

The Expression of p53 Protein in Chronic Lymphocytic Leukemia is Associated with Poor Response to Chemotherapy and Short Survival

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ABSTRACT

We have analyzed by immunocytochemistry (ICC) the frequency of p53 protein expression in 47 cases of chronic lymphocytic leukemia to assess the relationship between p53 and the disease stage as well as the possible impact of this protein on the response to treatment and survival. Of these, 42 samples were valid for interpretation. The overall frequency of p53 protein positivity in CLL was 16.7% (7 of 42 cases). The percentage of p53-positive cells increased with disease progression ($25 \pm 7\%$ in stage 3 vs $10.3 \pm 6.2\%$ in stage 1+2). This difference, however, did not reach statistical significance. A significantly poorer response to therapy was observed in p53-positive compared to p53-negative patients. Three out of 7 p53 (+ve) versus 31 out of 35 p53 (-ve) patients showed a response to therapy ($p = 0.05$). The overall survival rate was significantly shorter in patients with p53 protein expression ($p = 0.024$). The results of this study indicate that in CLL the expression of the p53 protein is associated with poor response to chemotherapy and short survival.

Key Words: *Chronic lymphocytic leukemia - p53 - Treatment response - Survival.*

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults. It is characterized by a highly variable clinical course, even in patients presenting at the same clinical stage. Several features that may predict disease progression and/or survival in CLL have been identified, but the biological mechanisms underlying this variability in disease behavior are not well understood [9].

Treatment is reserved for patients with low or intermediate risk disease who are symptomatic or have progressive disease, as evidenced

by increasing organomegaly or a lymphocytic doubling time of less than 12 months. Patients with high risk disease are usually treated, in an attempt to improve hemoglobin and platelets, although asymptomatic patients can be monitored and treatment only initiated upon disease progression [38].

Although the tumour specific action of most anticancer agents has been attributed to their effect on actively proliferating cells, an increased body of evidence suggests that anticancer drugs exert their action, at least partly, by triggering apoptosis. Of the factors controlling and regulating this process, p53 is believed to be of principal importance [14].

p53 is a 53-kD nuclear phosphoprotein, the product of a 20kb gene localized on the short arm of human chromosome 17, at position 17p13.1 [27]. The main physiological functions of p53 are cell cycle regulation, induction of apoptosis and stabilization of the genome. As each of them are indispensable gatekeeping devices of cellular homeostasis, alterations of the p53 gene may play a central role in multistep carcinogenesis process but also in the prognosis and response to therapy of a variety of tumours [20].

In the majority of tumours, p53 is inactivated by mutations which result in the production of a p53 protein with increased stability. This leads to the presence of positive p53 protein immunohistochemical staining of mutant cells, in

contrast to cells containing wild-type (wt) p53 which generally do not stain due to the relatively short half-life of the wt p53 protein [24].

In the present study, we analyzed by immunocytochemistry (ICC) the frequency of p53 expression in 47 CLL patients to investigate its relationship to the disease stage as well as its impact on the response to treatment and overall survival.

PATIENTS AND METHODS

Patients:

We have studied peripheral blood samples from 47 cases of CLL presented to the outpatient clinics of the Medical Oncology Department, National Cancer Institute, Cairo University and Unit of Hematology and Medical Oncology, Zagazig University Hospital during the period from January 1995 to April 1996. Diagnosis has been made between 1988 and 1996. All patients were subjected to the following evaluation:

- 1- Complete physical examination.
- 2- Complete blood count.
- 3- BM examination.
- 4- Immunophenotyping using monoclonal antibodies and the immunoperoxidase staining technique on the mononuclear cytopreps.
- 5- Bone marrow trephine to differentiate CR from PR-NOD.

CLL was diagnosed on the basis of the criteria recommended by the International Workshop on CLL [5] whereas clinical staging and response were based on the criteria recommended by NCI sponsored working group on CLL [10]. All the cases were classical CLL.

Twenty seven patients were men and 20 were women. Overall, 15 patients were on treatment at the time of this analysis, but all of them had greater than 80% peripheral blood lymphocytes. All patients received chlorambucil in a daily dose of 0.1 mg/kg plus prednisone which given in a daily dose of 1 to 2 mg/kg as first-line therapy [34]. Patients were followed-up for at least 42 months.

