

Neuronal Apoptosis Inhibitory Protein is Overexpressed in Patients with Unfavorable Prognostic Factors in Breast Cancer

Neuronal apoptosis inhibitory protein (NAIP) is a recently identified inhibitor of apoptosis protein. However, the clinical relevance of NAIP expression is not completely understood. In an attempt to determine the clinical relevance of NAIP expression in breast cancer, the levels of NAIP and survivin expression were measured in 117 breast cancer samples and 10 normal breast tissues using quantitative reverse-transcriptase-polymerase chain reaction. While there was no evidence of NAIP expression in the normal breast tissue, NAIP was expressed in all breast cancer samples. The level of NAIP expression in breast cancer was significantly higher (257 times) than in the universal tumor control. There was a strong correlation between the level of NAIP expression and the level of survivin expression ($p=0.001$). The level of NAIP expression in patients with a large tumor ($\geq T2$) and patients with an unfavorable histology (nuclear grade III) was significantly higher than in those patients with a small tumor (T1) and patients with a favorable histology (nuclear grade I, II) ($p=0.026$ and $p=0.050$, respectively). Although the level of NAIP expression was higher in patients with other unfavorable prognostic factors, it was not significant. The three-year relapse-free survival rate was not significantly the patients showing high NAIP expression and patients showing low NAIP expression ($86.47 \pm 4.79\%$ vs. $78.74 \pm 6.57\%$). Further studies should include the expressions of NAIP in a larger number of patients and for a longer period of follow-up to evaluate correlation with metastasis and treatment outcome. In conclusion, NAIP is overexpressed in breast cancer patients with unfavorable clinical features such as stage and tumor size, suggesting that NAIP would play a role in the disease manifestation.

Key Words : Breast Cancer; Neuronal Apoptosis Inhibitory Protein (NAIP); Apoptosis; Prognostic Factor; Clinical Relevance

Jaewon Choi, Yu Kyeong Hwang*,
Young Jin Choi[†], Ki Eun Yoo,
Jeong Han Kim[†], Seok Jin Nam[†],
Jung Hyun Yang[†], Sang Jin Lee[‡],
Keon Hee Yoo, Ki Woong Sung,
Hong Hoe Koo, Young-Hyuck Im[§]

Department of Pediatrics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul; Division of Immunotherapy*, Mogram Biotechnology Research Institute, Yongin; Division of Breast and Endocrine Surgery, Department of Surgery[†], Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul; Genitourinary Cancer Branch[†], National Cancer Center, Ilsan; Division of Hematology-Oncology, Department of Medicine[§], Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

Jaewon Choi and Yu Kyeong Hwang equally contributed to this work.

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Address for correspondence

Seok Jin Nam, M.D.
Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, Korea
Tel : +82.2-3410-3478, Fax : +82.2-3410-0040
E-mail : sjnam@smc.samsung.co.kr

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INTRODUCTION

Apoptosis is an active mechanism that leads to cell death. Tight regulation is essential to ensure a delicate balance between life and death, and the loss of apoptosis might proceed to a wide variety of diseases. Cancer also involves cellular defects that halt apoptosis in its development and progression of cancer (1). Hence, many studies have demonstrated the role of different apoptosis regulators in rendering tumor cells resistant to apoptosis both in vitro and in vivo. The upregulation of anti-apoptotic proteins would certainly assist the survival of tumor cells (2-5). Various anti-apoptotic proteins are expressed in different tumors, and their expression may be related with unfavorable prognostic features at diagnosis (6-14) and poor treatment responses (14-18).

