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Prognostic value of apoptosis in breast cancer (pT1-pT2). A TUNEL, p53, bcl-2, bag-1 and Bax immunohistochemical study

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Summary. Apoptosis or programmed cell death produces cells breaking into several fragments of nuclei, cytoplasm or both nuclei and cytoplasm, known as apoptotic bodies which can be visualized in haematoxylin-eosin staining. Some genes (promoters and suppressors) control this process and certain mutations may induce the expression of abnormal proteins, which can be detected by immunohistochemical staining.

Apoptosis can be detected by the TUNEL method either identifying apoptotic bodies or cells at the initial stages of the fragmentation process.

We have studied 186 cases of infiltrating ductal breast carcinoma, stages pT1-pT2, and analysed the prognostic significance of tumour recurrence and overall survival of apoptotic index (AI) through univariate and multivariate analysis. We have also studied the immunohistochemical protein expression of apoptosis promoter and suppressors gene (p53, nuclear expression; bcl-2 and Bax, cytoplasm expression; BAG-1, nuclear and cytoplasm expression). The results indicate prognostic significance of p53 and bcl-2 related to patient death and bcl-2 and tumour size to tumour recurrence, bcl-2 acting as a protector factor (apoptotic suppressor) in both situations.

On the other hand, we have not found useful prognostic information of AI either to tumour recurrence or overall survival in univariate or multivariate studies. In this study, Bax expression does not provide a new prognostic role in breast carcinoma, although it contrasts to the bcl-2 action and accelerates death.

Key words: Apoptosis, p53, Bax, BAG-1, bcl-2

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Introduction

Necrosis and apoptosis are two different forms of cell death. Tumour growth is determined by the result of equilibrium between proliferation and cell death (Jong et al., 2000).

Programmed cell death (apoptosis) is considered an important homeostatic mechanism to balance cell proliferation and keep the correct number of cells in physiological and pathological conditions (Wyllie et al., 1980; Thompson, 1995). Apoptosis consists of cell fragmentation into small pieces of cytoplasm or cytoplasm and nuclei known as apoptotic bodies which are phagocytosed by the neighbouring cells without inducing inflammatory reaction (Kerr et al., 1972). These apoptotic bodies can be seen in haematoxylin-eosin (HE)-stained tissue sections; however, they could not be detected at the initial apoptotic stages in which nuclear fragmentation has not taken place so far (Jong et al., 2000; Kato et al., 2002). Terminal deoxytransferasa (TdT)-mediated bio-dUTP nick-end-labelling (TUNEL) is a technique which allows us to detect these initial stages of apoptosis.

Disturbances in apoptosis regulation would contribute to tumour progression and oncogenesis (Williams, 1991). Apoptosis appears to be controlled by several genes. A group of genes with sequences homologous to bcl-2 modulate cell death and can be divided into two functionally antagonistic groups: suppressors, such as bcl-2, and cell promoters, such as Bax. Homo or heterodimerization are important for the apoptotic regulatory function of the bcl-2-related proteins. The ratio between Bax/Bcl-2 heterodimers appears to be essential in deciding the life or death of a cell. When Bax predominates, apoptosis is accelerated and the antiapoptotic activity of Bcl2 is antagonised (Reed, 1994; White, 1996).

p-53 tumour suppressor gene exerts antiproliferative effects, including growth arrest and apoptosis (Wyllie, 1992; Slooten et al., 1998). It is a key target for

inactivation in human cancer and the loss of p53 function may contribute to tumour development and resistance to chemotherapy treatment (Bellamy, 1997).

The purpose of this work was to investigate the prognosis significance of apoptosis by means of the TUNEL method, and the immunohistochemical protein expression of some genes implicated in the regulation of apoptosis (p-53, bcl-2, BAG-1 and Bax) in breast cancer.

Materials and methods

A total of 186 consecutive patients with invasive operable pT1 and pT2 (TNM-system of UICC) ductal breast carcinoma (NOS), based on the WHO criteria (WHO, 1981), were studied at the Joan XXIII University Hospital from Tarragona between 1988 and 2000. In our study, we have included infiltrating ductal breast carcinoma (pT1-pT2), the most frequent breast cancer, in order to avoid the dispersion of values into the different histological patterns.

Fresh tissue from tumourectomy was sent to the Pathology Department immediately after surgical removal. It was fixed in buffered formalin for up to 18 hours and afterwards embedded in paraffin.

Clinical study

Conventional clinical study features were evaluated, including age, treatment carried out, complementary treatment, presence of metastases (local and distant) and patient death (distinguishing between death from breast cancer and other causes). Follow-up information was extracted from patients´ charts or, in case of decease, by contacting the hospital and the attending physician at the time of death.

Histopathological characteristics

Sample tissues were studied in sections stained with HE. The histological grade was determined according to criteria established by Elston (1988), and histological type according to the WHO classification (1981). Classic pathological features were evaluated, including tumour size (in mm), tumour necrosis and node metastases. Tumour necrosis was ascertained when there was more than 10% of necrosis in the infiltrating component of the carcinoma. Presence or absence of lymph-node metastases was also evaluated and, if present, the number of nodes involved were counted.

Apoptotic index (AI)

Apoptotic cells were identified by the TUNEL method, according to the standard procedure included in the Apop Tag Plus In Situ Apoptosis detection system" (Oncor, Gaithersburg, MD). In brief, sections were deparaffinized with xylene and ethanol and rinsed with PBS; tissues were then digested with proteinase K for 15 minutes and washed with distilled water and in PBS. The sections were incubated in a reaction mixture containing terminal transferase and digoxigenin dUTP at 37 ºC for 1 hour. The specimens were then washed, followed by the addition of antidigoxigenin antibody coupled to horseradish peroxidase, and the tissues were incubated for 30 minutes at room temperature. Following rinsing with PBS, 3,3'-diaminobenzidine tetrachloride was added for 10 minutes and counterstained with hematoxylin and eosin.

