THE BCL-2 PROTEIN: A PROGNOSTIC INDICATOR STRONGLY RELATED TO ER AND PR IN BREAST CANCER

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Abstract

Bcl-2, the protein product of the Bcl-2 gene, is a member of the Bcl-2 family of proteins that play a crucial role in a complex mechanism of apoptosis. It was recently proposed that bcl-2 could inhibit cancer progression. In this study, we evaluated the expression patterns of Bcl-2, estrogen receptors (ER), progesterone receptors (PR) in 71 primary invasive breast carcinomas and their association with other clinicopathological parameters.
Samples from 71 patients with invasive breast cancer with follow-up ranging from 4-103 months (median 57 months) were included in the study. Forty-six patients (66%) obtained a complete response, while 5 (9%) were considered non-responders during the follow up period of 103 months. Eighteen (25%) patients died, 15 (21%) from primary disease and 3 (4%) from other disease. In unvaried analysis, tumor size (<2 cm), lymph node (<4 lymph nodes), hormonal status and Bcl-2 expression are correlated with longer overall (OS) and relapse-free survival (RFS). Patients with 4 or more positive axillary lymph nodes had significantly shorter OS (p=0.01) and RFS (p=0.009). Higher expression of Bcl-2 was associated with longer OS (p=0.02) and RFS (p=0.03), and this result were independent of axillary lymph nodes and tumor size in Cox multivariate analysis.

Introduction

Programmed cell death, apoptosis, is a physiological mechanism of cell death that plays an important role during development, metamorphosis and organ involution in many diseases, including cancer. (1) Regulation of apoptosis is a complex process and involves a number of genes, including Bcl-2 and related family members. (1-3) Abnormalities of apoptosis may lead to uncontrolled cellular proliferation and ultimately carcinogenesis. *BCL2*, first identified by its involvement in the t(14;18)(q32;q21) characteristic of follicular lymphomas (4) is a major negative regulator of apoptosis. The mechanism of the antiapoptotic function of BCL-2 is only partially understood, involving decreased mitochondrial release of cytochrome *c*, which is, in turn, required for procaspase-9 activation and initiation of the apoptotic cascade. (5)

The association of bcl-2 protein with a prognostically favorable phenotype is interesting and difficult to understand given its antiapoptotic function. One reason may be the expression of other members of the bcl-2 family that are proapoptotic such as Bax and can counteract the effect of bcl-2. High levels or aberrant patterns of bcl-2 expression occur in various tumors including breast cancer. (6,7). However, besides the experimental evidence showing the role of the bcl-2 protein as an inhibitor of apoptosis, some studies have suggested a growth-suppressing effect of bcl-2 associated with a retardation of mammalian cell proliferation. (8)

In this study, we wanted to evaluate the prognostic value of Bcl-2 protein in breast cancer and its correlation with OS and RFS. Also we wanted to evaluate the association between Bcl-2 protein and other prognostic clinical parameters.

Materials and Methods

Patient selection

The biopsy specimens from 71 patients with invasive breast cancer diagnosed at the Department of Pathology, University Hospital Sarajevo, Bosnia and Herzegovina, from January to December 1998 were randomly selected for this study. Clinical data were collected from the Department of Oncology, University Hospital Sarajevo, Bosnia and Herzegovina. Follow up ranged from 4 to 103 months (mean: 57 months). The last follow-up data were obtained in June 2004.
Three patients were excluded due to inadequate sample and one due to lost follow up. Breast cancer specimens were reviewed using morphologic and immunohistochemical criteria according to the WHO classification of breast cancer. (9) The degree of malignancy was assessed according to the Elston and Ellis grading system, which classifies tumors into grade I (well differentiated), grade II (moderately differentiated), and grade III (poorly differentiated). (10) All clinicopathological data together with clinical outcome are summarized according to treatment arm in **Tables 1.**

Table 1. Characteristic of 71 patients with breast cancer

Characteristic	Number (%)
Patients	71
female	71(100)
male	0(0)
Age	30-80 (53)
<40	6(8)
41-60	43(61)
>61	22(31)
Tumor type	
ductal	47(66)
lobular	9(13)
other	15(21)
Tumor size	
<2	19(27)
2-5	38(53)
<5	14(20)
Grade	
1	21(30)
2	22(31)
3	28(39)
Lymph node	
negative	30(42)
1-3	24(34)
4-9	12(17)
>10	5(7)
Overall survival	4-103 months
No evidence of disease	47(66)
alive with disease	6(9)
dead of disease	15(21)
dead of other disease	3(4)
Therapy	
Tamoxifen	29(41)
Chemotherapy	33(47)
Radiotherapy	9(13)

