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P53 Dysfunction in Chronic Lymphocytic Leukemia

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ABSTRACT

Introduction: Chronic Lymphocytic Leukemia (CLL) is characterized by a highly variable clinical course. Some of this variability can be attributed to the tumor suppressor protein p53 which regulates the transcription of a number of genes including the cycline-dependant kinase inhibitor of p21 and the antiapoptptic protein BCL-2. As p53 mutations occur in only 10% to 15% of patients with CLL, it is possible that p53 dysfunction occurs in the disease through alternative mechanisms. For example, 15 to 35% of patients have an extra copy of chromosome 12 which encodes the p53 inhibitory protein MDM2. Indeed over expression of MDM2 has been reported in CLL.

The Aim of this Study: is to determine p53 dysfunction in CLL patients as detected by impaired up regulation of p53 and p21 in response to chemotherapy, determine if p53 dysfunction is caused by MDM2 over expression, correlate CD38 and BCL-2 to the presence of p53 dysfunction and to correlate their percentage expression with other prognostic factors, treatment outcome and survival.

Results: This study included forty patients with CLL. In addition, ten subjects of matching age were used as a control group. Patients were grouped according to p53 response to chemotherapy into 3 groups: group "1", normal response (p53+, p21+). Group "2", Type A p53 dysfunction (p53+, p21–). Group "3", Type B dysfunction (p53–, p21–).

The highest p53 percentage expression was detected in group "2" (mean, 39.5±24.3SD) compared to group "1" (mean, 10.16±18.8SD) and group "3" (mean, 2.5±1.8SD) patients. The difference between groups was statistically highly significant.

P21 showed highest percentage expression in group "1" (mean, 16.1±6.6 SD) Compared to group "2" (mean, 2±1.3 SD) and group "3" (mean, 1.5±1.4 SD) patients (p<0.001).

Group 3 patients showed the highest MDM2 expression (mean, 2.36±2.21SD) Compared to group "1" (mean, 0.48±0.34 SD) and group "2" (mean, 0.23±0.36 SD) patients (p<0.001). On evaluating MDM2 by MFI, the highest value was detected in group "3" patients (a mean of 12.93±10.1 SD) Compared to groups "1" and "2" patients (mean, 6.95±6.03 SD and 1.25±0.22 SD respectively) (p=0.009).

There was no statistically significant correlation between CD34 expression and P53 function (p=0.2).

Among patients with advanced stages (III & IV) of the disease, a significantly higher percentage of patients was detected in group "2" and "3" compared to group "1" (p=0.008).

Patients with normal p53 response had a higher response rate and longer time to disease progression (mean 12 months ±5.1 SD) compared to patients with type A (mean 5.8 months ±2.3 SD; p=0.001) and type B (mean 9 months±4.4SD; p=0.08) p53 dysfunction.

The median overall survival for the whole group was 22.5 months (range 3-30 months). A higher percentage of dead patients was detected in group "2" (54.5%) and group "3" (45.5%) compared to normal response group (0%) patients (p=0.02). A negative correlation was found between percentage expression of p53 (r=–0.425, p=0.006), MDM2 (r=–0.61, p=0.07), BCL-2 (r=–0.09, p=0.57) and overall survival.

Conclusion: Causes of p53 dysfunction (other than p53 mutation) should be considered in CLL patients. High MDM2 expression level is associated with advanced stage of the disease, decreased response rate, TDP and overall survival. So MDM2 may be used as a prognostic and predictive marker for response and survival. Drugs that target MDM2-p53 interaction should be investigated for clinical applications in the treatment of CLL.

Key Words: *P53 dysfunction – CLL.*

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of predominantly non-dividing clonal mature B cells in the blood, bone marrow, lymph nodes, and

spleen and by a highly variable clinical course **[1]**. Some of this variability can be attributed to the tumor suppressor protein p53. Thus, p53 gene dysfunction in CLL is strongly associated with large cell transformation **[2]**, resistance to therapy with purine analogues **[3]**, and shortened patient survival **[4]**.

By triggering apoptosis or cell cycle arrest in response to DNA damage, p53 contributes to the cytotoxic action of many chemo therapeutic agents and protects the genome from mutagenic insult **[5]**. In quiescent cells, levels of p53 protein are low owing to its short half life. After DNA damage, the half life of p53 becomes prolonged **[6]**, and the protein accumulates in the nucleus **[7]**, where it regulates transcription of a number of genes, including the cyclin-dependant kinase inhibitor of $p21^{WAF1/CIP1}$, the proapoptotic protein BAX **[8]**, and the anti-apoptotic protein Bcl-2 **[9]**.

P53 mutations typically prolong the half life of the protein in the absence of DNA damage and are, therefore, associated with increased basal levels **[10]**. However, even when activated, mutant p53 protein cannot regulate gene expression because of its inability to bind to specific DNA sequence **[11]**. As P53 mutations occur in only 10% to 15% of patients with CLL **[12]**, it is possible that p53 dysfunction occurs in the disease through alternative mechanisms. For example, 15 % to 35% of patients have an extra copy of chromosome 12 **[4]**, which encode the p53 inhibitory protein MDM2. Indeed, MDM2 over expression has been reported in CLL and may be associated with triosomy 12 **[13]**.

The aim of this study is to determine p53 dysfunction in CLL patients as detected by impaired up regulation of p53 and p21 in response to chemotherapy, determine if p53 dysfunction is caused by MDM2 over expression, correlate CD38 and BCL-2 to the presence of p53 dysfunction and to correlate their percentage expression with other prognostic factors, treatment outcome and survival.

MATERIAL AND METHODS

The present study was carried out at the National Cancer Institute, Cairo University during the period between December 2003 and June 2006.

Forty patients with chronic lymphocytic leukemia (CLL) are included in the study. Their ages ranged between 35-79 years with a median of 66 years. In addition 10 normal subjects of matching age were used as a control group.

The studied groups were subjected to thorough history, physical examination, complete blood count, bone marrow examination and immunophentyping using flow cytometer partec III, to confirm the diagnosis of CLL using a wide panel of monoclonal antibodies purchached from DAKO (Denmark) and Santa Cruz Biotechnology (U.S.A), including FITC conjugated (CD45, CD5, CD3, CD4, CD20, FMC7, HLA-DR and Kappa light chains) and PRE conjugated (CD19, CD23, CD10, CD22, CD79b, CD8 and lambda light chains). Specific isotype control for FITC, PRE conjugated monoclonal antibodies were used. Results were expressed as a percentage of cells showing positive expression. The cut off values were calculated from the control group.