The Apoptotic Index (AI) was defined as the average number of apoptotic cells in 10 blindly selected high-power fields (x40 objective, 0.152 mm^2 per field) in a single histological tumor section from each patient.

Immunohistochemical study

A previously described methodology was used to determine p53 protein (mouse, clone DO7, Dako, Glostrup, Denmark), bcl-2 protein (mouse, clone 124, Dako, Glostrup, Denmark), BAG-1 protein (mouse, clone 3.10G3E2, NeoMarkers, Fremont, CA) and Bax protein (mouse, clone 2D2, NeoMarkers, Fremont, CA) (Sirvent et al., 1995, 2001). A positive protein expression was considered when there was more than 10% of cytoplasmic and/or nuclear staining.

Statistical analysis

Descriptive analysis: mean, standard deviation (SD) and minimum and maximum values were used for quantitative variables. Categorical variables were described in terms of absolute and relative frequency.
Spearman's rank-based correlation was used to assess the relationship between the variables.
Survival analysis: survival curves were calculated for recurrence and death using the Kaplan-Meier method.
Predictive-factor analysis: recurrence and death were used as independent variables.
Univariate analysis: the Cox proportional-risk analysis was used to calculate the relative risk (RR) of the different predictive variables. A 95% confidence interval (95% CI) was calculated for each RR.
Multivariate analysis: the Cox proportional-risk analysis was used to evaluate the independent predictive variables after they were adjusted. The results are given as RR with a 95% CI. After performing partial multivariate analysis, those variables which biologically and/or statistically could predict for the dependent variables were included in the final model.

The accepted level of statistical significance was $p<0.05$.

Data analysis was carried out using the BMDP package of statistical programs (Dixon, 1991).

Results

Patient characteristics are shown in Table 1. The mean age at diagnosis in this serie was 58.6 years (SD 13.1) (range 31-91).

Table 1. Clinical, histological and immunohistochemical findings.

Tumour size	
≤ 20 mm	91 (48.9 %)
21-50 mm	95 (51.1 %)
Tumour necrosis	
Presence	78 (41.9 %)
Absence	108 (58.1 %)
Histological grade	
I	57 (30.6 %)
II	63 (33.9 %)
III	66 (35.5 %)
Lymph-node status	
Negative	111 (59.7 %)
Positive	75 (40.3 %)
1-3	43
>3	32
Apoptotic index	
TUNEL (≤ 10)	92 (51.4 %)
TUNEL (>10)	87 (48.6 %)
Missing	7
p53	
Positive	36 (19.4 %)
Negative	150 (80.6 %)
Bcl-2	
Positive	133 (71.5 %)
Negative	53 (28.5 %)
BAG-1	
Positive	150 (80.6 %)
Negative	36 (19.4 %)
Bax	
Positive	76 (40.8 %)
Negative	110 (59.1 %)

The mean tumour size was 20.8 mm (SD 8.9) (range 1-46 mm). Tumour necrosis was seen in 78 cases (41.9%). Axillary nodes could be analysed in all 186 cases and 75 (40.3%) presented metastases in a variable number of nodes.

AI

Positive apoptotic cells or bodies showed brown staining in the nuclei or in their fragments (Fig. 1). Apoptotic index values had some variability, ranging between 0 and 300 (median 14.8, SD 9.8). An established cut-off value of 10 allowed us to split the whole group into two equally distributed subsets.

Immunohistochemical study

Specific staining was confined exclusively to the nuclei (p53), to the cytoplasm (bcl-2 and Bax) or both to the cytoplasm and nuclei (BAG-1) (Fig. 2-5).

Relationship between variables is expressed in Table 2.

Clinical outcome

The median follow-up for mortality was 54.6 months (SD 28.7) (range 1-138) and for relapse 53.1 (SD 29.03) (range 1-138). During this time there were 26 deaths resulting from breast cancer. The patients who died from unrelated causes were censored. The number of relapses was 37 cases.

Table 2. Correlation between parameters (r of Spearman).