Methods:

Mononuclear cells were isolated from heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation [15]. Air dried

cytopspins were prepared with a concentration of 5×10^4 cells per slide, fixed in acetone for 10 minutes, wrapped in aluminium foil and stored at 4°C until stained. Fixed cytopreparations were stained with a wide panel of primary monoclonal antibodies (MoAb) against B and T antigens. B-cell clonality was confirmed by the expression of either κ or λ light chains on the cell surface membrane. All the reagents were supplied by Becton Dickinson, Coulter and Dako Patts Laboratories. The MoAb used for the immunocytochemical detection of p53 was DO-7 (Dako, Patts laboratories, Denmark) which reacts with both wild-type and mutant p53 protein. DO-7 was used at a final concentration of 5 ug/ml. Immunostaining was performed using the ABC immunoperoxidase method as previously described [26] using the Vectastain kit (ABC Kit-PK 4002, supplied by Vector Laboratories, Burlingame, CA, USA).

The percentage of p53 positive cells (LI) was evaluated by light microscopy examining 500 lymphoid cells per sample. Normal lymphocyte preparations were always included and were persistently negative. p53 positivity appeared as intense brown nuclear staining, with good preservation of morphological details (Fig. 1). The reaction was always confined to the nucleus.

Statistical methods:

Statistical analysis system was used for data management and analysis. Harvard Graphics were used for drawing figures.

Comparisons between groups with respect to quantitative measurements were done using Mann-Witney test for small sample size. For comparisons between the different groups in the case of qualitative measurements, Fisher's exact test was used [16].

Survival estimates were done using Kaplan-Meier methods and comparisons between different groups of prognostic factors were done using the Log Rank test [28].

All *p*-values were two-sided. *p* values ≤ 0.05 were considered significant.

RESULTS

Patients' characteristics:

Twenty-seven patients were men and 20 were women. The mean age was 54.9 ± 11.1

years. According to the modified Rai staging system of CLL, only one patient was in stage 1 (low-risk), 32 in stage 2 (intermediate risk) and 14 in stage 3 (high risk) and according to the Binet's staging system, 8 patients had stage A, 25 had stage B and 14 patients had stage C disease.

According to the modified Rai staging system, as only one patient was in stage 1 (low-risk), therefore, patients in stages 1 and 2 were considered as one group in all analyses. There were no significant differences in age or sex distribution between the two groups. With regard to hematologic profile and immunologic studies, patients with stage 3 disease had a significantly lower hemoglobin level at presentation. All other parameters showed no differences between the two groups (Table 1).

p53 Expression in CLL:

Only 42 samples were valid for interpreta-

tion. The overall frequency of p53 protein positivity in CLL studied samples was 16.7% (7 of 42 cases). The percentages of positive cells (LI) ranged from 7 to 35%. Four positive cases were in stage 1-2 and another 3 were in stage 3. Two positive samples were found among the twelve newly diagnosed patients both were in modified Rai stage 2 disease (one of them was in Binet stage A disease).

Response to therapy and survival:

Overall, 36 patients showed a response while 9 showed no response. In the remaining 2 patients data were not available to determine treatment response (Fig. 2). p53 expression and stage 3 disease were associated with a significantly poorer response to chemotherapy ($p = 0.05$ & 0.014 respectively) (Table 2). The survival rates at one, two and three years were significantly shorter among patients with p53 positivity as well as those who did not respond to treatment (Table 3 and Fig. 2).

Table (1): Clinical and immunologic characteristics of 47 CLL patients.

Characteristic	CLL stage 1+2 (n=33)	CLL stage 3 (n=14)	p value
Sex (M/F)	19/14	8/6	0.097
Age (years)	55±9.4*	53±7.6	0.163
<i>Peripheral blood:</i>			
- Hb conc. (g/dl)	12.2±1.1	8.5±1.7	< 0.001
- TLC (x 10 ⁹ /L)	60.3±36.0	71.4±56.0	0.867
- Lymph. count (x 10 ⁹ /L)	50.39±33.28	64.89±56.16	0.738
- Platelet count (x 10 ⁹ /L)	179.1±44.7	123.7±60.9	0.006
<i>Bone marrow:</i>			
- Percentage of lymphocytes	71.4±12.8	69.9±13.7	0.719
- Cellularity			
Hypercellular n=13	7 (31.8)**	6 (46.1)	
Normocellular n=22	15 (68.2)	7 (53.9)	0.480
<i>Immunologic studies:</i>			
- Phenotype			
B-CLL n=39/42	26/28 (92.9)	13/14 (93.9)	
T-CLL n=3/42	2/28 (7.1)	1/14 (7.1)	1.000
- p53 (+ve) n=7/42	4/30 (13.3)	3/12 (25.0)	0.398

* Mean ± SD

** No (%)

Table (2): Response to therapy of CLL patients in relation to modified Rai staging system and p53 expression.

