Advances in Brief

Coordinate Expression of Apoptosis-associated Proteins in Human Breast Cancer before and during Chemotherapy1

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Abstract

**Purpose:** Induction of apoptosis is a key factor in the response of tumors to chemotherapy. Laboratory studies have established many of the factors that regulate and execute apoptosis, but the significance of these in human tumors is poorly understood. Therefore, the relationship between key components of this machinery was examined in primary human breast carcinomas before and 24 h after the initiation of chemotherapy.

**Experimental Design:** Apoptosis was measured using the terminal deoxynucleotidyl transferase-mediated nick end labeling assay, and proliferation was assessed using the anti-Ki67 antibody MIB-1. Monospecific polyclonal antibodies were used for immunohistochemical detection of Bcl-2, Bax, XIAP, activated (cleaved) caspase 3 and 6, and cleaved DNA Fragmentation Factor-40 (DFF40) using paraffin-embedded tissues.

**Results:** Before treatment, a significant correlation was found between apoptosis and proliferation \((r = 0.64, P < 0.0001)\), between caspases 3 and 6 \((r = 0.49, P = 0.004)\) and between cleaved DFF40 and active caspases 3 \((r = 0.66, P < 0.0001)\) or 6 \((r = 0.47, P = 0.006)\). Before treatment, expression of inhibitor of apoptosis protein, XIAP, also correlated positively with cleaved caspase 3 \((r = 0.64, P < 0.0001)\), caspase 6 \((r = 0.36, P = 0.04)\), and DFF40 \((r = 0.61, P = 0.0001)\). At 24 h after chemotherapy, significant increases in apoptosis and decreases in proliferation were observed, with the degree of increase in apoptosis inversely associated with decrease in proliferation. Chemotherapy-induced increases in Bax were correlated with increases in cleaved DFF40 \((r = 0.54, P = 0.0008)\), but no other variables showed significant change at 24 h after initiation of chemotherapy.

**Conclusion:** The pretreatment biomarker relationships suggest parallel cleavage and activation of these executioner proteins in breast cancer and that XIAP may maintain cell survival in the face of caspase activation. The findings provide in vivo evidence in human breast cancer that chemotherapy induces an apoptotic program characterized by up-regulation of Bax and cleavage of caspase substrate DFF40.

Introduction

The treatment of breast cancer has been substantially improved during recent decades by use of particular surgical approaches, endocrine manipulation, radiotherapy techniques, and, most recently, combination chemotherapy (1–4). Patients may be categorized according to tumor size, nodal and menopausal status, and pathological criteria, including histological subtype and grade. This has allowed individual prognosis to be assessed by reference to population databases (5). For endocrine therapy, ER3 has been a useful predictive guide to likely treatment benefit with Tamoxifen, and, recently, HER-2 status has promised the same for treatment with the monoclonal antibody Trastuzumab (6) and possibly for anthracycline-based combination regimens (7). However, at present, no biological indices are available that predict with confidence the clinical outcome to specific cytotoxic chemotherapy in all patients (8). Therefore, the search for predictive biological markers remains an important area for research.

Women with locally advanced or large operable breast cancer and no evidence of metastatic disease may be offered primary chemotherapy before definitive surgery (4, 9, 10). This allows an alternative to standard adjuvant approaches after surgery and results in reduced need for mastectomy without survival detriment (4). Primary chemotherapy also provides a unique opportunity to study the response of cancers at presentation, both clinically and pathologically (11), and a valuable clinical scenario for the identification of predictive biomarkers of response.

Given that apoptosis is the principle mechanism of chemotherapy-induced regression (12), we have hypothesized that the rate of apoptosis or proteins that regulate apoptosis may be predictive of responses to chemotherapy. Apoptosis is often increased in malignant tumors (12) and, along with proliferation, is more marked in pathologically high-grade tumors (13).
High proliferation, low apoptosis, and low necrosis predict for shorter survival in other solid tumors (14), whereas increased proliferation and reduced apoptosis in recurrent breast cancers in comparison to the primary lesion also predict for a worse prognosis (15). We have shown previously that apoptosis is increased in breast cancer during chemotherapy in the first 24 h (16) and that a significant fall in proliferation occurs (17). Preliminary clinical data indicate that this early increase in apoptosis with treatment may be predictive of subsequent favorable clinical response (18). A schematic diagram of the major pathways examined in the literature in mammalian cells leading to apoptosis is shown in Fig. 1.

