SPANDIDOS PUBLICATIONS

Intracellular localization of survivin determines biological behavior in colorectal cancer

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Abstract. Survivin is a bifunctional protein that suppresses apoptosis and regulates cell division and is highly expressed in various human cancers. Recently, the intracellular localization of survivin in tumors has been suggested as a prognostic marker, but the molecular mechanisms are not understood. The aims of the present study were to investigate the different localization of survivin expression in colorectal carcinoma and expression of survivin relationships with clinicopathological factors and patient survival. Immunohistochemical analyses of 142 cases of advanced colorectal cancer showed that 109 (76.8%) cases expressed survivin in the nucleus and 29 cases (20.4%) in the cytoplasm. Cytoplasmic survivin overexpression was associated with a poor prognosis, but nuclear survivin overexpression was associated with a better prognosis. Subcellular distribution of survivin in five cases of cancerous or surrounding normal tissues derived from fresh biopsy of non-fixed samples of colorectal cancer patients was further demonstrated by Western blotting. Survivin was primarily found in the insoluble fraction. Interestingly, regardless of survivin protein levels in the insoluble fraction, patients who had cancerous tissue expressing cytoplasmic and nuclear soluble survivin suffered from lymph nodes metastases. These data suggest that the function of cytoplasmic survivin might be important for malignant progress and the levels of cytoplasmic and nuclear soluble survivin might be more relevant for prognostic factors for colorectal cancer than the total amount of survivin.

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Introduction

Survivin is a member of the inhibitor of apoptosis protein (IAP) family (1) and plays an important role in the suppression of apoptosis by inhibiting the activity of caspase (2-4). Furthermore, it is also a subunit of the chromosomal passenger complex (CPC), which includes other subunits such as Aurora-B, INCENP (inner centromere protein) and Borealin to regulate cell division (5-8).

Survivin is unique in that it is expressed in fetal tissue and in a variety of human cancers (9-18), and several recent reports show evidence of survivin expression in specific adult tissues including colonic epithelium, normal endometrium, placenta, and bone marrow (19-22). On the other hand, intracellular localization of survivin in cancer cells has been reported to express biological features of cancer behavior. Survivin mRNA levels or cytoplasmic expression of the protein are associated with a poor outcome in various cancers (10-18). However, recent studies have reported opposing conclusions with regard to the significance and prognostic value of survivin nuclear expression (23-31). These findings suggest that differential localization of survivin may indicate different protein functions and affect patient prognosis. The aim of this study was to investigate the expression of survivin in a series of 142 colorectal carcinomas. In particular, we wished to examine the relationships between localization of survivin in the cytoplasm and the nucleus of the cancer cell and clinicopathological factors, and to clarify the biological meaning of its protein expression.

Materials and methods

Patients and tissue samples. A total of 142 advanced colorectal carcinomas (96 men and 46 women) including 71