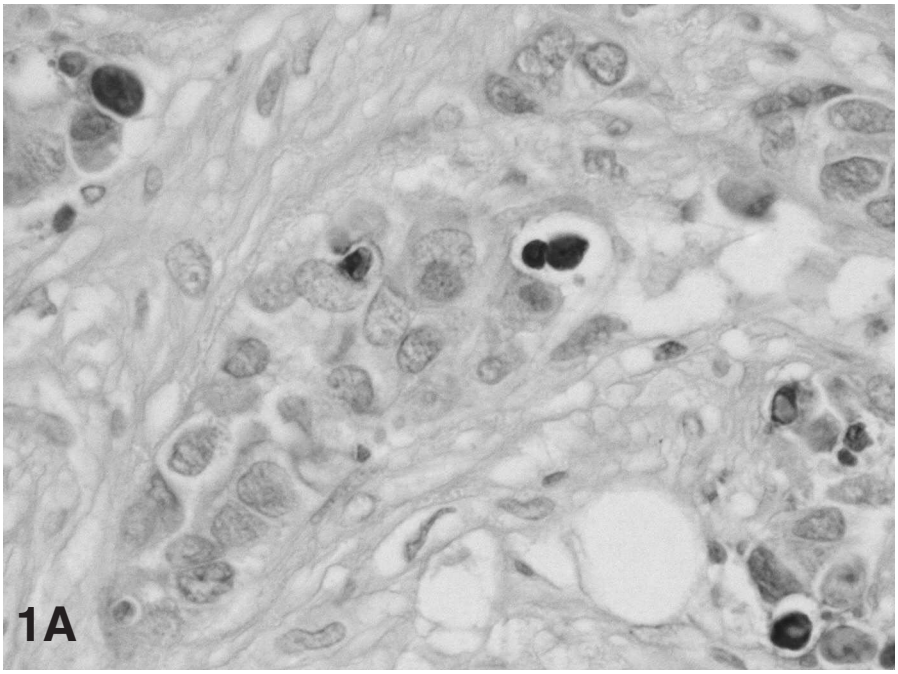
	T SIZE	H GRADE	TNECROSIS	LN STATUS	AI	P53	Bcl-2	bag-1	Bax
Tumour size	-								
Histol grade	0.28 p<0.001	- p<0.00							
Mitosis	0.3 p<0.000	0.86 p<0.00	0.36 p=0.001	0.21 p<0.01	0.4 p<0.001	0.41 p=0.001	-0.18 p=0.001	-0.20 p=0.01	0.19 p=0.02
Tumour necrosis	0.34 p<0.001	0.39 p<0.001	-						
Lymph-node status	0.33 p<0.001	0.2 p=0.01	0.2 p=0.01	-					
Apoptotic index	0.22 p<0.001	0.4 p<0.00	0.24 p=0.00	0.15 p=0.04	-				
P53		0.4 p<0.00	0.30 p<0.00		0.3 p<0.00	-			
Bcl-2		-0.24 p<0.00	-0.15 p=0.04			-0.17 p=0.02	-		
Bag-1	-0.21 p=0.005	-0.17 p=0.02	-0.19 p=0.02				0.3 p<0.001	-	
Bax	0.17 p=0.02	0.19 p=0.02	0.17 p=0.02				0.2 p=0.04		-

Mitosis: parameter included in histological grade.

The disease-free interval was 74.51 % (CI 95 %: 67-82) at 5 years. Survival time was 84.6 % (CI 95 %: 79-90) at 5 years. The results of Kaplan-Meier curves of p53, bcl-2 and lymph node stage for recurrence and survival are shown in Fig 6-8.

The results of univariate analysis are shown in Table 3. Tumor size, node metastases and p53 were positively associated with tumor recurrence and mortality. Tumor necrosis appeared to be associated only with mortality. Bcl-2 acted as a protector factor either for tumor recurrence or for mortality.

The multivariate analysis (Table 4) showed that tumour size was the only independent predictor for tumor recurrence, and p53 was the unique mortality predictor. Bcl-2 was a protector factor for both dependent variables.



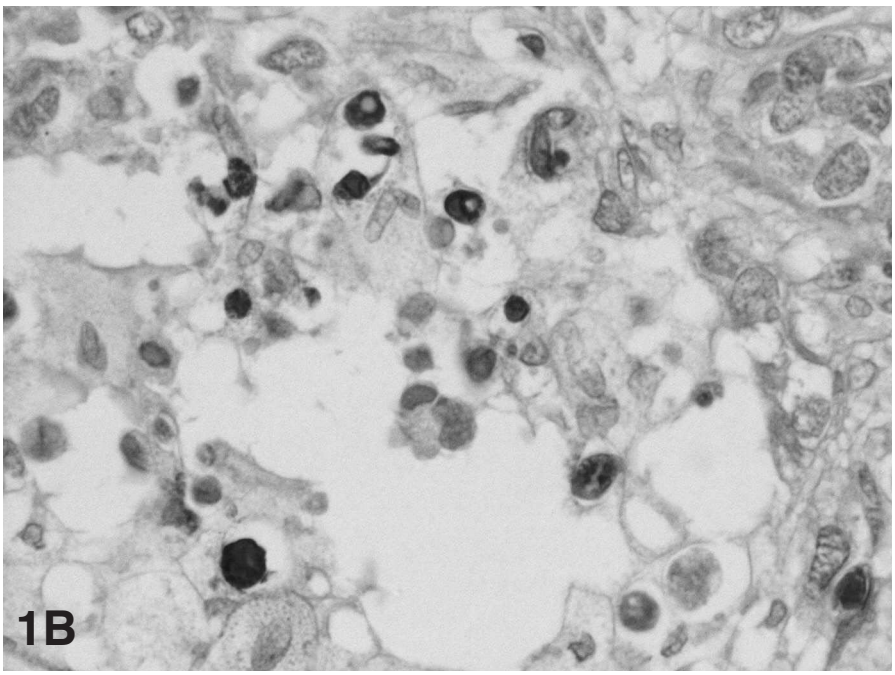


Fig. 1. A. TUNEL study: case with low apoptotic index. **B.** Tunel study: case with high apoptotic index. x 400

Discussion

In this study we have found that tumor size and p53 are predictive factors for tumor recurrence and mortality, respectively, in infiltrating ductal breast carcinoma. Bcl-2 comes out as a protector factor in disease evolution.