In this study, the relationships between selected proteins known to be involved in the regulation and execution of apoptosis in vitro were examined in breast carcinoma in vivo, during the first 24 h of treatment with chemotherapy. Bcl-2, Bax, XIAP, cleaved (active) caspase 3, cleaved caspase 6, and cleaved DFF40 were assessed for their relationship with apoptosis and the proliferation-associated antigen Ki67, before therapy and 24 h after initiation of chemotherapy. Associations with the established predictive biomarkers ER, PgR, and HER-2 were also assessed.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient demographics and treatment characteristics</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
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<tr>
<td>Menopausal status</td>
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<td>Pre</td>
<td>22</td>
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<td>5</td>
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<td>Invasive ductal carcinoma</td>
<td>30</td>
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<td>5</td>
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<td>2</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
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<tr>
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<tr>
<td>Median Tumour size (range cm)</td>
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<tr>
<td>Chemotherapy**</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>18</td>
</tr>
<tr>
<td>ECF</td>
<td>12</td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td></td>
</tr>
<tr>
<td>With chemotherapy (before 2nd biopsy)</td>
<td>19</td>
</tr>
<tr>
<td>After second biopsy</td>
<td>9</td>
</tr>
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</table>

*Grade according to the Bloom and Richardson Classification. N/A, not assessable.
**AC, Adriamycin and Cyclophosphamide; ECF, Epirubicin, Cisplatin, and infusional 5 Fluorouracil (cisplatin was substituted by carboplatin or cyclophosphamide in some patients); Other: MMM, Mitoxantrone, Mitomycin, and Methotrexate; ECU, Epirubicin, Cyclophosphamide, and UFT and leucovorin.

Materials and Methods

Patients and Tissue Samples. Women receiving primary chemotherapy for untreated breast cancer in the Royal Marsden Hospital from 1995 were invited to enter a 24-h breast tumor biopsy study. This entailed a pretreatment diagnostic biopsy and a second biopsy taken 24 h after the start of the first cycle of chemotherapy (Table 1). Thirty-five pairs of biopsies taken between February 1995 and April 1998, with sufficient tissue for the accurate measurement of apoptosis, proliferation, and biomarkers, were examined as described below. Both biopsies were taken using a 14-gauge Tru-cut core biopsy gun, and the tissue samples were fixed in 10% formal saline. After paraffin embedding, the stained sections were examined by a consultant histopathologist for diagnosis based on the WHO classification of breast tumors and graded according to the Bloom and Richardson classification (19). All patients went on to have an anthracycline-containing chemotherapy regimen. Response to chemotherapy was established by clinical assessment of the breast mass according to the criteria of the International Union against Cancer.

Tamoxifen was prescribed for 28 of the 35 patients, and in most patients, treatment started on the same day as the chemotherapy (Table 1).

Immunohistochemical Assays. The staining procedures used for ER, PgR, Ki67 (MIB 1) have been described previously (20). Similar methods were used for HER-2, Bcl-2, Bax, caspase 3, caspase 6, DFF40, and XIAP.

Antibodies for ER and PgR were purchased from Novocastra (Newcastle Upon Tyne, United Kingdom). MIB1 antibody (The Binding Site Ltd., Birmingham, United Kingdom)
was used at a dilution of 1:50 for 1 h for Ki67 immunostaining. The ICR 12 antibody was used to detect HER-2 membrane staining at a dilution of 1:800 (with a 1 mg/ml stock solution) as documented previously (21). It was found that at this concentration, normal tissue did not stain, and positive membrane staining was seen only in tumors with more than three copies of the HER-2 gene (21). No antigen retrieval procedures were conducted before HER-2 staining. For Bcl-2, a copper sulfate wash was used to enhance the stain before counterstaining. Anti-DFF40 antibody was purchased from ProSci, Inc. (Poway, CA) and used at a 1:1200 dilution. Known positive and negative tissue controls were used in each batch to determine the acceptability of the batch.