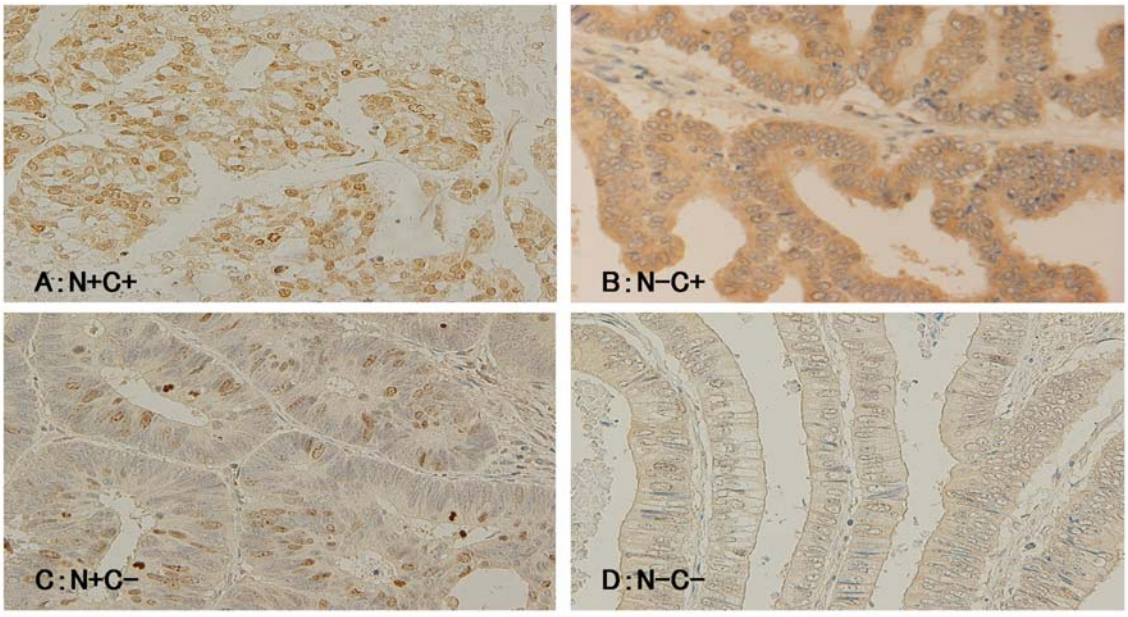


Figure 1. Survivin expression in colorectal cancer as shown by immunohistochemical staining. (A) Immunohistochemical staining of survivin (x200) shows both nuclear and cytoplasmic staining. (B) Immunohistochemical staining of survivin (x200) shows only cytoplasmic staining. (C) Immunohistochemical staining of survivin (x200) shows only nuclear staining. (D) Immunohistochemical staining of survivin (x200) shows neither nuclear nor cytoplasmic staining.

cases with lymph node metastasis and 25 cases with distant metastasis were obtained from the archive of Hiroshima University Hospital during 1984-2001 after surgical resection. The age of patients ranged from 37 to 84 years (mean, 63.8 years). Histologically, 52 cases were classified as well, 67 were moderately, and 23 were poorly differentiated colorectal carcinoma. For immunohistochemical examination, serial 4-μm sections were stained with hematoxylin and eosin and used for immunohistochemical analyses.

Immunohistochemical study and evaluation of staining. The sections were incubated with primary polyclonal anti-survivin antibody (NB500-201, Novus Biologicals, Littleton, CO, 1:1000) at 4˚C overnight after antigen retrieval by microwave treatment in citrate buffer (pH 6.0) and detection by the avidin-biotin peroxidase complex system using an ABC kit (Doko, Kyoto, Japan). The immunostaining was defined as positive when >20% of tumor cells were stained for survivin in the nuclei or the cytoplasm. The immunohistochemistry grade was defined as - to +++ according to the number of cells stained and to the intensity of the reaction to individual cells. Grades were defined as follows: -, at most no positive cells; +, 5-20% of tumor cells showed weak to moderate immunoreactivity; ++, 20-50% of tumor cells showed moderate immunoreactivity; +++, over 50% of tumor cells showed intense immunoreactivity. Cases with grade ++ and +++ were regarded as positive cases.

Statistical analysis. The Statcel software package was used for analysis. The χ^2 test and Fisher's test was used for comparison of data among groups. Survival analyses were conducted according to the Kaplan-Meier method and survival characteristics were compared using log-rank tests. A p-value <0.05 was considered to indicate statistical significance.

Subcellular fractionation. Nuclear and cytoplasmic extracts of cancerous or surrounding normal tissues derived from fresh biopsy non-fixed samples of colorectal cancer patients from Hiroshima Memorial Hospital were carefully prepared using a Nuclear Extraction Kit (Active Motif), according to the manufacturer's instructions. Insoluble fractions solubilized by SDS-sample buffer were also used. The fractionated protein samples were loaded onto an SDS-PAGE gel (20 μg/lane), and immunoblot analyses were performed. In order to evaluate the expression level of survivin in colorectal tissue protein samples by immunoblot analysis, we used three antibodies with different epitopes, monoclonal anti-survivin (1:1000, NB 500-237, Novus) antibody and rabbit polyclonal anti-survivin (1:1000, NB 500-201, Novus; 1:300, sc-10811, Santa Cruz) antibodies. Based on our unpublished experiments, we routinely used sc-10811 for immunoblot detection of human tissue-derived survivin. In order to examine the fractionation fidelity, monoclonal anti-α-tubulin (1:500, CLT-9002, Cedarlane Laboratories), anti-Lamin A/C (1:1000, sc-7292, Santa Cruz), rabbit polyclonal anti-NFκB (1:200, sc-372, Santa Cruz), and anti-phosphorylated histone H3 (32) antibodies were used.

