPT: 3 mL of peripheral blood (PB) was collected on EDTA divided in two sterile vacutainers. Another 2 mL blood was collected and left to clot for LDH. For cytogenetic analysis and FISH: 1 mL PB collected in sterile vacutainers coated with preservative free heparin. Adequate amount of BM aspirate and biopsy was used for BM examination from patients only. The PB samples were processed within 6–24 h of collection, preserved at room temperature (22–24 °C).

2.4. Statistical analysis

Data was analyzed using SPSS statistical package version 12. Numerical data were expressed as mean and standard error of mean (SEM). Qualitative data was expressed as frequency and percentage. Differences between groups were evaluated using Student's t-test for quantitative data and Chi-square (χ^2) test for qualitative variables. P value less than 0.05 was considered significant.

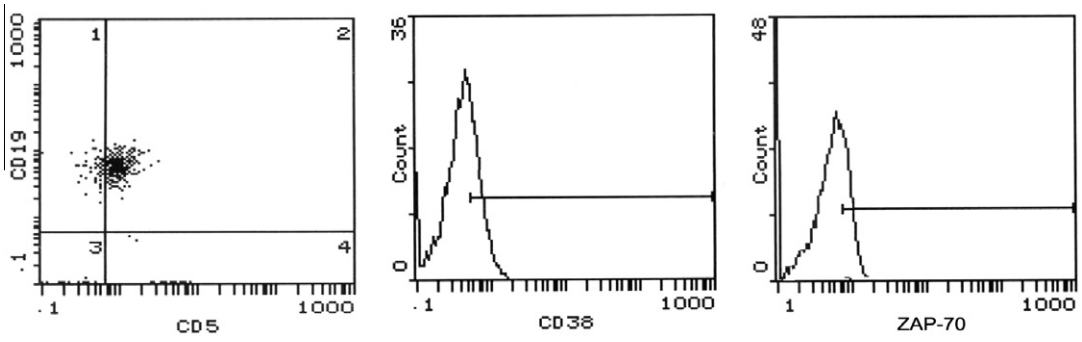

Figure 1 Flow cytometric representations of CD38 expression and ZAP-70 expression on $CD5^+CD19^+$ B-CLL cases.

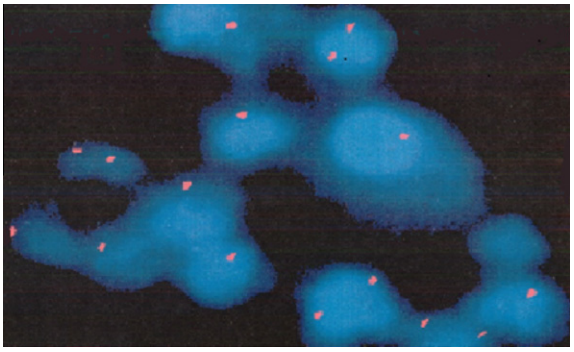

Figure 2 Interphase FISH analysis positive for p53 deletion (one red signal represents one copy of chromosome 17).

3. Results

3.1. Basic characteristics of study population

The results of this study are illustrated in Tables 1–3, Figs. 1–5.

Clinically, among the 40 studied patients 28 (70%) patients had lymphadenopathy, 24 (60%) had organomegaly. Regarding patients' clinical staging (Rai classification), 3 (7.5%) were stage 0, 4 (10%) were stage I, 10 (25%) were stage II, 8 (20%) were stage III and 15 (37.5%) were stage IV (Table 1).

After initial chemotherapy, 18 (45%) patients showed favorable response (CR or SD) while 22 (55%) showed unfavorable (PR or PD). During the follow-up period, patients were classified according to disease outcome into good 17 (42.5%) versus poor outcome 23 (57.5%). Six patients died (15%) due to causes related to CLL.

