Expression of *survivin* and Its Relationship to Loss of Apoptosis in Breast Carcinomas

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ABSTRACT

Aberrant inhibition of programmed cell death (apoptosis) prevents normal homeostasis and promotes tissue tumorigenesis, but whether it also influences the outcome of common cancers has remained arguable. The expression of a novel IAP apoptosis inhibitor, *survivin*, in breast cancer and its association with tumor cell apoptosis and overall prognosis were examined in this study. Immunohistochemical analysis showed that survivin expression was positive in 118 of 167 cases (70.7%) of breast carcinomas of histological stages I to III. In contrast, no expression of survivin in adjacent normal tissue was detected. Although survivin expression was not correlated with p53 mutations, survivin-positive cases were strongly associated with bcl-2 expression (78.0% versus 47.5%; *P* = 0.0005) and reduced apoptotic index (0.62% ± 0.51% versus 1.27% ± 1.37%; *P* < 0.0001). In addition, patients with low apoptotic index (<0.52%) had worse survival rates than the group with high apoptotic index (≥0.52%; *P* = 0.028), and multivariate Cox proportional hazard model analysis identified apoptotic index as an independent prognostic factor (*P* = 0.024). The results suggest that apoptosis inhibition by *survivin*, alone or in cooperation with bcl-2, is a significant prognostic parameter of worse outcome in breast carcinoma.

INTRODUCTION

Abnormalities in the control of programmed cell death (apoptosis) play an important role in tumorigenesis (1, 2). This process involves an evolutionarily preserved multistep cascade and is regulated by proteins that promote or counteract apoptotic cell death (3). bcl-2 was the first protein shown to lead to prolonged survival of cells by preventing apoptosis (4). Several apoptosis inhibitors related to the baculovirus *iap* gene have been identified in humans, mice, and Drosophila (5, 6). Highly evolutionarily conserved, IAP2 proteins contain two to three Cys/His BIRs and a COOH-terminal RING finger (4). Recombinant expression of IAP proteins counteracted various forms of apoptosis in vivo (7) and in vitro (6). These molecules are thought to block an evolutionarily conserved step in apoptosis. At least in the case of XIAP, this may involve direct inhibition of the terminal effectors caspase-3 and caspase-7 through a BIR-dependent recognition (8). Recently, a novel gene encoding a structurally unique IAP apoptosis inhibitor, designated as *survivin*, has been identified. Survivin is an *M* ~16,500 cytoplasmic protein with a single BIR and no RING finger. Recombinant expression of survivin prevents apoptosis induced by growth factor interleukin 3 withdrawal in a pre-B cell line (9). Unlike bcl-2 (10) or other IAP proteins (5, 6, 7), survivin is undetectable in terminally differentiated adult tissues but becomes notably expressed in the most common human cancers, including stomach (11), colorectal (12), lung, breast, pancreatic, and prostate cancers and high-grade non-Hodgkin’s lymphomas in vivo (9, 13). Our previous studies demonstrated that the expression of survivin was significantly associated with bcl-2 expression and reduced apoptotic indices, which were strongly correlated with poor prognosis after surgery in both gastric and colorectal cancers (11, 12). In this study, we assessed the expression of survivin in breast cancer and its potential effect on tumor cell apoptosis and overall survival.

