Original Article

mRNA in situ hybridization analysis of p-53 cancer suppression gene and Bcl-2 oncogene in chronic lymphocytic leukemia

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Summary:

Background: Several factors render chronic lymphocytic leukemia (CLL) an interesting subject for study by researchers. These include marked progress in understanding the molecular biology of normal and neoplastic lymphocytes and recent advances in molecular genetics techniques. Among molecular markers, p53 cancer suppressor gene and the antiapoptotic gene Bcl-2 have been widely studied.

Patients and methods: A retrospective cross-sectional study done on 60 patients with chronic lymphocytic leukemia compared with 20 controls (anemic patients), all recruited at the Medical City Teaching Hospital laboratories from January 2004 to December 2007. The bone marrow biopsy of each was re-examined histologically. In situ hybridization was performed utilizing biotin labeled p53 and Bcl-2 cDNA probes.

Results: The frequency of p53 positive signals in the study group was 28.3% (17of 60 cases). A significantly larger number of patients, with high score for p53 signal, were associated with high-risk clinical stage than patients with low score (p = 0.005). There was a significant direct positive correlation between increasing scores of p53-positive chronic lymphocytic leukemia cells and advancing clinical stage of the disease (p =0.002).

The frequency of Bcl-2 positivity was 50% (30 of 60 cases). No significant correlations found between Bcl-2 scores and clinical stage of the disease.

Conclusion: Although p53 alteration may occur early in the course of the disease, as shown by the p53 positivity in a proportion of patients in low and intermediate-risk stage of the disease, the highest frequency p53-positive cells, has been observed in high-risk stage of the disease. Therefore, p-53 score is an important prognostic variable in patients with chronic lymphocytic leukemia. However, Bcl-2, as assessed by ISH, is not regarded as an important prognostic marker.

Keywords: Chronic lymphocytic leukemia; p53; Bcl-2; in-situ hybridization.

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Introduction:

P53 gene, located on chromosome 17 band p13.1, is frequently mutated in a wide variety of human tumors, including leukemia. It encodes a 53-kD phosphoprotein that is normally present in the nuclei of the cells (1, 2, 3, 4). The p53 tumor suppressor protein is a transcription factor that is involved in the cell cycle arrest and induction of apoptosis in genetically damaged cells. Mutations or deletions of the p53 gene may facilitate the transmission of a genetic damage and the emergence of neoplastic clones with a survival-advantage 5.

Bcl-2 is localized at the outer mitochondrial and the nuclear membrane, as well as at the endoplasmic reticulum. Bcl-2 is known to belong to a family of apoptosis-regulatory gene products (6, 7). However, lack of apoptosis is considered a major component of the dysregulation of normal B-cell homeostasis in all subsets of this malignancy (7, 8).

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Patients, materials and methods

Selection of the patients: This is a retrospective cross-sectional study; whereby archival paraffin-embedded tissue blocks along with the clinical and hematological records of sixty patients with CLL were recruited at the Department of Hematology of the Medical City Teaching Laboratories in the period from January 2004 to December 2007. The patients were newly diagnosed and did not receive prior treatment. The bone marrow biopsies were performed at diagnosis. Paraffin-embedded tissue blocks of twenty control individuals (age and sex matched) along with their hematological reports were also collected. All the control bone marrows were negative for infiltrative lesions and were obtained from patients with anemia due to iron or vitamin B_{12} deficiencies. CLL patients were diagnosed and selected according to the criteria of the International Workshop on CLL (IWCLL) 9 which included: 1- Persistent absolute lymphocytosis of more than 10,000 mature-appearing lymphocytes/µL in the peripheral blood, 2- bone marrow aspirate smear with lymphocytes ≥ 30% of all

nucleated cells and 3- B-cell phenotype of peripheral blood. Criteria 1 with either 2 or 3 were needed for diagnosis. In this study diagnosis was based on criteria 1and 2. All patients had peripheral blood prolymphocytes of less than 10%. Clinical staging was done according to the modified Rai staging system 10.

In-situ hybridization: Sections, 5 µm in thickness were made on positively charged slides (Esco) from all the study sample and control groups and subjected for in-situ hybridization procedure to detect mRNAs of p53 and Bcl-2. Biotinylated cDNA liquid probes for p53 and Bcl-2 together with the in-situ hybridization/ detection kit were purchased from Maxim biotech, USA. The kit contains a house-keeping gene probe as a positive control. A target positive control was also used which included sections from oral squamous cell carcinoma already positive for p53 and from lymph node with follicular lymphoma already positive for Bcl-2. The procedure of in-situ hybridization was conducted according to the manufacturing company. It involved deproteinization of fixed tissue sections mounted on slides by proteinase-k enzyme, hybridization of a denatured biotinylated probe to the target sequence and denaturation of the target mRNAs in tissue sections. The hybridized probe was then detected by streptavidin– alkaline phosphatase (streptavidin-AP) conjugate. Upon addition of the substrate solution which is 5-brom-4 chloro-3 indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT), an intense blue signal appeared at the specific site of the hybridized probe.