Parameter	Response partial and complete		No response		p value
	N	%	N	%	
<i>Modified Rai staging system:</i>					
Stage 1+2 n=31	28	(90.3)	3	(9.7)	0.019
Stage 3 n=14	8	(57.1)	6	(42.9)	
p53 positive n=7	3	(42.9)	4	(75.1)	0.05
Negative n=35	31	(88.6)	4	(11.4)	

Table (3): Survival rates of CLL patients in relation to different studied parameters.

	No. of cases	Overall survival (%)			p value
		1 year	2 year	3 year	
Total	47	97.9	89.4	80.3	0.024
p53 (+ve)	7	100.0	71.4	42.9	
(-ve)	35	97.1	91.4	85.4	
<i>Modified Rai staging:</i>					
Stage 1+2	33	96.9	87.5	80.7	0.978
Stage 3	14	100.0	92.9	77.9	
<i>Response to therapy:</i>					
Responders	36	97.2	91.7	88.6	< 0.001
Non responders	9*	100.0	88.9	50.8	

* Means small sample size.

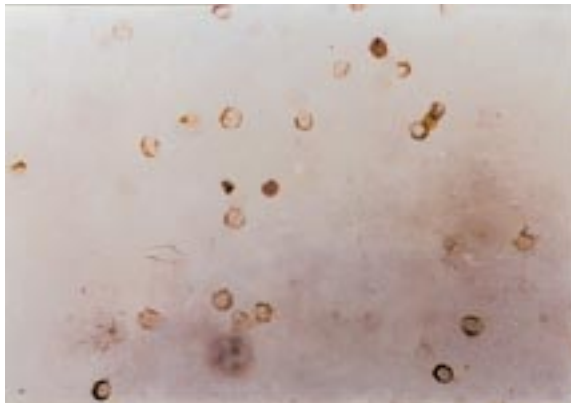


Fig. (1): Cells showing positive staining for p53 mAb (400C).

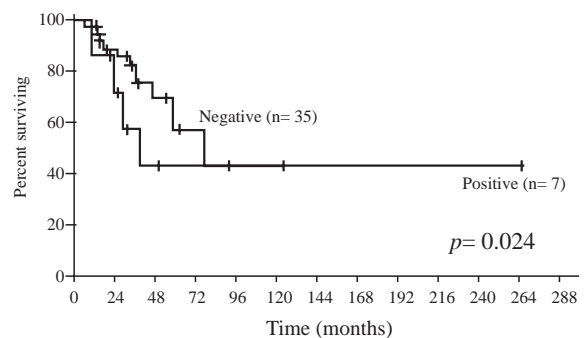


Fig. (2): Kaplan-Meier survival curves after diagnosis of CLL based on the expression of p53.

DISCUSSION

Mutations of p53 are the most common genetic abnormality in cancer [7,30]. They have been extensively studied in various malignancies of mature B cells, including NHL [1,4,33], CLL [18,19,22] and myeloma [13]. Less attention has been paid to the significance of p53 protein

expression in CLL, although an association with poor survival and nonresponse to therapy has been observed in a small series of CLL [2,29].

In this study, p53 protein expression was detected in 7 out of 41 samples (17%) using the immunoperoxidase method. Prevalence of pro-

tein expression detected in our study is in agreement with several studies documenting the existence of p53 gene mutation in 10-15% of cases of CLL [18,19,21,22].

Immunocytochemistry (ICC) for p53 is the simplest analysis for p53 integrity, but this approach cannot directly detect p53 mutations. Because of a short half-life of about 6-20 minutes, wt-p53 usually does not accumulate in most normal tissues in detectable amounts by ICC. However, most missense mutations of p53 prolong the half-life of the protein, permitting it to be immunocytochemically detectable. The technique is rapid and easily performed. Nevertheless, quantitation is difficult [25]. Several studies have, in fact, shown that in some high grade NHL the occurrence of positive immunostaining does not reflect point mutations in the p53 gene and vice versa [2,4,8,33,34,42]. Therefore, it is evident that the relationship between p53 protein detection and the existence of gene mutation is more complex than initially expected and that other mechanisms of p53 stabilization are frequently operating in NHL [35]. However, the association of p53 detection by ICC with poor prognosis would raise the question if this stabilization of the protein would still interfere with its normal function even in the absence of a mutation.