Other laboratory tests were also done including, liver and kidney function tests, Coomb's test, serum lactate dehydrogenase (LDH) level, B2 microglobulin and serum protein electrophoresis.

Radiological examination including chest X-ray, abdominal ultrasound and/or CT scan was done whenever needed for proper clinical staging of the disease.

The diagnosis of CLL was based on the criteria established by the International Work Shop on CLL and the National Cancer Institute-Sponsored Working Group Guidelines for CLL (NCI-WG) **[14]**. All cases were staged according to Rai system **[15]**.

Patients were treated by one of the following lines of chemotherapy depending on performance status and stage of the disease:

- *Chlorambucil (Clb) and prednisone:* Clb was given orally at a dose of 0.2mg/kg/day and prednisone 20mg/m^2/day for 14 days.
- *Cyclophosphamide, Vincristine, Prednisone (CVP):* Cyclophosphamide: 400mg/m^2 IV on days 1-3, Vincristine: 1.4mg/m^2 IV on day 1 and oral Prednisone 100mg/m^2 on days 1-5.
- *Fludarabine and cyclophosphamide (FC):* Fludarabine 25mg/m^2 IV on days 1-3 and

cyclophosphamide 250mg/m^2 IV on days 1-3 (for patients with refractory or resistant disease to CVP regimen).

Evaluation of response to chemotherapy had been made according to the following criteria:

- *Complete remission (CR):* Asymptomatic patients with no organomegaly or lymphadenopathy, lymphocyte count <$4x10^3$/µl, neutrophils >$1.5x10^3$/µl, hemoglobin (Hb) >11gm/dl, platelet count >$100x10^6$/µl and bone marrow lymphocytes <30%.
- *Partial remission (PR):* >50% decrease in organomegaly or lymphadenopathy plus one of the following: Neutrophils >$1.5x10^3$/µl hemoglobin (Hb) >11gm/dl, platelet count >$100x10^6$/µl.
- *Progressive disease (PD):* New lesion or >50% increase in organomegaly or lymphadenopathy, circulating lymphocytes revealing >50% increase.
- *Stable disease (SD):* Patients who do not fit the criteria for CR, PR or PD.

To screen for p53 dysfunction, CLL cells were examined (after 3 to 6 cycle of chemotherapy with a median of 4 cycles) for an impaired p53 response. To do this, we tested the effect of chemotherapy on the expression of p53 and other proteins reported to be transcriptionally activated (p21) or repressed (BCL-2) by p53. CD38 was also measured to be correlated with p53 dysfunction.

P53, MDM-2, BCL-2 and CD38 monoclonal antibodies were purchased from DAKO (Denmark). P53, MDM-2 and BCL-2 were measured intra-cytoplasmic using intrastain fixation and permebilization fit purchased from Dako. CD38 was measured as surface expression.

Results were expressed as a percentage of cells showing positive expression (Figs. 5-7). For MDM2 results were also expressed as mean fluorescent index, by dividing the mean fluorescent intensity of the monoclonal by that of the control. A cut off value of 10% and 30% were used for interpretation of BCL2 and CD38 positivity respectively **[16]**.

To establish the proper cut off value for either p53 or MDM2, a Roc curve was done (Figs. 2,3). Using this curve, a threshold of 4.95 and 0.09 percentage positivity was found to be appropriate for p53 and MDM2 respectively, above which the results were considered positive. A Roc curve was also done for MDM2 MFI and a cut off of 1.16 was established (Fig. 4).

P21 was measured by immunocytochemistry using mouse monoclonal antibody p21WAF1 Ab-5 (Clone HZ2) (Neo Markers) (REF: MS-387-Po, lot: 387 P405A). The detection kit used was Dako Envision system.

Quantification of positive cells was evaluated in 5 or more fields of each slide until a minimum of 1000 total cells had been examined. Percentage of cells showing p21 nuclear positivity was then calculated (Fig. 8).

A cut off values of 5% as previously reported in other studies **[17]** was used, above which the results were considered positive.

Statistical methods:

Data were analyzed statistically using SPSS (Statistical Package for Social Science) version 13. The following tests were done. Mean and standard deviation are descriptive values for quantitative data. Student t test for independent samples, and ANOVA (analysis of variance) for comparing means of more than two independents groups, post hoc test to detect the LSD (least significance difference). Chi-square compared impendent proportions. Pearson correlation coefficient (r) was used for correlation analysis. p value is Significant at 0.05. ROC curve was used for detection of the best cut off point.

RESULTS

This study included forty patients with chronic lymphocytic leukemia (CLL). Their ages ranged between (35-79) years with a median of 66 years. In addition 10 normal subjects of matching age were used as a control group.

The percentage expression of p53, p21, MDM2 (also by MFI), CD38 and BCL2 were significantly higher in the studied cases than that of the control group (Table 1).

A normal p53 response in which both p53 and p21 are positive in response to chemotherapy was observed in 6 out of the 40 studied

cases. Additional 6 cases showed p21 positivity but with negative p53 expression. P21 negativity was detected in 28 patients of them 13 patients had positive and 15 cases had negative p53 expression. So, patients were grouped according to p53 response to chemotherapy into 3 groups:

- *Group 1 (12 patients):* Normal p53 response (Positive or negative for p53 and positive for p21).
- *Group 2 (13 patients):* Type A p53 dysfunction (Positive p53, negative p21).
- *Group 3 (15 patients):* Type B p53 dysfunction (negative for both p53 and p21).

P53 percentage expression was highest in group 2 (mean, 39.5±24.3 SD) followed by group 1 (mean 10.16±10.8 SD). The lowest p53 percentage expression was detected in group 3 (mean 2.5±1.8 SD). The difference between groups was statistically significant (Table 2, Fig. 1).

P21 showed a mean percentage expression of 2±1.3 in group 2 and 1.5±1.4 SD in group 3. Those values were significantly lower than that of group 1 (mean, 16.1±6.6 SD) ($p<0.001$) (Table 2, Fig. 1).

To determine whether the type B p53 dysfunction was caused by MDM2 over expression, MDM2 expression was compared between groups.

The highest MDM2 percentage expression was detected in group 3 (mean, 2.36±2.21 SD) followed by group 1 (mean, 0.48±0.34 SD). Where as the lowest MDM2 expression was detected in group 2 (mean, 0.23±0.36 SD) ($p<0.001$) (Table 2).

Also on evaluating MDM2 expression by MFI, the highest MFI was detected in group 3 (mean, 12.93±10.1 SD) compared to a mean of 6.95±6.03SD and 1.25±0.22 SD for group 1 and 2 respectively. The difference between groups was highly statistically significant ($p=0.009$) (Table 2, Fig. 1).