Evaluation of the in situ hybridization signal: Quantification of in situ hybridization signal was evaluated under light microscopy (X100, X400 and X1000), whereas the counting of positive cells was performed at oil immersion (X1000). Counting of positive cells was conducted in 10 different fields taking their mean for each sample. ISH was given percentage scores, based on the number of stained cells. Percentage scores were assigned as: score 1 (low) = 1-25%, score 2 (intermediate) = 26-50%, and score 3 (high) = 51-100% 11. Statistical analysis was performed with the *SPSS16* statistical software program (SPSS Inc. Chicago, IL, USA). Associations between categorical variables were assessed via crosstabulation and chi-square. Spearman correlation was used to correlate variables when at least one variable was ordered. Exact tests were used to calculate the *p* value. In all statistical analyses, a *p* value < 0.05 was considered significant.

Results:

Hybridization signals (diffuse, granular or focal), were detected either in the nucleus or in the cytoplasm of CLL lymphoid cells of BM biopsies with good preservation of the morphological details. Normal lymphoid cells present in BM biopsies of control group did not show any signal for p53 and Bcl-2 (the median BM lymphocyte percentage was 11.5% with a range between 5 and 20%). Therefore, CLL was considered positive when at least 1% of lymphoid cells gave positive hybridization signal. The overall frequency of p-53 positivity in CLL was 28.3% (17 of 60 cases; 13 males and 4 females), with no statistically significant difference (table 1). The overall frequency of Bcl-2 positivity in CLL was 50% (30 of 60 cases; 22 males and 8 females), also with no statistically significant difference (Table 1). No significant difference was found between p-53-positive and p-53-negative patients when they are subdivided according to modified Rai staging system (table 2). No significant differences were found between the number of Bcl-2 positive and Bcl-2-negative cases when they are subdivided according to the modified Rai staging system (Table 2) The distribution of the different percentages of p-53 and Bcl-2 scores among the CLL patients is shown in table 3 and figures 1A & B respectively. No statistically significant difference was found between p-53 or Bcl-2 positive males and females regarding score. A significantly larger number of patients, with high score for p-53 signal, was associated with high-risk clinical stage than patients with low score ($p = 0.005$) (Table 4). At the same time there was a significant direct positive correlation between increasing scores of p-53-positive CLL cells and advancing clinical stage of the disease ($p = 0.002$) (Figure 2 A). No significant association was found between the number of patients with different Bcl-2 scores and the clinical stage of the disease (Table 4), also no significant correlations were found between Bcl-2 scores and the clinical stage of the disease (Figure 2 B).

Table 1. Distribution of P-53 and BCL-2 signals in CLL patients according to sex.

		Sex		
		Male	Female	Total
P53signal	Positive	13	4	17 (28.3%)
p = N.S.	Negative	32	11	43 (71.7%)
	Total	45	15	60 (100%)

Table2. Distribution of P-53 and BCL-2 signals according to modified Rai stage of CLL.

		Modified Rai stage			
		Low	Intermediate	High	Total
P53signal	Positive	1	5	11	17
p = N.S.	Negative	0	15	28	43
	Total	1	20	39	60
		Modified Rai stage			
		Low	Intermediate	High	Total
Bcl-2signal	Positive	*0*	10	20	30
p = N.S.	Negative	1	10	19	30
	Total	1	20	39	60

Table 3. Distribution of p-53 and BCL-2 scores according to sex.

		Sex		
		Male	Female	Total
Bcl-2 signal	Positive	22	8	30 (50%)
p =N.S.	Negative	23	7	30 (50%)
	Total	45	15	60 (100%)

		Modified Rai stage			
		Low	Intermediate	High	Total
P53score	Low(125%)	1	0	0	1
	Intermediate (26-50%)	0	2	0	2
P=0.005	High(51100%)	0	3	11	14
	Total	1	5	11	17
		Modified Rai stage			
			Intermediate	High	Total
Bcl-2 score	Low (1-25%)		5	8	13
	Intermediate (26-50%)		1	5	6
P = N.S.	High (51-100%)		4	7	11
	Total		10	20	30