Regarding the correlation analysis of the studied variables we have observed an association between the clasycally bad prognostic factors (large tumour size, high histological grade, lymph node-positive and tumor necrosis) and on the other hand between good prognosis parameters (small tumour size, low histological grade

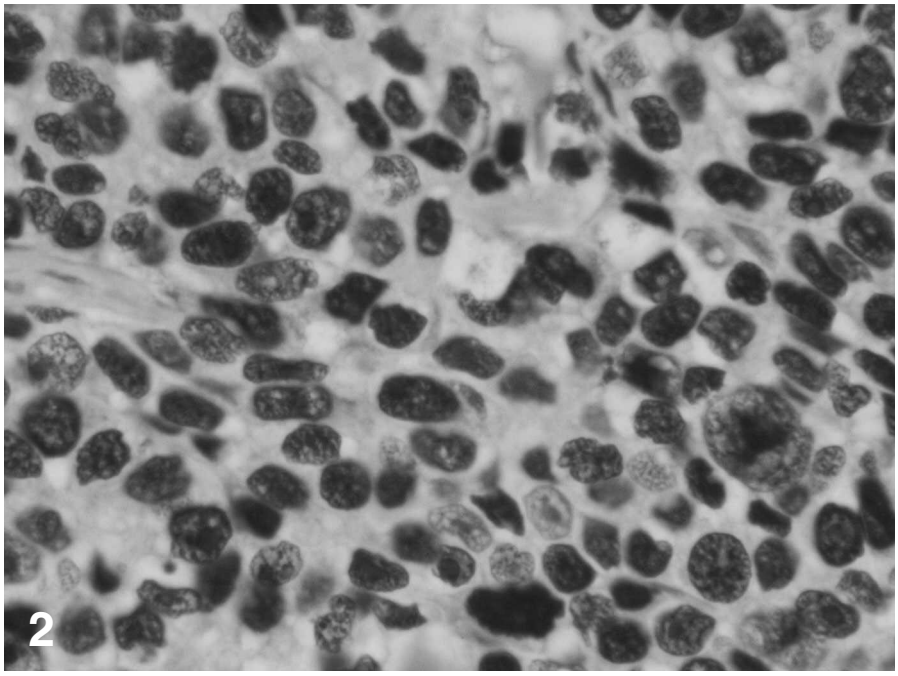


Fig. 2. p53. Strong positive expression in nearly all nuclei of tumoural cells (positive case). x 250

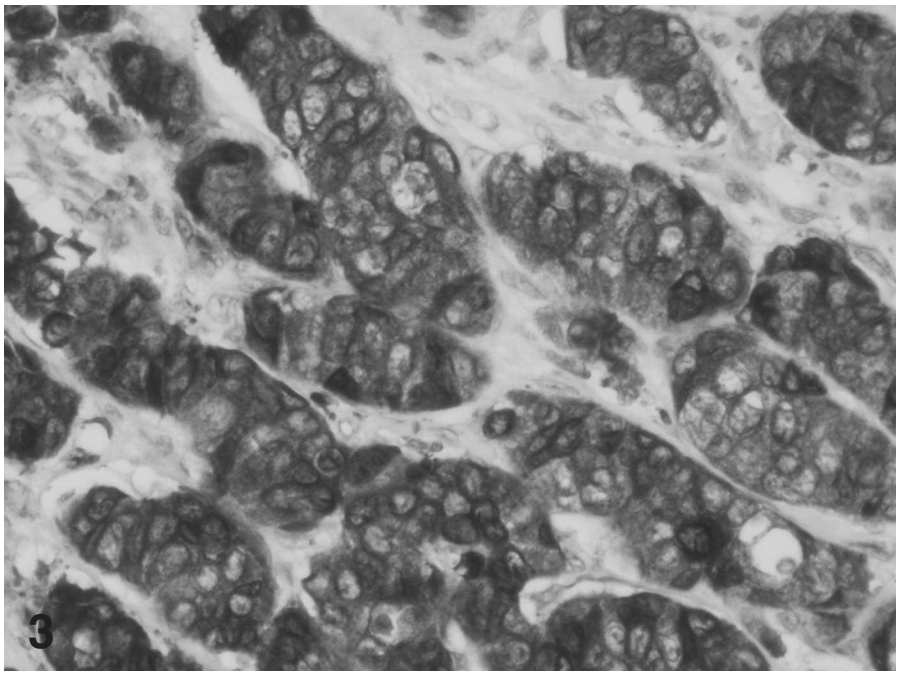


Fig. 3. Bcl-2. Strong positive expression in cytoplasm and membrane of tumoural cells (positive case) x 250

and lymph node-negative) (Table 2).

Among the methods to evaluate apoptosis, the TUNEL method allows the identification of cells that are in early stages of apoptosis giving a more reliable apoptosis measurement.

The value of AI can vary depending on the method used to count apoptotic cells, which may refer to the number of apoptotic cells per 1000 or 2000 tumour cells or to the percentage of apoptotic cells per 5 or 10 high-powered fields (Lipponen and Aaltomaa, 1994; Mustonen et al., 1997; Ellis et al., 1998; Slooten et al., 1998; Holmqvist et al., 1999; Kajiwuara et al., 1999; Kruger et al., 1999; Lipponen, 1999; Vakkala et al., 1999; Wu et el., 1999; Kymionis et al., 2001; Srinivas et al., 2002), or per square millimetre of neoplastic tissue (Schepop et al., 1996; Zheng and Zhan, 1998; Nishimura et al., 1999; Jong et al., 2000), or as in our case, per 10 high-powered tumour fields (Kato et al., 2002). We have found a positive correlation between AI and some well-known high risk clinicopathological factors for recurrence and shorter survival such as tumour size, necrosis, high histologic grade and lymph node positivity (Pillai et al., 1998; Zhang et al., 1998). On the other hand, the relationship between AI and mitosis number could suggest the existence of an interactive mechanism between tumoral proliferation and apoptosis which could be associated with bad prognosis (Allan et al., 1991; Lipponen and Aaltomaa, 1994; Slooten et al., 1996; Berardo et al., 1998; Lipponen, 1999; Zhang et al.,

