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Effective tamoxifen therapy of breast cancer involves both antiproliferative and pro-apoptotic changes

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Abstract

Despite knowledge of oestrogen receptor status, it is not always possible to predict which breast cancers will respond to tamoxifen. We have previously reported that decreased expression of Bcl-2 and/or Ki-S1 were associated with tumour response to neo-adjuvant tamoxifen in 50 elderly women with oestrogen receptor (ER)-positive breast cancer. In this study, we confirm that the expression of Bcl-2 and Ki-S1 are surrogates for the frequency of apoptosis and mitosis respectively, within these untreated breast cancers, with an inverse relationship between Bcl-2 expression and the apoptotic index ($P < 0.05$), and a positive relationship between Ki-S1 expression and the mitotic index ($P < 0.01$). However, after 3 months' tamoxifen treatment these relationships were no longer apparent. Moreover, amongst the 27 tumours in which Bcl-2 expression was reduced during the 3 months' therapy, there was a significant correlation between the response to therapy and the increase in apoptosis ($P < 0.05$), whereas in those tumours in which Bcl-2 did not fall with therapy, there was a significant correlation between response and the decrease in mitosis ($P < 0.05$). These data suggest there are at least two mechanisms for effective tamoxifen therapy: increased apoptosis as a consequence of reduced Bcl-2 expression, and decreased proliferation.

Keywords: Apoptosis; Bcl-2; Breast cancer; Mitosis; Response; Tamoxifen

1. Introduction

Tamoxifen is one of the most widely used drugs in the treatment of breast cancer, but although its efficacy in the adjuvant setting has been confirmed by overview analyses [1], not all patients benefit from this therapy. When given in the presence of clinically manifest disease, the majority of patients respond, particularly if their tumours are oestrogen receptor-positive (ER +ve) [2–4]. However, the benefit is not always maintained in the long-term. Thus, there is a need to identify with more precision which tumours are most likely to respond to therapy, and to understand the biological changes that occur during effective drug treatment.

Neo-adjuvant therapy with tamoxifen affords the opportunity to assess *in vivo* tumour sensitivity to the drug, by comparing and contrasting tumour samples taken before and during such treatment. We have previously described a series of 50 elderly women given tamoxifen for 3 months before definitive loco-regional surgery, in whom the expression of Bcl-2 and Ki-S1 were examined [5]. There was a fall in both markers with treatment, and a clear association between the degree of change and tumour response as assessed by ultrasound. Given that Bcl-2 may inhibit apoptosis [6], the relationship between Bcl-2 expression and the frequency of apoptosis has now been examined in these same patients, both before and after tamoxifen therapy. The frequency of mitotic figures was also assessed and compared with the expression of Ki-S1, a sensitive marker of cell-cycle activity and thus proliferation.

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2. Patients and methods

2.1. Patients

50 women over the age of 70 years with non-metastatic primary breast cancer presenting to the Edinburgh Breast Unit were entered into the study between October 1991 and October 1993. All had a tumour that was a maximum of 2 cm in diameter, which was ER +ve (>20 fmol/mg protein) and confirmed as invasive breast cancer by wedge biopsy. Each patient received 20 mg tamoxifen daily for 3 months. Tumour size was monitored by 4-weekly ultrasound measurements, and clinical response was defined as the percentage volume reduction between the initial and final tumour volumes [5].

2.2. Histology and immunohistochemistry

Tissue was available from the initial wedge biopsy and at definitive loco-regional surgery 3 months later. The material was stored in liquid N_2, and tumour pairs were thawed, fixed in 10% formaldehyde and then cut into 3 μm sections which were mounted onto lysine-coated slides. Sections were stained in a routine manner with haematoxylin and eosin. Apoptotic and mitotic indices were assessed using standard morphological criteria [7,8] by one observer, using the HOME microscope which records the positions of identified cells [9], and a proportion of sections were then re-examined by a second, independent observer. There were very good correlations between the two observers' assessments of both apoptosis and mitosis ($r=0.95$, $P<0.0001$). Results were expressed as the ratio of the number of cells that were apoptotic or mitotic to the total number of tumour cells examined. A minimum of 1000 (mean 1687) tumour cells were examined, although this was not possible in five tumours removed after 3 months' therapy, due to the reduced cellularity of the tumour. The apoptotic:mitotic ratio was defined as the number of apoptotic figures seen divided by the number of mitoses (although when this was 0, the ratio was potentially underestimated as being equal to the number of apoptotic figures identified).

