The impact of selected apoptotic markers and adhesion molecules on response to chemotherapy and prognosis of chronic lymphocytic leukemia

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Abstract: Aim: to assess the impact of selected apoptotic markers and adhesion molecules on the treatment outcome of CLL. **Patients and Methods:** Thirty newly diagnosed patients and 10 normal persons were studied. Proapoptotic markers (BCL2, P53, and CD95) and adhesion molecules (CD18, CD54) were assessed by flowcytometry (FCM) before and after treatment. P21 mRNA was assessed by RT- PCR. Patients received induction chemotherapy with CVP Regimen. **Results:** The study included 23 males and 7 females. Median age was 66years (Rage 53to79). There were increase of P53 and BCL2 and reduction of CD18 and CD54 in cases compared to control .CD95 didn't show significant increase compared to control. There were reduction in BCL2 after than before treatment and increase in CD18 and CD54 after than before treatment. Evaluable patients showed 11 complete remission (CR) (39.3%), 7 (25%) partial remission, 6 progressive diseases (21.4%) and 4 stable disease (14.3%). The observation after treatment was 2 to 13 months. Median time to disease progression (TTP) of the responding patients was 9 months. Death was reported in one case. There was increase in P53 and BCL2 expression in patients who did not achieve CR compared to those with CR and increase in CD18 level in CR than no CR cases. No Significant differences were found regarding the expression of CD95 and CD54 between both groups. Comparing the level of expression of the studied markers at initial presentation, there were increase in P53 and decrease in CD18 level in patients with no CR compared to those with CR. There was association in the expression of P53 and BCL2. Inverse relation between the expression of P53 and CD18 as well as between BCL2 and CD 18were reported at presentation. High BCL2 level after treatment was significantly associated with short TTP. There was inverse relation between P21 mRNA and P53 levels before treatment and decrease in P21 level was associated with bad prognosis and poor response to treatment. **Conclusion:** Measurement of P53 and CD18 level can define a group of patients with chemo refractoriness and disease aggressiveness, a group who may require different or more aggressive therapy. BCL2 level after treatment was correlated with TTP. [Nature and Science 2010; 8(8):229-235]. (ISSN: 1545-0740).

Key words: apoptotic markers; chemotherapy; chronic lymphocytic leukemia.

1. Introduction

B-cell CLL represents a neoplastic disorder caused primarily by defective programmed cell death (PCD), as opposed to increased cell proliferation. Defects in the PCD pathway play a vital role in the pathogenesis of the disease, thus creating a selective survival advantage for CLL clone (1, 2). The tumor suppressor gene P53 plays a vital role in apoptosis (PCD) through induction of G1 arrest thus preventing its entry to S phase following DNA damage (3). This occur through the influence of P53 over many genes including BCL2 and Bax genes, following DNA damage P53 up regulate the Bax gene (a proapoptotic gene) and suppresses BCL2 (an anti apoptotic gene) leading to apoptosis. Also increase in P53 level may lead to activation of Fas (CD95)/FasL interaction leading to apoptosis through activation of caspase cascade (4).

P21 (a recognized mediator of P53), one of the cyclin dependent kinase inhibitors, induces growth arrest in response to DNA damage, high level of P21 is found in cells arrested at G0/G1 phase and expression appears to correlate inversely with the cell proliferation index. This occurs by inhibiting protein kinase activation of G1 cyclin/CDK complex (5).

Leucocyte Function Antigen 1 (LFA-1) or CD18 expressed on all leucocytes and induces proliferation and T cell mediated cytotoxicity as well as B cell aggregation and immunoglobulin production through interaction with cell adhesion molecules as CD45, CD2, CD58, CD44 and CDW29. This role is also played by CD54 which together with LFA-1 are capable of facilitating adhesion during a secondary immune response (6).

Intracellular adhesion molecule 1 (ICAM1) or CD54 expression increases on malignant cells as

well as during acute and chronic inflammation (7). Both ICAM1 and LFA1 play an important role in cell adhesion although each one has its own legand (8).