3.2. Immunophenotypic analysis

All cases were positive for CD5, CD19, CD23 expression, as well as CD5/CD19 co-expression, and showed dim surface Ig expression. Out of them 12 (30%) were positive for FMC7 expression, 17 (42.5%) for CD38, 19 (47.5%) for ZAP-70.

3.3. Cytogenetic analysis

3.3.1. Conventional cytogenetic analysis

Successful cultures with well spread metaphases were encountered in 31 (77.5%) of patients, 11/31 (35.5%) patients showed normal karyotypes. While 20/31 (64.5%) patients showed

Table 1 Comparison between measured parameters in patients with positive CD38, ZAP-70 expression and presence of p53 deletion versus negative patients.

Parameters (*n*)	CD38				ZAP-70				Del 17p			
	+(17)*n* (%)	−(23)*n* (%)	χ^2	*P*	+(19)*n* (%)	−(21)*n* (%)	χ^2	*P*	+(14)*n* (%)	−(26)*n* (%)	χ^2	*P*
Sex												
M(24)	7(41.2)	17(73.9)	3.1	0.07	10(52.6)	14(66.7)	0.81	0.28	8(57.1)	16(61.5)	0.73	0.52
F(16)	10(52.8)	6(26.1)			9(47.4)	7(33.3)			6(42.9)	10(38.5)		
LN												
+(28)	13(76.5)	15(65.9)	0.58	0.44	15(78.9)	13(61.9)	1.38	0.21	10(71.4)	18(69.2)	0.02	0.59
−(12)	4(23.5)	8 (34.8)			4(21.1)	8(38.9)			4(38.6)	8(30.8)		
OM												
+(24)	13(76.5)	11(47.8)	3.34	0.06	13(68.4)	11(52.4)	1.06	0.31	8(57.1)	16(61.5)	0.07	0.52
−(16)	4(23.5)	12(52.2)			6(31.6)	10(47.6)			6(42.9)	10(37.5)		
Rai staging												
0(3)	–	3(13)			–	3(14.3)			1(7.1)	2(7.7)		
1(4)	–	4(17.4)			1(5.3)	3(14.3)			1(7.1)	3(11.5)		
2(10)	–	10(43.5)			–	10(47.6)			–	10(38.5)		
3(8)	6(35.3)	2(8.7)	21.8	0.0001*	5(26.3)	3(14.3)	22.5	0.0001*	4(28.6)	4(15.4)	8.57	0.07
4(15)	11(64.7)	4(17.4)			13(68.4)	2(9.5)			8(57.1)	7(26.9)		
BMinfl												
D(17)	12(70.6)	5(21.7)	7.65	0.002*	13(68.4)	4(19)	9.92	0.002*	10(71.4)	7(26.9)	7.37	0.007*
N(23)	5(29.4)	18(83)			6(31.6)	17(81)			4(28.6)	19(73.1)		
LDT (m)												
<12(23)	17(100)	6(26.1)	21.8	0.0001*	18(94.7)	5(23.8)	17.7	0.0001*	12(85.7)	11(48.3)	5.35	0.02*
>12(17)	–	17(73.9)			1(5.3)	16(76.2)			2(14.3)	15(57.7)		
Resp. to tt												
Favorable(18)	1(5.9)	17(73.9)	18.2	0.0001*	1(5.3)	17(81)	20.1	0.0001*	4(28.6)	14(53.8)	5.35	0.01*
Unfavorable(22)	16(94.1)	6(26.1)			18(94.7)	4(19)			10(71.4)	12(46.2)		
Outcome												
Good(17)	–	17(73.9)	21.1	0.0001*	1(5.3)	16(76.2)	20.5	0.0001*	2(14.3)	15(57.7)	2.65	0.103
Poor(23)	17	6(26.1)			18(94.7)	5(21.7)			12(85.7)	11(42.3)		

M: male; F: female; LN: lymphadenopathy; OM: organomegaly; BMinfl: bone marrow infiltration; D: diffuse; N: nodular; LDT: lymphocyte doubling time; m: months; Resp. to tt: response to treatment.

