

## Bcl-2 expression in breast carcinoma cells in vitro

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### SUMMARY

Apoptosis is a physiological form of cell death that occurs in all tissues. Deregulated expression of some oncogenes causes increased cell division and advances cell growth. The balance between *bcl-2* and *c-myc* and *c-jun* seems to be an important determinant of cellular sensitivity to the induction of apoptosis. *Bcl-2* gene promotes cell survival rather than cell proliferation. In our study *bcl-2* has been demonstrated to inhibit apoptosis in breast cancer cells in vitro (T47D). High expression of *bcl-2* was noticed to be strongly related to low rates of apoptotic and necrotic cell death. The mean value of the apoptotic index was 7.5% in *bcl-2*-negative tumours and 0.55% in *bcl-2*-positive tumours. High proliferation rate was associated with the absence of *bcl-2* expression. *C-myc* and *c-jun* accumulation were associated with the presence of *bcl-2* expression and with decreased apoptotic activity. The loss of *bcl-2* expression was strongly correlated with increased apoptotic and necrotic cell death. This establishes a model in which *bcl-2* not only mediates in cell death, but also cell division in breast carcinoma cells, and in which the regulation of cell division and cell death are strictly connected.

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## INTRODUCTION

One of the characteristic features of malignancy is an imbalance between the rates of proliferation and programmed cell death or apoptosis, which produces a steady increase in cell number. The CAS (cellular apoptosis susceptibility) gene participates in apoptosis as well as in cell proliferation. Behrens et al mapped CAS gene to chromosome 20q13. This region is known to harbor amplifications that correlate with aggressive breast cancer [1]. Both proliferation and survival are under the control of the growth factors, some of which play integral roles in transformation. One of the most important growth factors involved in the development of breast cancer is basic fibroblast growth factor.

Apoptosis is a physiological form of cell death that occurs in all tissues and in which cells intended to die undergo a series of characteristic morphological and biochemical changes [2,3]. The better known changes, are: DNA fragmentation, chromatin condensation and margination, membrane permeability changes and impaired mitochondrial activity [4,5]. The other characteristics of apoptosis include overall cell shrinkage, nonrandom internucleosomal DNA cleavage and the formation of membrane blebs and apoptotic bodies.

In normal breast, epithelial cell proliferation and apoptosis play complementary roles in maintaining tissue homeostasis. The reduction of apoptosis may lead to the preservation of genetically aberrant cells, thus favouring neoplastic development. Reduced apoptosis could aid the preservation of the cells that have undergone DNA damage, resulting in genetic changes associated with neoplastic development.

Deregulated expression of some oncogenes causes increased cell division and advances cell growth. Deregulation of the genes that control apoptosis can contribute to tumour progression and increased drug resistance. *Bcl-2* gene promotes cell survival rather than cell proliferation

[6]. Bcl-2 can protect the cells against apoptosis induced by agents with different mechanism of action and can increase resistance to a variety of anticancer drugs [7,8]. Bcl-2 and its anti-apoptotic function is associated with the intracellular membranes of the mitochondria, endoplasmic reticulum and nuclei. High expression of *bcl-2* was found to be strongly associated with low rates of apoptotic and necrotic cell death. The mean value of the apoptotic index was 7.5% in *bcl-2*-negative tumours and 0.55% in *bcl-2*-positive tumours. Cellular factors affecting sensitivity to the induction of apoptosis may modulate the resistance of tumour cells to cytotoxic drugs and irradiation. *Bcl-2* expression has been reported to be strongly associated with resistance to chemotherapy. Members of the *bcl-2* gene family play a crucial role in the regulation of apoptosis. The increased resistance to the induction of apoptosis leads to increased resistance to chemotherapy, which could imply that in breast tumours *bcl-2* has an impact on the sensitivity to induction of apoptosis. However, it may also be possible that the efficacy of *bcl-2* to inhibit apoptosis is determined by interactions with other proteins through post-translational modification, such as phosphorylation or mutations. We wanted to test the correlation between *bcl-2* staining and the proportion of apoptotic cells in the breast tumour cells in vitro (T47D). With the use of terminal transferase (TdT)-mediated dUTP nick end-labelling (TUNEL) it is possible to visualize single cells or areas in a tissue containing fragmented DNA. This establishes a model in which *bcl-2* not only mediates in apoptosis but also cell division in breast cancer cells, and in which the regulation of cell division and cell death are strictly connected.

## MATERIALS AND METHODS

The expression of *bcl-2*, *c-myc* and *c-jun* was determined immuno-histochemically using the monoclonal antibodies (*bcl-2* Boehringer Mannheim, Mannheim, Germany), *c-myc*, *c-jun* (Dako, Glostrup, Denmark).

Cell cultures were established in sterile plastic containers with an area of  $\approx 25 \text{ cm}^2$  in RPMI 1640 medium (Sigma) with 10% fetal bovine serum (FBS, Sigma) and penicillin and streptomycin in an incubator (temperature 37°C, 5% CO<sub>2</sub>, 90% air humidity). The cultures were incubated for a period of 72 hours.

