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# The pattern of expression of the apoptotic inducer Fas and the apoptotic inhibitor bcl-2 oncogenes immunohistochemically in bone-marrow invaded by the non-Hodgkin lymphomas

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**Turk J Haematol 2004;21(3): 141-147**

**Received:** 04.05.2004 **Accepted:** 13.05.2004

## ABSTRACT

Apoptosis is a feature commonly seen in tumors; it is an actively regulated cellular process that leads to cell death, in fact the ability to resist apoptosis may seem to offer an advantage to a growing tumor by slowing down the cell loss rate. The present study is a retrospective study aiming at evaluating the Fas/Apo-1, CD95 and bcl-2 oncoproteins immunohistochemically in 30 bone marrow tissue specimens invaded by non-Hodgkin lymphomas (stage IV), 60% of cases were under chemotherapy regimen, while the remainders were still. The study included the significance of the apoptotic inducer Fas/CD95 and apoptotic inhibitor bcl-2 immunoreactivity in relation to a number of clinicopathological variables including age, sex, pattern of malignant cell infiltration in bone marrow, type of malignant cells disseminated and the effect of chemotherapy in relation to apoptotic changes. Fas/CD95 immunoreactivity was positive in 23 cases; 76.7%, positive immunoreactivity was significantly associated with the chemotherapeutic effect ( $p= 0.0002$ ). On the other side bcl-2 immunoreactivity was positive in 7 cases; 23.3%. A significant association was found between bcl-2 positive immunoreactivity and sex ( $p= 0.03$ ), pattern of malignant cell infiltration in bone marrow ( $p= 0.02$ ) and lack of therapy effects ( $p= 0.0004$ ). As a conclusion (1) Apoptosis is a common feature in non-Hodgkin's lymphomas as confirmed by the high incidence of the apoptotic inducer Fas/CD95 positive immunoreactivity; (2) Fas/CD95 and bcl-2 as a tumor markers may work as a useful aid in establishing an apoptosis interpretation in cases of non-Hodgkin's lymphomas (stage IV); (3) Fas/CD95 expression translate the chemotherapeutic effects on malignant cells in non-Hodgkin's lymphomas; meanwhile the apoptotic inhibitor bcl-2 expression is an independent negative prognostic marker that has been shown to confer resistance to apoptosis.

**Key Words:** Apoptosis, Non-Hodgkin's lymphoma, Fas/CD95, Bcl-2 proto-oncogenes.

## ÖZET

### Non-Hodgkin lenfoma tutulumu olan kemik iliklerinde apoptozis indükleyici Fas ve apoptozis inhibitörü olan bcl-2'nin sunum şeklinin immünohistokimyasal olarak gösterilmesi

Apoptozis tümörlerde sık görülen bir olay olup, hücre ölümüne yol açan aktif olarak düzenlenmiş bir hücre sel süreçtir. Apoptozise direnç göstermek, hücre kaybını azaltarak tümöre bir avantaj sağlar. Bu çalışmada non-Hodgkin lenfoma evre IV olan ve kemik iliği tutulumu olan 30 hastanın kemik iliği dokusuna retrospektif olarak Fas/Apo-1, CD95 ve bcl-2 onkoprotein açısından immünohistokimyasal inceleme yapıldı. Olguların %60'ı kemoterapi almakta idi. Çalışmada, apoptozisi indükleyen Fas/CD95 inhibe eden bcl-2 immünreaktivitesi ile yaş, cinsiyet, kemik iliğinde malign hücre infiltrasyonu paterni, tipi ve kemoterapi ile olan ilgisi gibi klinikopatolojik değişkenler arasındaki ilişkiye bakıldı. Fas/CD95 23 olguda pozitif. Pozitif immünreaktivite kemoterapi etkisi ile yakından ilişkili idi ( $p= 0.0002$ ). Bcl-2 ise 7 olguda pozitif. Bcl-2 pozitifliği ile cinsiyet arasında ( $p= 0.03$ ), kemik iliğinde malign hücre infiltrasyon paterni arasında ( $p= 0.02$ ) ve kemoterapi etkisinin olmaması arasında ( $p= 0.0004$ ) yakın ilişki saptandı. Sonuç olarak non-Hodgkin lenfomada apoptozis sık görülen bir durumdur. Fas/CD95 ve bcl-2, kemik iliği tutulumu olan non-Hodgkin lenfomada apoptozis yorumu yapabilecek bir tümör işaretleyicisi olarak kullanılabilir. Fas/CD95 ekspresyonu kemik iliğine kemoterapinin etkisini gösterebilirken bcl-2 ekspresyonu apoptozise direnci gösteren bağımsız bir işaretleyici olarak kullanılabilir.