The terminal deoxynucleotidyl transferase-mediated nick end labeling assay was used to identify apoptotic cells in the biopsy samples (22). For Bax, active caspases 3 and 6 and XIAP sections, after microwave retrieval, sections were placed in acidic buffers (pH 5.5–6.0) and then exposed to monospecific PAB. These were generated against synthetic peptides or recombinant protein immunogens. The characterization of antibodies to Bax (PAB #1712) have been described previously (23).

Polyclonal antisera for caspases were generated in rabbits using synthetic peptides or recombinant protein immunogens (24). Among the peptides used as immunogens was the SGGVD-C peptide representing the cleaved subunit of caspase-3 (PAB #MN-1; Ref. 25). Affinity-purified His6-tagged pro-caspase-3 recombinant protein was used as an immunogen to produce an additional caspase-3 antiserum (PAB #1797).

An anticaspase-6 serum (PAB #1887), recognizing the active form of this caspase and antibodies specific for inhibitor of apoptosis protein XIAP (PAB #126) were generated in rabbits against relevant recombinant proteins, e.g., catalytic subunits of caspase 6 or Bir2 domain (baculovirus inhibitor repeat domain 2) of human XIAP, fused with a COOH-terminal His6-tag. These proteins were expressed in BL21 (DE3) cells by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside. After cell growth and lysis, the clarified cell lysates were applied to a nickel-nitrilotriacetic acid columns and eluted with an acidic buffers (pH 5.5–6.0) and then exposed to monospecific PAB. These were generated against synthetic peptides or recombinant protein immunogens. The characterization of antibodies to Bax (PAB #1712) have been described previously (23).

Table 2 Response to chemotherapy and further local treatment

<table>
<thead>
<tr>
<th>Response to chemotherapy</th>
<th>Total</th>
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<tbody>
<tr>
<td>CR</td>
<td>11</td>
</tr>
<tr>
<td>PR</td>
<td>20</td>
</tr>
<tr>
<td>NC</td>
<td>2</td>
</tr>
<tr>
<td>PD</td>
<td>1</td>
</tr>
<tr>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
</tr>
<tr>
<td>Conservative</td>
<td>16</td>
</tr>
<tr>
<td>Mastectomy</td>
<td>9</td>
</tr>
<tr>
<td>Radiotherapy to chest wall/nodes</td>
<td>32</td>
</tr>
</tbody>
</table>

CR, complete clinical response, with no palpable tumor until the completion of chemotherapy; PR, partial response defined as a >50% reduction in the product of two perpendicular diameters of the tumor which persisted until the end of chemotherapy; PD, progressive disease was either the appearance of a new metastatic lesion or a >25% increase in the sum of the products of two perpendicular diameters of the tumor; NC, no significant change by the above criteria; N/A, not assessible.

The proportion of staining cells (A) was categorized as follows: 1: 0–4%, 2: 5–19%, 3: 20–39%, 4: 40–59%, 5: 60–79%, and 6: 80–100%, and intensity of the stain (B) was graded: 0 = neg, 1 = weak intensity, 2 = intermediate intensity, and 3 = strong. The product of A × B gives a Quickscore between 0–18. The scoring was conducted independently by S. K. and M. P., who were blinded to treatment sequence and clinical outcome. Where discrepancies occurred, these were resolved by discussion between the analysts before statistical analysis.

Statistical Analyses. The significance of changes in expression of respective analytes was assessed by using the Wilcoxon signed rank test. Untransformed scores were used for all parameters except MIB1 and apoptosis. The latter were analyzed by investigating whether the log of the proportional change was nonzero, which is equivalent to examining whether the proportional change differs significantly from unity. CIs were calculated based on the logarithms of the proportional changes and were then back transformed. Spearman rank correlation was used to assess associations between parameters. Two-sided tests were used in all instances. Spearman rank correlation was used to examine trends between biomarker score and clinical outcome (complete clinical response, partial response, no change, and progressive disease).
Results

Tumor Pathology and Response to Treatment

The median age of the study group was 48 years (36–62), with 22 of the 35 patients being premenopausal (Table 1). Median tumor size was 6 cm (range 4–20), and 30 invasive ductal carcinomas and 5 lobular carcinomas were examined. Twenty were grade 3, and 13 were grade 2 (2 were not assessable from the core biopsies; Table 1).