Further 3 μm sections were cut and stained for Bcl-2 expression, using the 124 clone antibody (Dako, High Wycombe, Bucks, UK) and Ki-S1 expression (antibody provided by Kreipe, Keil University) as previously described [5].

Bcl-2 expression, seen as fine cytoplasmic granularity, and nuclear Ki-S1 expression were assessed independently by two pathologists, using a semi-quantitative scale: 0 (<5% of tumour cells stained); 1 (5–25% of tumour cells stained); 2 (26–75% of tumour cells stained); 3 (76–100% of tumour cells stained).

All determinations were done without knowledge of either the clinical response or whether the specimen was obtained before or after therapy.

2.3. Statistical analyses

Relationships between Bcl-2 expression, apoptosis and mitosis were analysed by the χ^2 test for trend using Excel for Windows version 5.0 (Microsoft Corporation, Redmond, WA, USA). Multiple regression analyses and non-parametric tests such as Mann–Whitney were performed on Minitab release 12.1 (Minitab Inc., State College, PA, USA). Apoptotic:mitotic ratios were log-normally distributed so the log-transformed ratio was used for statistical analyses. The correlation between two observer's scores was assessed as recommended by Bland and Altman [10,11].

3. Results

3.1. Clinical response

After three months' therapy, 31/50 (62%) of patients had shown at least a 25% reduction in tumour volume. One patient (2%) progressed on treatment with a volume increase of over 25%, and the remaining 18 (36%) were stable with a final tumour volume within 25% of the initial value.

3.2. Immunohistochemistry

The distribution of Bcl-2 expression is shown in Table 1, with the majority of pretreatment specimens being positive for Bcl-2 (44/50 (88%)), although the proportion fell to 36/49 (73%) in the post-treatment specimens, a reduction which approached statistical significance ($P=0.08$). The pattern of Ki-S1 expression is also shown in Table 1, with expression in 36/50 (72%) tumours before treatment, but only 23/50 (46%) after 3 months' therapy, this proportion being statistically significantly lower ($P<0.01$).

3.3. Histology

Morphologically apoptotic and mitotic cells were infrequent in the pretreatment specimens, with median indices of 0.45% (range: 0–1.5%) and 0.18% (range: 0–1.1%), respectively. After 3 months' therapy, there was a significant fall in the median number of mitotic figures to 0.1% (range: 0–0.6%, $P=0.025$), whereas there was no such change in apoptotic index (median: 0.37%, range: 0–1.6%). However, there was no overall change in the apoptotic:mitotic ratio with therapy, with an average value of 3.5 (range 0.3–14.0) being seen in both the pre- and post-treatment specimens. This is probably

Table 1
Bcl-2 and Ki-S1 expression before and after 3 months' tamoxifen therapy

	0 (< 5% cells)	1 (5–25% cells)	2 (26–75% cells)	3 (76–100% cells)
Bcl-2 staining index				
Pretreatment	6 (12%)	18 (36%)	12 (24%)	14 (28%)
Post-treatment[a,b]	13 (26%)	17 (34%)	11 (22%)	8 (16%)
Ki-S1 staining index				
Pretreatment	14 (28%)	20 (40%)	16 (32%)	0
Post-treatment	27 (54%)	15 (30%)	6 (12%)	2 (4%)

[a] One patient did not have suitable tissue available for assessment of Bcl-2 expression after therapy.
[b] 27 patients showed a reduced Bcl-2 expression after therapy compared with pretreatment values.

a statistical anomaly due to the small numbers of samples analysed. Furthermore, there were significant positive correlations between the apoptotic and mitotic frequencies in both the pre- and post-treatment specimens ($r = 0.38$, $P < 0.01$), such that tumours with a high apoptotic index tended also to have a high mitotic index.