Immunohistochemical staining

Formalin-fixed, paraffin-embedded tissue was cut at 5 m, dried overnight at 60oC and deparaffinized in xylene. Subsequently, sections were rehydrated through graded alcohols into water. Heat-induced epitope retrieval was achieved by boiling sections in the EDTA buffer at pH 8.9 in the Electrolux microwave oven at 1000W for 20 minutes (4x5min). After boiling, sections were allowed to cool at room temperature for 20 minutes, rinsed thoroughly with water and placed in Tris-buffered saline (TBS) for 5 minutes. Endogenous peroxidase was blocked with Peroxidase Block solution provided in the EnVison+® kit (DakoCytomation, Glostrup, Denmark) for 5 minutes and slides rinsed/washed with TBS. Primary antibodies used in the study are listed in **Table 2**.The visualization was performed using EnVision+® (DakoCytomation, Glostrup, Denmark) method according to the manufacturer's instructions. Appropriate positive and negative controls were used. Staining for **ER** and **PR** was evaluated semiquantitatively using the H score system according to the method described by *McCarty et al.* (11) which considers the intensity and percentage of cells. The score was calculated as follows: Hscore = (%3 + cell x 3) + (%2 + cell x 2) + (%1+ cell x1). The intensity of ER and PR immunostaining was visually estimated and stratified into 4 groups. **Bcl-2** expression was scored semiquantitatively: score 0 (0-10%), score 1 (10-20%), score 2 (20-50%), and score 3 (>50%) cells were positive.

Statistical Methods

Descriptive statistics comparing Bcl-2, ER and PR expression with conventional markers of tumor aggressiveness were analyzed by standard Chi-square tests and Pearson test. Estimates of relapse-free survival (RFS) and overall survival (OS) were calculated by the Kaplan-Meier product-limit method and the differences assessed by the log-rank test. Multivariate survival analysis using Cox's proportional hazard regression model was carried out to assess the independent contribution of each variable to survival. Probabilities of RFS and OS were calculated from the date of breast carcinoma diagnosis to either the date at which relapse from breast carcinoma was clinically identified or the date of last contact. All p-values were two-tailed and the 0.05 level was considered statistically significant. A computer program package (SPSS 11.5) was used for all statistical testing.

Table 2 Primary antibodies used for immunohistochemical staining

Antibody (Clone)	Dilution	Incubation time (Temp)	Source
ER (1D5)	1:20	30 min (RT)	DakoCytomation, Glostrup, Denmark
PR (PgR 636)	1:20	30 min (RT)	DakoCytomation, Glostrup, Denmark
Bcl-2 (124-BCL-2)	1:20	30 min (RT)	DakoCytomation, Glostrup, Denmark

RT room temperature

Results

Characteristic of seventy-one patients with breast cancer are shown in **Table 1**.

Forty-seven (66%) tumors were of ductal type, nine (13%) lobular and fifteen (21%) other types of breast cancer. Twenty-one (30%) tumors were grade I, 22(31%) were grade II and 28(39%) were grade III. The histologic distribution according to the WHO classification was as follows: grade 1, 21 cases (30%); grade 2, 22 cases (31%); and grade 3, 28 cases (39%).

Association between clinical and histological parameters with expression of blc-2, ER and PR

Grade 1 of breast cancer was associated with better OS (p = 0.001) and RFS (p = 0.01). (Figure 1) Tumor size <2 cm was associated with better OS (p=0.003) and RFS (p=0.007). (Figure 2) Also, patient with smaller number of positive lymph node than 3 had longer OS (p=0.003) and RFS (p=0.002). (Figure 3)

Distribution of H-scores for ER and PR, as well as percent of Bcl-2 positive cells with varying degrees of intensity is given in Table 3. The results of ER, PR, and bcl-2 expression are illustrated in Image. 1-9