MATERIALS AND METHODS

Patients and Samples. We studied a series of 167 patients with invasive breast carcinomas who did not receive any form of treatment prior to surgery. The surgically resected specimens used for this study were obtained from consecutive patients with breast carcinoma who underwent potentially curative resection at the Department of General and Gastroenterological Surgery, Osaka Medical College Hospital, during the period from 1988 to 1994. Clinicopathological factors, tumor histologies, and disease stage were assigned according to the General Rules for Clinical and Pathological Recording on Breast Cancer (14). The specimens consisted of 32 cases of stage 1, 104 cases of stage II, and 31 cases of stage III tumors. All of the patients were female, and the mean age of the patients was 53.1 years (SD, 11.84 years; range, 29–89 years). With regard to postoperative adjuvant therapy, the patients with four or more axillary lymph node metastases had six cycles of CMF (100 mg/m2 cyclophosphamide p.o. on days 1–14, 40 mg/m2 i.v. methotrexate on days 1 and 8, and 500 mg/m2 i.v. 5-fluorouracil on days 1 and 8, every 4 weeks) or CAF (100 mg/m2 cyclophosphamide p.o. on days 1–14, 30 mg/m2 Adriamycin i.v. on...
days 1 and 8, and 500 mg/m² 5-fluorouracil i.v. on days 1 and 8, every 4 weeks). The patients with three or less axillary lymph node metastases were treated with tegafur ([2-tetra-hydrofuryl]-5-fluorouracil] at an oral dose of 600 mg/day for 2 years after surgery. Node-negative premenopausal patients were followed without chemotherapy. Patients with ER-positive tumors and postmenopausal patients received adjuvant endocrine treatment with 20 mg/day oral dose of Tamoxifen. The median follow-up time in this study group was 96 months (range, 11–104 months). Routinely processed formalin-fixed, paraffin-embedded blocks containing the main tumor were prepared. Serial sections of 2–4 μm were cut from the blocks at the maximum cross-section of the tumor.

**Immunohistochemical Staining for survivin and the Scoring Method for Its Expression.** A pilot study using the anti-survivin antibody was conducted on various neoplasms, including high-grade non-Hodgkin’s lymphoma, pancreatic cancer, colorectal cancer, and gastric cancer to confirm the specificity in staining tumor cells and to determine an appropriate dilution for mAb 8E2 (IgG1 kindly provided by Dr. D. C. Altieri, Department of Pathology, Yale University, School of Medicine, New Haven, CT) for staining of breast carcinomas. One case of stage III gastric cancer was stained intensively and reproducibly for survivin expression in >30% of tumor cells, and this was used as a positive control throughout the present study. Negative control slides processed without primary antibody were included for each staining. For immunohistochemical detection of survivin protein, the standard avidin-biotin-peroxidase complex technique was carried out by using an LSAB kit (Dako A/S, Carpinteria, CA). Before using the LSAB kit, antigen retrieval was done by the pressure cooking method as described previously (11, 12). In brief, deparaffinized and rehydrated sections were bathed in a 10−3 m sodium citrate buffer (pH 6.0) after bringing the solution to a boil in a pressure cooker and boiled for 20 min while maintaining the pressure. After quenching in 3% hydrogen peroxide for 5 min, washing with PBS, and adding the equilibration buffer for 10 min, terminal deoxynucleotidyl transferase enzyme was pipetted onto the sections, which were then incubated at 37°C for 1 h. After stopping the reaction by putting sections in stop/wash buffer and washing, anti-digoxigenin-peroxidase was added to the slides. Finally, slides were washed with PBS, stained with diaminobenzine (DAKO A/S, Glostrup, Denmark) and a mouse antihuman p53 antibody (DO7, diluted 1:50; DAKO, Copenhagen, Denmark) were used as primary antibodies for bcl-2 and p53 immunostaining. Before addition of the primary antibodies, sections were heated in a microwave oven three times at 900 W for a total of 15 min in 10−3 m sodium citrate buffer (pH 6.0). The other staining procedures were the same as those for survivin. The scoring criteria for bcl-2 were the same as those for survivin, and cases with weighted scores of <1 were judged as negative. For p53 expression, cases with <5% positively stained tumor cells were defined as negative; otherwise, they were defined as positive.