3.4. Correlations between values

In pretreatment samples there was a significant inverse correlation between the degree of Bcl-2 expression and apoptotic index ($P < 0.05$) as shown in Fig. 1, and a significant positive correlation between Ki-S1 expression and mitotic index ($P < 0.01$) (see Fig. 2). After 3 months' tamoxifen therapy no significant relationships were apparent between Bcl-2 expression and the apoptotic index, or between the mitotic index and Ki-S1 (data not shown). No significant relationships were apparent between Bcl-2 expression and mitotic index, nor between Ki-S1 and apoptotic index, in tumours examined both before and after treatment (data not shown).

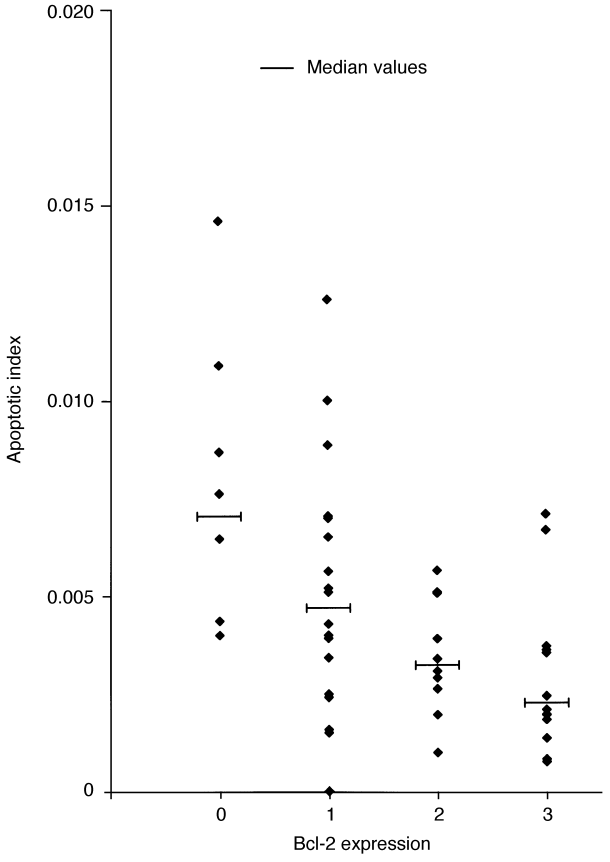

Fig. 1. Pretreatment Bcl-2 expression and apoptotic index (trend test $z = -2.1$, $P < 0.05$).

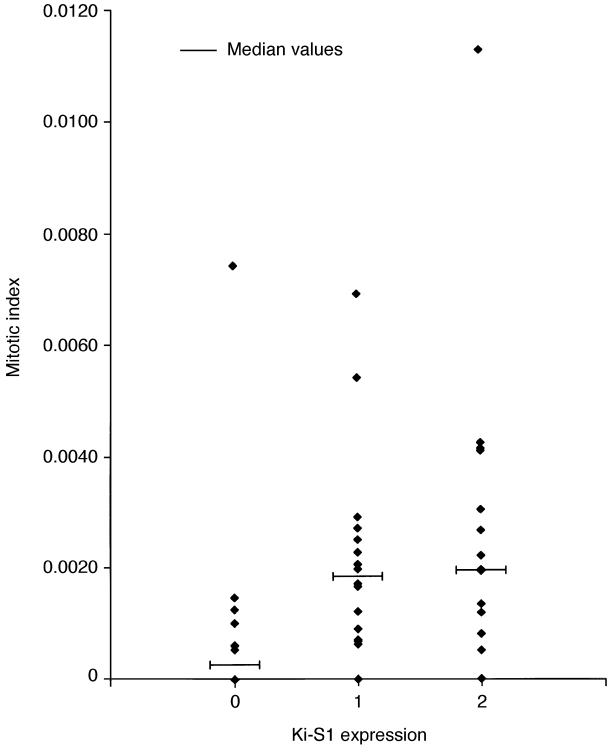

Fig. 2. Pretreatment Ki-S1 expression and mitotic index (trend test $z = -3.17$, $P < 0.01$).

