Survivin Expression Predicts Early Recurrence in Early-stage Breast Cancer

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Abstract. *Background: The apoptosis inhibitor survivin is one of the most specific proteins in breast cancer patients. The role of this protein in predicting prognosis is still controversial. Patients and Methods: Survivin mRNA was measured using quantitative TaqMan reverse transcription-PCR in 76 samples, including 48 early-stage breast cancer tissues and adjacent normal tissues, from patients with operable tumors, and was tested for correlation with established clinicopathological factors, or disease-free survival (DFS). Results: Comparing the survivin expression in 78 breast cancer patients with the clinicopathological factors (age, menopausal status, nodal category, tumor histology, tumor size, histological grade, ER and PgR status, and type of operation), T factor (T1-T4) was significantly associated with a high survivin mRNA ratio (p=0.0104). The proportion of tumors with a high survivin mRNA ratio was greater in node-positive than in node-negative tumors (p=0.0001), and in grade III tumors compared to grade I or grade II tumors (p=0.0001). Patients with low survivin expression showed significantly better disease-free survival than patients with high survivin expression in stage I and II breast cancer (p<0.0001, log-rank). Survivin expression alone is a powerful prognostic factor for disease-free survival of breast cancer patients without nodal involvement (HR: 0.024, 95%CI: 0.001-0.446, p=0.0123) using Cox multivariate regression analysis. Conclusion: Survivin is an indicator of the recurrence of early-stage breast cancer. Survivin might be used as a new marker to stratify early-stage breast cancer patients for more optimal treatment modalities, or it could be a promising target for therapy.*

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Approximately 25-30% of breast cancer patients with negative lymph nodes will develop distant metastases within 10 years after surgery (1). It is of great importance to find an improved marker to stratify breast cancer patients into different risk groups more accurately than can be achieved with current clinicopathological factors. Low-risk favorable patients can then be spared unnecessary treatment, avoiding side-effects and reducing the cost of treatment; furthermore, high-risk patients could be selected and offered treatment modalities customized (more aggressive) to their individual needs. A new method of gene profiling has recently been provided as a powerful tool in predicting clinical outcome.

Survivin is a member of the *IAP* gene family which has been implicated in both the inhibition of apoptosis and mitosis regulation (2). Survivin up-regulates genes in tumor tissues (3). High survivin expression in the primary tumor is related to poor prognosis in many cancer types (4-9). On the other hand, the correlation between survivin and prognosis in breast cancer patients is still controversial: previous studies have reported it to be associated with poor (10) or favorable prognosis (11); however, qualitative reverse transcription-PCR (RT-PCR) or immunohistochemistry using antibodies with different sensitivities toward the survivin variants were used in these studies. Quantitative survivin mRNA measured using real-time RT-PCR has the advantage of being more quantitative than classical RT-PCR and, in general, more specific and sensitive than immunohistochemical assays. As survivin concentrations are largely controlled at the level of gene transcription (2, 12), quantitative RT-PCR should have advantages for comparing survivin protein concentrations.

In the present study, we measured survivin mRNA using quantitative TaqMan RT-PCR in 48 early-stage breast cancer tissues and coupled normal tissues with established clinicopathological factors, and disease-free survival (DFS).

Patients and Methods

Patients. This study was approved by the Institutional Review Board. A series of 76 patients with unilateral, resectable breast cancer who underwent surgery of their primary tumor between April 1999 and December 2001 were selected on the basis of the availability of frozen tissue at the National Hospital Organization Kumamoto Medical Center, Japan. The histological examination of tumors was performed on sections stained with hematoxylin and eosin. Each tumor was typed according to the classification of the Japanese Breast Cancer Society. The clinical data were collected retrospectively.