Over the last decade, a complex network of pro- and anti-

apoptotic proteins that govern the tight regulation of the apoptosis pathways have been revealed (19-22). Among anti-apoptotic proteins, a group of proteins, known as the inhibitor of apoptosis protein (IAP), are the only cellular factors that act both on the initiator and effector caspases (23-26). To date, eight human IAPs have been identified: NAIP, cIAP1, cIAP2, XIAP, survivin, apollon, ILP-2, and livin (27). As their name implies, the IAP family proteins can inhibit the apoptosis induced by a variety of stimuli. Therefore, the overexpression of various IAPs is regarded as an unfavorable factor in various malignancies. However, in breast cancer, the clinical relevance of IAP overexpression has not been evaluated with the exception of survivin. The overexpression of survivin in breast cancer is associated with the presence of unfavorable prognostic factors at diagnosis and a poor clinical outcome.

Neuronal apoptosis inhibitory protein (NAIP), which is a member of the IAPs, is expressed in mammalian cells and inhibits the apoptosis induced by a variety of signals. This gene is homologous to two baculovirus IAPs (28). NAIP has been linked to the inherited disease, spinal muscular atrophy (SMA), which occurs in children and manifests as a degeneration of the motor neurons (29). NAIP may play a role in the mechanisms of resistance of tumor cells to various chemotherapeutic agents. Moreover, the strong expression of IAPs, particularly Survivin and NAIP, is observed in the bone marrow of AML (30, 31). However, little is known about the clinical relevance of NAIP expression in breast cancer.

In this context, this study examined the clinical relevance of NAIP expression in breast cancer using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The result showed that overexpression of NAIP was associated with the unfavorable clinical features of breast cancer.

MATERIALS AND METHODS

Patients and clinical evaluations

One hundred and seventeen patients, who were newly diagnosed as breast cancer at Samsung Medical Center from August 2003 to December 2004, were enrolled in this study. All the patients were diagnosed with a breast carcinoma preoperatively by radiological findings and tissue biopsy, and they did not receive any form of treatment prior to surgery. All the patients underwent a potentially curative resection, and the tumor specimens were sent to a pathologist for an evaluation of various prognostic factors including histological subtype, lymph node involvement, expression of estrogen receptor (ER), and progesterone receptor (PR). A bone scan was performed routinely for an evaluation of the stage. The Institutional Review Board in the Samsung Medical Center approved this study, and informed consent was obtained from the patients or guardians.

RNA isolation and cDNA synthesis

The tumor tissue specimens were taken from the periphery of the tumor mass resected in the operating room and stored at -70°C in a RNAlater reagent (Ambion, Austin, U.S.A.). The tumor tissue specimens were homogenized using a rotor-stator homogenizer, DIAX 900 (Heidoph, Schwabach, Germany). The total RNA was extracted using a Qiamp kit (Qiagen, Chatsworth, U.S.A.) according to the manufacturer's protocol. After treatment with DNA-free[®] (Ambion) to remove the chromosomal DNA, the complementary DNA (cDNA) was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, U.S.A.) with oligo (dT) 15-mer primer and stored at -20°C until use.

Quantitative Real-time RT-PCR

The mRNA expression levels of the IAPs and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by quantitative RT-PCR using the ABI PRISM 7000 sequence detector system (Applied Biosystems, Foster City, CA, U.S.A.). The real-time PCR amplification was carried out using the pre-developed assay-on-demand gene expression set for the NAIP gene (Hs00244967_m1, GeneBank accession number NM_004536, Applied Biosystems), Survivin gene (Hs00153353_m1, GeneBank accession number NM_001168, Applied Biosystems), and TaqMan[®] GAPDH Control Reagents (Applied Biosystems) for the GAPDH gene in combination with the TaqMan[®] Universal PCR Master Mix (Applied Biosystems).

All the reactions were performed in triplicate using a 20 μL sample containing 50 ng of cDNA. The reaction protocol involved heating at 50°C for 2 min and then at 95°C for 10 min, followed by 40 cycles of amplification cycles (15 sec at 95°C and 1 min at 60°C). The analysis was performed using ABI PRISM 7000 Sequence Detection software (Applied Biosystems). The expression level of the IAP genes in the unknown samples was calculated as the ratios of IAP versus GAPDH. The IAP and GAPDH mRNA levels were quantified using a standard curves made from known serial dilution of Universal Human Reference RNA (Invitrogen, Carlsbad, U.S.A.). The standard curves were generated by assuming a linear relationship between the first cycle number, at which the fluorescence signal increased significantly (Ct value), and the logarithm of the starting quantity. A negative control without the template was included in each experiment.