The mean CD38 expression was 14.5±16.2 SD in group 1, 7.1±6.9 SD in group 2 and 8.6± 9.8 SD in group 3. The difference between the groups was statistically non-significant ($p=0.2$) (Table 2).

The mean BCL2 expression was 42.6±41.4 SD in group 1, 45.4±36.8SD in group 2 and 31.6±20.6 SD in group 3. The difference between the groups was statistically non – significant ($p=0.51$) (Table 2).

A negative significant correlation was found between the percentage expression of BCL-2 and CD38 ($r=–0.386$ and $p=0.014$).

Prognostic impact of p53 dysfunction:

A significantly higher percentage of patients with advanced stages (III & IV) of the disease was detected in group "2" (54.5%) and "3" (45.5%) compared to group "1" (9.5%) ($p=0.008$).

Pearson correlation coefficient showed positive correlation between p53 percentage expression and LDH level ($r=0.342$; $p=0.03$). No significant correlations were found with age ($r=0.221$; $p=0.1$), hemoglobin concentration ($r=–0.105$; $p=0.5$), total Leucocytic count ($r=–0.236$; $p=0.1$), platelet count ($r=–0.65$; $p=0.6$) or percentage of lymphocytes in peripheral blood ($r=0.019$; $p=0.6$).

As regard treatment outcome:

Patients with normal p53 response tend to have a higher complete response rate and less treatment failure with statistically significant longer time to disease progression (mean 12 months ±5.1 SD) than either those with type A (mean 5.8 months ±2.3 SD; $p=0.001$) or type B (mean 9 months ±4.4 SD; $p=0.08$) p53 dysfunction (Fig. 9).

As regard overall survival:

The median overall survival for the whole group was 22.5 months (range 3-30 months). A higher percentage of dead patients was detected in group "2" (54.5%) and group "3" (45.5%) compared to normal response group (0%) ($p=0.02$) (Table 3).

A negative correlation was found between percentage expression of p53 ($r=–0.425$, $p=0.006$), MDM2 ($r=–0.61$, $p=0.07$), BCL-2 ($r=–0.09$, $p=0.57$) and overall survival (Table 4).

A positive significant correlation was found between p21% expression and overall survival ($r=0.325$ and $p=0.041$).

Table (1): Comparison of profile of p53 and its related markers among control group and studied group.

	Percentage expression (Mean ± SD*)		*p*-value
	Cases (N=40)	Control (N=10)	
p53 %	16.8±21.9	0.9±0.2	0.02
p21 %	6.1±7.6	0±0	0.01
MDM2 %	1.5±1.6	0.038±0.04	0.05
MDM2 (MFI)**	7.34±8.44	1.18±0.16	0.05
Bcl2	37.9±32.2	0.8±0.2	<0.001
CD38	9.9±11.5	1.9±0.7	0.036

*SD: Standard Deviation. **MFI: Mean fluorescent intensity.

Table (2): Comparison of profile of p53 and its related markers among the three subgroups of the study group.

	Percentage expression (Mean ± SD*)			*p*-value
	Group 1 (N=12)	Group 2 (N=13)	Group 3 (N=15)	
p53 %	10.16±10.8	39.5±24.3	2.5±1.8	0.008* <0.001** <0.001***
p21 %	16.1±6.6	2±1.3	1.5±1.4	<0.001
MDM2 %	0.48±0.34	0.23±0.36	2.36±2.21	<0.001
MDM2 (MFI)	6.95±6.03	1.25±0.22	12.93±10.1	0.009
Bcl2	42.6±41.4	45.4±36.8	31.6±20.6	0.51
CD38	14.5±16.2	7.1±6.9	8.6±9.8	0.2

Comparison between groups: 1 & 3*, 2 & 3** and 1 & 2***.

Table (3): Comparison between percentage expression of P53 and different prognostic factors, treatment outcome and survival in the studied 40 cases.

	Group 1 (No. %)	Group 2 (No. %)	Group 3 (No. %)	*p*-value
Age (years):				
<60	4 (28.6)	4 (28.6)	6 (42.9)	0.8
≥60	8 (30.8)	9 (34.6)	9 (34.6)	
Stage (Rai):				
II	10 (52.6)	3 (15.8)	6 (31.6)	0.008
III & IV	2 (9.5)	10 (47.6)	9 (42.9)	
LDH:				
Low	10 (43.5)	5 (21.7)	8 (34.8)	0.07
High	2 (11.8)	8 (47.1)	7 (41.2)	
Response rate:				
Complete Remission	3 (60)	1 (20)	1 (20)	
Partial Remission	9 (36)	7 (28)	9 (36)	0.2
Stable Disease	0 (0)	2 (66.7)	1 (33)	
Progressive Disease	0 (0)	3 (42.9)	4 (57.1)	
TDP (months):				0.001*
(Mean ± SD)	12±5.1	5.8±2.3	9±4.4	0.08** 0.07***
Survival Status:•				
Dead	0 (0)	6 (54.5)	5 (45.5)	0.02
Alive	12 (41.4)	7 (24.1)	10 (34.5)	
OS (months):				0.001*
(Mean ± SD)	24.3±4.6	16.8±9.03	21.1±9.5	0.3** 0.17***

• At 30 months. TDP: Time to Disease Progression. OS: Overall Survival.
Comparison between groups: 1& 2*, 1 & 3** and 2 & 3***.

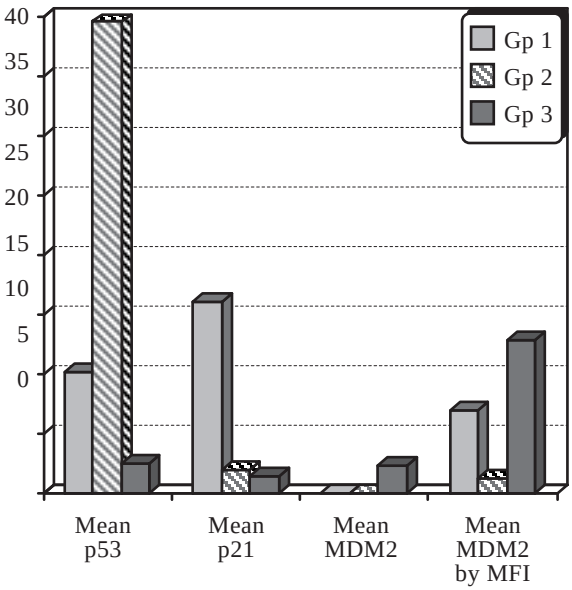


Fig. (1): Means of p53, p21 and MDM2 % expression in groups 1, 2 and 3 within the studied group.