Table 3. Univariate analysis in pT1 and pT2 breast-cancer patients: Cox proportional-risk analysis

	RECURRENCE		MORTALITY	
	RR	95% CI	RR	95% CI
Tumor size	**2.5**	**1.2-5.5**	**2.9**	**1.1-7.7**
Tumor necrosis	1.66	0.8-1.6	**2.11**	**1.02-4.4**
Histological grade	1.22	0.9-1.7	1.4	0.9-2.3
Node metastases	**2.45**	**1.3-4.5**	**2.5**	**1.2-5.4**
AI	1.34	0.8-2.2	1.4	0.7-1.9
p53	**1.5**	**1.03-2.15**	**2**	**1.3-3.1**
Bcl-2	**0.67**	**0.46-0.9**	**0.53**	**0.33-0.85**
BAG-1	0.82	0.8-1.04	0.72	0.4-1.2
Bax	0.9	0.8-1.1	1	0.6-1.7

Table 4. Multivariate analysis in pT1 and pT2 breast cancer patients: Cox proportional-risk analysis

	RECURRENCE		MORTALITY	
	RR	95% CI	RR	95% CI
Tumour size	**1.05**	**1.01-1.09**	1.03	0.9-1.1
Tumour necrosis	1.6	0.4-1.4	1.2	0.35-4.22
Node metastasis	0.7	0.6-3.4	1.8	0.6-5.12
p53	1.07	0.9-1.23	**1.16**	**1.03-1.35**
Bcl-2	**0.88**	**0.7-0.9**	**0.84**	**0.72-0.96**
BAG-1	0.99	0.8-1.14	0.99	0.8-1.14
Bax	0.97	0.8-1.16	0.96	0.8-1.16

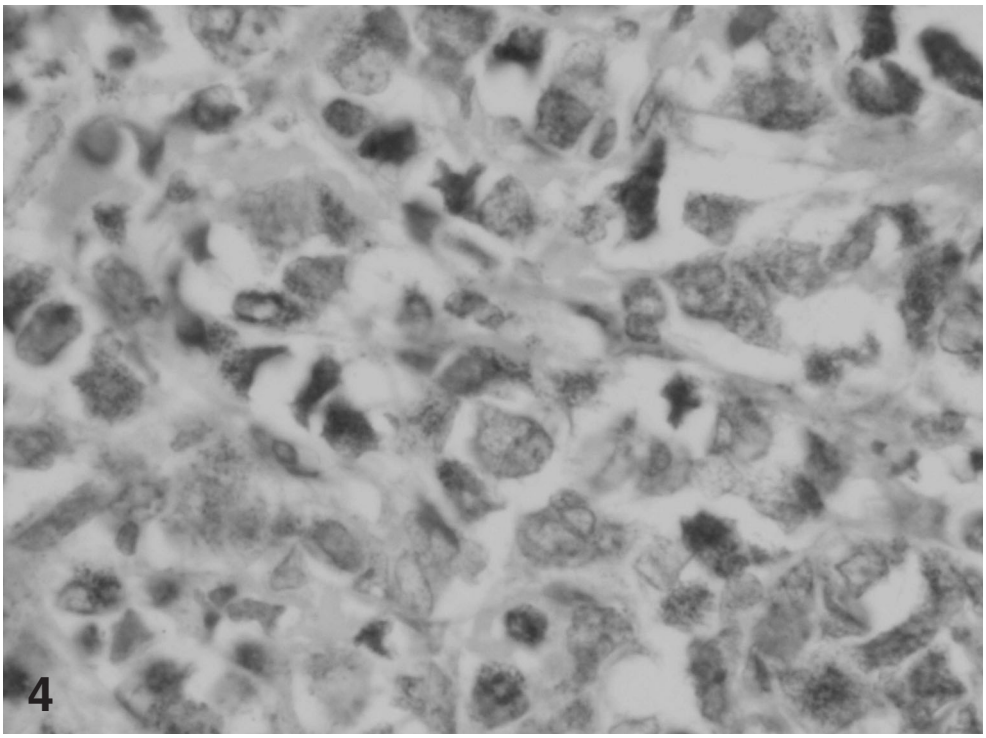
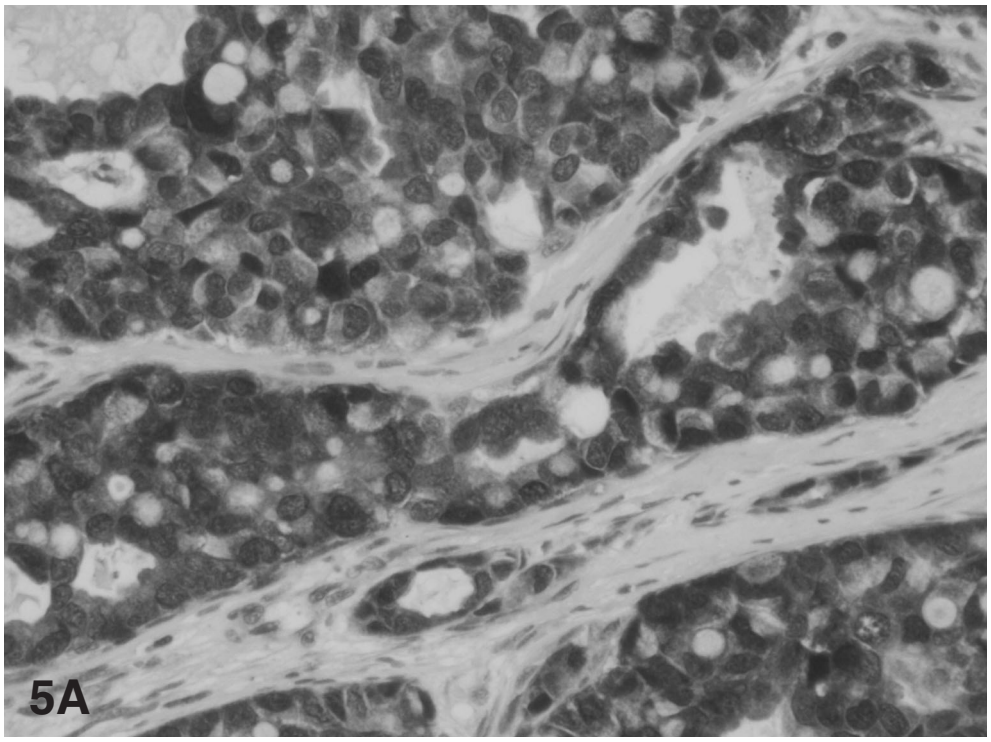