**Histochemical Detection of Apoptosis and Determination of the AI.** Apoptotic cells and apoptotic bodies were detected by *in situ* labeling using an ApopTag *in situ* Detection kit (S7101-KIT; Oncor, Gaithersburg, MD). In brief, deparaffinized and rehydrated sections were digested with proteinase K (20 µg/ml in PBS; Wako, Osaka, Japan) for 20 min at room temperature and washed. After quenching in 3% hydrogen peroxide for 5 min, washing with PBS, and adding the equilibration buffer for 10 min, terminal deoxynucleotidyl transferase enzyme was pipetted onto the sections, which were then incubated at 37°C for 1 h. After stopping the reaction by putting sections in stop/wash buffer and washing, anti-digoxigenin-peroxidase was added to the slides. Finally, slides were washed with PBS, stained with diaminobenzine (DAKO A/S, Glostrup, Denmark) substrate, and counterstained with methyl green. A positive control was prepared by nicking DNA with DNase I (0.7 µg/ml; Stratagene Co., La Jolla, CA) for the first staining procedure. A specimen known to be positive for apoptotic cells was used as positive control for subsequent staining. Substitution of terminal deoxynucleotidyl transferase with distilled water was used as negative control. The AI was expressed as the ratio of positively stained tumor cells and bodies to all tumor cells, given as a percentage for each case, and determined according to the criteria described previously (11, 12, 16). In brief, a minimum of 3000 cells was counted at 400-fold magnification. Positively staining tumor cells with the morphological characteristics of apoptosis were identified using standard criteria, including chromatin condensation, nuclear disintegration, and formation of crescent caps of condensed chromatin at the nuclear periphery.

**Statistical Analysis.** All statistical analysis was performed using the SPSS 6.1 J software package for Macintosh (SPSS, Inc., Chicago, IL). Variables associated with survivin expression as well as the correlation between survivin and p53 or bcl-2 expression were analyzed by the χ² test. Differences in tumor cell AI for groups differing according to survivin expression were checked by the independent Wilcoxon method. The survival curves were plotted according to Kaplan-Meier method and checked by the log-rank test. A value of *P* < 0.05 was considered statistically significant.
RESULTS

State of Expression of Survivin in Breast Carcinomas. Immunohistochemical staining revealed that anti-survivin mAb 8E2 specifically reacted with breast carcinoma cells, with positive staining of the cytoplasm of tumor cells (arrows; ×400), whereas no expression of survivin was observed in adjacent normal tissues. Unlike bcl-2, no reactivity of the anti-survivin mAb 8E2 with infiltrating lymphocytes and other stroma cells was detected. The intensity of survivin staining was usually homogeneous within a given case, but the number of positive tumor cells stained by the anti-survivin mAb varied between 20 and 100%, depending on the case investigated. After multiplying the weighted survivin score, 118 cases of breast carcinoma in this series were defined as positive (70.7%), with a weighted survivin score from 1 to 12. The distributions of weighted survivin scores were 0 for 49 cases, 1 for 17 cases, 2 for 26 cases, 3 for 30 cases, 4 for 14 cases, 6 for 13 cases, 8 for 8 cases, 9 for 7 cases, and 12 for 3 cases.

Relationship Between Expression of Survivin and Clinicopathological Factors. Table 1 shows the correlation between expression of survivin and clinicopathological factors. It shows that none of the prognostic parameters analyzed, includ-
Expression and Apoptosis in Breast Carcinomas

The mean AI in survivin-positive tumors (n = 118) was 0.62% (SD, 0.51%), which was significantly lower than the mean AI of 1.27% (SD, 1.37%) observed in survivin-negative tumors (n = 49; P < 0.0001). However, when the cases with survivin weighted scores <2 were defined as negative and otherwise as positive, the mean AI for survivin-positive tumors (n = 101) was 0.63%, which was also significantly lower than the mean AI of 1.08% for survivin-negative tumors (n = 66; P = 0.0016). The mean AI in bcl-2-positive tumors was significantly lower than that in bcl-2-negative tumors (P = 0.0013). In survivin-positive tumors (n = 118), 99 cases were also positive for bcl-2 expression, and the mean AI in these cases was 0.56% (SD, 0.44%). In bcl-2-negative tumors (n = 40), the mean AI of 19 survivin-positive tumors was 0.94% (SD, 0.72%), which was lower than the value of 1.57% (SD, 1.86%) observed in survivin-negative tumors (P = 0.233).

Prognostic Analysis in Patients with Breast Carcinoma.