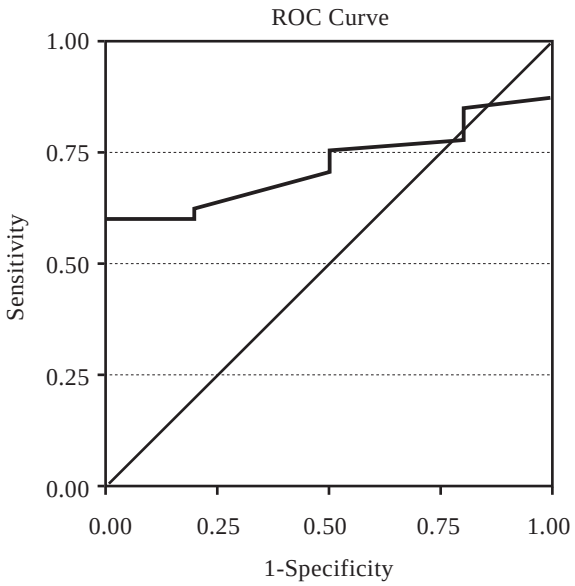


Fig. (2): ROC curve for p53 expression.

Diagonal segments are produced by ties.
1- Area under curve (AUC) = 0.72
2- Significance: <0.05 is significant.
3- The best cut off was 4.95% at which: Sensitivity = 60% and specificity = 100%.

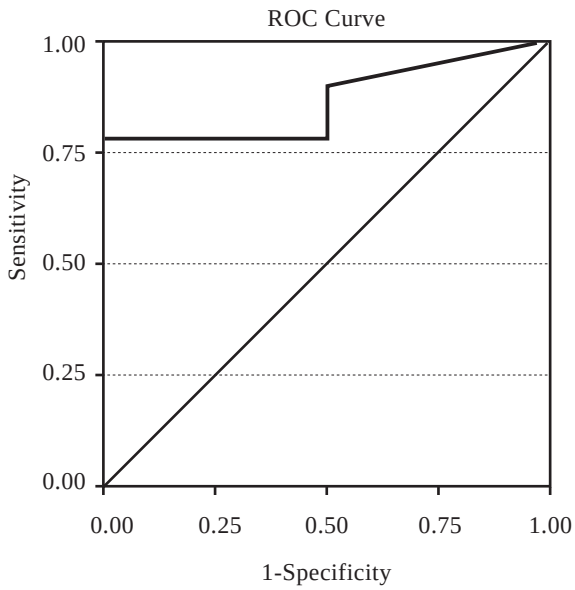


Fig. (3): ROC curve for MDM2 % expression.

Diagonal segments are produced by ties.
1- Area under curve (AUC) = 0.862
2- Significance: <0.05 is significant.
3- The best cut off was 0.09% at which: Sensitivity = 77% and specificity = 100%.

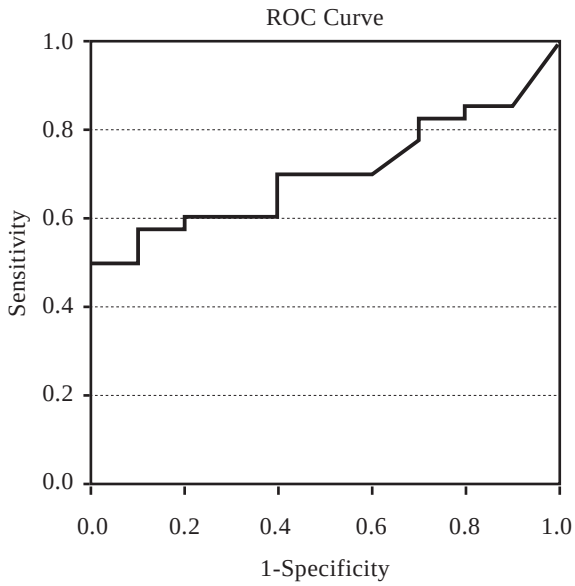
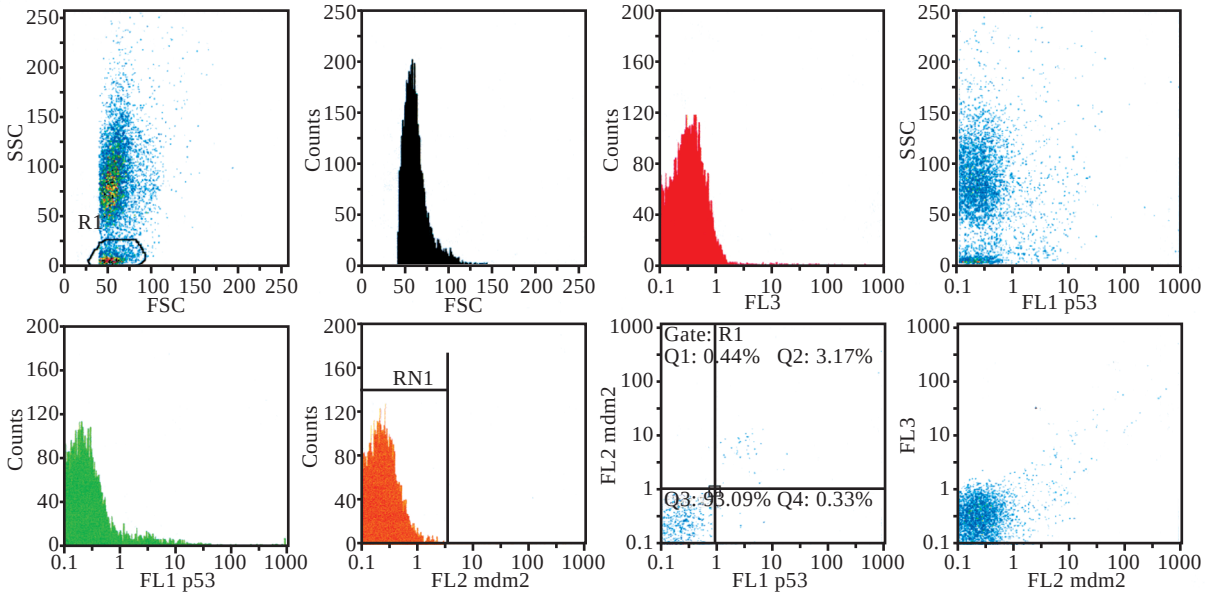