The aim of this study was to evaluate the clinical significance of CD18, CD54 BCL2, P53 and CD95 by FCM, in monitoring the patients' prognosis and to evaluate their possible role in mediating disease progression and response to chemotherapy in CLL cases. Also to evaluate expression of P21 mRNA in cases with high P53 levels by RT-PCR to assess its significance as a prognostic factor.

2. Patients and Methods

This study was conducted at the Medical Oncology and Clinical Pathology Departments of the National Cancer Institute (NCI) – Cairo University. Thirty newly diagnosed CLL patients were included, they were 23 males (76%) and 7 females (24 %), their ages ranged between 53to79 years old with a median of 66 years. Ten age and sex matched normal volunteers were selected as controls. The diagnosis was established according to the International Workshop on CLL (9). Immunophenotyping had been assessed to confirm the diagnosis. Patients were eligible for treatment according to the following criteria: ECOG performance status 0 -II with high risk category (Rai stage III-IV) or Rai stage I-II if they have at least one of the followings: one or more of the disease related symptoms, progressive marrow failure, massive splenomegaly or lymphadenopathy or progressive lymphocytosis > 50 % in 2 months or lymphocyte doubling time < 6 months**.**

Eligible patients were subjected to full history taking and clinical examination (lymphadenopathy and hepatosplenomegaly taken into consideration). Chest x ray and abdominal and pelvic ultrasonography were performed to all cases.

Full blood picture and bone marrow examination were done. Immunophenotyping was assessed using flowcytometery partec III from DAKO cytomation to confirm the diagnosis of CLL using FITC conjugated; CD5, CD3, CD4, CD20, FMC7, HLA-DR , kappa light chains and RPE conjugated CD19, CD23, CD10, CD22, CD79b, CD8 and lambda light chain. Patients as well as the control groups were evaluated for the expression of apoptotic markers BCL2 (clone 124), P53 (clone D0-7), CD 95(clone β-G34), and adhesion molecules, CD18 (clone MEM48) and CD54 (clone 65β5) from Dako. In addition to isotopic control which were obtained from Becton Dickinson (Mountain view, California). Fixation and permeabilization kits (Dako, Denmark) were used.

Results were expressed as a percentage of cells showing positive expression. Re-evaluation of all markers was done after 3 cycles of treatment.

Ten cases with high P53 level (mean value was 8.4%) were tested for relative quantitation of P21 mRNA before and after 3 cycles of treatment according to the following principle:

mRNA was extracted using. QIAmp® RNA minikit supplied by (QIAGEN company) from 2 ml whole blood on EDTA followed by reverse transcription using Taq Man Gold RT- PCR kit (N808-O232).Using the following thermal cycler parameters.

- 25°c for 10min.
- 37°c for 2hrs.
- 95°c for 5min.
- 4°c pause

Five µl of the extruded DNA sample mixed with 1.25ul of the primer probe Solution and 12.5µl of master mix supplied by (QIAGEN company) (The Taq Man EZ (N808-O236) RT-PCR master mix core reagent kit and the mixture was transferred to micro Amp optical 96 well reaction plate and transferred to the thermal cycler block ABI prism 7000 sequence detection system supplied by (Applied Biosystems AB) according to the following thermal protocol.

- 95°c for 10min. (DNA polymerase activation)
- 95°c for 15sec. (Denaturation step)
- 60°c for 1 min. (PCR annealing and extraction).

Eligible patients were treated with CVP regimen which was given as following:

Cyclophosphamide 400 mg/m^2 I.V. D1 to D3, Vincristine 1.4 mg/m^2 D1 and Prednisone 100 mg D1 to D5 P.O. Cycles to be repeated every 21 days**.**

Evaluation of response to chemotherapy was done according to the following criteria:

Complete remission (CR): Asymptomatic patients with no organomegaly or lymphadenopathy, lymphocyte count≤ 4x 10^9/l, neutrophils ≥ 1.5x10^9/l, Hemoglobin (HB) level ≥11gm/dl, platelet count >100x 10^9/l and bone marrow (BM) lymphocyte <30%.