Statistical analysis

The differences in the level of IAP expression with respect to the established clinicopathological prognostic factors and treatment outcome (occurrence of a relapse) were analyzed using a Mann-Whitney U test. The Spearman's rank correlation test was used to assess the gene co-expression patterns of the NAIP and survivin in breast cancer tissues. The patients were categorized into two groups according to the NAIP expression levels (\geq median or $<$ median). The relapse-free survival rates (RFS) in each group were estimated using the Kaplan-Meier method and compared using the log-rank test. p values <0.05 were considered significant.

RESULTS

Patient characteristics

One hundred and seventeen patients were enrolled in this study, and their clinical characteristics are listed in Table 1.

The median age was 59 yr (range 24-76). Thirteen patients (11.1%) were younger than 35 yr old. Ductal type was the most common histological subtype (77.8%). The tumor size was larger than T1 in 91 patients (77.8%). A lymph node metastasis was present in 57 patients (48.7%). The stage was higher than IIa in 52 patients (44.4%). The nuclear grade

Table 1. Relationship between levels of *NAIP* and *survivin* expression and the clinicopathological prognostic factors at diagnosis

	No.	(%)	NAIP		Survivin	
			Median	<i>p</i> value	Median	<i>p</i> value
Age						
≥35	104	(88.9)	216.0		0.805	
<35	13	(11.1)	324.0	0.485	2.621	0.004*
Tumor size						
T1	26	(22.2)	102.0		0.704	
T2-4	91	(77.8)	319.0	0.026*	0.890	0.657
Node involvement						
Negative	60	(51.3)	202.0		1.084	
Positive	57	(48.7)	264.5	0.604	0.654	0.030*
Stage						
≤IIa	64	(54.7)	190.5		0.974	
≥IIb	52	(44.4)	287.0	0.532	0.654	0.057
Nuclear grade						
I, II	40	(34.2)	198.5		0.381	
III	74	(63.2)	306.0	0.050	0.975	0.033*
Histologic grade						
I, II	43	(36.7)	324.0		0.574	
III	58	(49.6)	221.0	0.768	1.225	0.003*
ER expression						
Positive	55	(47.0)	257.0		0.735	
Negative	62	(53.0)	244.5	0.692	1.060	0.026*
PR expression						
Positive	46	(39.3)	211.5		0.805	
Negative	71	(60.7)	314.0	0.253	0.944	0.234

Differences in expression of NAIP and survivin according to established clinicopathological prognostic factors were analyzed using a Mann-Whitney U test. The expression levels are presented as median values. *p* values of <0.05 were considered significant.

was III in 74 patients (63.2%) and the histological grade was III in 58 patients (49.6%). There were 62 (53.0%) and 71 (60.7%) patients with ER- and PR-positive tumors, respectively.

Expression levels of NAIP were very high in breast cancer

While there was no evidence of NAIP expression in the normal breast tissue, NAIP was expressed in all the breast cancer samples. The level of NAIP expression in breast cancer was significantly higher than in universal tumor control. Fig. 1 shows the relative levels of NAIP and survivin expression compared with the universal tumor cell control. While the median levels of survivin expression were 0.8 times that of the control, the median level of NAIP expression was very high (257 times that of the control) (Fig. 1A). In addition, the level of NAIP expression was strongly correlated with that of survivin (*p*<0.001, Fig. 1B).

NAIP expression was associated with the presence of unfavorable prognostic factors

Table 1 and Fig. 2 show the level of NAIP expression with respect to the prognostic factors. The level of NAIP expression in patients with a large tumor (≥T2), and an unfavorable histology (nuclear grade III) was significantly higher than in the patients with a small tumor (T1) and a favorable histology (nuclear grade I, II) (*p*=0.026 and *p*=0.050, respectively). Although the level of NAIP expression was higher in patients with the other unfavorable prognostic factors (age <35 yr, positive node involvement, ≥IIb stage, and negative PR expression), it was not significant.