**Anahtar Kelimeler:** Apoptozis, Non-Hodgkin lenfoma, Fas/CD95, Bcl-2.

## INTRODUCTION

Apoptosis is a feature commonly seen in tumors; it is an actively regulated cellular process that leads to cell death, in fact the ability to resist apoptosis may seem to offer an advantage to a growing tumor by slowing down the cell loss rate<sup>[1,2]</sup>. The pharmacological manipulation of apoptosis offers new possibilities for prevention and treatment of cancer<sup>[3]</sup>. Apoptosis (programmed cell death) plays a crucial role in tumor response to chemotherapy, it render the cells susceptible to therapeutic intervention<sup>[4]</sup>. Most anti-cancer drugs agent produce apoptosis in sensitive cells, this observation influence the response of chemotherapy<sup>[5]</sup>.

Bcl-2 is unique among proto-oncogenes by its ability to block programmed cell death without promoting cell proliferation, which lead its categorization as a member of a new category of oncogenes: regulators of cell death<sup>[6,7]</sup>. Bcl-2 is only one member of an expanding family of genes involving in cell death whose co-expression and balance seems important in controlling apoptosis<sup>[8]</sup>. Bcl-2 blocks programmed cell death and thus represent the first step before malignant transformation, it is probably responsible for the

high rate of cells that remain in the G0 phase of the cell cycle, above this, recent studies demonstrated the role of bcl-2 resistance to chemotherapy induced-apoptosis which possibly accounts for the well documented inability of conventional chemotherapy to cure disseminated diseases<sup>[9,10]</sup>.

Fas/Apo-1, CD95 is a membrane glycoprotein belonging to the tumor necrosis factor/nerve growth factor receptor family and which can trigger apoptosis in some lymphoid cell lines<sup>[11]</sup>. The CD95 (Fas)/CD95 ligand (CD95L) system is an important mechanism triggering apoptosis<sup>[12]</sup>. Fas (CD95, Apo-1) mutations were found in some lymphomas, suggesting impairment of Fas-mediated cell death signaling that may cause tumor development<sup>[13]</sup>. CD95 mediated apoptosis is at least partially inhibited by expression of bcl-2 proto-oncogene<sup>[5]</sup>. The CD95 death receptor and its ligand have been implicated in the control of apoptosis and so drug induced apoptosis was suggested to depend in part on activation of Fas/CD95<sup>[14,15]</sup>.

### Aim of Study

The present study is a retrospective study aiming at evaluating the Fas/Apo-1, CD95

and bcl-2 oncoproteins immunohistochemically in bone marrow tissue specimens invaded by non-Hodgkin lymphomas (stage IV) in relation to common clinicopathological variables including those who did not received any therapy and those who were already under chemo-therapy regimen.

#### **MATERIALS and METHODS**

Thirty bone marrow tissue biopsy specimens were collected from patients with disseminated non-Hodgkin lymphomas invading the bone marrow (stage IV). They were collected from the Hematology Department, Medical Research Institute, Alexandria University from January 2001 to December 2001.

Specimens collected were fixed in 10% formalin and processed by the original methods as paraffin blocks. All paraffin blocks were cut at 3-5  $\mu$ m sections on glass slides for conventional haematoxylin and eosin as well as for immunohistochemical staining.

Conventionally stained sections were examined microscopically for evaluation of the pattern of malignant non-Hodgkin cell infiltration as well as for type of malignant cell disseminated in bone marrow.

#### **Immunohistochemical Staining**

The immunohistochemical staining procedures and interpretations were performed at the: Tumor Marker Unit; Department of Histopathology, Damanhour Medical Institute; The General Organization for Teaching Hospitals and Institutes (GOTHI). Prepared sections were dewaxed in xylene and rehydrated in graded alcohol. The endogenous peroxidase was blocked by hydrogen peroxide 0.1% for 10 min. at room temperature. Non-specific binding was blocked by incubating the slides in 20% FCS in PBS for 20 min. at room temperature. Monoclonal antibodies against Bcl-2 (clone-124) and Fas/Apo-1, CD95 (clone Apo-1) were obtained from Dako; immunohistochemical procedures were performed as described previously<sup>[16]</sup>.