Patients had no previous diagnosis of carcinoma and were without distant metastases at the time of diagnosis. The median age was 58 years (range 37-90 years). Patients underwent modified radical mastectomy (n=58) or breast-conserving surgery (n=18). Sixteen patients underwent postoperative radiotherapy of the breast after breast-conserving surgery. Lymph node involvement was found in 17 patients. Subsequent systemic adjuvant therapy was given based on established clinicopathological criteria at that time. Patients treated with adjuvant endocrine therapy (n=64) received 20 mg of tamoxifen daily for at least 5 years. In total, 16 patients received adjuvant chemotherapy with 4 cycles of 5-fluorouracil, epirubicin and cyclophosphamide (FEC). Sixteen patients received both endocrine and chemotherapy. The median follow-up time of surviving patients was 75 months (range 2-93 months). Patients were checked (history, physical examination, routine laboratory investigations) once every 3 months during the first 2 years, once every 6 months for 5 years, and once a year thereafter. Once a year, a CT scan or bone scintigram was performed. During follow-up, 15 patients had recurrence and 9 patients died. Contralateral breast cancer and second malignancies were not considered as recurrent disease.

Tissue processing. After primary surgery, a representative part of the tumor and adjacent normal tissue were immediately frozen with RNA Later™ (QIAGEN KK, Tokyo, Japan), stored at –80˚C until RNA extraction, and routinely determined for ER and PgR status with immunohistochemical staining. The samples were coded and clinical information was unavailable to the technicians performing the mRNA quantification. Total RNA was purified with TRIzol reagent (Invitrogen, Tokyo, Japan) according to the manufacturer's protocol. RNA concentrations were determined spectrophotometrically based on absorbance at 260 nm (Genequant; Amersham Biosciences Corp., 800 Centennial Ave, Piscataway, NJ08855 USA).

RT-PCR. Purified total RNA (1.0 μg) was denatured for 5 min at 70˚C and immediately cooled on ice. Reverse transcription was performed with the Reverse Transcription System (Promega KK, Tokyo, Japan) according to the manufacturer's protocol. Quantitative PCR was performed as reported elsewhere (13), with both survivin and GAPDH mRNA concentrations expressed in absolute copy numbers.

The survivin was amplified by the following set of primers: reverse: ggggacttc tcaggtcgtgt; forward: tggacttctt catgccccga. The survivin internal probe was ttgcggtgcgagtcgtgg aagtaa.

PCR amplification conditions were one cycle at 50˚C for 2 min, and 95˚C for 10 min, followed by 50 cycles at 95˚C for 15 sec and 60˚C for 1 min. The measured value was calculated by comparative Ct methods (13) and GAPDH gene amplification was used as the control. The amount of survivin mRNA was expressed as n-fold survivin mRNA levels relative to that of adjacent normal breast tissue. A tumor/normal ratio of survivin mRNA expression greater than 1 was identified as high expression, all and the others as low.

Table I. *Association between survivin expression and clinicopathological status.*

Characteristic	No. of cases	High survivin (%)	*p*
Age			
<50	21	9 (42.8)	0.7538
≥50	55	21 (38.2)	
Menopausal			
pre	15	5 (33.3)	0.6478
post	61	25 (40.9)	
Primary tumor (T)			
1	32	8 (25.0)	0.0104
2	33	11 (33.3)	
3	7	7 (100)	
4	4	4 (100)	
Nodal status			
negative	59	14 (23.7)	0.0001
positive	17	16 (94.1)	
Histological grade			
grade I	31	5 (16.1)	0.0001
grade II	27	9 (33.3)	
grade III	18	16 (88.8)	
Histological type			
papillo-tubular	16	5 (31.2)	0.63
solid-tubular	20	8 (40.0)	
scirrhous	27	14 (41.8)	
others	10	3 (30.0)	
Estrogen receptor			
negative	58	20 (34.5)	0.179
positive	18	10 (55.5)	
Progesterone receptor			
negative	52	18 (34.6)	0.2834
positive	24	12 (50.0)	
Operation			
BCS	18	11 (61.1)	0.179
mastectomy	58	9 (15.5)	

BCS: breast-conserving surgery.