Bcl-2 positive immunoreactivity was detected in fifty one (72%) tumor samples. Six (9%) of this showed immunoreactivity in 10-20% of the tumor cells, scored as 1+, 10 (14%) samples showed staining in 21-50% of the tumor cells, scored as 2+, and 35 (49%) samples revealed immunoreactivity in more than 51% of the tumor cells, scored as 3+. Twenty (28%) samples showed immunoreactivity in <10% of the tumor cells, and were scored as 0. (Table 3)

Survival analysis revealed a favorable OS (p=0.02) and relapse free survival (p= 0.03) for patients with strong expression of bcl-2. (Figure 4) In general, more lower-grade of breast cancer expressed Bcl-2, ER and PR (Table 4), than the high-grade breast cancer did. Grade of breast cancer (p>0.000, linear-by-linear Association) and tumor size (p=0.01, linear-by-linear Association) were negatively associated with bcl-2 expression. (Table 4-6)

Also, Bcl-2 protein expression was positively associated with ER (r=0.408, p<0.001) and PR expression. (r=0.413, p<0.001, Pearson test). (Table 7 and 8)

Table 3. Imunohistochemical results of, ER, PR and Bcl-2

	Percent bcl-2 (%)	H-score ER (%)	H-score PR (%)
0	20 (28)	20(28)	25(35)
1	6(9)	6(9)	12(17)
2	10(14)	20(28)	6(9)
3	35(49)	25(35)	28(39)
Total	**71 (100)**	**71(100)**	**71(100)**

Table 4. Correlation between clinical parameters and Bcl-2, ER and PR expression

	Age	Diagnosis	Grade	Size	LNS*
ER	P=0.023	P=0.517	P<0.001	P=0.285	P=0.648
PR	P=0.389	P=0.167	P<0.001	P=0.04	P=0.105
Bcl-2	P=0.315	P=0.915	P<0.001	P=0.01	P=0.230

*LNS-Lymph node status

Table 5. Negative association between bcl-2 expression and Grades of Breast cancer*

		GRADE			Total
		1	2	3	
bcl-2	0	2 (10)	4 (20)	14 (70)	20(100)
	1	1(17)	2 (33)	3 (50)	6(100)
	2	4(40)	2(20)	4 (40)	10(100)
	3	14 (40)	14(40)	7 (20)	35(100)
Total		21 (30)	22 (31)	28 (39)	71(100)

* Data are given as number (percentage of row total). P<0.001, linear-by-linear Association.

Table 6. Negative association between bcl-2 expression and size of breast cancer*

		SIZE			Total
		<2	2-5	>5	
bcl-2	0	2(10)	11(55)	7(35)	20(100)
	1	2(33)	2(33)	2(33)	6(100)
	2	3 (30)	6(60)	1(10)	10(100)
	3	12 (34)	19(54)	4(12)	35(100)
Total		19(27)	38(54)	14(20)	71(100)

* Data are given as number (percentage of row total). P=0.01, linear-by-linear Association.

Table 7. Positive association between bcl-2 and ER expression*

		ER				Total
		0	1	2	3	
bcl-2	0	13(65)	2(10)	1(5)	4(40)	20(100)
	1	2(33)	1(17)	3(50)		6(100)
	2	1(10)	1(10)	5(50)	3(30)	10(100)
	3	4(11)	2(6)	11(32)	18(51)	35(100)
Total		20(28)	6(9)	20(28)	25(35)	71(100)

* Data are given as number (percentage of row total). P<0.001, linear-by-linear Association.

Table 8. Positive association between bcl-2 and PR expression*

		PR				Total
		0	1	2	3	
bcl-2	0	14	1	2	3	20
	1	3	2		1	6
	2	2	3	1	4	10
	3	6	6	3	20	35
Total		25	12	6	28	71

* Data are given as number (percentage of row total). P<0.001, linear-by-linear Association.