#### **Statistical Study**

Immunoreactivity for both the CD95 and bcl-2 were studied in relation to clinicopathological variables including age, sex, pattern of malignant cell infiltration in bone marrow, type malignant cells disseminated and the effect of chemotherapy in relation to apoptotic changes. To evaluate the statistical significance in present study Chi-square according to Mantel Haenzel was applied as appropriate. A p-value of < 0.05 was considered significant.

#### **RESULTS**

The thirty cases included in the present study were 18 male and 12 females with a ratio (3:2); the age incidence ranged from 15 y to 76 y. Eighteen cases; 60% were under chemotherapy regimen, while the remainders were still.

Microscopic examination of all cases revealed different patterns of infiltration's in bone marrow biopsy specimens they included: focal pattern in 8 cases; 26.7%, interstitial pattern in 9 cases; 30%, paratrabeular in 3 cases; 10%, diffuse pattern in 7 cases; 23.3%, and finally mixed pattern was seen in 3 cases; 10%.

The types of malignant non-Hodgkin lymphoma cells disseminated in the bone marrow were small cell type in 7 cases; 23.3%, large cell type in 13 cases; 43.3%, lymphoblastic cell type in 2 cases; 6.7% and mixed cellularity in 8 cases; 27.3% (Table 1).

The CD95 immunoreactivity was positive in 23 cases; 76.7% while 7 cases; 23.3% were negative, the staining reaction when present was cytoplasmic and along cell membrane; nuclei were clearly negative (Figures 1,2) on the other side bcl-2 presented positive immunoreactivity in 9 cases; 30% and it was negative in 21 cases; 70%, the immunoreactivity was always cytoplasmic extending to nuclear membrane with various degrees of intensity and inter-tumor heterogeneity of staining (Figure 3,4).

Table 1. The Immunoreactivity for both the Fas/Apo-1, CD95 and bcl-2 as studied in relation to clinicopathological variables

Variables	Fas/CD95 immunoreactivity		Chi-square	p-value	Bcl-2 immunoreactivity		Chi-square	p-value
	Negative	Positive			Negative	Positive		
Age								
> 50 y	5 (16.7%)	12 (40%)	0.7	0.376	13 (43.3%)	4 (13.3%)	0.76	0.385
≤ 50 y	2 (6.6%)	11 (36.7%)			8 (26.7%)	5 (16.7%)		
Sex								
Female	3 (10%)	9 (30%)	0.03	0.864	11 (36.7%)	1 (3.3%)	4.32	0.037
Male	4 (13.3%)	14 (46.7%)			10 (33.3%)	8 (26.7%)		
Pattern			4.08	0.395			11.17	0.0247
1. Focal	3 (10%)	5 (16.7%)			7 (23.3%)	1 (3.3%)		
2. Interstitial	0	9 (30%)			7 (23.3%)	2 (6.6%)		
3. Paratrabeular	1 (3.3%)	2 (6.6%)			1 (3.3%)	2 (6.6%)		
4. Diffuse	2 (6.6%)	5 (16.7%)			6 (20%)	1 (3.3%)		
5. Mixed	1 (3.3%)	2 (6.6%)			0	3 (10%)		
Type			2.63	0.453			6.68	0.0828
1. Small	3 (10%)	4 (13.3%)			6 (20%)	1 (3.3%)		
2. Large	3 (10%)	10 (33.3%)			9 (30%)	4 (13.3%)		
3. Mixed	1 (3.3%)	7 (23.3%)			4 (13.3%)	4 (13.3%)		
4. Lymphoblastic	0	2 (6.6%)			2 (6.6%)	0		
Chemotherapy			11.24	0.0002742			12.38	0.000434
1. Before	7 (23.3%)	5 (16.7%)			4 (13.3%)	8 (26.7%)		
2. After	0	18 (60%)			17 (56.7%)	1 (3.3%)		

p&lt;0.05 is considered significant.

