

## Low expression of *bcl-2* in *Brca1*-associated breast cancers

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**Summary** Little data are available concerning the molecular mechanisms of action of *Brca1* and *Brca2* in breast oncogenesis. Recent experimental results suggest that *Brca1* plays a role in the regulation of apoptosis. In order to determine whether the analysis of human tumours would provide data supporting this hypothesis, we have assessed the expression of the antiapoptotic *bcl-2* and of the proapoptotic *p53* genes in *Brca1*- and *Brca2*-associated breast carcinomas. The levels of expression of these genes were compared to those observed in controls and to the mitotic and the apoptotic indexes. Our series were composed of 16 cases of breast carcinoma in women with a germline *Brca1* gene mutation, and of four cases with *Brca2* mutation. A group of 39 patients aged under 36 years and for whom the search for *Brca1* gene mutations was negative, and a group of 36 cases of sporadic cancers without data on their *Brca* status were used as controls. Immunohistochemistry was used to detect *p53* and *bcl-2* gene products. Mitotic and apoptotic indexes were higher in *Brca1*-associated tumours than in controls. No significant difference in *p53* immunostaining was observed between the four groups of patients. In contrast, the rate of *bcl-2*-positive tumours was lower (31%) in *Brca1*-carcinomas than in carcinomas without *Brca1* mutation (90%) ( $P < 10^{-3}$ ). A strong *Bcl-2* expression was found in the four cases of *Brca2*-associated carcinomas. No significant correlation was observed between *p53* and *Bcl-2* immunostainings, either in cases or in controls. The association between *Brca1* status and *Bcl-2* expression remained significant after adjustment for the oestrogen receptor status. Our study shows that a low expression of *bcl-2* characterises most *Brca1*-associated breast carcinomas, a biological trait which seems not to be shared by *Brca2*-associated tumours nor to be related to oestrogen receptor and/or *p53* status. *bcl-2* might thus be one of the target genes involved in the oncogenesis related to *Brca1* and its down-regulation may account for the increased apoptosis and the high proliferative rate observed in *Brca1*-associated carcinomas. © 2000 Cancer Research Campaign

**Keywords:** *Brca1*; *Brca2*; *bcl-2*; proliferation; apoptosis; breast cancer

*Brca1* and *Brca2* germline mutations are frequently observed in women presenting breast carcinoma as part of an hereditary cancer susceptibility syndrome (Ford et al, 1998). The histological phenotype of *Brca*-associated breast tumours differs from that of sporadic cases: *Brca1*-associated tumours generally present as poorly differentiated and highly mitotic carcinomas (Jacquemier et al, 1995; Lakhani et al, 1998) whereas *Brca2* tumours were reported to present a high (Agnarsson et al, 1998) or a low (Lakhani et al, 1998) mitotic index. However, no characteristic immunophenotype of these lesions has been reported so far (Robson et al, 1998), and little data are available regarding the biological mechanisms involved in the development of tumours associated with *Brca1* or *2* gene mutations. Using animal models, it was recently reported that the disruption of the *Brca1* alleles specifically in mouse mammary epithelium leads to increased apoptosis and to tumour formation (Xu et al, 1999). These findings prompted us to analyse the expression of the antiapoptotic *bcl-2* and of the proapoptotic *p53* genes in a series of *Brca1*- and *Brca2*-associated breast carcinomas, together with mitotic and apoptotic indexes, and to compare these values to those obtained in sporadic cancers.

This analysis showed that, whereas no difference in *p53* expression was observed between the different groups, *Brca1*-associated tumours displayed a low rate of *bcl-2* expression and high mitotic and apoptotic indexes. The decrease in *bcl-2* expression was found to be independent from hormone receptor status and may account for the high apoptotic and proliferative rates observed in *Brca1*-associated tumours.

### MATERIAL AND METHODS

Sixteen women affected with a breast carcinoma before the age of 36 were detected to be carriers of a germline *Brca1* mutation through the *Brca1* analysis of a series of 123 early-onset consecutive cases as reported in a previous study (Ansquer et al, 1998). The corresponding 16 tumours were analysed and correspond to the group of cases. A first control group was constituted by a series of 39 women from the early-onset series identified as non-*Brca1* carriers. Tumours of 36 sporadic cases, untested for *Brca1* and *Brca2* mutation, constituted a second control group. The former group has been chosen to avoid a possible bias related to the fact that tumours occurring in young women might present a characteristic immunophenotype independently from their *Brca* status. In addition, the tumours of four women belonging to the early-onset breast carcinomas series and identified as carrier of a germline *Brca2* mutation were also studied. *p53* and *bcl-2* gene expression was assessed by immunohistochemistry using anti-*p53* (D0-7) and

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**Table 1** Clinicopathological characteristics of patients and tumours according to the *Brca* status