Eleven patients had a complete clinical response, 20 had partial response, 2 had no change, 1 had progressive disease, and 1 was not evaluable for response because of early death. Most patients (33 of 35) went on to have breast surgery, conservative surgery, or mastectomy, with axillary dissection. Thirty-two patients went on to have adjuvant radiotherapy (Table 2).

Relationship between AI and Ki67

Pretreatment. Highly significant correlation was observed between Ki67 and AI in pretreatment samples (r = 0.64, P < 0.0001; Fig. 2A). No statistically significant correlation was found between AI and the pretreatment level of any of the biomarkers surveyed.

After Chemotherapy. Comparisons of pre and post-treatment specimens demonstrated that AI rose from a mean of 0.75% (95% CI 0.71–0.78) to 1.17% (95% CI 1.11–1.23; P < 0.001; Fig. 3). Ki67 score fell from 24.1% (95% CI 22.5–25.8) to 18% (95% CI 16.8–19.3; P < 0.001; Fig. 4). A negative correlation (r = −0.36; P = 0.04) was seen between pretreatment Ki67 and relative rise in AI. A significant positive correlation was observed between the relative fall in Ki67 score and rise in apoptosis score (r = 0.4; P = 0.02; Fig. 5). Thus, it is clear that for most patients, an increase in apoptosis and/or a decrease in proliferation was induced by chemotherapy. Although decreases in apoptosis and/or increases in proliferation were noted for a minority of patients, the proportional change was below unity in only 3 patients.

The correlative relationship between Ki67 score and AI was maintained in the post-treatment samples (r = 0.4; P = 0.02).

Correlation of Pretreatment Biomarker Values

Before chemotherapy, cytoplasmic Bcl-2 staining in breast tumor was seen in 93% of the patient specimens examined (65 of 70 slides), Bax in 92%, cytoplasmic cleaved (active) caspase 3 in 87%, cytoplasmic cleaved caspase 6 in 99%, and cleaved nuclear DFF40 and cytosolic XIAP in 100%. Significant correlations were observed among caspase 3, 6, and DFF Table 3A. Caspase 3 correlated with caspase 6 (r = 0.34; P = 0.66), Bcl-2 (r = 0.54; P = 0.006), but neither caspase 3 nor Bcl-2 showed such changes at 24 h (Fig. 6). Consistent with the strong positive correlation between XIAP and caspase 3 (r = 0.66; P < 0.0001; Fig. 2B) and 6 (r = 0.47; P = 0.006). There was also a strong positive correlation between XIAP and caspase 3 (r = 0.64; P < 0.0001; Fig. 2C) and DFF40 (r = 0.61; P = 0.0001; Fig. 2D) and caspase 6 (r = 0.36; P = 0.04) in pretreatment specimens. MIB-1 proliferation-associated antigen failed to show direct correlation with any of these apoptosis-related proteins.

Change of Biomarkers 24 h after Chemotherapy

Compared with pretreatment specimens, Bax showed a significant increase at 24 h after initiation of chemotherapy (P = 0.05). Immunostaining for cleaved DFF40 also increased after chemotherapy (P = 0.02). Caspase 6 showed a tendency to rise with chemotherapy (P = 0.06), but neither caspase 3 nor Bcl-2 showed such changes at 24 h (Fig. 6). Consistent with the significant increases seen in Bax and DFF40 at 24 h after chemotherapy, the change in the scores for these two parameters was significantly correlated (r = 0.54; P = 0.0008; Fig. 7). DFF40 maintained its significant relationship with caspase 3 and XIAP after 24 h of chemotherapy (r = 0.60; P = 0.0002 and r = 0.54; P = 0.001, respectively; Table 3B).
Significance of Hormone Receptor and HER2 Status