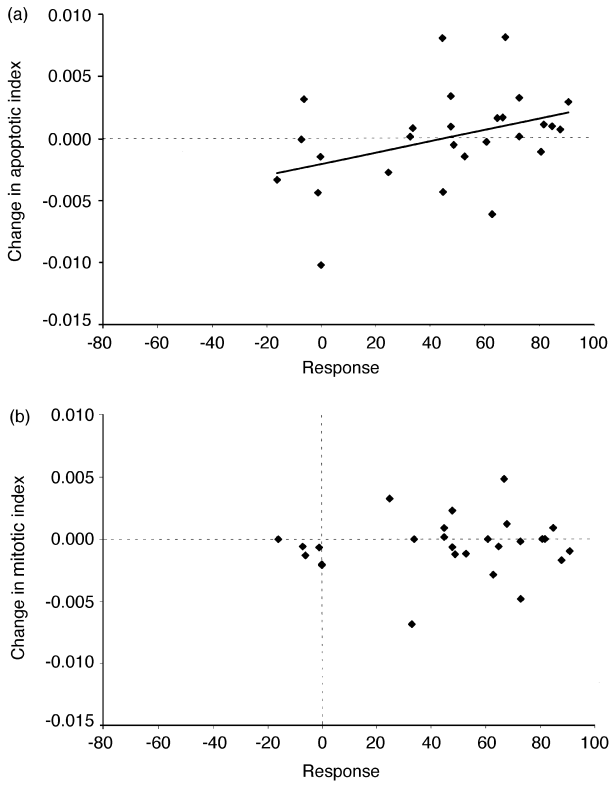

Fig. 3. Tumour response in those tumours ($n=27$) with reduced Bcl-2 expression after 3 months' tamoxifen therapy and: (a) change in apoptotic index ($r=0.382$, $P<0.05$); (b) change in mitotic index ($r=-0.1$, $P>0.5$).

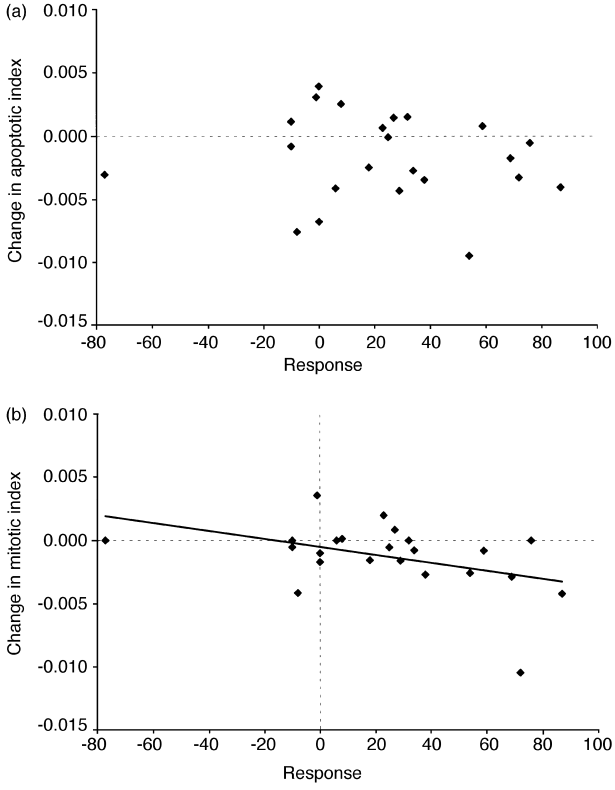

Fig. 4. Tumour response in those tumours ($n=23$) without reduced Bcl-2 expression after 3 months' tamoxifen therapy and: (a) change in apoptotic index ($r=0.096$, $P>0.5$); (b) change in mitotic index ($r=0.424$, $P<0.05$).