* Significance level <0.05.

Table 2 Correlation between CD38, ZAP70 and del 17p expression and prognostic factors.

Parameters	CD38		ZAP-70		Del 17p	
	r	*P*	*r*	*P*	*r*	*P*
Age (years)	−0.71	0.66	−0.265	0.09	−0.37	0.01*
TLC ($\times 10^9$/L)	0.45	0.785	0.39	0.01*	0.05	0.75
Hb (g/dL)	−0.386	0.01*	-0.62	0.0001*	−0.39	0.01*
Plat ($\times 10^9$/L)	−0.34	0.02*	−0.53	0.0001*	−0.21	0.18
AbsLym ($\times 10^9$/L)	0.07	0.66	0.47	0.02*	0.104	0.52
BMLym (%)	0.21	0.20	0.58	0.0001*	0.22	0.16
LDT (m)	−0.58	0.0001*	−0.78	0.0001*	−0.41	0.007*
LDH (mg/dL)	0.62	0.0001*	0.77	0.0001*	0.62	0.0001*
ZAP-70 (%)	0.77	0.0001*	–	–	0.54	0.0001*
Del 17p	0.431	0.005*	0.547	0.0001*	–	

Hb: hemoglobin; Plat: platelets; TLC: total leukocytic count; Abs. lymph: absolute lymphocyte count; BMlym: bone marrow lymphocytes; LDT: lymphocyte doubling time; LDH: lactic dehydrogenase.
* Significance level < 0.05.

Table 3 Correlation between combined expression of CD38 and ZAP and some studied parameters.

Parameters(*n*)	CD38 + ZAP− (1)	CD38 − ZAP+ (3)	CD38 + ZAP+ (16)	CD38 − ZAP− (20)	χ^2	*P*
Sex						
M(24)	–	2(66.7)	8(50)	14(70)	30.5	0.33
F(16)	1(100)	1(33.3)	8(50)	6(30)		
LN						
+(28)	–	2(66.7)	13(81.2)	13(65)		
−(12)	1(100)	1(33.3)	3(18.8)	7(35)	3.55	0.29
OM						
+(24)	1(100)	2(66.7)	11(68.8)	10(50)		
−(16)	–	1(33.3)	5(31.3)	10(50)	2.42	0.49
Rai staging						
0(3)	–	–	–	3(15)		
1(4)	–	1(33.3)	–	3(15)		
2(10)	–	–	–	10(50)	31.9	0.001*
3(8)	1(100)	–	5(31.2)	2(10)		
4(15)	–	2(66.7)	11(68.8)	2(10)		
BMinfl						
D(17)	–	1(33.3)	12(75)	4(20)		
N(23)	1(100)	2(66.7)	4(25)	16(80)	11.9	0.008*
LDT						
<12(23)	1(100)	2(66.7)	16(100)	4(20)		
>12(17)	–	1(33.3)	–	16(80)	24.2	0.0001*
Del 17p						
+(13)	1(100)	2(66.7)	9(56.2)	1(10)		
−(27)	–	1(33.3)	7(43.8)	19(90)	11.8	0.008*
Resp. to tt						
Response(18)	1(100)	1(33.3)	–	16(80)	24.3	0.0001*
No resp.(22)	–	2(66.7)	16(100)	4(20)		
Outcome						
Good(17)	–	1(33.3)	–	16(80)	24.1	0.0001*
Poor(23)	1(100)	2(66.7)	16(100)	4(20)		

M: male; F: female; LN: lymphadenopathy; OM: organomegaly, BMinfl: bone marrow infiltration, LDT: lymphocyte doubling time; Resp. to tt: response to treatment.
* Significance level < 0.05.

cytogenetic abnormalities; either numerical abnormalities in the form of trisomy 12 in 6/31 (19.3%) patients or structural abnormalities as follows: del 13q14 in 5/31 (16.1%) patients, del 17p13 in 3/31 (9.6%), del 6q in 2/31 (6.5%) patients. 2/31 (6.5%) patients with 11q rearrangement and 2/31 (6.5%) had 14q rearrangement.