Partial remission (PR) : ≥50%decrease in organomegaly/or lymphadenopathy plus one of the following: neutrophil ≥ 1.5x10^9/l, HB ≥ 10gm/dl or ≥ 50% improvement, platelet count> 100x10^9/l or ≥ 50% improvement. Criteria for CR and PR should be maintained for two months.

Progressive disease (PD): new lesion or ≥50% increase in organomegaly or lymphadenopathy, circulating lymphocyte >50% increase.

Stable disease (SD): All other than the above.

Statistical Methods

(SPSS) version 12 was used for data analysis t-test and chi-square test was used for comparative analysis, ANOVA for comparing means for more than 2 independent groups. Pearson correlation coefficient (r). P-value is significant at 0.05 levels.

3. Results

The clinical and hematological characteristics of the patients and control groups presented in table (1). As shown in table (2), there was significant increase of P53 and BCL2 in patients as compared to control group (P= 0.04 and 0.001 respectively). There was also significant reduction of CD18 and CD54 in CLL cases compared to control (P= 0.001). Although CD95 showed increase expression level in the patients, it did not reach a statistical significant level compared to control (P= 0.06).

Table (3) shows the correlation between the studied markers and different hematological parameters.

There was highly significant reduction in BCL2 after than before treatment (P= 0.001) but high significant increase in CD18 and CD54 have been reported after than before treatment (p=0.001 and o.o1respectively).

Response to treatment:

Out of twenty eight cases who were evaluable for response, 11 patients achieved complete remission CR (39.3%), 7 patients (25%) had partial remission (PR), 6 showed progressive disease (PD) (21.4%) and 4 had stable disease(SD) (14.3%). The observation period after the end of treatment ranged between 2 to 13 months. The time to disease progression of the responding patients (CR and PR) showed a median of 9 months (Range 4 to 13). TTP showed negative correlation to TLC(r =- 0.5 and P=0.02) and positive correlation to HB level and platelets count (r=0.5 and P=0.02, and r=0.5and P=0.02 respectively). Death was reported in one case only due to progressive disease. No relation was found between different stages and the response to chemotherapy (P= 0.1).

There was significant increase in P53and BCL2 expression levels in patients who did not achieve CR compared to those with CR (P= 0.02 and 0.001 respectively). Also there was significant increase in CD18 expression level in patients with CR than those who didn't achieve CR (p=.001). No Significant differences were found regarding the expression of CD54and CD95 between both groups.

Comparing the level of expression of the studied markers at initial presentation in the CR group and those who did not achieve CR (PR, SD and PD), it was found that there is a significant increase in P53 expression level in patients who didn't achieve CR compared to those with CR. The level of expression increases in an ascending manner from PR to PD. There is statistically significant decrease in CD18 in patient with no CR compared to those with CR and the level of expression showed more decrease in non responding cases (SD, PD) table 4.

There was significant increase in P53 and BCL2 expression levels after treatment in patients with poor response (PR, PD and SD) compared to those with CR. Again the post treatment BCL2 levels were higher in less responding cases. The after treatment results of CD18 showed a statistical significant decrease in CD18 in patients with poor response(PR, PD and SD) compared to those with CR with lower levels in less responding cases (SD,PD) Table 5.

A significant positive correlation between the expression of P53 and BCL 2 (r= 0.56 and P=0.002) and a significant negative correlation between the P53 and CD18 (r= 0.67 and P= 0.001) as well as between BCL2 and CD 18(r= 0.8 and P= 0.001) were reported. There was negative correlation between TTP and P53 expression as well as between BCL2 expression level after treatment and TTP (r=-0.5, P= 0.01).

A negative correlation was found between P53 expression and P21 mRNA before treatment (r= 0.675, P= 0.04) however no correlation was found between both parameters after treatment.

Table (6) showed the expression of p21 mRNA before and after treatment with different response criteria.