Fig. 2a shows the Kaplan-Meier curves for patients with breast carcinoma categorized according to survivin expression. The overall 5-year survival rate for patients with survivin-positive breast carcinomas (87.3%; 103 of 118) was less than that for patients with survivin-negative tumors (95.9%; 47 of 49), but the difference was not significant. To study a possible correlation between AI and prognosis, the patients were dichotomized by the median value of 0.52 for AI, which provided the most sensitive parameter of survival difference in the present patient series. However, AI dichotomized by the median value (0.54%) was also proved to be prognostically significant in the multivariate analysis (P = 0.044).

Association of Expression of survivin and bcl-2 with Tumor Cell Apoptosis.

Every case of breast carcinoma examined showed apoptotic cells and apoptotic bodies that were detected by in situ labeling (Fig. 1b). The relationships between AI and expression of survivin and bcl-2 were examined (Table 3). The mean AI of the 167 cases was 0.81% (SD, 0.90%; range, 0.10–8.61%), with a median value of 0.54%. No significant correlation was observed between the AI and the tumor stage. The mean AI in survivin-positive tumors (n = 118) was 0.62% (SD, 0.51%), which was significantly lower than the mean AI of 1.27% (SD, 1.37%) observed in survivin-negative tumors (n = 49; P < 0.0001). However, when the cases with survivin weighted scores <2 were defined as negative and otherwise as positive, the mean AI for survivin-positive tumors (n = 101) was 0.63%, which was also significantly lower than the mean AI of 1.08% for survivin-negative tumors (n = 66; P = 0.0016). The mean AI in bcl-2-positive tumors was significantly lower than that in bcl-2-negative tumors (P = 0.0013). In survivin-positive tumors (n = 118), 99 cases were also positive for bcl-2 expression, and the mean AI in these cases was 0.56% (SD, 0.44%). In bcl-2-negative tumors (n = 40), the mean AI of 19 survivin-positive tumors was 0.94% (SD, 0.72%), which was lower than the value of 1.57% (SD, 1.86%) observed in survivin-negative tumors (P = 0.233).
DISCUSSION

The results of the present study suggest that decreased apoptosis integrated partly by survivin expression (9, 13) is a new predictive indicator of poorer prognosis in breast carcinoma. Among the recently described IAP family, survivin is characterized by a unique structure with a single BIR and no zinc-binding domain known as the RING finger, and by a selective distribution in common human cancers but not in normal adjacent tissue in vivo (9). In this study, specific staining for survivin was detected in 118 cases (70.7%), with a variable proportion of positive tumor cells (20–100%). In contrast, the adjacent normal tissue or the infiltrating lymphocytes did not express survivin, in accordance with our prior studies (11, 12). One of the intriguing findings in this study was that the expression of survivin in breast carcinoma was associated with significantly reduced apoptosis, as compared with that in survivin-negative tumors. Apoptosis is a feature commonly seen in tumors, and in fact the ability to resist apoptosis may seem to offer an advantage to a rapidly growing tumor, by slowing down the cell loss rate, but potential effector molecules responsible for these aberrations have not been identified. In this study, we demonstrated that low AI, which is correlated with survivin expression, functions as a predictive/prognostic indicator of poor overall survival in breast carcinoma ($P = 0.028$), although this will have to be validated by additional clinical studies. It was independently confirmed by multivariate Cox proportional hazard model analysis, which identified the AI as a significant independent predictor of overall survival ($P = 0.024$), although the decrease in survival rate of survivin-positive patients did not reach statistical significance. However, in contrast to our findings, several studies, using AI assessment with a microscopy at 200–400-fold magnification, have demonstrated that apoptotic cell death is increased in tumors with high-grade malignancy in comparison with tumors with low-grade malignancy in various

![Fig. 2 a](image_url)  
Kaplan-Meier curves for overall survival rates of patients with breast carcinoma categorized according to survivin expression. No significant difference was found between the groups ($P = 0.108$; log-rank test).  