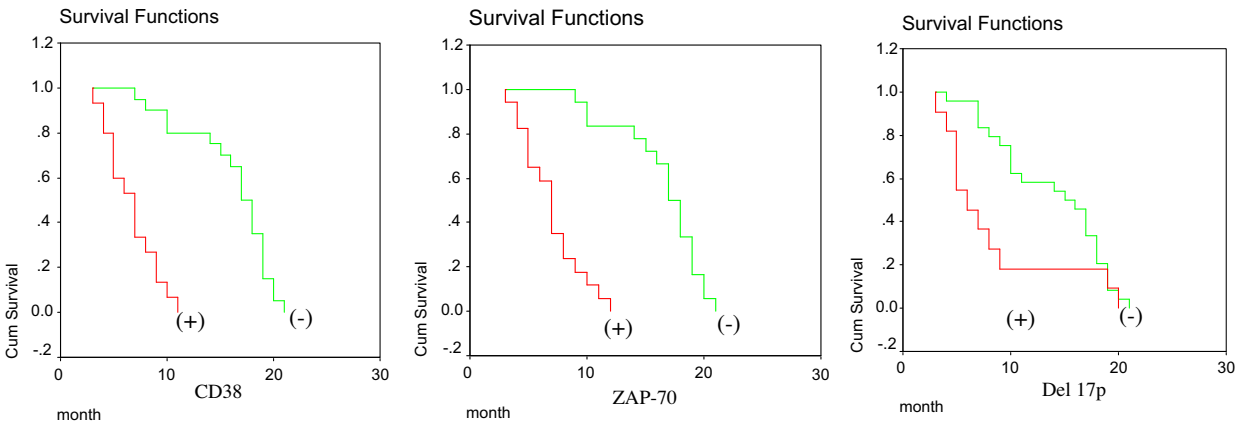

Figure 3 Kaplan–Meier curve TDP for CD38, ZAP-70 and Del 17p expression.

3.3.2. FISH analysis

All the 40 patients were re-evaluated using metaphase and interphase FISH analysis, 14 (35%) patients were positive for deletion of 17p.

3.3.3. Comparative and survival study

According to CD38, ZAP expression and presence of del 17p, patients were stratified into positive or negative expressor.

High CD38 expression was associated with advanced Rai staging, diffuse bone marrow infiltration and low lymphocyte doubling time (LDT) ($P < 0.05$; Table 1). CD38 levels were correlated negatively to Hb levels, platelet counts, LDT and positively to BM lymphocytes and LDH ($P < 0.05$; Table 2).

ZAP-70 expression was significantly associated with advanced Rai stages as 94.7% of patients expressing ZAP-70 were in stages 3 and 4 ($P = 0.0001$). Positive expression was significantly associated with diffuse pattern of bone marrow infiltration ($P = 0.007$) and with LDT <12 months ($P = 0.0001$; Table 1). ZAP-70 levels were significantly positively correlated to TLC, absolute lymphocytes in PB, BM lymphocytes and LDH values, while it was negatively correlated to Hb levels, platelet counts and LDT ($P < 0.05$; Table 2).

Regarding response to treatment and outcome, CD38 and ZAP-70 expression were significantly associated with non-responsiveness to initial chemotherapy and poor outcome ($P = 0.000$; Table 1).

CD38 and ZAP-70 levels were correlated to each other with a high statistical significant difference ($P = 0.0001$; Table 2).

Patients harboring 17p deletion had statistically significant association, as regards the pattern of BM infiltration ($P = 0.007$), LDT ($P = 0.02$), but not with the Rai staging ($P = 0.07$). 71.4% of patients with 17p deletion showed unfavorable response to initial chemotherapy which is statistically higher than those not harboring the deletion ($P = 0.0001$). However, the presence of 17p13 deletion was not statistically associated with disease outcome ($P = 0.103$; Table 1). Correlation with bad prognostic criteria of CLL showed a significant negative correlation between del 17p and age, Hb, LDT and a significant positive correlation with LDH levels ($P = 0.000$). Again, a positive significant correlation was detected between del 17p13 and both CD38 and ZAP-70 ($P = 0.005$ and 0.000, respectively; Table 2).