Statistical analyses. Statistical analyses were carried out with STATVIEW software (V.5.0, 1998; Abacus Concepts Inc., Berkeley, CA, USA). The tumor and normal tissue variables were analyzed with Chi-square tests or Fisher's exact tests. Disease-free survival time (defined as the time from surgery until diagnosis of recurrent disease) and overall survival time (defined as the time between date of surgery and death by any cause) were used as follow-up end-points. The Cox proportional hazards model was used to assess the prognostic value of the survivin expression ratio (tumor/normal), in addition to other clinicopathological factors. Kaplan-Meier survival curves were generated and the equality of survival distributions was determined by log-rank testing. Two-sided *p*-values <0.05 were considered statistically significant.

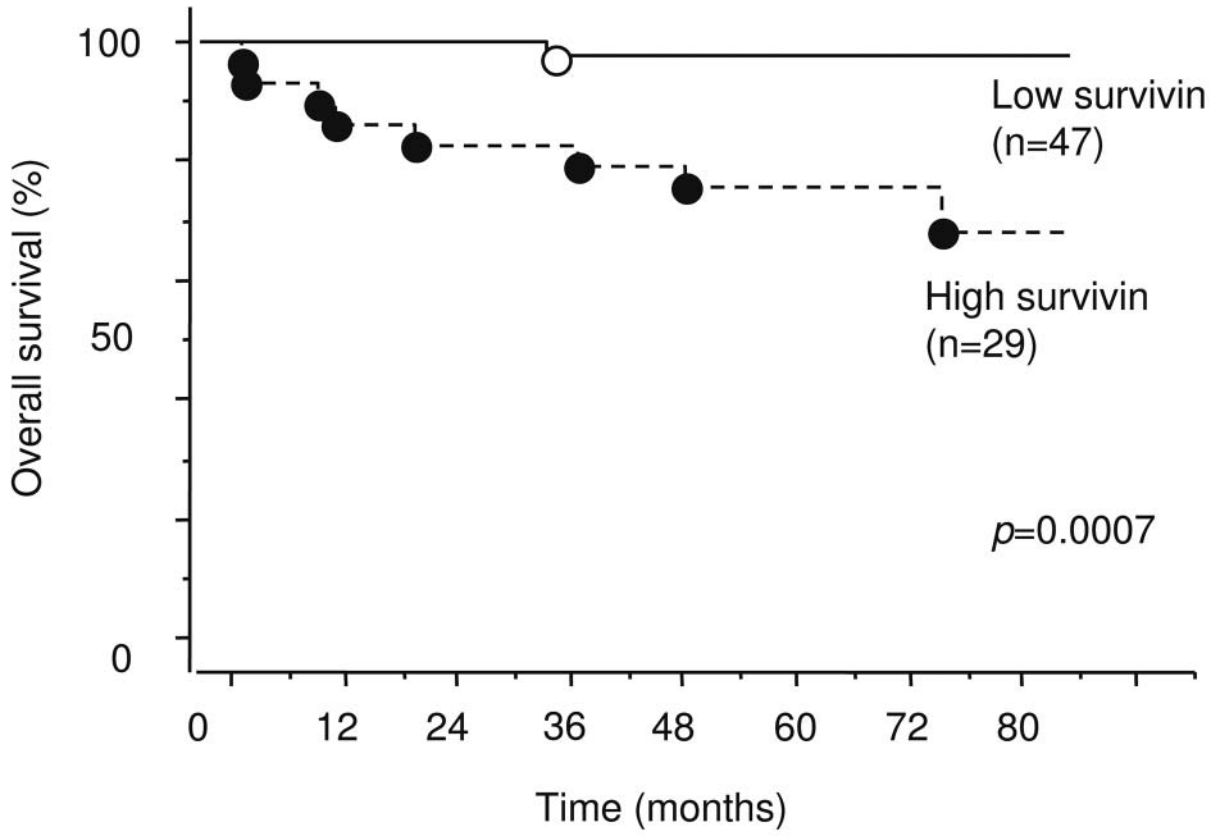

Figure 1. *Overall survival curves according to survivin gene expression. Differences are statistically significant (p=0.0007). Number of patients in each group: high, 47; low, 29.*