The cultures with T47D cells not treated and after paclitaxel treated were heated in 10 nM

citrate buffer pH 6.4 90°C for 10 min and cooled down for 2 h. After washing in double-distilled water followed by phosphate-buffered saline (PBS), the slices were incubated for 10 h with primary antibodies (*bcl-2*, *c-myc*, *c-jun* 1:200). After a thorough washing in PBS, the cells were incubated with biotinylated secondary antibodies (Dako) followed by StreptABCComplex (Dako). The staining was visualized using diaminobenzidine (Sigma, St Louis, MO, USA). The mitotic index was determined by counting the number of mitoses per 500 cells in high-power fields in H&E staining slices. The apoptotic cells were visualized by staining of fluorochromes – DAPI, Hoechst.

The cells were defined as apoptotic if the whole nuclear area of the cell stained positively. The number of apoptotic cells was calculated in the 100 high-power fields and were then divided by total number of cells giving the percentage of apoptotic events per cell population [9].

## RESULTS AND DISCUSSION

Table 1 shows the relationship between *bcl-2* expression, apoptotic activity and proliferative activity. As shown, *bcl-2* expression was correlated with a low apoptotic index (AI) and low proliferative activity as determined by mitotic index (MI). The median value of the AI was 7.5% in *bcl-2*-negative tumours and 0.55 in *bcl-2*-positive tumours. Figure 1 shows representative examples of *bcl-2* and fluorochromes staining in *bcl-2* positive and *bcl-2*-negative tumour cells.

Table 2 shows a cross-tabulation of apoptotic index and the number of mitoses.

Of the cell cultures with 0 – 9 mitoses, had high rates of apoptosis (18%), whereas in the cells with counts greater than or equal to 10 the rate of apoptosis was low (4 and 2%). Apoptotic and mitotic activity showed a good positive correlation. The smallest apoptotic index was noticed in fast proliferation cells.

These data confirm that in the breast cancer cells the absence of immunohistochemically detectable *bcl-2* correlates not only with the presence of apoptotic cells, but also with the presence of large areas of necrosis. This can suggest that *bcl-2* affects not only the threshold for the induction of apoptosis but also the sensitivity to necrosis. It has been reported that in vitro hypoxia may induce both necrosis and apoptosis, and that both types of cell death can be partially prevented by the expression of *bcl-*

2, as well as by inhibitors of enzymes known to be involved in the execution of apoptosis. They are caspases [10].

In vitro experiments clearly showed that *bcl-2* overexpression confers resistance to apoptosis induced by paclitaxel treatment. A number of apoptosis-inducing agents used in cancer therapy (etoposide, doxorubicin, arabinofuranosylcytosine) as well as the proapoptotic second messenger ceramide, induce a disruption of the mitochondrial transmembrane potential, which produces nuclear DNA fragmentation. Moreover, it seems that *bcl-2* is able to prevent the early mitochondrial features of apoptosis [11]. *Bcl-2* did not predict response to chemotherapy in women with node-negative early breast cancer. However, the strong inverse correlation between *bcl-2* expression and cell death suggests that *bcl-2* is an important regulator of apoptosis in breast carcinoma cells. The efficacy of *bcl-2* to inhibit cell death depends on its binding to other proteins, for example to the proapoptotic protein bax. The latter led to proteolytic cleavage of poly(ADP-ribose) polymerase and fragmentation of nuclear DNA.

The apoptosis in breast carcinoma seems to correlate with proliferative activity assessed by the mitotic index and supports the hypothesis that apoptosis may play a role in the selection of clonal subpopulations with high growth potential but not regulated by the p53 system. The mechanism of p53-mediated cell death is

possibly associated with the gene's function of transcriptional modulation and thus may be regulated by the activation or inhibition of the transcription of other genes [12]. P53 expression may occur early in breast cancer development and increases during progression. Substantial experimental evidence suggests that *bcl-2* promotes cell survival by preventing the onset of apoptosis induced by a variety of stimuli [13]. Apoptosis and the homeostatic control is very important factor in the immune responses [14].

It is becoming increasingly clear that certain genes needed for proliferation and transformation play a double role. Overexpression of *c-myc* and *c-jun*, for example, increases the rates of proliferation but decreases the rate of apoptosis.

The strong correlation between cell division and cell death in breast carcinomas suggests that, similar to *c-myc* and *c-jun* overexpression, deregulated expression of genes critical to proliferation leads to conflicting signals in many cells in vivo, which in turn may induce apoptosis. In this study, *c-myc* and *c-jun* accumulation seemed to be associated with decreased apoptotic activity, suggesting that many of the apoptotic events observed are *c-myc* and *c-jun*-dependent.

**Conflict of interest:** The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of the paper.

**Tab. 1.** Expression of *bcl-2*, *c-myc* and *c-jun* in breast cancer cells

	Apoptotic index	P-value
<i>Bcl-2</i> -negative	7.50	
<i>Bcl-2</i> -positive	0.55	< 0.001*
<b><i>c-myc</i> expression</b>		
<i>Bcl-2</i> -negative	26.40	
<i>Bcl-2</i> -positive	15.50	< 0.05*
<b><i>c-jun</i> expression</b>		
<i>Bcl-2</i> -negative	34.60	
<i>Bcl-2</i> -positive	15.80	< 0.05*

\* t-Student test

**Tab. 2.** Association of apoptotic index and mitotic counts

Number of mitoses	Apoptotic index	P-value
0–9	18	
10–19	4	< 0.001*
> 20	2	< 0.001*

\* t-Student test

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