Parameters	Cases		Controls		P <sup>a</sup>
	<i>Brca1</i> mutation	<i>Brca2</i> mutation	No <i>Brca1</i> mutation	Sporadic tumours	
Number of cases	16	4	39	36	–
Patient's age					
median	34	50.5	33	62.5	–
mini–maxi	25–47	49–52	21–36	31–80	
Tumour size					
≤20 mm	5 (31.2%)	2 (50%)	17 (43.6%)	9 (25%)	
>20 mm	11 (68.8%)	2 (50%)	22 (56.4%)	27 (75%)	ns
Node status					
N0	6 (37.5%)	3 (75%)	26 (66.7%)	26 (72.2%)	
N1	10 (62.5%)	0	13 (33.3%)	9 (25%)	4.6×10 <sup>-2</sup>
N2	0	1 (25%)	0	1 (2.8%)	
Histological grade					
I	0	0	3 (7.7%)	7 (19.4%)	
II	4 (25%)	1 (25%)	27 (69.2%)	16 (44.4%)	3.10 <sup>-4</sup>
III	12 (75%)	3 (75%)	9 (23.1%)	13 (36.2%)	
Mitotic index <sup>b</sup>	14.5	5.5	4	8	9.10 <sup>-3</sup>
Apoptotic index <sup>b</sup>	10	9	7	nd	2.10 <sup>-2</sup>
Hormonal status					
ER-positive	3 (21.4%)	4 (100%)	27 (71.1%)	26 (74.3%)	10 <sup>-3</sup>
PR-positive	7 (46.7%)	3 (75%)	28 (73.7%)	23 (65.7%)	ns
nd	3		1	1	
Bcl-2 status					
positive cases	5 (31%)	4 (100%)	35 (90%)	27 (77%)	<10 <sup>-3c</sup>
P53 status					
positive cases	9 (56.2%)	0	24 (61.5%)	13 (38.2%)	ns
nd				2	

<sup>a</sup>*Brca1* cases vs no *Brca1* mutation; <sup>b</sup>median of indexes; <sup>c</sup>Chi-squared test with Yate's correction; nd = not determined; ns = not significant

anti-Bcl-2 (124) monoclonal antibodies (Dako A/S, Glostrup, Denmark). The threshold of positivity was 5% of malignant stained cells for p53 and 1% for Bcl-2. Proliferation rate was determined by counting the number of mitotic features in ten successive microscopic fields at G × 400, as recommended for the evaluation of the Nottingham histological grade (Elston and Ellis, 1991; Genestie et al, 1998). Apoptotic index was assessed on haematoxylin–eosin-coloured histological sections by counting nuclear and/or cytoplasmic features characterising the apoptotic process (Bessis, 1964; Cummings et al, 1997). In every case, a tumour area presenting the highest number of apoptotic features was determined at low magnification, then analysed at high power field (G × 400). After image capture, apoptotic features were pointed on the screen and scored using an image analysis system (Perfect Image; Clara Vision, Orsay, France). The apoptotic index was defined as the ratio between this count and the total number of invasive malignant cells. The counting was performed on successive fields when less than 100 invasive malignant cells were present on a single field.

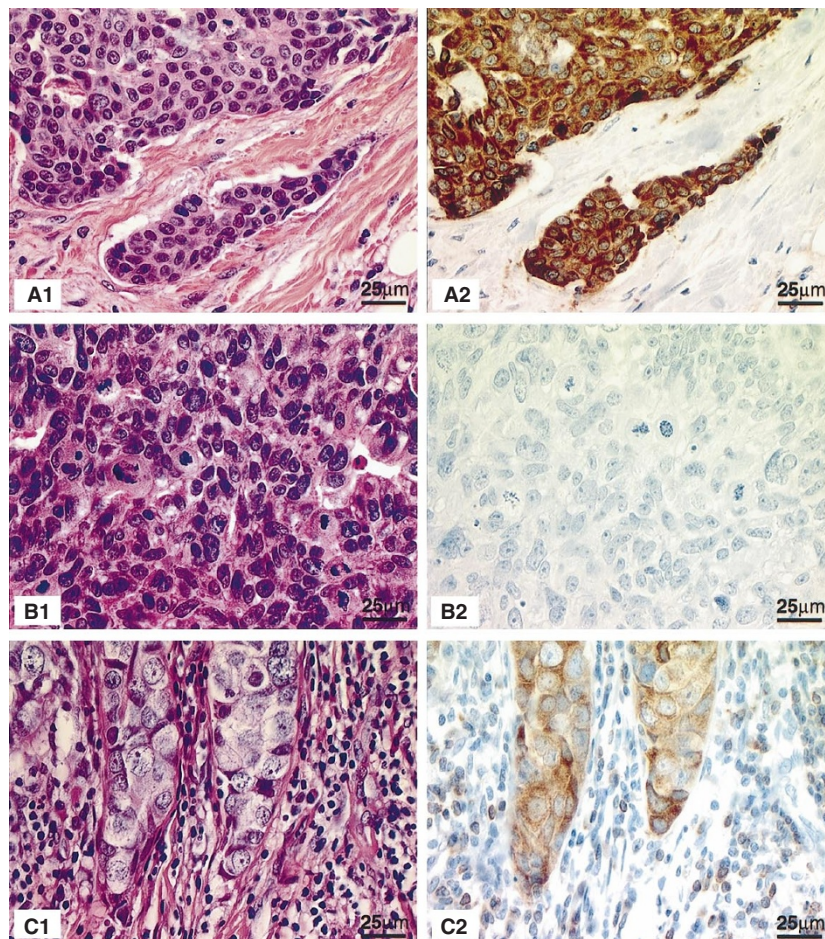
The association between *Brca1* gene mutation and tumour characteristics was evaluated using the Chi-squared test with Yate's correction when necessary for discrete variables (tumour size, nodal status, histological grade, hormonal status, *bcl-2* status, *p53* status) and the Mann–Whitney test for continuous variables (age, mitotic index, apoptotic index). Logistic regression was used to assess the independent association of factors for *Brca1* gene mutation identified in univariate analysis, in a group of tumours for which all clinical and pathological data were available (52 tumours), i.e. 13 cases with *Brca1* mutation and the 39 controls with no *Brca1* mutation. All variables with *P* value < 0.05 were