Results

Survivin expression and clinicopathologicalal factors. We investigated the proportion of high survivin mRNA ratios (tumor mRNA/normal tissue mRNA) in 76 breast cancer patients with other clinicopathological factors: age, menopausal status, nodal category, tumor histology, tumor size, histological grade, ER and PgR status, and type of operation (Table I). T factor (T1-T4) was significantly associated with a high survivin mRNA ratio, with higher T having a greater proportion of tumors with high survivin expression ($p=0.0104$). The survivin mRNA ratio was higher in node-positive than in negative tumors ($p=0.0001$), and grade III tumors had a significantly greater proportion of high survivin mRNA ratios than grade I or grade II tumors ($p=0.0001$); however, no association was found between survivin mRNA ratio and age, menopausal status, ER or PgR status, or type of operation.

Table II. *Multivariate Cox proportional hazard model.*

Characteristic	Risk rate	95% CI	*P*
T; primary tumor [T1-T4]	0.874	0.383-1.992	0.7485
N [negative *vs.* positive]	0.770	0.188-3.148	0.716
ER [positive *vs.* negative]	0.069	0.0015-30.904	0.3907
PgR [positive *vs.* negative]	0.001	0.001-206.173	0.8267
Stage	5.044	1.829-13.914	0.0018 *
Survivin [low *vs.* high]	0.568	0.025-13.033	0.7235

Prognostic factors. To investigate survivin expression and prognosis, Kaplan-Meier survival curves classifying tumors as high *vs.* low showed the most significant difference in overall survival ($p=0.0007$, log-rank test; Figure 1). When we evaluated whether survivin was associated with a poor prognosis, several factors were subsequently investigated in