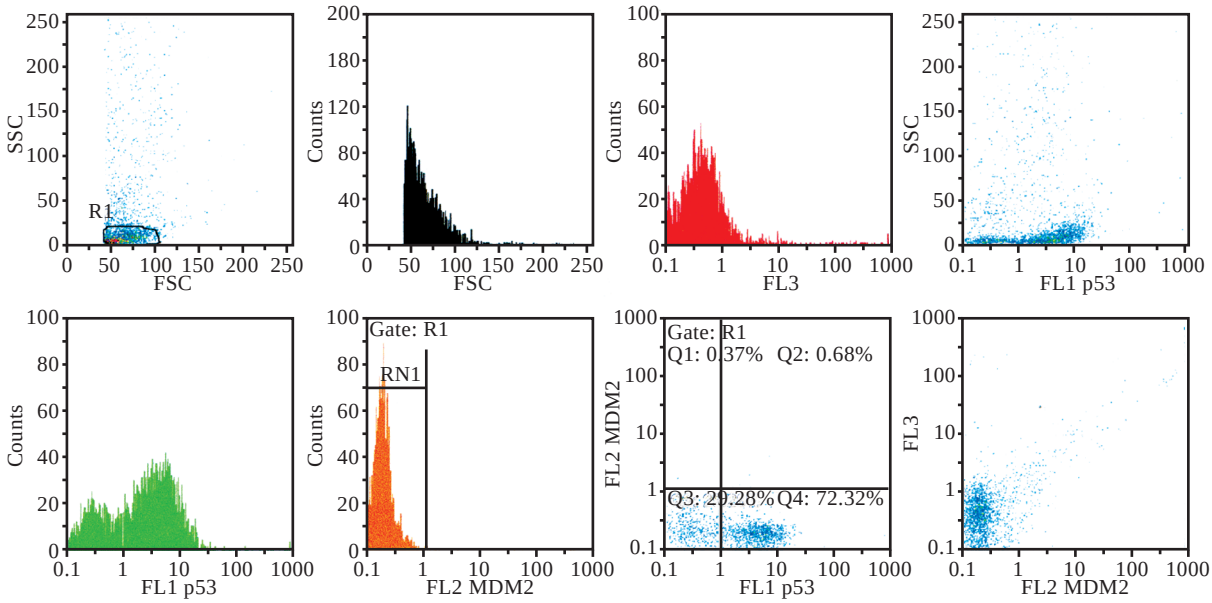
Fig. (4): ROC curve for MDM2 expression by MFI.

1- Area under curve (AUC) = 0.701
2- Significance: <0.05 is significant.
3- The best cut off was 1.16 at which: Sensitivity = 70% and specificity = 60%.



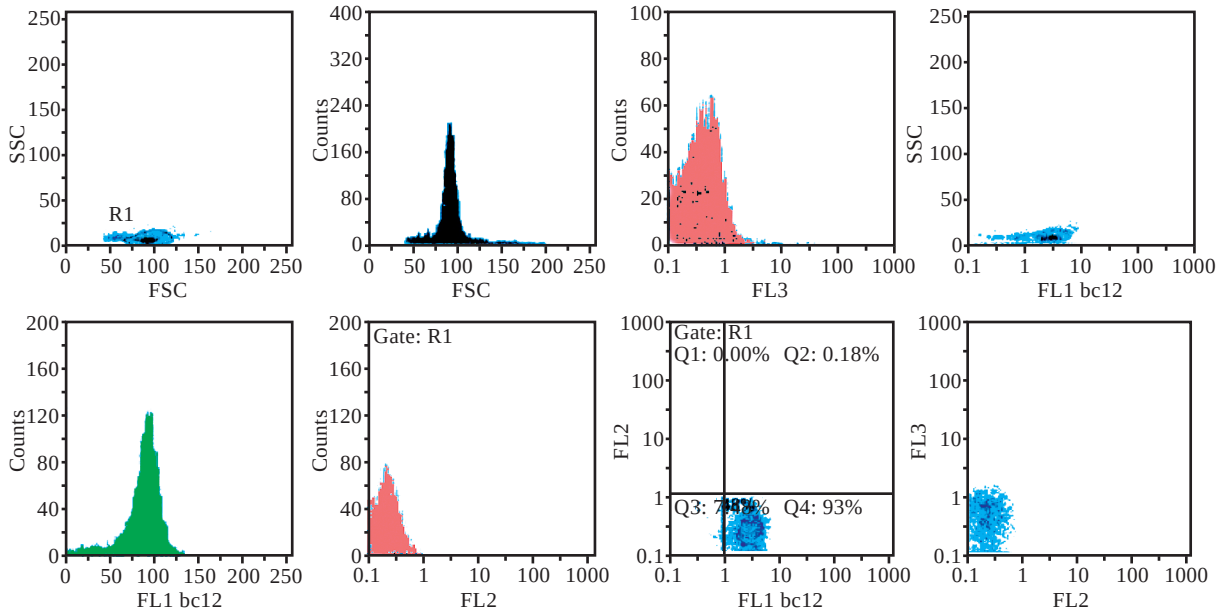
Region	Gate	Ungated	Count	Count/ml	% Gated	GMn-x	Mean-x	CV-x%	GMn-y	Mean-y	CV-y%
R1	<None>	160.7	1607	–	19.24	56.70	57.54	17.66	0.00	7.60	73.88
Q1	R1	61	7	–	0.44	0.13	0.20	131.31	1.33	1.76	109.79
Q2	R1	284	51	–	3.17	4.47	40.95	447.00	6.23	15.53	325.31
Q3	R1	7672	1496	–	93.09	0.16	0.20	75.68	0.16	0.20	76.66
Q4	R1	334	53	–	3.30	2.03	2.37	63.67	0.22	0.29	81.89
RN1	<None>	8216	8216	–	98.38	0.18	0.25	118.02	–	–	–

Fig. (5): Flow Cytometric analysis of a case of CLL, MDM2 positive and p53 negative.



Region	Gate	Ungated	Count	Count/ml	% Gated	GMn-x	Mean-x	CV-x%	GMn-y	Mean-y	CV-y%
R1	<None>	1909	1909	–	60.34	61.66	63.14	22.47	7.00	8.33	58.25
Q1	R1	30	7	–	0.37	0.23	0.27	61.37	41.14	281.58	93.96
Q2	R1	179	13	–	0.68	11.96	94.95	286.25	25.79	315.52	147.73
Q3	R1	922	559	–	29.28	0.30	0.36	60.79	0.23	0.26	55.18
Q4	R1	2033	1330	–	69.67	4.51	6.31	386.06	0.18	0.20	46.50
RN1	R1	2963	1891	–	99.06	0.20	0.22	54.17	–	–	–

Fig. (6): Flow Cytometric analysis of a case of CLL, which is p53 positive.