Among the breast cancer specimens examined here, 63% of the tumors were ER positive (22 of 35), and 31% (11 of 35) were PgR positive. All PgR-positive tumors were ER positive. Low pretreatment MIB1 scores were associated with ER positivity ($P = 0.0008$). ER-positive tumors were also more likely to have greater changes with chemotherapy ($P = 0.01$). No significant association of ER and PgR with pretreatment AI or changes in AI were seen. Pretreatment ER and PgR positivity were strongly associated with pretreatment Bcl-2 ($r = 0.003$; $P = 0.004$). HER-2 positivity was found in 26%. Higher pretreatment AI ($P = 0.01$) and Ki67 scores ($P = 0.02$) were seen in HER-2-positive samples.

Relationship of Biomarkers with Clinical Outcome

Higher MIB-1 levels were associated with better clinical response ($r = 0.42$; $P = 0.02$). No other associations with response, disease-free interval, or overall survival were found.

Discussion

This is the first study to investigate associated changes in the levels or activation status of Bax, XIAP, caspases 3 and 6, and DFF40 in breast cancer before and during chemotherapy in vivo. The proteins examined represent some of the key initiators, effectors, and regulators of the apoptotic pathway. There are few published data on any of these biomarkers during chemotherapy. This study had two related aims: (a) to establish the relationships between these parameters and apoptosis in human breast cancer in vivo; and (b) to assess whether any of these might predict biological response (i.e., change in apoptosis and/or proliferation) or clinical response to therapy.

Pretreatment apoptosis correlated strongly with proliferation, a finding consistent with our previous work (27) and that of others (13, 28). High AI has also been found to correlate with high pathological grade, overexpression of p53, and low expression of Bcl-2 and ER (13, 29-31). Apoptosis rose within 24 h of chemotherapy, and proliferation fell significantly over the same time, both of which we have noted previously (16, 32). Al-
though univariate analysis has shown high AI to be associated with poor prognosis, it has not been an independent prognostic marker in any previous multivariate analyses (13, 33, 34). In the present study, we could not confirm the relationship between apoptosis and caspases found previously by another study (35). However, others have also found no significant association (36). Moreover, the antisera we used selectively recognize the proteolytically cleaved forms of caspases 3 and 6, whereas prior studies used antibodies that recognized the uncleaved protein (35).

A negative correlation was found between proliferation and change in apoptosis \( r = -0.36; \ P = 0.04 \), which suggests that low pretreatment levels of proliferation are associated with a larger rise in apoptosis after treatment. This finding is of uncertain significance; it may relate to biopsies with low proliferation also having low pretreatment AI, such that identical absolute changes in AI are relatively greater. Indeed, high proliferation pretreatment was associated with an improved response to chemotherapy. This is a consistent finding with other studies using the same proliferation-associated antigen (37, 38) and other techniques for the assessment of proliferation (39, 40). Such findings may relate to a greater effect of chemotherapy in highly proliferative tumors. The fall in proliferation observed in our study within 24 h of starting treatment has been shown to persist to the end of chemotherapy in most patients (27, 37).

ER positivity was associated with low proliferation scores, consistent with earlier studies (13, 37, 41). As established prevously, ER- and PgR-positive tumors were associated with Bcl-2 immunostaining (42, 43). This correlation between hormone receptors and Bcl-2 has been suggested to reflect regulation of Bcl-2 via the ER (44) and appears to be indicative of slowly proliferating tumors (43). Bcl-2 has failed to emerge as a prognostic marker independent of other markers, such as p53 positivity, but in univariate analysis, Bcl-2 has been found to indicate a favorable prognosis (43, 45). This paradoxical correlation with outcome for an apoptosis inhibitor, which \textit{in vitro} confers apoptosis resistance (46), could be explained by the inverse relationship with p53 immunopositivity, as well as by its association with ER (45), and by other factors (47). No association between Bcl-2 and apoptosis, caspase 3, caspase 6, or DFF40 was found in the present study. This may be in part because of the presence of the other members of the Bcl-2 family and the interactions among them.