Combined analysis of ZAP-70 and CD38 yielded that 40% of patients were $CD38^+/ZAP\text{-}70^+$ and 50% were $CD38^-/ZAP\text{-}70^-$; $P < 0.001$), while the remaining 10% were discordant ($CD38^+/ZAP\text{-}70^-$ or $CD38^-/ZAP\text{-}70^+$) (Table 4). $CD38^+/ZAP\text{-}70^+$ patients were characterized by being associated with advanced Rai stages, diffuse type of BM infiltration, shorter lymphocyte doubling time (LDT); and show more frequent abnormalities in chromosome 17 than the other subsets of patients with a high statistical significant difference ($P < 0.05$). While $CD38^-/ZAP\text{-}70^-$ were significantly associated with earlier Rai stages, nodular BM infiltration, longer LDT (>12 m), good response to treatment and better outcome ($P < 0.05$), while the discordant groups ($CD38^+/ZAP\text{-}70^-$ and $CD38^-/ZAP\text{-}70^+$) showed variable associations (Table 4). Thus, ZAP-70 and CD38 expression analyses provided information identifying three patient subgroups with good, intermediate and poor prognosis.

The time to disease progression (TDP) of CLL patients was assessed by Kaplan–Meier curves and the Log-Rank test (Fig. 3). Patients with CLL cells that were $CD38^-$ had longer TDP (16.1 months, 95% CI 14.2–17.9), whereas those with $CD38^+$ CLL cells had shorter TDP (6.6 months, 95% CI 5.4–7.8) (Log-Rank test = 31.3; $P = 0.0001$). Again, patients with $ZAP\text{-}70^-$ CLL cells had a TDP of 16.5 months 95% CI (14.8–18.1), whereas those with $ZAP\text{-}70^+$ cells had a TDP of 6.9 months and 95% CI (5.5–9.1) with a Log-Rank test of

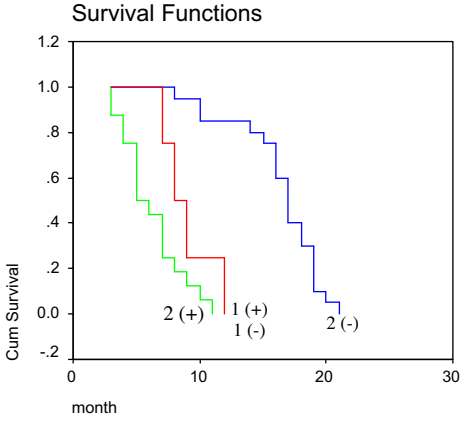

Figure 4 Kaplan–Meier curve TDP for combined CD38/ZAP-70 expression.

33.1 and a high statistical significance ($P = 0.0001$). While del 17p carriers showed a TDP of 8.2 months with a 95% confidence interval of (4.8–11.7) while non-carriers showed a TDP of 13.7 month (CI: 11.7–15.8) with a non-significant Log-Rank test ($P < 0.05$).

CLL patients with $CD38^+/ZAP\text{-}70^+$ had a mean survival time of 6.1 months with a 95% CI of (5.01–7.36), whereas those with $CD38^-/ZAP\text{-}70^-$ had a TDP of 16.3 months a 95% CI (14.7–17.8). On the other hand, patients with discordant results had a TDP of 9 months with a 95% CI (6.01–11.9) (Fig. 4).

Combined analysis of CD38, ZAP-70 and del 17p13 yielded that the three parameters were positive in nine patients (22.5%), 10 patients (25%) were positive for two parameters, three patients (7.5%) were positive for one parameter only, whereas 18 patients (45%) were negative for all parameters and were shown to run a more benign course (Table 4).