Results

Nuclear (N+, N-) and cytoplasmic (C+, C-) survivin expression and clinicopathological factors in colorectal cancer. We examined the expression of survivin in 142 cases of colorectal cancer cases by immunohistochemistry. Survivin expression was observed in nuclei and/or cytoplasm of colorectal cancer cells (Fig. 1). Nuclear survivin expression was detected in 109 cases (Table I) and cytoplasmic survivin expression was detected in 29 cases (Table II). We examined the correlation between survivin nuclear or cytoplasmic expression and clinicopathological factors. Nuclear survivin

Table I. Correlation between expression of nuclear survivin and clinicopathological factors in colorectal cancer.

	Nuclear survivin expression				
Total	N+ 109	(%) 76.8	N- 33	(%) 23.2	p-value
Tumor size (mm)					
≥50	48	44	14	42.4	
<50	61	56	19	57.6	
Histological differentiation					
Poor	22	20.2	4	12.1	
Well/Moderate	87	79.8	29	87.9	
Lymph node metastasis					
Negative	59	54.1	12	36.4	
Positive	50	45.9	21	63.6	
Distant metastasis					
Negative	96	88.1	21	63.6	<0.01
Positive	13	11.9	12	36.4	
Tumor stage					
B, C	93	85.3	19	57.6	<0.01
D	16	14.7	14	42.4	

Table II. Correlation between expression of cytoplasmic survivin and clinicopathological factors in colorectal cancer.

	Cytoplasmic survivin expression				
Total	C- 113	(%) 79.6	C+ 29	(%) 20.4	p-value
Tumor size (mm)					
≥50	44	38.9	18	62.1	<0.05
<50	69	61.1	11	37.9	
Histological differentiation					
Poor	20	17.7	6	20.7	
Well/Moderate	93	82.3	23	79.3	
Lymph node metastasis					
Negative	61	54	10	34.5	
Positive	52	46	19	65.5	
Distant metastasis					
Negative	96	85	21	72.4	
Positive	17	15	8	27.6	
Tumor stage					
B, C	93	82.3	19	65.5	<0.05
D	20	17.7	10	34.5	

negative cases were significantly increased compared with positive cases in primary colorectal cancer with distant metastasis ($p<0.01$), stage D ($p<0.01$, Table I). Cytoplasmic survivin positive cases were significantly increased in stage D ($p<0.05$) compared with negative cases, and the tumor size was also larger in cytoplasmic positive cases than in negative cases (Table II).

Survival analysis. We used a Kaplan-Meier method to examine the survival rate of 142 patients who showed expression of cytoplasmic and nuclear survivin (Fig. 2). The 5-year survival rate of nuclear positive cases (N+) was higher than that of nuclear negative cases (N-) ($p=0.002$, Fig. 2A), and the 5-year survival rate of cytoplasmic positive cases (C+) was lower than that of cytoplasmic negative cases (C-)