3.5. Correlations between biological values and clinical tumour response

There were no significant correlations between the percentage tumour volume reduction after 3 months' tamoxifen therapy and the initial or final values of the apoptotic or mitotic indices, or with their ratio. Furthermore the change in these indices did not correlate with response (data not shown). However, there was, within the subgroup of 27 tumours expressing less Bcl-2 expression after treatment, a significant positive correlation between the degree of response and the change in apoptotic index ($P<0.05$), as shown in Fig. 3(a), but not with the change in mitosis (Fig. 3(b)). In contrast, for the remaining 23 tumours in which Bcl-2 expression did not decrease, there was no correlation between the degree of response and the change in apoptosis, but there was a significant negative correlation with the change in mitotic index ($P<0.05$), as seen in Fig. 4(a, b). Further confirmation that increased apoptosis and decreased mitosis are in general observed in different tumours is to be found in Table 2. Here it can be seen that the co-occurrence of these changes is found in only 6/31 (19%) of responding tumours, a proportion that is not significantly different from the 3/18 (17%) observed in the non-responsive tumours. It is interesting to note, that when comparing this small group of tumours manifesting changes in both apoptosis and mitosis with those tumours that had a change in only one of these indexes, there is, as might be expected, a significantly higher increase in the apoptotic:mitotic ratio ($P<0.025$). Furthermore, when this comparison is restricted to tumours that responded to treatment, then there is a trend for the degree of that response to be higher in

Table 2
Changes in apoptosis and mitosis and clinical response[a]

	No.	↓ Mitosis only (%)	↑Apoptosis only (%)	Both ↓ mitosis and ↑ apoptosis (%)
Progressive disease (volume ↑ of >25%)	1	0	0	0
Stable disease (volume ↑ or ↓ of <25%)	18	8 (44)	3 (17)	3 (17)
Response (volume ↓ of >25%)	31	11 (35)	9 (29)	6 (19)

[a] The apoptotic or mitotic index is considered to have changed when the absolute difference in pre- and post-treatment values is ⩾0.1%.

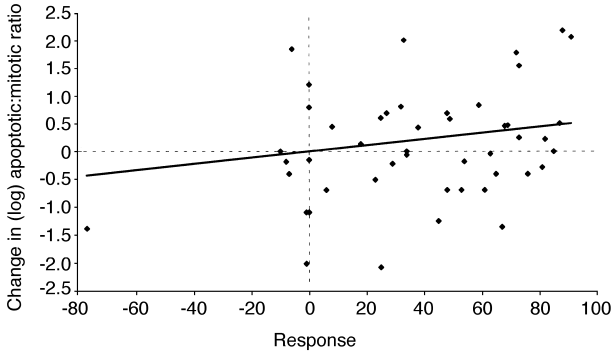

Fig. 5. Change in log-transformed apoptotic:mitotic index and response to 3 months' tamoxifen therapy ($r = 0.313$, $P < 0.05$).

those tumours with a change in both indices as compared with those tumours with a change in only one (median reduction of 73% as compared with 51%, $P < 0.08$).

There was also a significant quantitative relationship between the degree of tumour response and the change in the log-transformed apoptotic:mitotic ratio ($P < 0.05$), as seen in Fig. 5.

Finally, the possibility was considered that the actual level of ER in the initial tumour specimen might influence the biological changes during therapy. Although the initial tumour level of ER was a very significant predictor of the subsequent response to tamoxifen ($r = 0.537$, $P < 0.001$), it did not appear to influence the manner of that response, in that there were no differences in the initial tumour ER scores according to whether or not there were changes in Bcl-2 expression, apoptotic index, mitotic index or their ratio. These calculations were carried out using the Mann–Whitney test using 100 fmol/mg as the cut-off point between tumours with low or high ER values.

4. Discussion

This study was conducted in order to determine the relationship between effective tamoxifen therapy and changes in the frequency of proliferation and cell death in clinical breast cancers. It follows on from a previous study [5], which found that the response of breast cancer after 3 months' tamoxifen correlated both with the pre-treatment level of Bcl-2 expression, considered a surrogate for the level of apoptosis, and the post-treatment level of Ki-S1 protein, considered a surrogate marker of proliferation. In that earlier work an even stronger correlation was evident between clinical response and the combined index obtained by summing the changes in both markers, used as a surrogate for the relative balance between cell growth and death in the tumour.