Region	Gate	Ungated	Count	Count/ml	% Gated	GMn-x	Mean-x	CV-x%	GMn-y	Mean-y	CV-y%
R1	<None>	3411	3411	–	79.68	93.89	94.21	8.23	5.41	5.88	39.46
Q1	R1	0	0	–	0.00	–	–	–	–	–	–
Q2	R1	52	6	–	0.18	10.78	24.31	135.03	2.30	3.14	84.75
Q3	R1	557	255	–	7.48	0.38	0.47	56.61	0.17	0.19	55.22
Q4	R1	3672	3150	–	92.35	2.76	2.99	39.76	0.20	0.24	60.46

Fig. (7): Flow Cytometric analysis of a case of CLL, which is BCL2 positive.

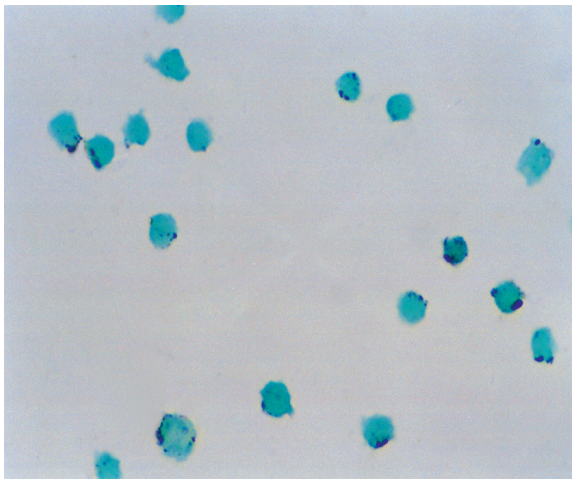


Fig. (8): p21 Positive nuclear expression by immunocytochemistry.

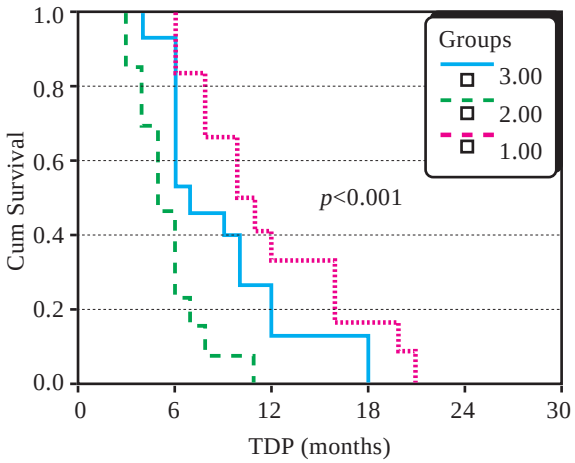


Fig. (9): Time to disease progression of the studied group according to p53 response to chemotherapy.

Table (4): Correlation with overall survival.

	(*r*)	*p* value
P53	–0.425	0.006
MDM2	–0.61	0.07
P21	0.325	0.041
BCL2	–0.09	0.57
CD34	0.116	0.4

r = Pearson correlation coefficient.

DISCUSSION

B-cell chronic lymphocytic leukemia (B-CLL) is characterized by the accumulation of long-lived CD5 (+) B lymphocytes. Several drugs currently used in the therapy of B-CLL act, at least partially, through activation of the P53 pathway. DNA-damaging agents increase p53 levels by posttranslational stabilization and induce p53-dependent cell death **[18]**. Importantly, B-CLL cells from patients with p53 mutations or deletions are associated with drug resistance and short survival **[19]**. As TP53 mutations occur in only 10% to 15% of patients with CLL **[12]**, it is possible that p53 dysfunction occurs in the disease through alternative mechanisms. MDM2 over expression has been reported as an alternative cause of p53 dysfunction **[20]**. MDM2 was suggested to abrogate the transactivating and growth inhibitory functions of the wild type p53 in tumor cells expressing this gene by binding to the acidic activation domain of p53 **[21]**.

In this study, p53/p21 response after chemotherapy was estimated in 40 CLL cases to detect the patient group having p53 dysfunction. MDM2 expression was measured as a trial to correlate its over expression to p53 dysfunction and decreased response to chemotherapy observed in those patients.

Of the 40 CLL studied cases, p53 and p21 measured after chemotherapy, showed a higher mean percentage expression than that of the normal control. These differences were statistically significant. This is in agreement with previous studies, which reported that chemotherapy of patients with chronic lymphocytic leukemia induces a p53-dependent gene expression response **[2]**. p53 accumulates in response to DNA damage and coordinates the cellular response to such damage by cell cycle arrest (by transcriptional activation of p21) or inducing apoptosis (by repressing BCL2) **[10]**.

However, in this study BCL2 was not repressed and was significantly higher than the control group. In accordance to our results, other researchers have found that, in response to DNA damaging effect, p53 regulates the expression of p21, but not BAX or BCL2 **[13]**.

A negative correlation was found between percentage expression of BCL-2 and CD38, with a significant correlation value. In contrast, other researchers suggested that CD38 confers to CLL cells a more malignant cellular phenotype. They explained that CD38 antigen has an important role as a modulator of intracellular signals and that cross-linking of CD38 up-regulates BCL-2 and inhibits apoptosis **[16]**.

MDM2 measured either by; percentage expression or MFI of the studied group was higher than that in the normal control. Several previous articles have reported that MDM2 protein is over expressed in a variety of neoplasms, including leukemia and lymphoma **[22,23]**.

Several mechanisms have been proposed for how MDM2 decreases the therapeutic benefits of cytotoxic drugs. The most obvious explanation is the role that MDM2 plays in p53 degradation. Because p53 is up regulated by DNA damaging agents, including chemotherapy and radiotherapy, the level of MDM2 is increased as a result of its role in feedback control. As a result, p53 degradation increases resulting in no subsequent increase in p21 so, preventing cell cycle arrest. MDM2 may also have a direct inhibitory effect on p21 **[24]**. In accordance, in this study a negative correlation was found between p21 and MDM2 percentage expression with a significant correlation value.

Another possible role for MDM2 in decreasing response to chemotherapy is by increasing expression of the multi drug resistance gene **[25]**. Thus there are many possible p53 dependants and independent mechanisms of action for the MDM2 mediated resistance to radiation therapy and chemotherapy. In this study we focused on MDM2 effect on p53 and its related molecules.