Patients with CLL cells positive for one parameter had a TDP of 12 months with a 95% CI (4.2–19.8), whereas those with two positive parameters had a TDP of 8.2 months with a 95% CI (7.0–9.5). On the other hand, patients who had CLL cells expressing the three parameters had a TDP of 4.5 months with a 95% CI (3.8–5.2), whereas those with negative CD38, ZAP-70 del 17p had a TDP of 16.7 months with a 95% CI (15.3–18.1) (Fig. 5).

4. Discussion

The current challenge is the identification of CLL patients at high risk, at time of diagnosis, for therapy-tailoring [13]. Molecular-targeted therapy, aiming to elucidate the existing genetic aberration, was found to play a crucial role in the improvement of patient response [14]. In recent years it has become evident that CLL patients with unmutated IgV_H generally have more aggressive disease and shorter overall survival [1]. CD38, a marker associated with CLL, was found to correlate with IgHV mutational status [7]. Recently, ZAP-70 was shown to be highly expressed in CLL patients with unmutated status and to be correlated with disease progression, and overall survival [4]. Thus assays for CD38 and ZAP-70 expression would yield important prognostic information for patients with CLL [5].

In the current study, 42.5% of patients showed high levels of CD38. Similar results were reported recently [15] suggesting that high expression of CD38 simply reflects an increased cellular turnover due to loss of CD38 when CLL cells enter the circulation. While other reports showed low percentage of CD38 positivity [2,7]. This discrepancy may be attributed to the fact that CD38 expression can change with time and after receiving chemotherapy which selectively eliminate the CD38 clone. Thus it was proposed that, for accurate assessment of

Table 4 Correlation between combined expression of CD38, ZAP-70 and del 17p and some studied parameters.

Parameters (*n*)	All positive (9)	2 positive (10)	1 positive (3)	All negative (18)	χ^2	*P*
Sex						
M(24)	4(44.4)	6(60)	2(66.7)	12(66.7)	1.29	0.37
F(16)	5(55.6)	4(40)	1(33.3)	6(33.3)		
LN						
+(28)	7(77.8)	8(80)	1(33.3)	12(66.7)	2.75	0.45
−(12)	2(22.2)	2(20)	2(66.7)	6(33.3)		
OM						
+(24)	5(55.6)	8(80)	2(66.7)	9(50)	2.54	0.45
−(16)	4(44.4)	2(20))	1(33.3)	9(50)		
Rai staging						
0(3)	–	–	1(33.3)	2(11.1)		
1(4)	–	1(10)	–	3(16.7)		
2(10)	–	–	–	10(55.6)	30.2	0.006*
3(8)	3(33.3)	3(30)	–	2(11.1)		
4(15)	6(66.7)	6(60)	2(66.7)	1(5.6)		
BMinfl						
D(17)	8(88.9)	5(50)	1(33.3)	3(16.7)	13.1	0.004*
N(23)	1(11.1)	5(50)	2(66.7)	15(83.3)		
LDT						
<12(23)	9(100)	9(90)	2(66.7)	3(16.7)	23.3	0.001*
>12(17)	–	1(10)	1(33.3)	15(83.3)		
Resp. to tt						
Response(18)	–	2(20)	2(66.7)	14(77.8)	20.6	0.0001*
No resp.(22)	9(100)	8(80)	1(33.3)	4(22.2)		
Outcome						
Good(18)	–	2(20)	2(66.7)	14(77.8)	20.8	0.0001*
Poor(22)	9(100)	8(80)	1(33.3)	4(22.8)		

M: male; F: female; LN: lymphadenopathy; OM: organomegaly, BMinfl: bone marrow infiltration, LDT: lymphocyte doubling time; Resp. to tt: response to treatment.

* Significance level <0.05.