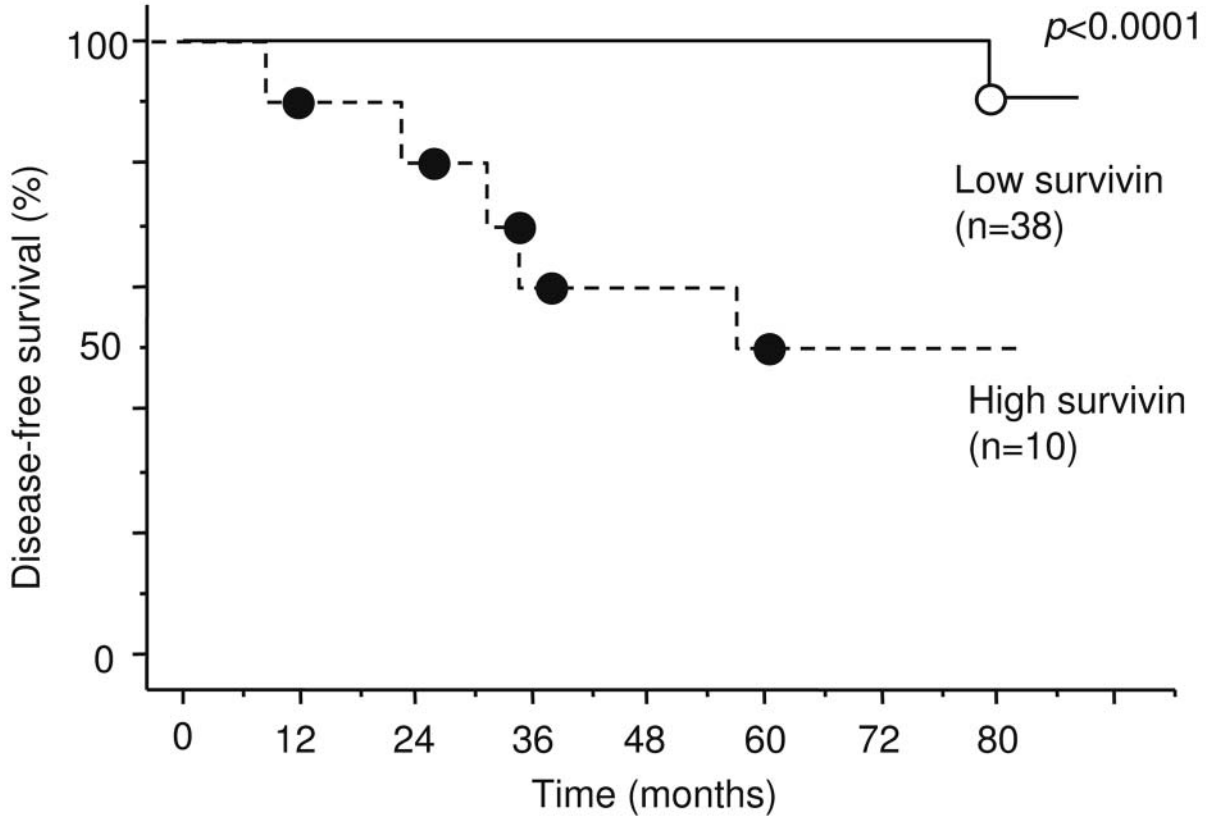

Figure 2. *Disease-free survival curves in T1-2N0 early-stage breast cancer patients according to survivin gene expression. Differences are statistically significant (p<0.0001). Number of patients in each group: high, 38; low, 10.*

univariate survival analyses. T factor (primary tumor, hazard ratio (HR): 2.814, 95% CI: 1.565-5.060, $p=0.0006$), nodal status (HR: 3.724; 95% CI: 2.085-6.653; $p<0.001$), ER (HR: 0.124; 95% CI: 0.031-0.0502; $p=0.0034$) and PgR status (HR: 0.195; 95% CI: 0.048-0.787; $p=0.0217$), stage (HR: 2.733; 95% CI: 1.853-4.030; $p<0.0001$) and survivin expression (HR: 0.068; 95% CI: 0.008-0.543; $p=0.0112$) were significantly powerful prognostic factors in univariate Cox regression analysis for overall survival; however, in multivariate analyses, we could find significance only in the clinical stage (HR: 5.044; 95% CI: 1.829-13.914: $p=0.0018$; Table II).

Prognostic factor of early-stage (T1 or T2N0M0) breast cancer. When we investigated the relationship between survivin expression and disease-free survival in early-stage, T1or T2 node-negative cancer patients, we found significant differences between the high and low expression groups ($p<0.0001$, log-rank test; Figure 2). Patients with low survivin expression showed better disease-free survival than patients with high survivin expression. Overall survival curves of patients in these stages are not shown because no disease-related deaths occurred in either group during the follow-up period.

Table III. *Multivariate Cox proportional hazard model (T1-2 node negative tumor).*

Characteristic	Risk rate	95% CI	*P*
T; primary tumor [T1-T2]	0.209	0.013-3.492	0.2761
Ly [negative *vs.* positive]	0.487	0.086-2.773	0.4174
V [positive *vs.* negative]	2.123	0.368-12.232	0.3995
Grade [II, III *vs.* I]	1.468	0.208-10.348	0.6997
ER [positive *vs.* negative]	0.414	0.022-7.658	0.5532
Survivin [low *vs.* high]	0.024	0.001-0.446	0.0123

Multivariate regression analysis. The independent relationship of survivin with disease-free survival was studied with Cox multivariate regression analysis. This analysis was performed including tumor size (T1, T2), Ly (lymphatic invasion), V (vascular invasion), nuclear grade, hormone receptor status, and survivin expression; only survivin expression showed significantly better disease-free survival of breast cancer patients with T1or T2 node-negative tumors (HR: 0.024; 95% CI: 0.001-0.446; $p=0.0123$; Table III).