The study group was further subdivided according to p53 functional status into three subgroups, This categorization was suggested previously that impaired up regulation of p21 in response to DNA damaging effect defines a state of p53 dysfunction, while level of p53 itself determine the type of dysfunction Thus in type A defect, mostly associated with p53 mutation, p53 levels are increased, reflecting the prolonged half life of mutant p53 as compared with the wild type protein **[13]**.

In contrast, in the type B defect (suspected to be caused by MDM2 over expression), p53

levels were not increased in response to DNA damaging effect **[13]**. P53 percentage expression was highest in group 2 followed by group 1. Lowest expression was detected in group 3. The difference between groups was statistically significant.

In type A p53 dysfunction, the high percentage of p53 expression was suggested to be caused by p53 mutation. P53 mutation typically prolong the half-life of the protein, therefore increasing its level. This abnormally prolonged half-life enables the detection of p53 expression by flowcytometer or immunohistochemistry using anti p53 monoclonal antibody. However, even when activated, mutant p53 protein cannot regulate gene expression because of its inability to bind to specific DNA sequence **[11]**. This explains the low level of p21 observed in this group of patients.

The high p53 percentage expression detected in normal p53 response was explained in earlier studies. In quiescent cells, levels of p53 protein are low owing to its short half life. After DNA damage, the half-life of p53 becomes prolonged **[6]** and the protein accumulates in the nucleus **[7]**.

However, p53 level was higher in type A, compared to that of normal response group, suggesting a more stabilizing effect of p53 mutation on p53 protein level. This is in agreement with previous reports, which suggested that, the high p53 protein detected should be considered as a marker of p53 gene mutation **[5,26]**.

P21 showed a mean percentage expression in type A and in type B dysfunction group significantly lower than that of normal response group ($p<0.001$). This is in agreement with what was previously documented. The expression and function of p21 after DNA damage appear to be strictly dependent on the presence of functional wild type p53 **[27]**. So, the decrease in p21 level in both type A and type B dysfunction groups confirms their categorization as having p53 dysfunction.

Level of MDM2% expression or MFI was highest in patients with type B p53 dysfunction (suggested to be caused by MDM2 over expression) followed by those with p53 normal response. The lowest expression was detected in patients with type A p53 dysfunction (suggested to be caused by p53 mutation). This is in accordance of previous reports, which stated that an excess of MDM2 protein could abrogate transcriptional activation by wild type p53 **[28]**.

The difference of mean percentage CD38 expression between normal response, type A and type B dysfunction groups was statistically not significant. Our results suggest no correlation between CD38 expression and p53 functional status. This is in accordance with what was reported earlier **[16]**.

Also no significant difference was found on comparing the 3 groups regarding BCL2 percentage expression. Previous reports have suggested that p53 was regulating the expression of p21, but not BCL2 **[13]**.

A positive correlation was found between LDH level and p53% expression with a significant correlation value. Also, a negative correlation was found between p53% expression and over all survival with significant correlation value.

P53% expression showed no significant correlation with the other known prognostic factors such as age, Hb concentration or platelet count. Previous studies have reported significant direct correlations between the percentage of p53 positive staining cells and other CLL aggressive features including ß2-microglobulin, lower hemoglobin level and increased age **[29]**.

Among patients with advanced stages of the disease (stage III and IV), 47.6% were of type A p53 dysfunction, 42.9% were of type B p53 dysfunction and only 9.5% were of normal p53 response group. This difference was statistically significant. These data are consistent with that reported by others **[30,31]**.

In our study, Patients with normal p53 response tend to have a higher complete response rate and less treatment failure with statistically significant longer time to disease progression (mean 12 months ±5.1 SD) than either those with type A (mean 5.8 months ±2.3 SD; $p=0.001$) or type B (mean 9 months ±4.4SD; $p=0.08$) p53 dysfunction. In accordance, previous studies, had reported that MDM2 overexpression influences the cellular response to cytotoxic/DNA damaging agents and as negative regulator of p53 is related to decreased response

to both chemotherapy and radiation therapy and increased risk for relapse **[29,31]**. Others had reported that MDM2 expression become markedly reduced or absent during remission **[32]**. On comparing the TDP between type A and type B dysfunction, it was shorter in type A than in type B. In accordance, Francis et al., 2003 **[16]**, reported that positive p53 percentage expression was strongly associated with p53 gene mutation and progressive disease.

A higher percentage of dead patients was detected in group "2" (54.5%) and group "3" (45.5%) compared to normal response group (0%) (p=0.02). On comparing OS of patients with type A versus that of type B dysfunction, no statistically significant difference was obtained. However, a high significant decrease of overall survival was observed on comparing type A with normal response group (p=0.001). In agreement with our results, other investigators reported that positive expression of p53 protein as measured by immunohistochemistry was strongly associated with p53 gene mutation, refractoriness to therapy and reduced survival **[29]**.

On comparing OS of type B dysfunction to that of normal response group, no statistically significant difference was obtained (p=0.3). However, a negative correlation was found between MDM-2% expression and over all survival, where r=–0.61 and p=0.07 with borderline significant correlation value. Previous studies reported that on separating patients according to their p53 functional status, those with demonstrable p53 dysfunctional status had a much shorter disease specific survival. On subdividing the p53 dysfunctional cases, patients with the type A p53 defect, had a significantly shorter survival than patients with type B defect **[16]**.

From this study we conclude that:

- P53 dysfunction groups had a decreased response rate, a more advanced stage of the disease and less TDP than that of p53 with normal function group. This goes with the established importance of the p53 pathway in maintaining genomic integrity and mediating the action of certain cytotoxic agents including purine analogues.
- A decrease in overall survival was observed in groups with p53 dysfunction. This suggests that p53 function test is a powerful predictor of outcome in CLL.
- On comparing the two methods used for evaluating MDM2 expression, both percentage expression and MFI revealed a significant increase of MDM2 in group 3. However, using percentage as cut off point is subjective. So the use of both MFI in association with percentage positivity can be a better predictor of disease progression and outcome of the disease than percentage alone.
- Type B p53 dysfunction group (with high MDM2 expression level) showed a higher percentage of patients in advanced stage of the disease, decreased response rate and decreased TDP. So MDM2 may be used as a marker for advanced stage. Cut off values of 0.09% and 1.16 by MFI was suggested in this study.

Recommendations:

- Considering results of this study and the established oncogenic potential of over expressed MDM2 proteins, a possible role of MDM2 proteins in promotion of CLL disease remains to be further evaluated.
- Drugs that target MDM2-p53 interaction could provide a novel therapeutic strategy for CLL should be investigated for clinical applications in the treatment of CLL.