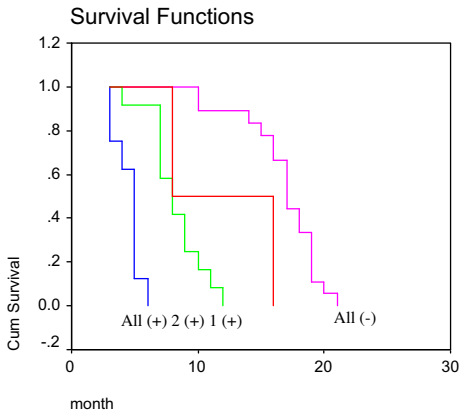

Figure 5 Kaplan–Meier curve TDP for combined CD38, ZAP-70, del 17p expression.

its prognostic significance, only samples close to or at the time of presentation was tested. Again in this study, a significant correlation was found between CD38 levels and some of B-CLL poor prognostic parameters. In accordance to earlier reports [3,16] suggesting that CD38 measurement is a powerful prognostic tool that should be considered as a standard clinical test for B-CLL patients.

Furthermore, in this study 47.5% of the patients were ZAP-70 positive as previous reports [5,6]. Analysis of the clinical characteristics of our CLL patients revealed that those who had clinical progress (more advanced clinical stage, diffuse type of BM infiltration or a LDT <12 months) also had higher ZAP-70 levels compared to patients with stable disease and that its levels were significantly correlated to some of B-CLL poor prognostic parameters. This is in accordance with earlier studies reporting that ZAP-70 expression is correlated to disease progression and aggressiveness [17]. Conceivably, some studies have not found ZAP-70 to be an independent risk factor for aggressive disease [18]. This might be related to the methods used for determining the expression of this intracellular tyrosine kinase, thus standardization using optimized flow cytometry methods can yield reproducible and consistent results as ZAP-70 was found to be a stable disease marker [19].

The present work showed the superiority of FISH over CCA in detection of minute genetic aberrations and its capability of analyzing interphase as well as metaphase, besides the CLL cells often exhibit low yields of viable metaphase despite the use of B-cell mitogens consequently 14 (35%) cases were positive for del 17p by FISH using LSI, compared to only 3 (7.5%) of all cases detected by CCA. The ability of detection of the deletion by CCA might be hampered by failure of mitosis, fuzzy chromosomal morphology, poor quality of metaphase spread, poor staining chromosomal pattern, in addition to low index and low density [8]. Again, previous works showed variable results of detected 17p deletion by FISH [14,20–22]. This discrepancy could be attributed to different methods of detection, sample size and/or various studied ethnic groups.

In this study, diffuse BM infiltration and shorter LDT were found to be significantly associated with the presence of del 17p. Again the deletion was correlated negatively with hemoglobin and positively with LDH. This deletion appear to contribute to disease progression with survival of less than 2 years [20,22].

Regarding response to treatment, this study showed that high levels of CD38 or ZAP-70 were significantly associated with non-responsiveness to initial chemotherapy indicating that these biological factors might be used to predict the chemo-sensitivity of B-CLL patients as previous studies [1,17]. Other investigators added that the overall response rate and response duration could be worse in those patients [23].

Again, our patients with del 17p were significantly more resistant to therapy than non-deleted cases. Similarly previous studies [13,24] showed that abnormalities of 17p convey resistance and alter the sensitivity of CLL cells to chemotherapeutic agents with lower response rate and early relapse after autologous stem cell transplantation. Others showed evidences on the efficiency of anti-monoclonal antibody therapy in CLL with 17p gene abnormality [1].

In the present study, disease outcome was statistically associated with high expression of CD38 or ZAP-70 on the lymphocytes as previously reported [23]. The expression of these markers was associated with adverse outcome in CLL and was considered as an important risk factors in these patients. However, despite having 17p13 deletion, no statistically significant association was detected with disease outcome in our patients. This was also previously documented [21]. On the contrary, the association between 17p13 deletion and poor outcome has been previously reported [8,13,14,22].