In most cases, survivin overexpression was associated with the presence of unfavorable prognostic factors. However, for the lymph node metastasis, and stage, the level of survivin overexpression was higher in early staged disease than in advanced disease (*p*=0.030 and 0.057, respectively).

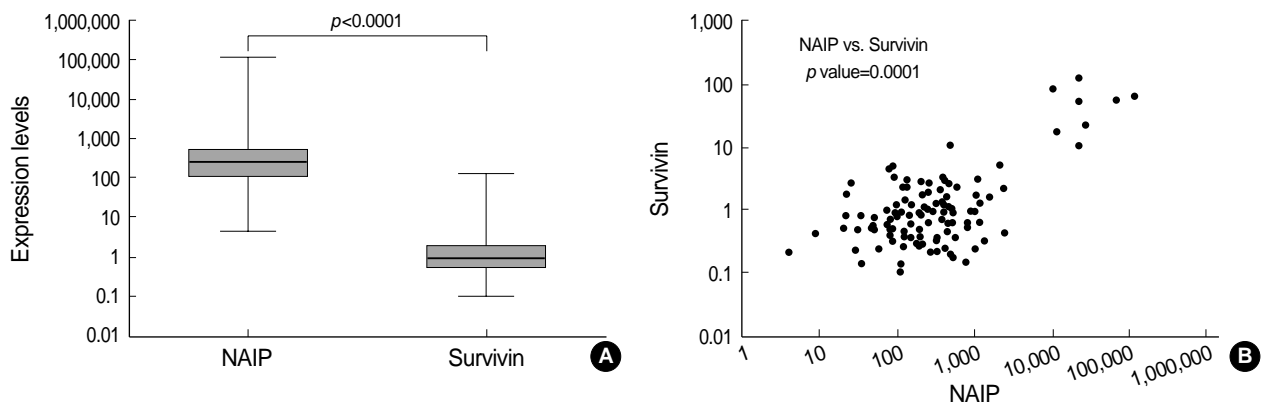


Fig. 1. Relative expression levels of NAIP and survivin mRNA compared with universal human reference RNA. The level of NAIP expression was 257 times higher than the control, whereas the median level of survivin expression was 0.8 times that of the control (A). The correlations between NAIP and survivin expression in each patient were analyzed by a Spearman's rank correlation test. Positive correlation was observed between NAIP and survivin (B, *p*=0.0001).