Table 9. Cox regression test results

	B	SE	Wald	df	Sig.	Exp(B)
GRADE	-.123	.628	.039	1	.844	.884
ER	.004	.005	.624	1	.430	1.004
PR	-.006	.006	1.144	1	.285	.994
Bcl-2	-.029	.014	4.181	1	.041	.972
THERAPY	-.213	.114	3.529	1	.060	.808
LNS*	.815	.379	4.627	1	.031	2.260
SIZE	1.675	.821	4.161	1	.041	5.341

LNS-Lymph node status

Figure 1. Grade of breast cancer was associated with better overall survival (p=0.001) (**A**) and RFS (p=0.01; log rank test) (**B**)

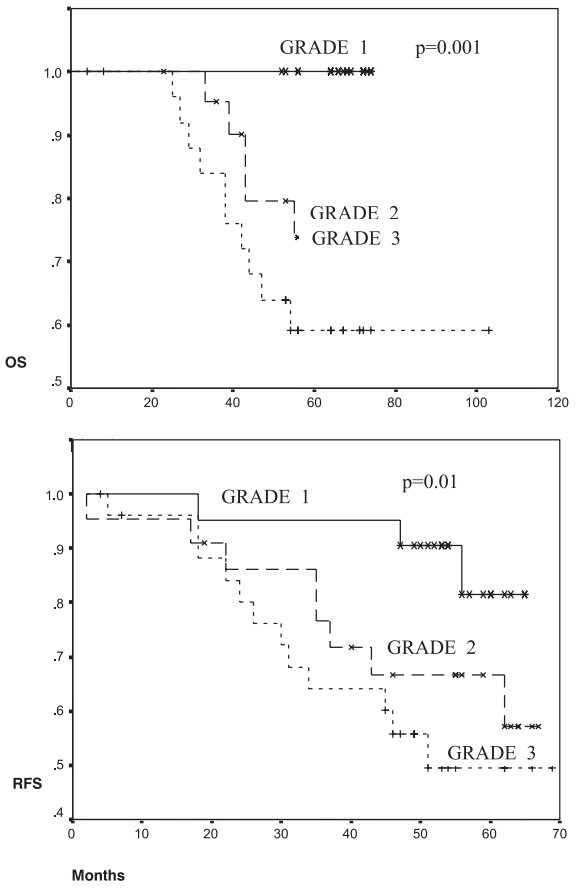
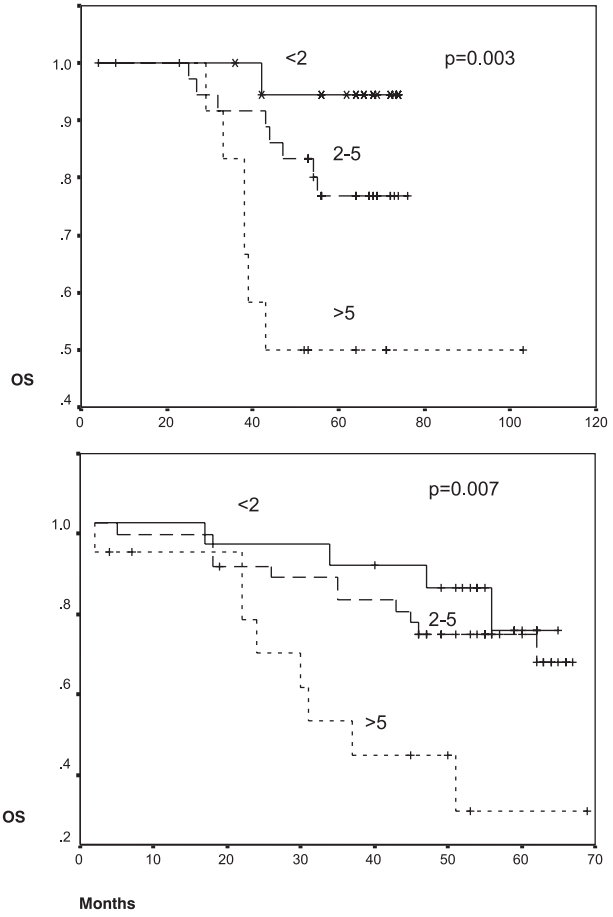


Figure 2. Tumors size <2cm was associated with better overall survival (p=0.003) (**A**) and RFS (p=0.007; log rank test) (**B**)



Bcl-2 expression in breast cancer was independent of tumor size and lymph node status in multivariate analysis. However, strong ER and PR expression was not independent of size and lymph node status as a favorable prognostic factor for OS and RFS in multivariate analysis. (Table 9)

Discussion

Bcl-2 is an oncogene that contributes to malignancy by inhibiting apoptosis and thereby extending cell survival and is one of the most studied apoptotic genes in breast cancer.