4. Discussion

In our study we found that the TTP showed positive correlation with the HB level and platelets count and negative correlation to TLC which was reported by others (10, 11). We encountered an increase in BCL2 expression in all patients as compared to control group which is also reported by other studies (12, 13). Patients who attained CR showed significant reduction of BCL2 expression level while those who didn't achieve CR showed variable levels of expression the highest of which was encountered in advanced cases. This was in agreement with Schimmer et al (13) and Khorshed etat (14). A strong negative correlation was found between TTP and BCL2 expression after treatment, this is in agreement with other studies (14, 15).

Our results showed that negative or faint P53 level of expression was associated with good prognosis and CR, while higher levels of P53 were expressed in non responding cases(SD ,PD) this is in agreement with other Studies (16, 17). We found no significant difference in level of expression of P53 before or after treatment indicating that P53 mediated apoptosis signals might be defective in these

leukemia cells. Recent studies showed the presence of del (17p13) and/or abnormal function of TP53 gene have consistently been shown to identify CLL patients who are unlikely to respond to purine nucleoside analogues (Fludarabine, Pentostatin or Cladribine), Alkylating agents (Chlorambucil, Cyclophosphamide or Bendamustine) and combinations of these drug classes with or without Rituximab (18,19,20,21).

Individuals with TP53gene abnormalities do respond to other therapies with different apoptotic pathway such as high-dose methylprednisolone and alemtuzumab. Therefore, TP53abnormalities is considered a useful tool to identify patients with aggressive disease particularly younger patients who may be in need of different and/or more intensive therapeutic strategies including allogeneic transplantation (22, 23). We found significant correlation between TTP and high P53 expression level which is in agreement with Cordone et al (24) and Shanafelt et al (25) who found that high P53 expression to be associated with short lymphocyte doubling time and rapid relapse. There was significant reduction of CD18 as compared to control group and this is in agreement with Nadkarni et al. (26). There was a further reduction of the level of expression of CD18 in patients who didn't show CR (PD and SD) compared to those who achieved CR indicating that low CD 18 expression level was associated with advanced disease and chemorefractoriness. Angelopoulou et al (27) reported low CD 18 expression to be associated with diffuse bone marrow infiltrate and advanced disease.

In our study significant relations were found between high P53 and low CD18 expression levels and short TTP and this was in accordance with others (25, 28).

The significant inverse relation between P53 and CD18 level of expressions and their strong correlation to TTP suggest it as one of the prognostic models in CLL.

Our study showed significant reduction of CD54 expression level compared to control group this was consistent with the results of others (29).

We found that B CLL cells express very low level of CD95/Fas before and after treatment regardless of their response to chemotherapy suggesting no or defective fas/CD95 mediated apoptosis pathway in CLL and indicating that CD95 does not play an important role as a prognostic marker this was also reported by Romano et al. (30)

We found significant negative correlation between levels of expression of P53 and P21 mRNA before treatment and this was association with disease progression. Cobo et al (31) reported that high p53 expression with low p21level was exclusively found among p53 mutated CLL and this was associated with progressive cases. Similar finding was reported also by Cordone et al who stated that p21 gene is induced by wild type p53 but not mutant p53 and p21 seems a reliable strategy of p53 gene status assessment. (24).

5. Conclusions

BCL2 was expressed in all cases which highlight the importance of its incorporation in the studied panel of CLL cases. BCL2 level of expression after treatment is correlated to TTP**.**

Measurement of P53 and CD18 level can define a group of patients with aggressive disease and chemorefractoriness who may require different or more aggressive treatment modalities. Our study didn't show a beneficial impact of measuring CD95 in predicting the patients' response to chemotherapy suggesting no or defective fas/CD95 mediated apoptosis pathway in CLL. Larger numbers of cases are needed to assess the exact significance of measuring P 21 mRNA level in CLL

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Table (1): Shows clinical and hematological characteristics of both patients and controls.