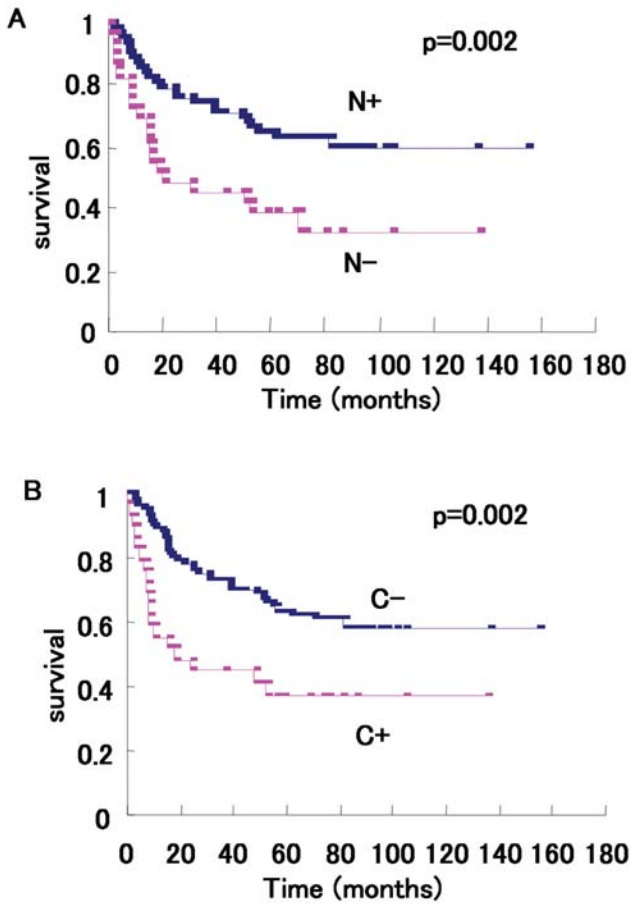


Figure 2. Kaplan-Meier survival curves of patients with colorectal cancer according to survivin expression. (A) N+ vs. N- 5-year survival was 63.9% vs. 38.5% (p=0.002). (B) C+ vs C- 5-year survival was 37.2% vs 63.4% (p=0.0019). These data showed that nuclear survivin controls death and cytoplasmic survivin promotes death.

(p=0.0019, Fig. 2B). In conclusion, nuclear survivin-positive cases showed a good prognosis, but cytoplasmic survivin-positive cases showed a poor prognosis.

Subcellular fractionated survivin is found in the cytoplasmic and nuclear soluble fraction in metastatic cancerous tissues. We examined the subcellular distribution of survivin in cancerous or surrounding normal tissues derived from fresh biopsy non-fixed samples of colorectal cancer patients. The cellular proteins were fractionated and the subcellular distribution of survivin was examined by immunoblot analysis. The fidelity of fractionation was evaluated by immunoblot analysis using antibodies against α-tubulin, NFκB, Lamin A/C, and Ser10-phosphorylated histone H3. α-tubulin was fractionated into a cytoplasmic soluble fraction (Fig. 3A). NFκB, a transcription factor, was primarily found within the cytoplasmic and nuclear soluble fractions (Fig. 3A). Lamin A/C and Ser10-phosphorylated histone H3 were found only in the insoluble fraction (Fig. 3A). Survivin in both normal and cancerous tissues was primarily found in the insoluble fraction (Fig. 3B). Survivin was up-regulated in cancerous tissue in four patients. In one patient, there was no significant up-regulation of survivin compared with normal tissue from the same patient. Interestingly, regardless of survivin protein levels in the insoluble fraction, patients who had cancerous tissue expressing cytoplasmic and nuclear soluble survivin suffered from lymph nodes and other metastases (Fig. 3B).