The percentage of cells positive for p53 staining ranged from 7-32%. The coexistence of p53-positive and negative cells within the same leukemic population supports the hypothesis that p53 dysregulation can be a late event in the progression of the disease [12]. This is in agreement with previous studies, which have shown a strong correlation between p53 mutations and progression in hematologic malignancies. In our study, the p53 per cent positivity was higher in stage 3 disease compared to stage 1+2, however, this difference was not statistically significant probably due to the limited number of our cases. Low-grade NHL rarely have p53 alterations, but their progression to high grade lymphoma can be associated with p53 mutations. For example, serial biopsies of patients with follicular NHL who underwent histologic transformation showed that one-third of the transformed samples acquired a p53 mutation that was not detected in the follicular stage of the disease [36]. Another study found that 4 of 5 cases of transformation of follicular to diffuse large-cell NHL were associated with

p53 mutations [31]. Interestingly, in this study, one sample of follicular NHL had regions of transformation to high-grade NHL; cells of this region, but not those of the follicular areas, contained a mutant p53 [31]. The evolution of CML and MDS to myeloid blast crisis and AML, respectively, has been associated with loss of the short arm of chromosome 17 and mutation of the remaining p53 allele [25]. p53 mutations have also been associated with progression of solid malignancies such as the transition from benign adenoma to malignant colon carcinoma [3], evolution of gliomas [40] and development of metastatic prostate cancer [25].

On the other hand, mutations of p53 have also been found in the precancerous phases of adenocarcinomas, including adenomatous polyps of patients with familial polyposis coli suggesting that p53 mutation may occur as an early event in carcinogenesis as well [25]. In support of this theory we observed that among the 7 samples showing p53 positive staining, two patients were newly diagnosed and one of them was in Binet stage A. Similarly, Cordone et al. [12] reported the occurrence of p53 alteration in the so-called smouldering CLL, as shown by the p53 positivity in a proportion of patients studied at diagnosis and in stage A.

In our study, p53 protein expression had strong implications for the clinical course of the disease. p53-positive patients showed a significantly poorer response to therapy and shorter overall survival compared with p53-negative patients. In the study of El-Rouby et al. [18], patients with p53 gene mutations were found to have an aggressive form of B-CLL disease characterized by advanced Rai stage, rapid lymphocyte doubling time (LDT) and resistance to chemotherapy. In this study, while 27 of 29 treated patients (93%) without p53 mutations achieved a partial remission, only one of seven treated patients (14%) with p53 mutations achieved a partial remission ($p = 0.00009$). In the same study, patients with p53 gene mutations had a 13-fold greater risk of death than patients without p53 mutations ($p = 0.013$). Similarly, Cordone et al. [12] reported that 16 of 18 p53-negative patients treated with fludarabine achieved a PR ($n=9$) or CR ($n=7$), whereas 2 p53-positive patients treated with this purine analog achieved only a short-lived PR. Mutations of the p53 gene have been associated with decreased survival in NHL [35] and in other

types of tumours, including colon [39], breast [41], bladder, gastric, lung and prostatic cancers [37].

The mechanisms whereby p53 mutations induce resistance to chemotherapy can only be hypothesized. First, it has been shown that normal p53 suppressed the multidrug resistance (*mdr1*) gene promoter, whereas mutated p53 could stimulate it [11]. Expression of the *mdr1* gene has been correlated to resistance to chemotherapy in many tumour types including AML [6] and MDS [29]. Therefore, mutated p53, by activating *mdr1* expression, could interfere with response to chemotherapy. However, three reports in AML [44], CLL [18] and MDS [25] failed to show a correlation between p53 mutations and *mdr1* gene expression. A second hypothesis can be made by considering the role of p53 in apoptosis. It has been suggested that some chemotherapeutic agents, including anthracycline derivatives and Ara-C [23] could induce leukemic cell death, at least in part, by triggering apoptosis. p53 appears to be required for the induction of apoptosis induced by irradiation or heat shock in cell lines [32]. Absence of p53 could explain resistance to chemotherapy.

In our work, p53 showed an apparent impact on prognosis. However, it was detected in a few number of cases which did not allow its analysis in context with known other prognostic parameters, namely BM trephine morphology, lymphocytosis at diagnosis, lymphocyte doubling time, cytogenetics and beta 2 microglobulin. Accordingly, we have to collect more p53 positive CLL cases to be able to perform such an analysis.

In conclusion, our findings indicate that p53 expression in CLL is associated with poor response to therapy and short survival. In the context of a heterogeneous condition like CLL, this simple, inexpensive and reliable ICC method appears to offer a useful prognostic tool capable of identifying patients who may be considered candidates for more intensive therapeutic strategies. This is of particular relevance for younger patients for whom more eradicated approaches are becoming more frequently used.

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