Fig. 4. Bax. Strong positive expression in cytoplasm and membrane of tumoural cells (positive case). x 260

1999).

In our series, we have not found AI to be a prognostic factor for recurrence or mortality, either in the univariant or multivariant models, as has been reported by other authors (Slooten et al., 1996; Kato et al., 2002) and in contrast to that suggested by other works (Lipponen and Aaltomaa, 1994; Lipponen, 1999; Vakkala et al., 1999; Jong et al., 2000; Villar et al., 2001;



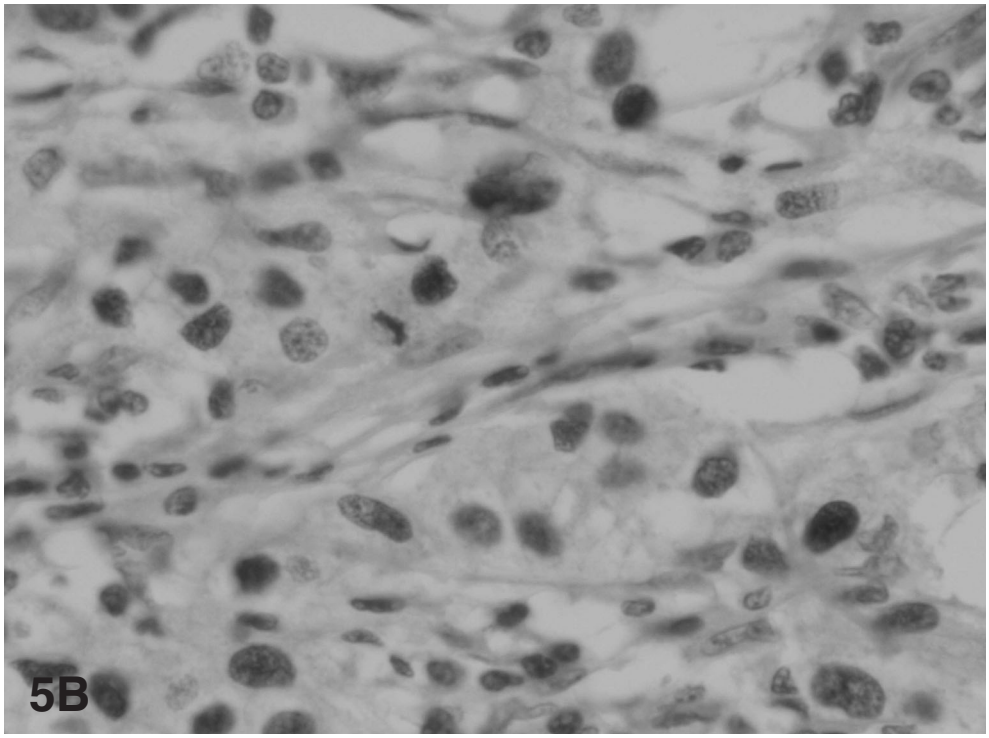


Fig. 5. A. BAG-1. Strong positive expression in both, nuclei and cytoplasm. x 250. **B.** BAG-1. Positive expression predominantly in nuclei of tumoural cells. x 400

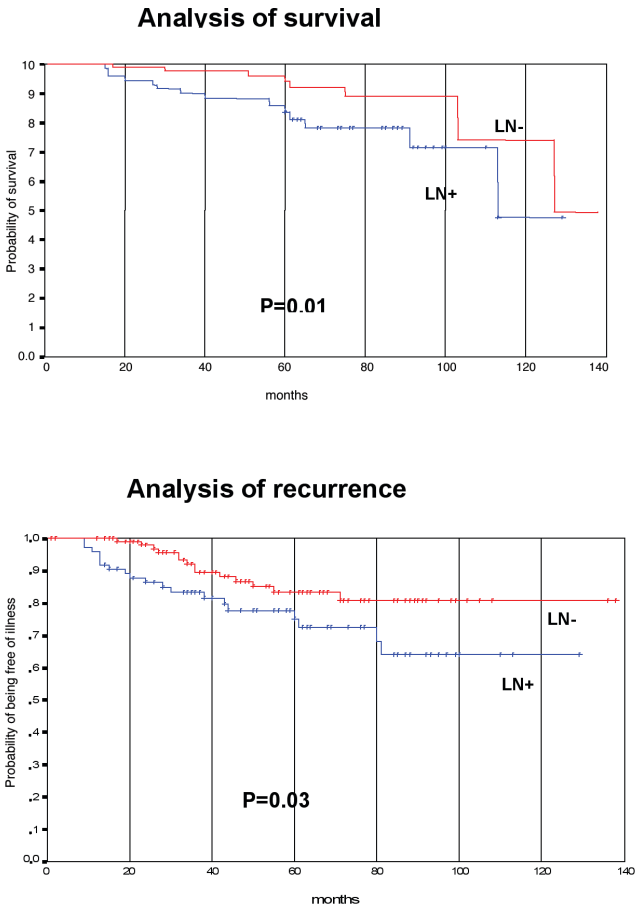


Fig. 6. Kaplan Meier of survival and recurrence in patients with positive versus negative lymph-node.