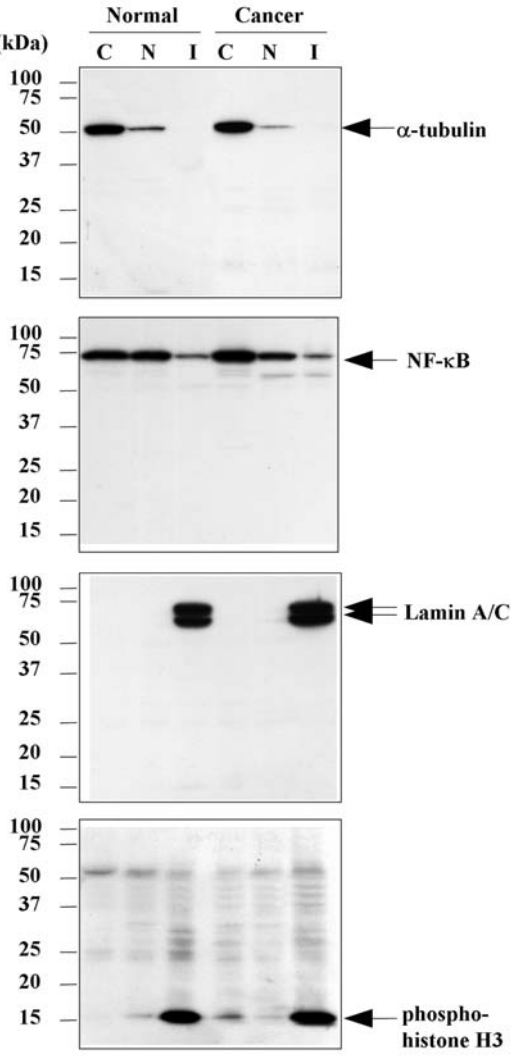
Discussion

Survivin mRNA levels or cytoplasmic expression protein are associated with a poor outcome in various cancers, including breast cancer (10), lymphoma (11), non-small cell lung cancer (12), liver cancer (13), gastric carcinoma (14), ovarian carcinoma (18), and colorectal cancer (15-17). In this study, we also found that overexpression of cytoplasmic survivin showed a poor prognosis with a high frequency of stage-D. Cytoplasmic survivin has cytoprotective activity, and is associated with microtubules and mitochondrion, which can interfere with the function of caspases (2-4,33). In our study, we also found that survivin was highly expressed in the nucleus in colorectal cancer cases. In particular, we showed that the prognosis of colorectal cancer was dependent on the difference in survivin expression between the nucleus and the cytoplasm of the cancer cell. The cases with nuclear survivin expression demonstrated a better prognosis and a lower incidence of distant metastasis and stage-D. Our findings suggest that nuclear and cytoplasmic localization of survivin may have different protein functions, which can affect patient outcome (28).

A previous study has shown that cytoplasmic and nuclear survivin are independently modulated during cell cycle progression and only cytoplasmic survivin is associated with p34cdc2 and is phosphorylated on Thr34 (34). Phosphorylation by p34cdc2 may increase the affinity of survivin for active caspase-9 and apoptosis inhibition (35). If nuclear survivin is not associated with p34cdc2, it may induce apoptosis.

Furthermore, the expression of nuclear survivin in gastric cancer (23), breast carcinomas (24), osteosarcoma (25), transitional cell carcinoma of urinary bladder (26), pancreas cancer (27), and non-small-cell cancer (28), is correlated with a good prognosis. These previous reports are consistent with the results from our study. However, in hepatocellular carcinoma, esophageal squamous cell carcinoma, and in epithelial ovarian tumors, the expression of nuclear survivin is correlated with an unfavorable prognosis (29-31). The reason for these different prognostic results of different subcellular locations of survivin in different cancers is unclear. Nuclear survivin may have a different role in epithelial cancer and adenocarcinoma.

We also examined the expression of survivin in the fraction of cancerous or surrounding normal tissues derived from fresh biopsy non-fixed samples of colorectal cancer patients by immunoblot analysis. This fraction included the nuclear and cytoplasmic soluble and insoluble fraction (primarily nuclear matrix). We found that survivin was up-regulated in cancerous tissue compared with normal tissues and was primarily found in the insoluble fraction in both normal and cancerous tissues. Interestingly, the expression of cytoplasmic and nuclear soluble survivin was found in the cancerous tissue of lymph nodes patients. These findings suggest that the different localization of survivin may have different patient outcomes.



Survivin is a nuclear shuttling protein and has nuclear export signals, and its subcellular distribution is regulated by active import into the nucleus and CRM1-mediated export to the cytoplasm. This suggests that active transport between the nucleus and cytoplasm may constitute an important regulatory mechanism for survivin function (36). In dividing cells, the CRM1-dependent nuclear export signal is essential for tethering survivin and survivin/Aurora-B kinase complex to the mitotic machinery and is essential for proper cell division (37). Nuclear export seems to be required for the cytoprotective activity of survivin, but nuclear accumulation inhibits cytoprotective function (37). These results suggest that: i) cytoplasmic soluble survivin is likely to have a cyto-protective function; ii) cytoplasmic soluble survivin is probably active because Crm1-dependent nuclear transportation is essential for survivin function as both a chromosome passenger and anti-apoptotic protector; and iii) the levels of cytoplasmic and nuclear soluble survivin might be more relevant for prognostic factors for colorectal cancer than the total amount of survivin.