![Fig. 2 b](image_url)  
Kaplan-Meier curves for overall survival rates of patients with breast carcinoma categorized by high versus low AI ($P = 0.028$; log-rank test).
survivin in situ morphic nuclei may be extremely difficult (21). Bedi et al. have used in this study. In addition, the morphological technique used in this study, including insufficient numbers of patients studied and the heterogeneous character in terms of adjuvant therapy, second-line and third-line treatments for recurrence and/or distant metastasis, who responded variably to combinations of chemotherapy and/or endocrine therapy.

Table 4 Univariate Cox proportional hazard model

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Risk rate</th>
<th>95% CI*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size (cm) (≥2 vs. &lt;2)</td>
<td>1.292</td>
<td>0.37–4.50</td>
<td>0.688</td>
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<tr>
<td>Lymph node metastasis (positive vs. negative)</td>
<td>18.073</td>
<td>2.39–136.87</td>
<td>0.005</td>
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<tr>
<td>Lymphatic invasion (positive vs. negative)</td>
<td>2.842</td>
<td>0.93–8.72</td>
<td>0.067</td>
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<tr>
<td>Venous invasion (positive vs. negative)</td>
<td>1.824</td>
<td>0.52–6.36</td>
<td>0.346</td>
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<tr>
<td>ER status (positive vs. negative)</td>
<td>0.332</td>
<td>0.13–0.86</td>
<td>0.023</td>
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<tr>
<td>p53 expression (positive vs. negative)</td>
<td>2.363</td>
<td>0.90–6.22</td>
<td>0.081</td>
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<tr>
<td>bcl-2 expression (positive vs. negative)</td>
<td>0.631</td>
<td>0.22–1.80</td>
<td>0.389</td>
</tr>
<tr>
<td>survivin expression (positive vs. negative)</td>
<td>3.093</td>
<td>0.71–13.53</td>
<td>0.134</td>
</tr>
<tr>
<td>AI (≥0.52 vs. &lt;0.52)</td>
<td>0.328</td>
<td>0.12–0.93</td>
<td>0.037</td>
</tr>
</tbody>
</table>

* CI, confidence interval.

Table 5 Multivariate Cox proportional hazard model

<table>
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<tr>
<th>Characteristic</th>
<th>Risk rate</th>
<th>95% CI*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node metastasis (positive vs. negative)</td>
<td>22.518</td>
<td>2.86–177.54</td>
<td>0.003</td>
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<tr>
<td>ER status (positive vs. negative)</td>
<td>0.224</td>
<td>0.08–0.62</td>
<td>0.004</td>
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<tr>
<td>AI (≥0.52 vs. &lt;0.52)</td>
<td>0.300</td>
<td>0.10–0.86</td>
<td>0.024</td>
</tr>
</tbody>
</table>

* CI, confidence interval.

human tumors (17, 18), including breast carcinoma (19). Although morphological studies using light microscopy are useful for the detection of some apoptotic bodies, they may not reveal the early apoptotic stages (20), i.e., “normal-looking” apoptotic cells, which can be detected by the DNA nick end labeling technique used in this study. In addition, the morphological identification of apoptotic nuclei within large numbers of polymorphic nuclei may be extremely difficult (21). Bedi et al. (22) have shown by in situ end-labeling technique that the transformation of colorectal epithelium to carcinomas is associated with a progressive inhibition of apoptosis. Consequently, although it has been shown that biological processes other than apoptosis may lead to positive in situ end-labeling (23, 24), this technique of simplifying the identification of apoptotic nuclei in routinely processed tissue sections seems more reliable than morphological assessment alone. Nevertheless, identification of prognostic relevance of tumor cell apoptosis seems problematic in various aspects of this study, including insufficient numbers of patients studied and the heterogeneous character in terms of adjuvant therapy, second-line and third-line treatments for recurrence and/or distant metastasis, who responded variably to combinations of chemotherapy and/or endocrine therapy.