Overexpression of Bcl-2 occurs in 40% to 80% of human breast tumors (12, 7). In primary human breast cancer specimens, high Bcl-2 expression is associated with good prognosis as well as the expression of estrogen and progesterone receptor and low tumor grade. (12-18). In our study bcl-2 expression correlated positively with other predictors of favourable clinical course of the patient and less aggressive behaviour of tumour such as ER and PR positivity, smaller tumour size, lymph node negativity and lower tumour grade. Also, Bcl-2 expression in breast cancer was independent of tumor size and lymph node status in multivariate analysis. However, strong ER and PR expression was not independent of size and lymph node status as a favorable prognostic factor for OS and RFS in multivariate analysis.

According to *Sjöström et al.* (19) low bcl-2 expression was associated with shorter time to progression and shorter overall survival. Yang et al. (20) demonstrated that bcl-2-positivity was associated with favorable prognosis and their Cox proportional hazard model demonstrated that bcl-2 protein is an independent prognostic factor in invasive breast cancer, which is similar to our results.
One possible explanation for the phenomenon that bcl-2 is associated with better outcome (in clinical studies of prognostic factors in breast cancer) is that bcl-2 positive tumours often have ERs and, therefore more favourable prognosis. This could partially explain a favourable prognostic value of bcl-2, due to endocrine treatment that followed operative procedure.

We must also stress the possible inhibitory effects of bcl-2 on the progression of tumour which might explain why in our study bcl-2 overexpression is inversely associated with tumour size and grade. This seems paradoxical be-

Figure 3. Patient with smaller number positive lymph nodes had longer overall survival (p=0.003) (**A**) and RFS (p=0.002; log rank test) (**B**)

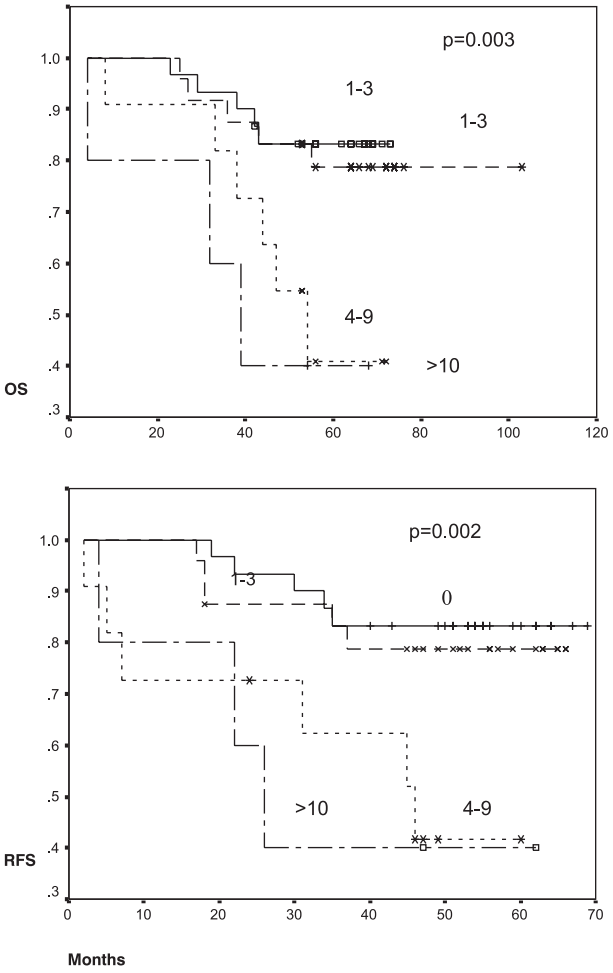
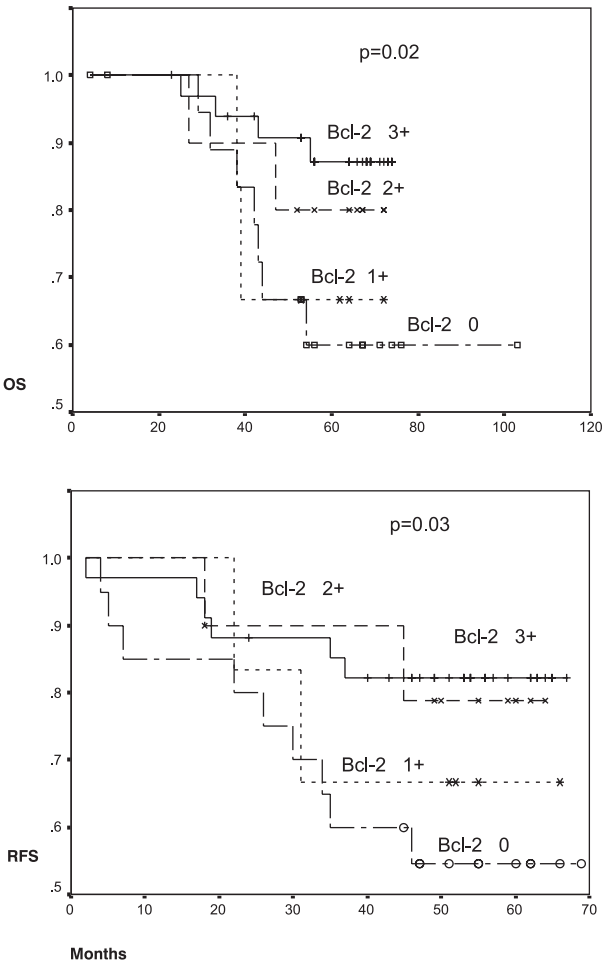