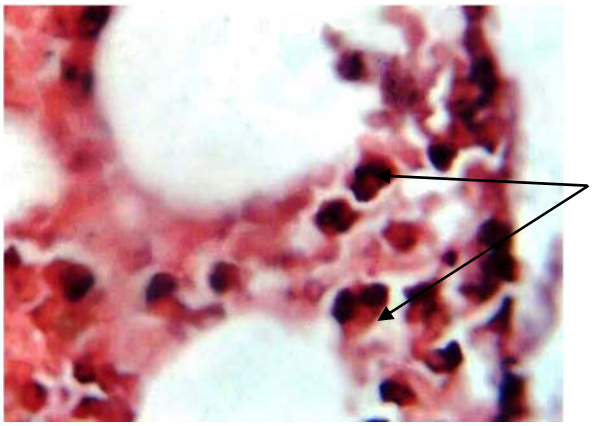

Figure 1A. CLL: BM biopsy. Positive p-53 ISH signal. The lymphocytes show blue cytoplasmic staining (arrows); high score (× 1000).

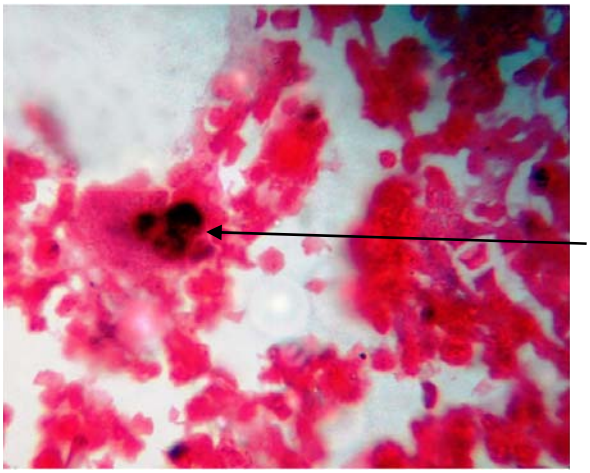

Figure 1 B. CLL: BM biopsy. Positive Bcl-2 ISH signal. The lymphocytes show blue cytoplasmic staining (arrow); low score (× 1000).

Table 4. Distribution of P53 and BCL-2 scores according to the clinical stage of the disease.

		Sex		
		Male	Female	Total
P53 score	Low (1-25%)	1	0	1 (5.9%)
	Intermediate (26-50%)	2	0	2 (11.8%)
P = N.S.	High (51-100%)	10	4	14 (82.3%)
	Total	13	4	17 (100%)
		Sex		
		Male	Female	Total
Bcl-2 score	Low (1-25%)	10	3	13 (43.3%)
	Intermediate (26-50%)	5	1	6 (20%)
P = N.S.	High (51-100%)	7	4	11 (36.7%)
	Total	22	8	30 (100%)

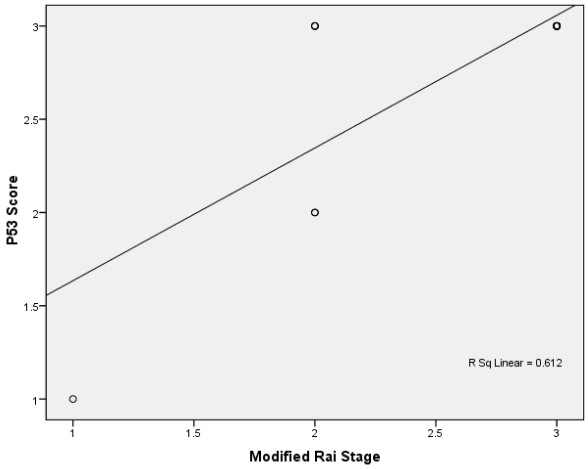

Figure 2 A. Scatter-plot showing the correlation between p-53 score and clinical stage.

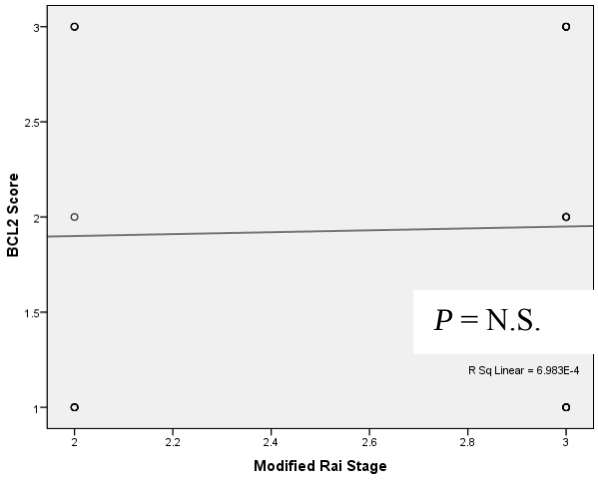

Figure 2 B. Scatter-plot showing the correlation between BCL-2 score and clinical stage.