Discussion

In this report, we studied survivin mRNA expression, as measured using quantitative RT-PCR, in the primary tumor and compared it with paired normal breast tissue, evaluating whether the expression of this gene is a possible independent predictive factor in human breast cancer. As previously reported, the quantitative TaqMan RT-PCR method is more sensitive than immuno-histochemical staining and most tumor cells express survivin mRNA (14). As we found a high expression of survivin mRNA in normal adjacent tissue in some cases, it is worth comparing the survivin expression of tumor cells with that of normal adjacent tissue in order to dichotomize the comparatively high or low expression. Therefore, if a tumor/normal ratio of survivin mRNA expression was higher than 1 or less than 1, it was identified as a high or low expression, respectively. We do not know why the expression level of normal adjacent tissue should be higher than that of tumors; however, one possibility is detection bias because of the greater sensitivity of quantitative RT-PCR.

As previously reported, our results for survivin expression being higher in node-positive than node-negative breast cancers led us to the previous findings (15, 16), similar to the relationships between the histological grade, tumor size and survivin expression (15); however, age, menopausal status, histological type, ER and PgR status, and operation procedure have not shown a relationship with survivin expression.

In previous reports, it has been shown that survivin relates to poor prognosis in a variety of cancers, for example, colorectal cancer (4), non-small cell lung cancer (5), hepatocellular carcinoma (6), esophageal cancer (7), rectal cancer (8) and bladder cancer (9); however, these results remain controversial (10, 11). The prediction of poor prognosis with survivin mRNA expression involves the function of survivin as an inhibitor of apoptosis.

At the beginning of this study, we investigated the possibility of a prognostic value for survivin at all stages of breast cancer as previously reported; initially it seemed that survivin expression would be such a possible prognostic marker. However, we failed to elucidate these properties in multivariate analyses because of several confounding factors. This might be due to the sensitivity of our methods compared to other studies which used immunohistochemical staining or qualitative RT-PCR. As some patients develop distant metastases even if the tumor stage is T1N0M0 or T2N0M0, a much more powerful predictive marker is essential to be identified to distinguish between favorable and unfavorable prognoses. Therefore, we focused on its potential as a predictor of early recurrence in the subgroup without nodal metastases and T1 or T2 stage. Subgroup analyses showed that survivin expression was the most powerful prognostic indicator for early recurrence of T1-2N0 tumors. It appears that the prognostic role of survivin expression might be more important for early-stage breast cancer, such as S100A4 (17). Barnes *et al.* (18) have reported that survivin expression may be an early event in the malignant process, and our findings support the hypothesis that it is not only an early event but is involved in the recurrence of early-stage breast cancer.

Several splice variants having different potentials have been reported in breast cancer tissues; Kennedy *et al.* (11) reported the survivin Ex3 splice variant. It might be assumed that nucleus-localized survivin is due to the survivin Ex3 splice variant, which has an anti-apoptotic function. This splice variant lacks the nuclear export signal, because of a frame shift induced by exon 3 skipping (2). Thus, the prognostic value of nucleus-localized survivin is not necessarily different from that of cytosolic survivin. Survivin 2B is localized in the cytoplasm, while this variant is probably an antagonist of survivin. In this study, we reported that survivin is related to the early recurrence of node-negative breast cancer, but the relationship with the other variants and prognosis is not clear. Further study should be performed to elucidate the functions of the several forms of survivin.

Conclusion, survivin mRNA, as measured with quantitative RT-PCR, in early-stage breast cancer is a strong and independent prognostic marker. Survivin mRNA concentrations in tumors can be dichotomized between different risk groups. Therefore, low-risk patients can avoid unnecessary treatment and high-risk patients can be offered more aggressive treatment, even for early-stage breast cancer. In the future, gene profiling including survivin will be an important tool for assessing prognosis and may lead us to a promising target for breast cancer therapy.

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