Srinivas et al., 2002).

The percentage of p53 positivity in breast cancer oscillates between 11 and 58% in the different reported series (Friedrichs et al., 1993; Lipponen et al., 1993; Martinazzi et al., 1993; Charpin et al., 1995; Gohring et al., 1995; Rosanelli et al., 1995; Rosen et al., 1995; Sirvent et al., 1995; Horne et al., 1996; Katoh et al., 1996; Chen et al., 2002; Kato et al., 2002; Volkmann et al., 2002). A negative correlation between p53 and bcl-2 was observed in this study, as was previously reported by Yang et al. (1999); however we do not observe any association between this protein and BAG-1 and Bax expression.

Mutations in the p53 gene, which favour the overexpression of mutant forms of the protein, have been associated with a shorter survival (Thor et al., 1992; Bergh et al., 1995; Kovach et al., 1996; Turner et al., 2000; Sirvent et al., 2001; Thirion et al., 2002). P53 inactivation produces a loss of control in the cell cycle, increases cell proliferation and favours apoptosis, as has reported by many authors (Slooten et al., 1999, Lipponen and Aaltomaa, 1994; Pietiläinen et al., 1995; Mustonen et al., 1997; Berardo et al., 1998; Zhang et al., 1998; Jong et al., 2000). An association between p53 expression and necrosis has also been described elsewhere (Kato et al., 2002).

The observed percentage of positive cases of bcl-2 (71.5%) was comparable, or slightly greater, to that reported in the literature when using similar techniques (Bhargava et al., 1994; Joensuu et al., 1994; Leek et al., 1994; Silvestrini et al., 1994; Gasparini et al., 1995; Kobayashi et al., 1997; Wu et al., 2000; Bukholm et al., 2002; Sierra et al., 1998; Ioachin et al., 2000; Kymionis et al., 2001; Villar et al., 2001;). However, the positivity

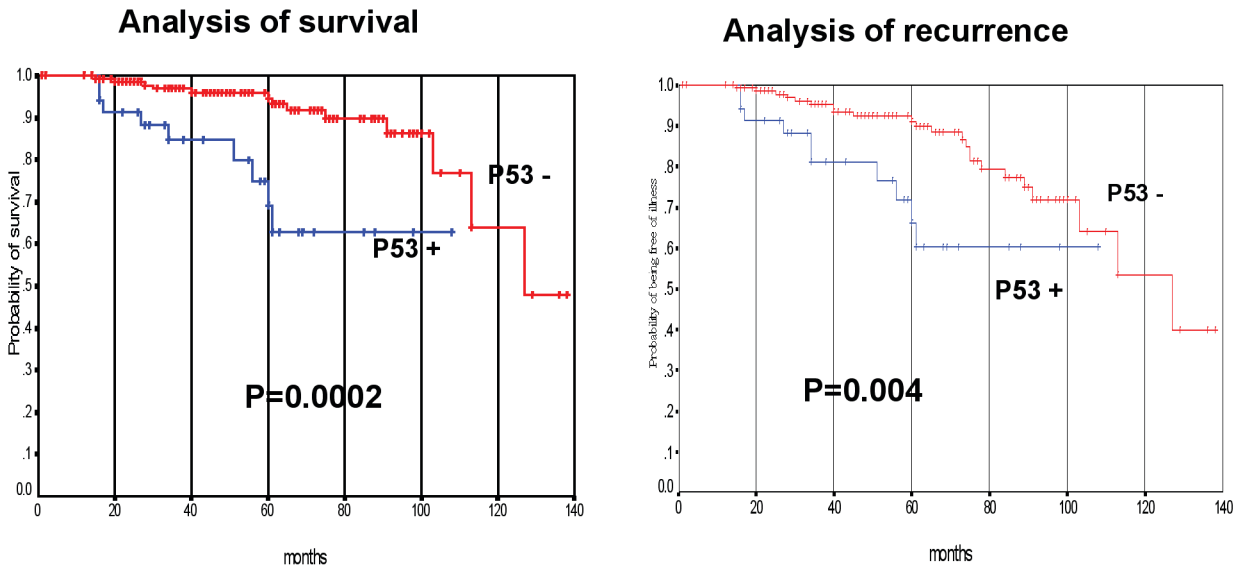


Fig. 7. Kaplan Meier of survival and recurrence for p53.

for BAG-1 (80.6%) and Bax (40.8%) differ from that observed by other authors (Tang et al., 1999; Turner et al., 2001; Sierra et al., 1998; Ioachin et al., 2000; Rehman et al., 2000; Wu et al., 2000; Kymionis et al., 2001; Bukholm et al., 2002) which is probably attributable to the methods used and how the cases are evaluated.