introduced in the initial multivariate model (excepted mitotic and apoptotic indexes) and deleted through a backward procedure. Variables with a significance level of 0.05 were kept in the final model.

## RESULTS

Our analysis showed that a higher histological grade ( $P = 3.10^{-4}$ ), a higher mitotic index ( $P = 9.10^{-3}$ ), a higher apoptotic index ( $P = 2.10^{-2}$ ) and a lower rate of oestrogen receptor positivity ( $P = 10^{-3}$ ) were observed in *Brca1*-associated breast cancers than in controls (Table 1). A slight positive correlation was found between mitotic and apoptotic indexes ( $r = 0.28$ ,  $P = 0.045$ ).

The immunohistochemical analysis found no significant difference in p53 immunostaining between the four groups of tumours. In contrast, a significant difference in *bcl-2* expression was found between cases and either control groups (Figure 1). The rate of *bcl-2*-positive tumours was lower (31%) in *Brca1*-associated carcinomas than in sporadic tumours (77%) ( $P = 2.10^{-3}$ ). This difference was even more striking in comparison with the rate of *bcl-2*-positive tumours observed in young women without *Brca1* mutation (90%) ( $P < 10^{-3}$ ). The four cases of *Brca2*-associated carcinoma were found to be positive. The mean number of labelled cells was 56% in the group of sporadic tumours and 45% in tumours with no *Brca1* mutation. In contrast, a complete absence of labeling was observed in 11 of the 16 cases of *Brca1*-associated carcinomas. A weak staining of 5% of the tumour cells was observed in one case whereas the remaining four cases exhibited 40–80% of labelled cells. The negative association of *bcl-2* expression and *Brca1*-mutation remained significant after



**Figure 1** Histological aspects of sporadic invasive ductal carcinoma (A1), *Brca1*-associated (B1) and *Brca2*-associated (C1) carcinomas. Immunostainings of *bcl-2* in these cases. High level of *bcl-2* expression in sporadic cancer (A2) and in *Brca2*-associated carcinoma (C2). No expression detected in *Brca1*-associated carcinoma (B2)

adjustment on oestrogen receptor status. In multivariate logistic regression analysis, low expression of *bcl-2* (odds ratio (OR) 13.9; 95% CI 2.6–76.9) and high histological grade (OR 7.1; 95% CI 1.3–38.8) were significant predictive variables associated with the presence of *Brca1* gene mutation (Table 2). No significant correlation was observed between p53 and Bcl-2 immunostaining, either in cases or in controls.

## DISCUSSION

Our study shows that most *Brca1*-associated breast carcinomas are characterized by high mitotic and apoptotic rates, and by a decrease of *bcl-2* expression. These biological traits seem not to be shared by *Brca2*-associated tumours and not related to oestrogen receptor and/or to p53 status. In an analysis of the immunophenotype of *Brca*-associated tumours, only a slight decrease of *bcl-2* expression was found in familial cancers in comparison with the level observed in controls (Robson et al, 1998). However, in this work, *Brca1*- and *Brca2*-tumours were included in the same group, and this could account for the negativity of the result. Recent data emphasize that different molecular pathogenetic processes characterize *Brca1*- and *Brca2*-associated tumours (Armes et al, 1999), and this is in agreement with our data on distinctive *bcl-2*-expression in these two tumour types.