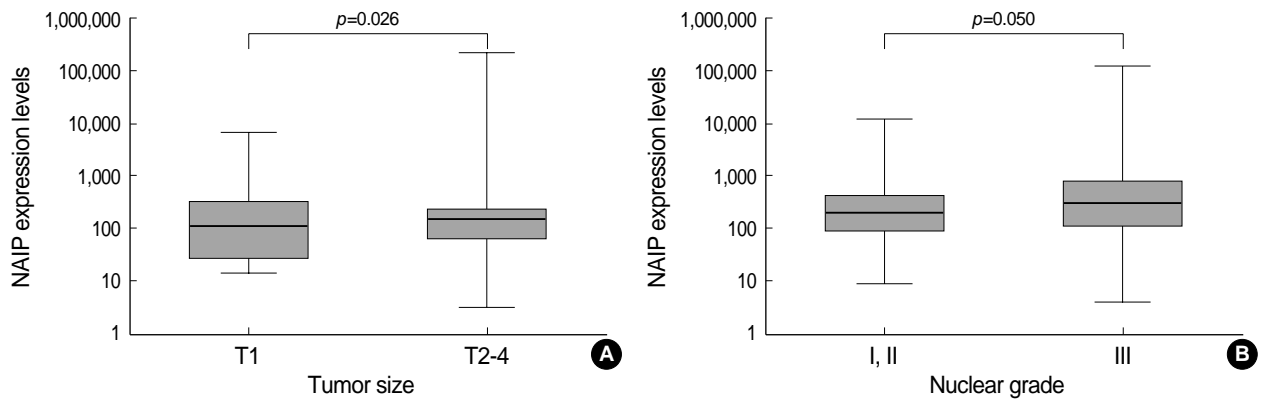


Fig. 2. Levels of NAIP expression according to clinicopathologic prognostic factors. NAIP overexpression was correlated with the presence of unfavorable prognostic factors, T2 (A) and nuclear grade III (B).

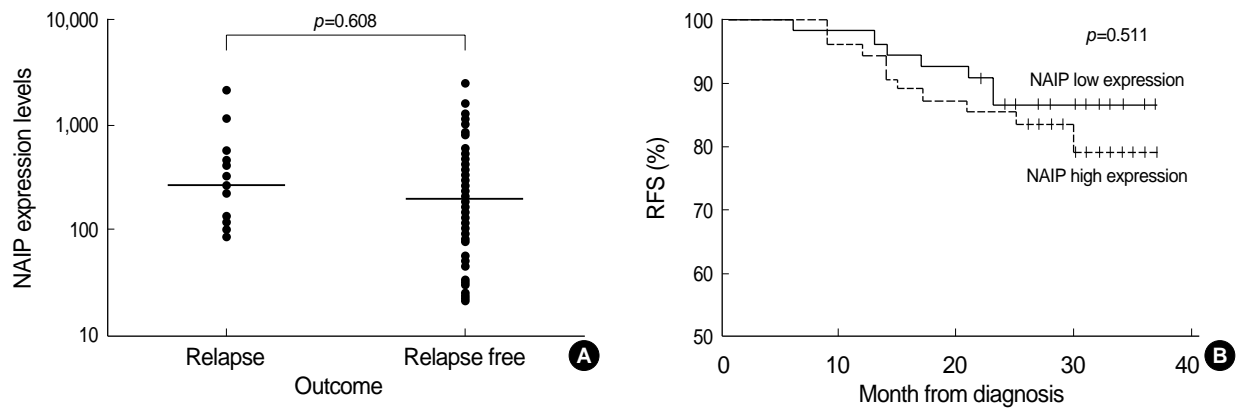


Fig. 3. Expression of NAIP was slightly higher in relapsed patients than in relapse-free patients (266 vs. 202 folds of control, $p=0.608$). (A) The relapse-free survival rate also was slightly higher in patients with a high level of NAIP expression ($86.47 \pm 4.79\%$) than in patients with a low level of NAIP expression ($78.74 \pm 6.57\%$) (B). This three-year relapse-free survival according to NAIP expression did not show a statistical significance ($p=0.511$).

NAIP expression was high in the patients with a poorer treatment outcome

The median follow-up duration was 28 months (range, 1-75). The tumor relapsed in 16 patients, and treatment-related mortality occurred in 6 patients. The 3-yr overall survival (OS) and RFS rates (\pm SE) were $82.2 \pm 7.0\%$ and $76.0 \pm 6.8\%$, respectively.

Higher levels of NAIP expression were found to be associated with a less favorable treatment outcome, but this was not significant. The median levels of NAIP expression in the relapsed patients ($n=16$) and relapse-free patients ($n=84$) were 266 and 202, respectively ($p=0.608$, Fig. 3A). Similarly, the 3-yr RFS rate was lower in the patients showing NAIP overexpression (\geq median) than in those not showing NAIP overexpression ($78.74 \pm 6.57\%$ vs. $86.47 \pm 4.79\%$, $p=0.511$, Fig. 3B). Survivin overexpression was not associated with an unfavorable treatment outcome in this study (data not shown).