In this study, Bcl-2 proved to be an independent protector prognostic factor for recurrence and mortality (Table 4). These findings were in accordance with some works reported in the literature (Bhargava et al., 1994; Leek et al., 1994; Silvestrini et al., 1994; Gasparini et al., 1995; Sierra et al., 1998; Slooten et al., 1998; Veronese et al., 1998; Wu et al., 2000; Sjöström et al., 2002); however this disagrees with the series published by Joensuu et al. (1994).

Recent studies have found that bcl-2 expression may have a favourable prognostic value for breast cancer treated with chemo- and endocrine therapies (Yang et al., 2003), nevertheless other authors have attributed the positive effect of bcl-2 expression to depend on the type of anticancer drug used (Del Bufalo et al., 2002). In some studies, it has been observed that there exists an association between bcl-2 and Bax decrease with the existence of lymph node positivity (Bukholm et al., 2002) whereas strong bcl-2 and weak Bax expression have been associated with lymph node negativity (Jalava et al., 2000 Wu et al., 2000). Bax overexpression could accelerate death cell by apoptosis by counteracting the bcl-2 action, which inhibits apoptosis (Bucci et al., 2001). Thus, bcl-2 and Bax expression along with apoptosis could differentiate patients into two groups: patients with good or bad prognosis (Wu et al., 2000).

One study, which analyses the effect of adjuvant therapy in metastatic breast cancer, proves that the presence of Bax expression represents a prognostic indicator of good response to therapy (Sjöström et al., 1998). Thus, the prognostic significance of Bax in breast cancer may be limited to settings where chemotherapy is involved in the treatment of patients (Krajewsky et al., 1999) and may not have prognostic significance in cases of early-stage of breast cancer treated by local surgery and radiotherapy (Sierra et al., 1998; Slooten et al., 1998; Veronese et al., 1998). It has also been described that loss of Bax expression is an indicator of bad response to chemotherapy in patients with advanced disease (Krajewsky et al., 1995). However, any of these factors (bcl-2, Bax or BAG-1) could anticipate the response to chemotherapy (Sjöström et al., 2002).

BAG-1 is a multifunctional protein that blocks apoptosis. From all the genes studied, BAG-1 is the unique protein that has expression in both nuclei and cytoplasm. This is probably as a result of its association with other proteins that enter the nuclei (Liu et al., 1998; Takayama et al., 1998; Turner et al., 2001), but most tumours exhibited cytoplasmic BAG-1, while a smaller proportion also had nuclear immunostaining (Townsend et al., 2002). Patients with high levels of BAG-1 correlated with longer overall survival in a group of patients with early-stage cancer (Krajewsky et al., 1999). It has also been described that high levels of cytosolic BAG-1 immunostaining correlated with better prognosis (Turner et al., 2001) than nuclear BAG-1 immunostaining (Tang et al., 1999; Townsend et al., 2002). It is difficult for us to separate cases with nuclear or cytosol BAG-1 positivity because, in general, we have observed a mixed (nuclear and cytosolic) positivity in the studied samples.

In conclusion, in our study we have found that tumour size and bcl-2 have inverse independent

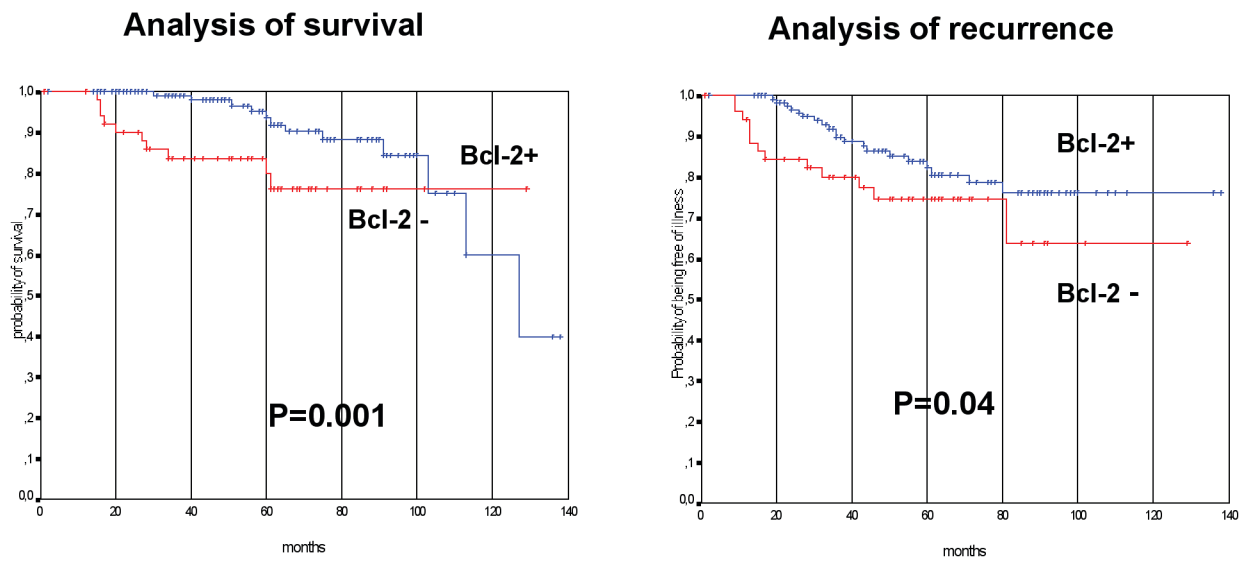


Fig. 8. Kaplan Meier of survival and recurrence for Bcl-2.

prognostic values for recurrence, and in a similar manner p-53 and bcl-2 for mortality. However, we have not found prognostic significance either with AI, Bax or BAG-1.

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