The objective therefore, of the present study, was to confirm the relationship between effective tamoxifen therapy and its known antiproliferative [12–14] and pro-apoptotic actions [13,15,16]. Therefore, the data were first examined to determine whether the levels of expression of Bcl-2 and Ki-S1 were representative of the underlying processes of cell growth and death, using morphological assessment in order to avoid further indirect comparisons. Fig. 1 confirms the anticipated inverse relationship between Bcl-2 expression and the level of apoptosis, a consequence of the inhibitory effect of Bcl-2 on apoptosis [6], consistent with prior reports [17,18]. In the same manner, Fig. 2 confirms that Ki-S1 is a marker of tumour proliferation, as it correlates directly with the frequency of mitoses. The frequency of mitoses before treatment, and the reduction observed with therapy, are both consistent with other reports on the level of proliferation in breast cancer [17,19–21] (although using different techniques to assess proliferation). However, in the tumour material removed after 3 months' therapy, no clear relationships were observed between proliferation and apoptosis and their respective surrogate markers, Ki-S1 and Bcl-2. The data were therefore studied further to identify possible reasons for this. Two hypotheses were considered: firstly that the response seen in some tumours was a consequence of reduced proliferation, whereas in others it was as a result of increased apoptosis, and secondly, that the more impressive tumour responses might be associated with the more significant changes in tumour biology.

Bcl-2 is an oestrogen-regulated protein [22] whose expression can be inhibited by tamoxifen [23]. Overall, there was a trend towards reduced Bcl-2 expression after therapy, as previously reported [5], although the level did not fall in all tumours, and was not associated with an overall reduction in apoptosis. Considering, therefore, the subgroup of 27 tumours in which the level of Bcl-2 fell with 3 months' tamoxifen therapy, an increase in apoptosis would have been anticipated, which itself could be associated with a clinical response. This is illustrated in Fig. 3(a), in which can be seen a significant correlation between the increase in apoptosis and the percentage tumour volume reduction for this subset of tumours. In contrast, in the subgroup of 23 tumours which did not express less Bcl-2 after therapy, Fig. 4(b) demonstrates that there was a significant correlation between the reduction in mitosis and the clinical response. These data are consistent with the hypotheses that there may be different routes to tumour response to tamoxifen, and that the degree of biological change is reflected in the extent of the clinical response. Table 2 suggests that in the majority of tumours only one of these two mechanisms operates to induce tumour response, since one or other effect was seen in 20 (65%) of responding tumours, but changes in both apoptosis and proliferation only occurred in 6 (19%). However, there is a suggestion that this minority might manifest a greater degree of tumour response. Furthermore, given

that the rates of proliferation and apoptosis appear to be biologically related, as suggested both by our data and observations in breast and other cancers [24–26], it is the net effect of reduced proliferation and/or increased apoptosis that will determine tumour response. The relative balance between these two processes can be approximated using the apoptotic:mitotic ratio [27]. We have previously shown that in tamoxifen-sensitive breast cancer cell line xenografts this ratio is increased during therapy, with no change occurring in ER-negative, insensitive xenografts [28]. Fig. 5 confirms that in the present study of clinical breast cancers there was not only a similar correlation between clinical tumour response and change in the apoptotic:mitotic ratio, but that the extent of that clinical response is paralleled by a larger change in this ratio. The apparent imprecision in the relationship may in part be due to the heterogeneity of the behaviour of residual tumour after 3 months' therapy. Previous experience has shown that some of these tumours would have continued to shrink, had they not proceeded to surgery, whereas others might have remained static or even progressed. It could be conjectured therefore that the latter group are represented by those tumours which manifested a fall in the ratio at 3 months. The fairly large scattering of data in Fig. 5 is probably also due to the small numbers in the study.

There are, however, other possible explanations for the lack of a clear relationship after therapy between Bcl-2 expression and apoptosis. It must be recalled that there are many other members of the Bcl-2 family, such as Bax, Bcl-X_l etc., which in combination with Bcl-2 play important roles in determining the propensity for a cell to undergo apoptosis [29,30]. A recent study has reported a shift in the Bax/Bcl-2 ratio as patients with leukaemia responded to therapy [31]; one could anticipate that similar patterns might occur in tamoxifen therapy of breast cancer. This study has not determined possible changes in the levels of other members of the Bcl-2 family, although such studies are currently underway.

Thus, effective tamoxifen therapy appears to cause tumour regression by two, possibly separate, mechanisms: on the one hand, reduced Bcl-2 expression associated with increased apoptosis, and on the other, reduced proliferation with no significant change in Bcl-2 expression. The clinical relevance of this observation remains to be determined, but further studies into the mechanisms of effective endocrine therapy in breast cancer could help optimise our current therapeutic approaches.

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