Proapoptotic Bax, another member of the Bcl-2 family, induces mitochondrial permeability transition and activation of downstream effector caspases, such as caspases 3, 6, and 7 (48). DNA fragmentation is a terminal event in the apoptotic pathway and is induced largely by the DFF. DFF40/CAD is activated by caspases leading to the induction of nuclease activity and DNA fragmentation (49–51). Although no association with cleaved caspase was found, Bax correlated with cleaved terminal nuclear

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Change in AI in breast biopsies during the first 24 h of chemotherapy. The data points represent the pretreatment and 24-h post-treatment apoptotic indices for individual patients.}
\end{figure}
protein DFF40 both before and at 24 h after initiation of chemotherapy when AI rose.

IAPs are an evolutionarily conserved family of proteins that prevent cell death by directly inhibiting caspases (52). The group includes XIAP, cIAP1, cIAP2, NIAP, and Survivin. XIAP has emerged as one of the most potent of the inhibitors in the IAP family (52, 53). Previous in vitro work with XIAP has shown selective binding to and inhibition of caspases 3, 7, and 9 but not caspases 1, 6, or 8 (53, 54). In this study, a strong correlation was found between pretreatment XIAP and caspase 3 levels and, with the cleaved product of DFF40, a substrate for caspase 3. A weaker but significant correlation ($r = 0.36; P = 0.04$) was also found between XIAP and cleaved caspase 6.

These associations may indicate a significant role for XIAP in the regulation of apoptosis in breast cancer. IAPs in human cancer cell lines and human acute myeloid leukemia suggested that IAPs are widely, although differentially, expressed in different tumor cell lines. XIAPs, along with cIAP1, were the most widely expressed, and XIAP protein in acute myelogenous leukemia was associated with an adverse prognosis (55). In the present study, XIAP was not a predictive marker of response, but this may relate to the small sample size of the study.

Caspases are a family of cysteine-dependent aspartate-directed proteases, which contribute to the morphological and biochemical changes that characterize apoptotic cell death. Thus far, 11 human caspases have been identified. Caspases can act as both initiators and effectors of the apoptotic pathway. Caspase 3 is capable of catalyzing the specific cleavage of many key cellular proteins involved in apoptosis and is required for some typical hallmarks of apoptosis, particularly for apoptotic chromatin condensation and DNA fragmentation in many cell types (56). Caspase 6, also an executioner caspase, probably acts downstream of caspase 3. Both have been identified in breast cells undergoing apoptosis in vitro (15). Active, cleaved caspases 3 and 6 were identified in the cytoplasm of nearly all of the study samples. No correlation of these immunoscores for these caspases was observed with AI or Ki67. This may be in part because of the way staining for these parameters was assessed. By exclusion of granular staining and grouping the scores (an additive score of intensity and quantity of staining) in three broad categories, Vakkala et al. (35) were able to see a correlation of caspases 3, 6, and 8 with apoptosis. Despite the lack of correlation with AI in our study, there was a strong relationship between caspases 3 and 6, caspase 3 and DFF40, and caspase 6 and DFF40, which suggests an integrated role for these late-stage effectors in apoptosis in human breast cancer.

The high clinical response rates as seen in this cohort (88% objective response) were similar to those reported elsewhere for neoadjuvant chemotherapy (4, 10), but this limits the assessment of markers for their relationship with clinical response. The small size of the cohort would also allow only very strong predictors of response to be revealed.

In conclusion, in this study, using human breast biopsies before and 24 h after chemotherapy, we observed a significant rise in apoptosis, fall in proliferation, rise in Bax, and increase in cleaved DFF40 within 24 h of starting chemotherapy. Pretreatment tumors expressed cleaved caspases 3 and 6 and DFF40 in a coordinate manner. Of particular interest, XIAP also correlated strongly with these effectors of apoptosis, suggesting...
feedback between positive and negative regulators of the apoptotic process. The expression of these biomarkers should be studied further in larger patient cohorts to examine their role as predictive markers for long-term treatment outcome.

Acknowledgments

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References

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