High levels of *bcl-2* expression have been observed in 75–80% of cases of sporadic breast cancers (Leek et al, 1994; Silvestrini et al, 1994; Hellemans et al, 1995). This expression was found to be associated with the presence of favourable prognostic features (Joensuu et al, 1994) and with the expression of oestrogen and progesterone receptors (Barghava et al, 1994; Gee et al, 1994; Leek et al, 1994; Silvestrini et al, 1994; Hellemans et al, 1995; Charpin et al, 1997).

Since *Brca1*-associated breast carcinomas are frequently oestrogen receptor negative, poorly differentiated tumours, and since a positive association exists between the respective expression of oestrogen receptor (ER) and *bcl-2*, the down-regulation of *bcl-2* in *Brca1*-associated carcinomas might merely reflect the down regulation of ER in this type of tumour. In our study, the loss of *bcl-2* expression characterizing *Brca1*-associated carcinomas behaved as a biological trait independent from the ER status of the tumours. Definitive conclusions cannot be drawn from this multivariate analysis considering the limited number of cases included in this series. However, it is worth mentioning that in multivariate analyses of prognostic factors characterizing large series of axillary node-positive breast tumours, the lack of *bcl-2* expression was found associated with a poor outcome of the disease, independently from the ER status (Hellemans et al, 1995; Berardo et al, 1998).



**Table 2** Association of biological parameters with *Brcal* gene mutation in logistic regression model<sup>a</sup>

Biological parameters	OR	95% CI	P value
<i>Bcl-2</i> expression			
high	1		
low	13.9	2.6–76.9	0.002
histological grade			
I/II	1		
III	7.1	1.3–38.8	0.02

OR = adjusted odds ratio; CI = confidence interval; <sup>a</sup>*Brcal* mutated and not mutated tumours

The molecular mechanisms of action of *Brcal* in the tumour process are poorly understood. The observation of increased apoptosis in mouse mammary tissue triggered by the inactivation of the two copies of the *Brcal* gene (Xu et al, 1999) suggest that the *Brcal* gene products could function as transcriptional regulators of (Yang and Lippman, 1999). The anti-apoptotic *bcl-2* might be one of the target genes for *Brcal* in such mechanisms. Our present data reporting a down-regulation of *bcl-2* and a high apoptotic index of *Brcal*-associated carcinomas support this hypothesis. Another line of evidence indicates that *Brcal* plays a positive role in the control of cell growth (Gudas et al, 1996). *bcl-2* gene product could also act as a second messenger in this control: an inhibitory effect of entry into the cell cycle has been reported for *bcl-2* (O'Reilly et al, 1996). and this effect was found genetically separable from the functions of the protein in cell survival (Huang et al, 1997). The high proliferative rate observed in *Brcal*-, but not in *Brcal2*-associated tumours (Armes et al, 1999), might thus be related to the respective levels of *bcl-2* expression in these tumours. It is worth mentioning that a positive link between proliferation and apoptosis has already been observed in carcinomas of the breast (Dowsett et al, 1999), and that the loss of *bcl-2* has been found to be associated with both increased apoptosis and increased proliferative rates in sporadic breast cancers (van Slooten et al, 1998). In such cases, it would be of interest to determine whether this loss was associated with a down-regulation of *Brcal*, the implication of which in the progression of sporadic breast carcinomas is also a matter of speculation (Yang and Lippman, 1999).

The similarity in p53 immunostaining between *Brcal*- and non *Brcal*-associated tumours is in agreement with our previous results reporting a similar pattern in p53 mutations in these two groups of tumours (Schlichtholz et al, 1998). These results are at discrepancy with data reporting a high rate of p53 mutation in a small group of *Brcal*-associated carcinomas (Crook et al, 1997). It is worth mentioning that a very large diversity of p53 mutational patterns is observed among cohorts of sporadic breast cancers (Hartmann et al, 1997). These variations may also concern familial tumours and thus account, at least in part, for the discrepancies observed between the rates of p53 alterations in these tumours. Furthermore, the biological consequence of p53 cell accumulation, as determined by immunohistochemistry, is unclear and the link between p53-positive immunostaining and gene mutation is inconstant in breast cancers (Sjögren et al, 1996). No correlation was observed between p53 and *bcl-2* immunostaining in our study, a result already reported by others (Silvestrini et al, 1996).

In conclusion, a low *bcl-2* expression and high proliferative and apoptotic rates characterize most *Brcal*-associated breast carcinomas. The analysis of a large series of cases including the assessment of the expression of *bcl-2*, of *Brcal* and of biological parameters associated with cell death and proliferation, should help to determine whether alterations of the functions of *Brcal* gene product were responsible for the down-regulation of *bcl-2*, and thus for the high proliferative rate and increased apoptosis observed in *Brcal*-associated carcinomas.

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