Overall, these data suggest that the biological behavior of colorectal cancer may differ according to both the cellular expression level and the intracellular localization of survivin. The function of cytoplasmic survivin might be important in relation to malignant progress and the levels of cytoplasmic and nuclear soluble survivin might be more relevant for prognostic factors for colorectal cancer than the total amount of survivin. We believe that the inhibition of cytoplasmic localization of survivin could be a new means of suppressing survivin function in cancer therapy.

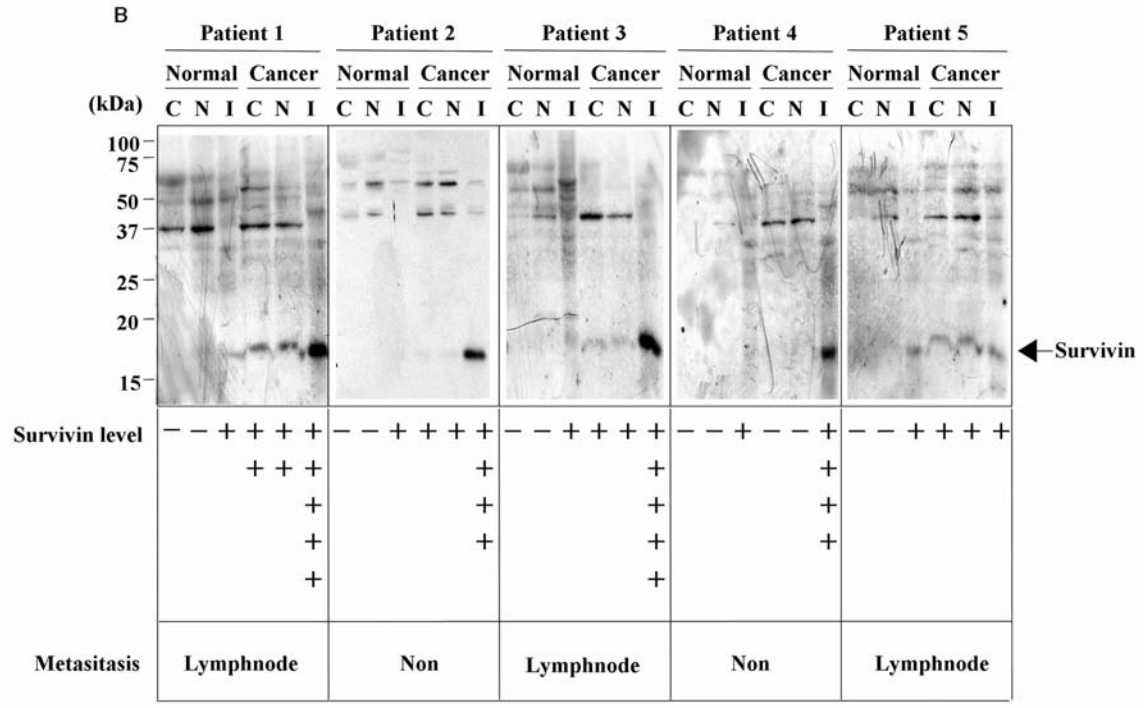


Figure 3. Subcellular fractionated survivin is found in the cytoplasmic and nuclear soluble fraction in metastatic cancerous tissues. (A) The fidelity of fractionation was evaluated by immunoblot analysis using antibodies against α-tubulin, NFκB, Lamin A/C, and Ser10-phosphorylated histone H3. (B) The expression level of survivin in the nuclear and cytoplasmic soluble and insoluble fraction of cancerous or surrounding normal tissues derived from fresh biopsy samples of colorectal cancer patients was obtained by immunoblot analysis.

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