Figure 4. Bcl-2 expression was associated with better overall survival (p=0.02) (**A**) and RFS (p=0.03; log rank test) (**B**)



cause, in experimental models, bcl-2 overexpression protects cells from apoptotic death and decreases cell-cell adhesion leading to the lost of the contact inhibition. (6, 21, 22) In this way, bcl-2 is supposed to enhance cancer cells survival and promote tumorogenesis. (6,22,23) But, some other studies found inverse correlation between Bcl-2 expression and proliferation index in invasive tumours. (e.g. malignant melanoma). This suggests the possible role of bcl-2 in the regulation of cell proliferation. (24-26)

Also, we found that absence of Bcl-2 expression is strongly associated with high proliferation rates and high tumor grade. A number of mechanisms by which Bcl-2 exerts its positive growth-supporting effects and its anti-apoptotic function have been proposed. It has been shown that Bcl-2 plays a role in the regulation of cell division. As in our study, Bcl-2 overexpression (27) has previously been shown to be associated with negative axillary lymph node status. We found that 50% patients with negative lymph node as well as 58% patients had bcl-2, 3+ expressions, without significant correlation. However, besides the experimental evidence showing the role of the bcl-2 protein as an inhibitor of apoptosis, some studies have suggested a growth-suppressing effect of bcl-2 associated with a retardation of mammalian cell proliferation. (8)

A study by *Hellemans et al.* (13) showed no prognostic significance for bcl-2 expression in node-negative patients, but bcl-2 negativity correlated with reduced survival among node-positive patients. The evaluation of bcl-2 expression and extent of apoptosis may provide useful prognostic information on breast cancer patients; however while increased apoptosis is strongly associated with the progression from primary carcinomas to lymph node metastases; bcl-2 does not seem to play a significant role in this process. (28) However, lymph node metastases are not only the result of altered apoptosis, but are caused by several other genetic alterations, for example alterations in adhesion proteins, (29) cell motility and angiogenesis, among others. The way in which these proteins affect the other parameters involved in lymph node metastasis is not known.

Briasoulis E et al. (30) were recently found that quantita-

tive assessment of bcl-2 expression constitutes a new approach in early breast cancer with potential clinical implications. We consider that molecular sub-staging of patients with stage II breast cancer by level of bcl-2 expression provides additional important prognostic information and prompts for investigation of its clinical significance on the issue of adjuvant systemic therapy.

Recently it was proposed that bcl-2 could inhibit cancer progression (8). The level of bcl-2 protein in T cells has been connected with the retardation of the G1/ S transition through the sustained level of p27 (8) or through dephosphorylation of RB (31). Therefore, the protective effect of bcl-2 against cell death may be accomplished by slowing down the cell cycle progression (by increasing the length of the G1phase) what in the end has positive effect on the overall survival of the patients with breast cancer.

We confirm with studies *Coradini D et al* (32) that no single biomarker was able to identify patients with the best (or worst) prognosis or those which would be responsive to a given therapy. Novel findings derived from gene-expression analysis indicate that the simultaneous consideration of molecular alterations contributing to the hallmarks of cancer might provide clinically useful prognostic, and perhaps therapeutic, information.

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