CD95 immunoreactivity was significantly associated with chemotherapeutic effect ( $p=0.0002$ ), while there were not any significant difference for other clinicopathological variables that includes age, sex, pattern of malignant cell infiltration in bone marrow, and type malignant cells disseminated.

A significant association was found between bcl-2 positive immunoreactivity and sex ( $p=0.03$ ), pattern of malignant cell infiltration in bone marrow ( $p=0.02$ ) and lack of therapy effects ( $p=0.0004$ ) (Table 1).

### DISCUSSION

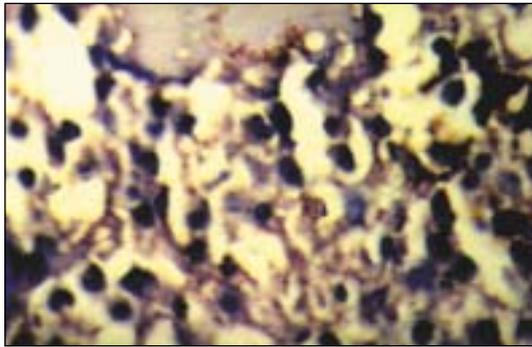
In the recent years, tumor biology is quickly becoming an important issue in pathology as it is related to the prognosis and treatment of cancer<sup>[17]</sup>. There has been an increasing interest in the role of apoptosis in tumorigenesis<sup>[18]</sup>. An imbalance between cellular apoptosis and survival may be critical for pathogenesis of lymphoma<sup>[1]</sup>.

The present study is a retrospective study aiming at evaluating the pattern of the apoptotic inducer CD95/Apo-1 and the apoptotic inhibitor bcl-2 oncogene immunohistochemically in bone marrow tissue specimens invaded by non-Hodgkin lymphomas (stage IV). The study included the significance of Fas/CD95 and bcl-2 immunoreactivity in relation to a number of clinicopathological variables including age, sex, pattern of malignant cell infiltration in bone marrow, type of malignant cells disseminated and the effect of chemotherapy in relation to apoptotic changes.

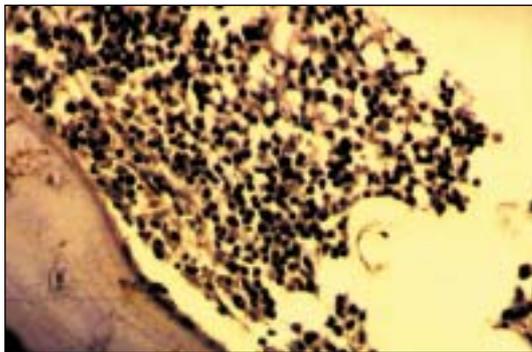
Fas/CD95 immunoreactivity was positive in 23 cases; 76.7%. Carson et al, 1993, reported that apoptosis usually affects scattered individual cells, rather than all the cells in a particular area and once initiated, it proceeds quickly<sup>[3]</sup>. Positive immunostaining showed a marked high incidence in relation to sex, where 14 male cases represented 60.9% of all positive cases. According to the pattern of infiltration in the bone marrow, all the cases of interstitial pattern of infiltration

(9 cases) were Fas/CD95 positive representing 39.1% of positive cases. Large cell type presented the highest incidence among all cell types disseminated (10 cases), representing 43.5% of the positive cases this finding was concomitant with Sigel et al, 2000 study, in which Fas/CD95 expression was slightly higher in the large atypical cells; moreover Soini et al 1998 mentioned that, high-grade malignant non-Hodgkin's lymphoma show a significantly marked apoptosis<sup>[19,20]</sup>. The effect of chemotherapy presented a high incidence for Fas/CD95 positive immunoreactivity, 18 cases representing 78.3% of positive cases; moreover, positive immunoreactivity was significantly associated with the chemotherapeutic effect ( $p=0.0002$ ). Fas/CD95 receptor activation that subsequently activates the procaspase-8 have been implicated in the execution of drug induced apoptosis<sup>[21]</sup>. Inman et al, 2000, found that induction of apoptosis was detected as early as eight hours after treatment<sup>[22]</sup>. Meanwhile, there were not any significant difference for other clinicopathological variables that includes age, sex, pattern of malignant cell infiltration in bone marrow, and type malignant cells disseminated.