DISCUSSION

While the expression of various IAPs and their prognostic significance has been examined in different cancers (6-18), survivin is the only IAP that has been evaluated for its expression and clinical relevance in breast cancer to date (32-35). To the best of our knowledge, there are no reports of the expression of the other IAPs other than survivin in breast cancer tissues. This study is the first to evaluate the level of NAIP expression in breast cancer using quantitative RT-PCR in an attempt to determine a possible association with the established clinicopathologic prognostic factors.

While NAIP was not expressed in the normal breast tissue but was expressed at high levels in breast cancer compared with the universal tumor control. This suggests that quantitative RT-PCR for NAIP can be used to find a minimal tumor in the regional lymph node or bone marrow. Because RT-PCR is more sensitive than either immunohistochemistry or a conventional pathologic examination, quantitative RT-PCR for NAIP might be valuable in detecting minimal disease if NAIP expression is still not detected in

the further experiment on a large number of normal breast, lymph node and bone marrow tissues.

Expression of survivin and other IAPs can be measured by immunohistochemistry (IHC) using antibodies, conventional RT-PCR, and quantitative RT-PCR. Although IHC appear to be a more specific method for detecting biologically and clinically significant cancer micrometastases in histologically normal specimen in some cancers, RT-PCR appears to be a more sensitive method maintaining a reasonable specificity (36-39). The IHC method has some advantages in detecting specific antigen protein including histological observations while RT-PCR guarantees high sensitivity and quantitative analysis in a total amount of RNA specimen. However, RNA expression itself does not reflect the protein expression exactly. Therefore, complementary use of two methods is recommended.

While survivin overexpression is known to be strongly associated with the unfavorable clinical features and RFS rate in breast cancer (32-34), survivin overexpression was not significantly correlated with RFS rate in this study. The NAIP expression level was strongly correlated with survivin overexpression, however, poorer treatment outcomes were not significantly correlated with NAIP overexpression. We assume that a small number of patients and a relatively short follow-up duration might have resulted in an insignificant correlation between NAIP expressions and clinical outcome.

Interestingly, survivin expression was inversely correlated with the disease extent (lymph node metastasis and stage), while NAIP expression was not significantly associated with the disease extent. These results are partly similar with those reported by Span et al. (33) in that overexpression of survivin was correlated with unfavorable prognostic factors (young age, unfavorable histologic grade, and negative ER expression). However, unlike those studies, our study showed that overexpression of survivin was correlated with negative node involvement and less advanced stage. Three splicing variants of survivin mRNA were detected in breast cancer tissue, and levels of both survivin-2B and survivin-DeltaEx3 but not survivin were significantly higher in nodal metastasis than primary carcinomas (40). Similarly the overexpression of other IAPs showed strong correlations with negative lymph node metastasis and less advanced stage in our study (data not shown).

The IAP family proteins inhibit apoptosis induced by a variety of stimuli, and therefore, their overexpression is expected to be associated with the unfavorable clinical features in a variety of malignancies including AML. However, the clinical significance of IAP overexpression in acute leukemia is not completely consistent with what was expected from previous in vitro studies. For example, IAP overexpression was not always associated with the unfavorable clinical features in acute leukemia (26). Furthermore, it was recently reported that the high expression of Livin, also a member of IAP family proteins, is an independent favorable prognostic fac-

tor in childhood ALL (27). This suggests that the role of IAP in leukemogenesis or in the maintenance of leukemic cells might be different from what has been previously recognized. We assume that a complex network of pro- and anti-apoptotic proteins might have a pivotal role in the controlling apoptosis pathways and its cellular factors.

To the best of our knowledge, there have been no reports on the expression and clinical relevance of IAPs other than survivin in breast cancer. This study is the first to show an association between NAIP overexpression and the unfavorable clinical features in breast cancer even though there was no significant association between NAIP overexpression and an unfavorable treatment outcome. There were a small number of patients and a relatively short follow-up duration in this study, which might have confounded the results. Therefore, a further study on more patients and for a longer follow-up duration will be needed to elucidate the association between NAIP overexpression and the treatment outcome.

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