	Patients	Controls
Number	30	10
Sex (Male/Female)	23/7	6/4
Age range (years)	53-79	40-70
Rai staging: Stage II Stage III Stage IV	 3 20 7	
Mean HB concentration (g/dl)	9.7±2.3	13.2±5.1
Mean TLC($X10^6$/L)	123.6±71.7	9.2±3.1
Mean Platelets count ($x10^6$/L)	192.6±135.8	443±180
ALC (10^6/L)	76.8±18.3	4.3±1.2
Lymphocytes in BM%	70.6±15.1	Not done

TLC (total leucocyte count), BM bone marrow, ALC (absolute lymphocytes count).

Table (2): Comparison of the studied markers in CLL patients before treatment with the control group

parameter	CLL (mean ±SD %)	Control group (Mean ±SD %)	P-value
p53	7.3±11.1	0.01±0.01	0.04
Bcl-2	76.2±15.2	9.6±0.4	0.001
CD95	0.6±0.4	0.2±0.2	0.06
CD18	23.4±21.4	72.9±7.1	0.001
CD54	2.9±2.9	32.6±4.9	0.001

Table (3): Correlation between the studied markers and different hematological parameter.

		TLC	ALC	Hb	Platelets	B.M. Lymph
p53	r	0.699	0.4	-0.1	-0.2	0.3
	p	0.001	0.02	0.4	0.2	0.08
BCL-2	r	0.2	0.4	-0.1	-0.3	0.4
	p	0.1	0.02	0.4	0.06	0.02
CD95	r	0.07	0.1	0.2	-0.2	0.06
	p	0.7	0.5	0.28	0.2	0.7
CD18	r	-0.67	-0.7	0.3	0.3	-0.3
	p	0.001	0.001	0.1	0.06	0.03
CD54	r	-0.2	-0.2	0.3	0.04	-0.3
	p	0.2	0.1	0.09	0.8	0.058

TLC (total leucocytes count), ALC (absolute lymphocytes count), and B.M. lymph (bone marrow lymph)

Table (4): comparison between the studied markers at presentation with different response to treatment

		CR	PR	SD	PD	P value
No		11	7	3	7	
P53	Mean ±SD	0.3±0.1	4.4±4	15.7±6.4	19.5±15.6	0.001
Bcl-2	Mean ±SD	69.5±17.4	80.1±7.5	94.8±4.6	75.7±16.5	0.7
CD95	Mean ±SD	0.6±0.5	0.4±0.3	0.9±0.1	0.7±0.4	0.3
CD18	Mean ±SD	44.2±18.3	14.3±16.4	4.1±0.86	7.7±2.4	0.001
CD54	Mean ±SD	3.9±1.8	0.5±0.4	1.4±0.92	3.9±4.9	0.06

CR (complete remission), PR (partial remission), SD (stable disease), and PD (progressive disease).

Table (5): Comparison between the studied markers after treatment with different response criteria.

		CR	PR	SD	PD	P value
no		11	7	3	7	
P53	Mean ±SD	0.3±0.3	3.8±3.1	14.6±5.7	17±10.4	0.001
Bcl-2	Mean ±SD	3.6±3.8	49.4±16.5	57.9±24	73.9±19.3	0.001
CD95	Mean ±SD	0.7±0.7	0.8±0.2	0.9±0.1	0.7±0.3	0.9
CD18	Mean ±SD	58.5±15.3	28±4.7	8.6±2.4	11.2±5.8	0.001
CD54	Mean ±SD	5.9±2.5	14.1±15.3	2.9±1.4	6.3±6.1	0.1

CR (complete remission), PR (partial remission), SD (stable disease), and PD (progressive disease).

Table (6): Expression of p21 mRNA before and after treatment with different response criteria

Response	P21 before treatment, (mean ±SD) (range) pg/µl	P21 after treatment, (mean ±SD) (range) pg/µl
PR	52	71
PD	5.378. ± 11.1(0.1-14.1)	4.1±3.7 (1.03-11.3)
SD	8.7±11.1 (0.5-21.5)	6.6±6.5 (1.6±14.1)
Total	11.6±16.5 (0.1-52)	11.6±21.4 (1.03-71.4)