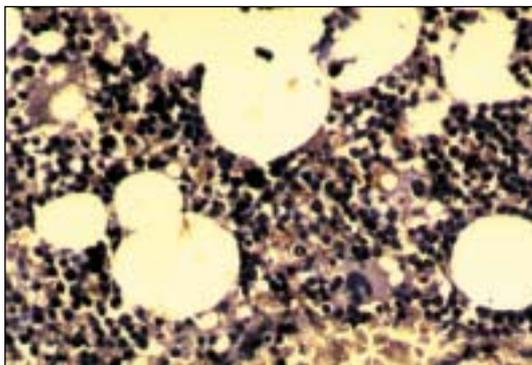
On the other side bcl-2 immunoreactivity was positive in 9 cases; 30%. A significant association was found between bcl-2 positive immunoreactivity and sex ( $p=0.03$ ), pattern of malignant cell infiltration in bone marrow ( $p=0.02$ ) and lack of therapy effects ( $p=0.0004$ ). Varana et al, 2002, concluded in their study that cells expressing pro-survival bcl-2 family are frequently resistant to a variety of chemotherapeutic agents<sup>[23]</sup>. High levels of bcl-2 expression is an independent negative prognostic marker that has been shown to confer resistance to apoptosis induced by chemotherapeutic drugs, presumably accounting for the association of bcl-2 with low sensitivity to chemotherapy and poor prognosis, thus forming a major obstacle for cure of lymphoid malignancies (5,9,14). Drugs that target bcl-2 function, will therefore act to render such cells susceptible to therapeutic intervention by re-opening the



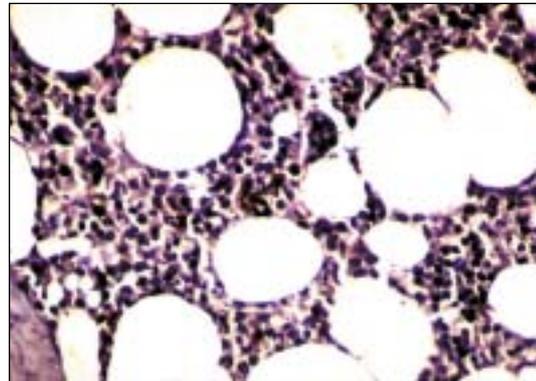
**Figure 1.** The pattern of CD95 positive immunoreactivity of large cell type of non-Hodgkin's lymphoma in paratrabeular pattern of invasion under chemotherapeutic effect (400x).



**Figure 2.** Diffuse CD95 positive immunoreactivity of small cell type of non-Hodgkin's lymphoma (paratrabeular pattern), also under therapy effect (400x).



**Figure 3.** Mixed cell type of non-Hodgkin's lymphoma (diffuse pattern) presenting marked diffuse bcl-2 positive cytoplasmic immunostaining (400x).



**Figure 4.** Small cell type of non-Hodgkin's lymphoma (interstitial pattern) presenting focal bcl-2 positive immunoreactivity (400x).

apoptotic pathway that was closed by the bcl-2<sup>[24]</sup>.

Fas/CD95 expression is generally associated with a favorable prognosis, Fas/CD95 mediated apoptosis is at least partially inhibited by expression of bcl-2 proto-oncogene<sup>[13]</sup>. Loss of Fas/CD95 expression or function by the neoplastic cells had been associated with more aggressive clinical behavior; meanwhile expression of bcl-2 was related to poor prognosis<sup>[25]</sup>. Thus, it is a challenge to devise approaches for inducing the death of tumor cells in which the expression of pro-survival family members is elevated or deregulated<sup>[23]</sup>.

#### **As a conclusion:**

1. Apoptosis is a common feature in non-Hodgkin's lymphomas as confirmed by the high incidence of the apoptotic inducer Fas/CD95 positive immunoreactivity.

2. Fas/CD95 and bcl-2 as a tumor markers may work as useful AIDS in establishing an apoptosis interpretation in cases of non-Hodgkin's lymphomas.

3. Fas/CD95 expression translate the chemotherapeutic effects on malignant cells in non-Hodgkin's lymphomas; meanwhile the apoptotic inhibitor bcl-2 expression is an independent negative prognostic marker that has been shown to confer resistance to apoptosis.

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