REFERENCES

1- Caligaris-Cappio F, Hamblin TJ. B-cell chronic lymphocytic leukemia: A bird of a different feather. J Clin Oncol. 1999, 17: 399.

2- Lens D, Dyer M, Garcia -Marco JM. P53 abnormalities in CLL are associated with excess of prolymphocytes and poor prognosis Br J Haematol. 1997, 99: 848-857.

3- El Rouby S, Thamas A, Castin D. p53 gene mutation in B-cell chronic lymphacytic leukemia is associated with drug resistance and is independent of MDRl/ MDR3 gene expression. Blood. 1993, 82: 3452-3459.

4- Döhner H, Stilgenbauer S, James MR. 11 q deletions identify a new ~ subset of B-cell chronic lymphocytic leukemia characterized by extensive nodal involvement and inferior prognosis. Blood. 1997, 89: 2516.

5- Levine AJ. p53, the cellular gatekeeper for growth and division. Cell. 1997, 323-331.

6- Kastan MB, Onyekwere 0, Sidransky D, Vogelstein B, Craig R. Participation of p53 protein in the cellular response to. DNA damage. Cancer Res. 1991, 51: 6301- 6311.

7- Fritsche M, Haessie C, Brandner G. Induction of clear accumulation of the tumor suppresser protein P 53 by DNA-damaging agents. Oncogene. 1993, 78: 307.

8- Martinez J, Georgoff I, Levine AJ. Cellular localization and cell cycle regulation by a temperature sensitive p53 protein. Gene Dev. 1991, 5: 151.

9- Marschitz L, Tinhofer I, Hittmair A. Analysis of Bcl-2 protein expression in chronic lyinphocytic leukemia: A comparison of three semiquantitation techniques. Am J Clin Pathol. 2000, 113: 219.

10- Lane DP. P53 and human Cancers. Br Med Bull. 1994, 50: 582-599.

11- Prives C. How loops, B sheets and helices help us to understand P53. Cell. 1994, 78: 543-546.

12- Newcomb EW. p53 gene mutations in lymphoid diseases and their possible relevance to drug resistance. Leukemia and Lymphoma. 1995, 17: 211.

13- Andrew R, Paul D, Shernington S. P53 dysfunction in B- Cell chronic lymphocytic leukemia: Inactivation of ATM as an alternative to TP53 mutation. Blood. 2001, 98: 814-822.

14- Cheson BD, Bennett JM, Grever M. National Cancer Institute-sponsored working group guidelines for chronic lymphocytic leukemia: Revised guidelines for diagnosis and treatment. Blood. 1996, 87: 4990.

15- Rai K, Sawitsky A, Cronkit E, Wolf S, Litcher P. Clinical staging of chronic lymphocytic leukemia. Blood. 1975, 46: 219.

16- LinK, Sherrington P, Dennis S. Relationship between p53 dysfunction, CD38 expression and IgVH mutation in chronic lymphocytic leukemia. Blood. 2002, 100 (4): 1404.

17- Said E, Antoine M, Anne M. High expression of MDM2 protein and low rate of $p21^{waf1/cip1}$ expression in SCIP Mice Epstein Barr Virus induced lymphoproliferation, Journal of Histochemistry and cytochemistry. 1999, 47 (10): 1315.

18- Shich S, Taya Y, Prives C. DNA damage-inducible phosphorylation of P53 at N-terminal sites including a novel site, ser 20 requires tetramerization. EMBO J. 1999, 18: 1815.

19- Bellosillo B, Villamor N, Colomer D, Pons G, Montserrat E, Gil J. In vitro evaluation of fludarabine in combination with cyclophosamide and/or mitoxantrone in B-cell chronic lymphocytic leukemia. Blood. 1999, 94 (8): 2836.

20- Kojima M. Leukemia p53 mediated apoptosis induced by MDM2 inhibitor nutlin 3a. Blood. 2006, 108 (3): 993.

21- Oliner J, Pretenphol J, Thiagalingam S. Oncoprotein MDM2 conceals the activation domain of tumor suppressor p53. Nature. 1993, 362: 857.

22- Mohamed A, Hamid E, Carlos E. Expression profile of MDM2 proteins in chronic lymphocytic leukemia and their clinical relevance. Am J Hematol. 1997, 54: 189.

23- Zhang R, Wang H. MDM2 oncogene as a novel target for human cancer therapy. Curr Pharm Design. 2000, 6: 393.

24-Yanamoto S, Kawasakai G, Yoshitemi I. p53, MDM2 and p21 expression in oral squamous cell carcinomas: relationship with clinicopathologic factors. Oral Surg Oral Med Oral Pathol Oral Radiol Erdod. 2002, 94: 593.

25- Cocker H, Hobbs SM, Tiffin N. High levels of the MDM2 oncogene in pediatric rhabdomyosarcoma cell lines may confer multi drug resistance. Br J Cancer. 2001, 85: 1746.

26- Lepelley P, Preudhomme C, Vanrumbeke M. Detection of p53 mutations in hematological malignancies: Comparison between immunocytochemistry and DNA analysis. Leukemia. 1994, 8: 1342.

27- Dulic K, Kaufmann WK, Wilson SJ. p53 independent inhibition of cyclin dependent kinase activities in human fibroblasts during radiation induced G1 arrest. Cell. 1994, 79: 1013.

28- Momand J, Zambetti GP, Olson DC, George D. The MDM2 oncogene products form a complex with the p53 protein and inhibit p53-mediated transactivation. Cell. 1992, 69: 1237.

29- Francis J, Nebiyou B, Susan O. A prognostic model for survival in chronic lymphocytic leukemia based on p53 expression. Br J Haematol. 2003, 121: 578.

30- Ichikawa A, Hotta T, Takagi N. Mutations of p53 gene and their relation to disease progression in B-cell lymphoma. Blood. 1992, 79: 2701.

31- Takahashi Y, Oda Y, Kawaguchi K, Tamiya S. A hereditary expression and molecular abnormalities of cell cycle regulatory proteins in rhabdomyosarcoma. Mod Pathol. 2004, 17: 660.

31- Federl S, Kantarjian H, Estey E, Manshowri T. The prognostic significance of p16 (INK 4a) / p14 (ARF) locus deletion and MDM2 protein expression in adult acute myelogenous leukemia. 2000, 1: 1976.

32- Zhou M, Gu L, Abshire T, Homans A. Incidence and prognostic significance of MDM2 oncoprotein over expression in relapsed childhood acute lymphoblastic leukemia. 2000, 14: 61.