CD38 is a cell-surface receptor mediating interactions which sustain proliferation and survival [9]. Co-expression of CD38 and ZAP-70 in CLL was suggested to form part of the same signaling pathway or that they intersect in some steps. CD38 ligation directly triggers the cytoplasmic tyrosine kinase ZAP-70 phosphorylation [23]. It was proved that CD38 pathway is selectively active in $CD38^+/ZAP\text{-}70^+$ cells, while $CD38^+/ZAP\text{-}70^-$ patients were consistently unable to signal via CD38 [25].

The existence of a functional link between the two molecules in neoplastic B-cells prompted us to evaluate the effects of their coexpression. In this study, combined analysis of ZAP-70 and CD38 yielded discordant results in 10% of patients, whereas 50% of the patients were concordantly negative and 40% of them were concordantly positive for ZAP-70 and CD38 expression in accordance to a previous study [19]. Clinically, advanced Rai stages, diffuse BM infiltration together with LDT less than 12 months were mostly associated with $CD38^+/ZAP\text{-}70^+$ group followed by the discordant group and were least with $CD38^-/ZAP\text{-}70^-$ group. Thus ZAP-70 and CD38 expression provided complementary prognostic information identifying three patient subgroups with good, intermediate and poor prognosis. Similar results were obtained in a previous report suggesting that concordantly positive group was associated with an active and aggressive disease with greatest proliferative potential [3] thus it was proposed that simultaneous testing of CD38 and ZAP-70 provides a tool for risk stratification of patients [1].

In this study, $CD38^+/ZAP\text{-}70^+$ group was found to be more significantly resistant to chemotherapy as previously reported [1,3] that these patients required treatment with polychemotherapy or combined immunochemotherapy compared with $CD38^-ZAP\text{-}70^-$ patients who were either untreated or treated with chorambucil monotherapy.

Our $CD38^+/ZAP\text{-}70^+$ patients had a mean time to disease progression of 6.1 months, whereas those with $CD38^-/ZAP\text{-}70^-$ had a TDP of 16.3 months, on the other hand patients who where discordant, had a TDP of 9 months as previously reported [16].

In this study, there was a significant association between CD38%, ZAP-70 expression and presence of del 17p. The three parameters were detected on the lymphocytes of 22.5% of patients and were all absent in 45% of patients. While, two of them were present in 25% and only one parameter was positive in 7.5% in accordance to previous reported data [23].

Again, $CD38^+$/$ZAP\text{-}70^+$ patients who harbored del(17p) were significantly associated with advanced stages of Rai staging, diffuse type of BM infiltration, lower LDT, and were more resistant to treatment than those expressing low levels, in excellent agreement with the published literature [24]. It was also shown that cases with high CD38, ZAP-70 and harboring del(17p) had significantly increased resistance to chlorambucil and fludarabine [1].

Kaplan–Meier analysis of the patients showed significantly lower TDP (4.5 months) in patients with higher ZAP-70, CD38 expression and carrying del 17p, while those with negative CD38, ZAP-70 and del 17p had a TDP of 16.7 months, whereas TDP was 8.2 months in patients with two parameters positive, and was 12 months in patients with cells positive for one parameter only. Similar results were previously shown by other investigators [23] who added that over representation of the high-risk genomic aberration 17p deletion in B-CLL discordant for CD38/ZAP-70 pointed towards a distinct biologic background of the observed disease subgroups. This finding was also supported by gene expression profiling where the expression of 37 genes differed significantly between the groups defined by their expression of ZAP-70 and CD38, including genes that are involved in regulation of cell survival and chemotherapy resistance [13].

In conclusion, the combined expression of CD38 and ZAP-70 together with del 17p in CLL is a precise diagnostic tool for identifying high-risk patients and convey rapid progression; they are accurate predictors of clinical outcome thus could be used to indicate when more novel chemotherapeutic approaches are needed and provided help in guiding individual patient treatment.

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