Discussion

P-53: Structural alterations and point mutations of the p-53 tumor suppressor gene have been shown in 10% to 15% of CLL; they have been associated with poor survival and nonresponse to therapy, suggesting that p-53 may play a role in the clinical course of the disease (1, 12). No association was found between p-53 signal positivity and the sex of patients (table 1). Similar results were reported by other workers (13, 14, 15). No association was found between p-53 signal positivity and the clinical stage of the disease (table 2). However, Lens et al, found that CLL cases with p53 abnormalities were characterized by a higher incidence of Binet stage C than patients without p53 abnormalities 14. The frequency of p-53 positivity was 28.3% (17 of 60 cases) (table 1), which was higher than the frequency of p-53 expression reported by Cordone et al. 13 and Mahdi 15, who gave a frequency of 15% and 15.8 % respectively of p-53 protein positivity in CLL using immunohistochemical method to detect the expression of p-53 protein. In this study, most of the patients (65%, n =39/60) were in high-risk clinical stage (table 2) while only 1 patient (1.7%) was in low-risk stage. Previous studies in Iraq showed similar findings of advanced clinical stage of CLL in Iraqi patients at diagnosis (15, 16). While in the study of Cordone et al 13, 59.7% of their CLL patients were in Binet stage A and only 13.2% were in Binet stage C. Similar studies in the Western world had shown that most of their CLL patients were in early clinical stage of the disease at the time of diagnosis 17. A difference between the two techniques is an important factor, since ISH detects the mRNA and not the protein. A point mutation can abolish protein synthesis, thus no p-53 protein is made in spite of the presence of its mRNA. However, this mRNA is regarded as an immature one since such mutations usually affect the introns with defective splicing of the immature mRNA (18, 19). No statistically significant difference was found between p-53-positive males and females regarding score (table 3). Other workers reported similar results (13, 14). The association between the number of cases with different p-53 scores and clinical stage was statistically significant; being higher with advanced disease stage (Table 4). This may explain the higher frequency of p-53 positivity in this study than that found in Western countries. This study also revealed that p-53 score, as assessed by ISH, is significantly correlated with the clinical stage of the disease (Figure 2 A), and thus it is an important prognostic variable in patients with CLL. These data concur with those reported by other workers, in their analyses of p-53 expression in patients with CLL. They reported that significantly higher number of their p-53-positive patients are associated with advanced clinical stage of the disease than patients in early clinical stages, and also showed that the score of p-53 protein expression, as measured by IHC was strongly correlated with p-53 gene mutations, advanced disease, progressive disease, refractoriness to therapy and reduced survival (13, 20). Advancing clinical stage of CLL is associated with increasing number of CLL cells carrying mutated p-53 mRNA as shown in figure 2 A, i.e. there is expansion of an initially minor subclone with a mutated p-53 gene during disease progression. Cordone et al reported similar result 13. Regarding the forty three (71.7 %) CLL cases, which were p53-negative by ISH technique, the RNA-ISH procedure, cannot exclude p-53 gene deletion in CLL cells. This deletion can be confirmed by the application of FISH technique on chromosomal preparations.The absence of a positive fluorescent signals confirm gene deletion 21. The application of DNA-ISH technique is not always helpful in discriminating between cells carrying a wild type of gene and those with a mutated one, those with a deleted gene and a mutated one and those with wild type of gene and a deleted one 22. **Bcl-2:** Although the Bcl-2 gene is rarely translocated in CLL, the Bcl-2 protein is constantly over- expressed, and high levels have been noted using immunoblot analysis in CLL patients. The mechanisms responsible for the high levels of Bcl-2 in CLL cells remain unclear but possibly could be related to gene hypomethylation and *trans*-acting regulatory factors 23. The frequency of Bcl-2 positivity was 50% (30 of 60 cases) (table 1). This was similar to the frequency of Bcl-2 expression in CLL reported by other workers who found that Bcl-2 protein is constantly over- expressed, using immunoblot analysis, in 47-60% of CLL patients (23, 24, 25). No association was found between Bcl-2 signal positivity and the sex of patients (table 1). Similar results were reported by other workers (23, 24, 26). No association was found between Bcl-2 signal positivity and the clinical stage of the disease (table 2). Other workers reported similar results (26, 27). No statistically significant difference was found between Bcl-2-positive males and females regarding score (table 3). Similar results were reported by other workers (23, 24). No correlation was found between Bcl-2 score in CLL cells and clinical stage of disease (Table 4 & figure 2 B). Similar findings were reported by other workers (23, 28). However other workers had observed in their study that cells from patients at stages A and B have lower levels of Bcl-2 as compared to patients at stage C with an advanced disease and the association was statistically significant (25, 26).

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