We found compelling evidence that the presence of survivin in breast carcinoma was strongly associated with expression of bcl-2 and with reduced AI (Tables 2 and 3). Our results agree with the findings of previous investigations, which showed a similar association between survivin and bcl-2 expression in neuroblastoma (25), gastric cancer (11), colorectal cancer (12), and high-grade non-Hodgkin’s lymphoma.3 The percentage of bcl-2-positive cases in our series of breast cancer (76.0%) was comparable with those reported by other investigators (26). The accumulation of mutated p53 protein was detected in 23.6% of the present series of patients. The rate of p53 accumulation was within the range of values reported by others (27–29). The survivin gene is encoded at chromosome 17q25 (30), whereas the bcl-2 gene is located at chromosome 18q21 and may be involved in the tumorigenic t(14;18) translocation (10). These data imply that other transcriptional factors may contribute to the coregulation of both gene products in the progression of cancer. In this context, both survivin and bcl-2 genes are regulated by TATA-less, GC-rich promoter sequence in similar manners, and both are markedly transcribed in actively proliferating cell types (9), suggesting common mechanism(s) of transcriptional activation. However, regardless of the pathway of simultaneous coexpression, it appears that survivin and bcl-2 proteins may mediate nonoverlapping, antiapoptosis mechanisms. Although bcl-2 is an integral inner mitochondrial membrane protein implicated in counteracting cytochrome c release from the mitochondria, IAP molecules, potentially including survivin, prevent apoptosis by targeting the terminal effectors caspase-3 and caspase-7 (8, 31, 32). Survivin is expressed in the G2-M phase of the cell cycle in a cell cycle-regulated manner and associates with microtubules of the mitotic spindle. Disruption of survivin-microtubule interactions results in loss of survivin’s antiapoptosis function and increased caspase-3 activity during mitosis. The overexpression of survivin in cancer may obliterate this apoptotic check point and allow aberrant progression of transformed cells through mitosis (32). In breast carcinoma and in many of the most common human cancers, inhibition of apoptosis may be a general feature, and expression of survivin alone or survivin plus other antiapoptosis genes like bcl-2 may cause more pronounced antiapoptotic effects, as reflected in the significantly reduced apoptotic index observed in our series.

Although decreased AI associated with survivin expression was shown to be an indicator of poor prognosis in breast carcinoma, the predictive value was not as strong as was observed in colorectal carcinoma (P = 0.024 versus P = 0.0001; Ref. 12). One of the factors relevant to this issue may be the limited number of patients studied in this trial and the various responses to chemotherapy and/or endocrine therapy (case by case) that were given postoperatively to the present series of patients. Clark et al. (33, 34) has recently proved that either mitosis expression or telomerase activity, a regulator of S-phase fraction, is an independent prognostic factor in node-negative and node-positive breast cancers. Therefore, identification of prognostic significance of apoptotic index should be clarified.

3 Unpublished observations.
with additional use of these prognostic markers. bcl-2 has been reported to be frequently expressed in breast cancer and to be associated with positivity for ERs in both node-negative and node-positive breast cancers (35, 36). These findings are in agreement with our finding of a significant positive relationship between bcl-2 immunoreactivity and ER status (P = 0.024).

However, unlike survivin proteins, the association of bcl-2 with a favorable clinical prognosis in breast cancer is reported (26, 36). One explanation for this paradox is that bcl-2 may have still unrecognized and other nonapoptotic functions. In clinical studies, bcl-2 expression is inversely correlated with S-phase fraction and tumor size in breast cancer (37, 38). Furthermore, bcl-2 has proved to have potential to lead to prolongation of the cell cycle as well as a decrease in vitro breast cancer growth (39).

It is well known that there are multiple genetic pathways that control apoptosis, a part of which is probably regulated by survivin and/or bcl-2. Therefore, changing the level of expression of these proteins may not necessarily have an effect on outcome of therapy. A recent in vitro study demonstrated, however, that antisense survivin RNA down-regulated expression of endogenous survivin in transformed cells and resulted in increased apoptotic cell death (32). In this context, in addition to chemotherapy and endocrine therapy, targeted antagonists of survivin may be beneficial as apoptosis-based therapy for breast carcinoma.

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