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Nuclear Survivin in pN2 Non-small Cell Lung Cancer : Prognostic and Clinical Implications

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Running Head : Nuclear Survivin in pN2-NSCLC

Key words : Non-small cell lung cancer, nuclear, pathological N2, prognosis, survivin

Abbreviations: CT, computed tomography; EBUS-TBNA, endobronchial ultrasound guided transbronchial needle aspiration; IAP, inhibitor of apoptosis; IHC, immunohistochemistry; KDa, Kilo Dalton; LI, labelling index; LN, lymph node; MRI, magnetic resonance imaging; NSCLC, non-small cell lung cancer.

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Abstract

Patients with N2 non-small cell lung cancer (N2-NSCLC) represent heterogeneous groups. Survivin is a member of the inhibitor of apoptosis (IAP) family. If N2-NSCLC patients could be stratified, based on survivin expression and/or its relation to cell cycle proteins, into homogeneous subgroups, certain therapies could be selected for those patients.

Survivin expression in 78 surgically-resected primary pN2-NSCLC tumors, was evaluated using immunohistochemistry. Relationships of survivin expression to overall survival, clinical features, and 6 cell cycle-related proteins' expressions (pRb, cyclin D1, $p16^{INK4A}$, p53, $p21^{Waf1}$, ki-67) were analyzed.

Nuclear survivin and the number of mediastinal lymph node (LN) stations were independent prognostic factors. Patients' group with combined negative survivin/single mediastinal LN station were the most favorable prognostic group, and was related to the clinical nodal factor. Indeed, patients with negative survivin/low Ki-67 labelling indices had the best survival, especially in non-squamous histopathology.

We conclude that, nuclear survivin is strongly related to LN metastasis and proliferative potentials in pN2-NSCLC patients. Preoperative N2-NSCLC patients with combined negative nuclear survivin and single mediastinal LN station, or low proliferative indices, particularly in clinical N0-1 disease and non-squamous histopathology, respectively, are expected to have a favorable postoperative prognosis and may be candidates for primary resection.

Introduction

Lung cancer is the most common cause of cancer-related mortality. Although surgical therapy remains the primary treatment for resectable disease, the composite 5-year survival is only 16% [1]. In particular, patients with stage IIIA-N2 non-small cell lung cancer (NSCLC) represent incredibly heterogeneous groups, with regards to both prognosis and treatment [2,3]. Attempts to define homogeneous subgroups of N2-NSCLC patients, have identified some prognostic factors [3,4].

Neverthless, if we could identify, molecularly-based, factors that can be used as criteria for deciding whether to conduct surgery in patients with clinical N2-NSCLC, we could improve the oucome of these patients [4].

Survivin is a 16.5 KDa protein that is overexpressed in almost all malignancies, but rarely detected in normal differentiated adult tissues [5]. Functionally, survivin has been shown to inhibit apoptosis, regulate cell division and enhance angiogenesis [5,6]. Deletion of survivin resulted in a catastrophic defect of microtubule assembly, with absence of mitotic spindles, disorganized tubulin aggregates and multinucleation, in the survivin knock-out mice [7]. Nucleocytoplasmic localization of survivin in tumor cells, determined by immunohistochemistry, has been reported to provide prognostic information in several types of cancer [8]. However, the prognostic significance of nuclear survivin expression remains controversial in different tumor types [9], including NSCLC [10,11].

In our previous work [4], we could classify pN2 NSCLC patients into homogeneous prognostic subgroups, based on the expression of 6 cell cycle-related markers (pRb, cyclin D1, $p16^{INK4A}$, p53, $p21^{Waf1}$, ki-67). In the current study, we evaluated immunohistochemically, the expressions of both nuclear and cytoplasmic survivin in pN2-NSCLC patients as well as their relations to overall survival and clinical features. Moreover, we searched for possible relation(s) between survivin and

cell cycle proteins expressions in N2-NSCLC patients. We hypothesized that N2-NSCLC patients could be stratified, based on survivin expression and/or its relation to cell cycle proteins, into homogeneous subgroups, for whom certain therapeutic strategies could be selected.

Materials and Methods

Study design

To accomplish the aims of the current study, an immunohistochemical (IHC) analysis for survivin expression in primary surgically-resected specimens of patients with pN2-NSCLC, was performed. Then we studied the prognostic implications of survivin expression with regards to overall survival and clinical features of those patients. Lastly, we searched for possible relation (s) between survivin expression and that of 6 cell cycle-related proteins [4] in 61/78 patients.

Patients and tissue samples

Archived tissue blocks from 1990-1996, of surgically-resected primary tumors with histologically-proven N2-NSCLC (pN2-NSCLC) were retrieved from the files of the Department of Thoracic Surgery, Chiba University, Chiba, Japan. The study comprised 78 patients who had undergone surgical resection with curative intent. They were 58 men and 20 women with a mean age of 62.8 years. Histopathological diagnosis was carried out according to the World Health Organization specifications [12]. The tumors included 51 adenocarcinomas, 23 squamous cell carcinomas, and 4 large cell carcinomas. All patients received neither induction chemotherapy nor preoperative radiotherapy. Patients were excluded if they had metastatic NSCLC, a prior history of metastatic malignancy, or neuroendocrine tumors. Sixty-two patients underwent lobectomy, 14 patients underwent pneumonectomy, and 2 patients underwent segmentectomy. Deaths caused by operative complications were excluded. Primary tumors were staged as T1, T2, and T3 in 20, 38, and 20 patients, respectively.

Preoperative staging included a computed tomography (CT) scan of the chest, a CT scan of the abdomen, fiber-optic bronchoscopy, brain magnetic resonance imaging (MRI), and bone scintigraphy for all patients. Abdominal ultrasound was obtained only from patients who had an abnormality on their abdominal CT. Mediastinal lymph nodes (LNs) with a short-axis dimension

≥1 cm on a CT scan were considered abnormal. All patients underwent mediastinal LN dissection at the time of surgery. Complete resection was defined as resection of all macroscopic tumor with the resection margins free of tumor on microscopic analysis. All patients had positive ipsilateral mediastinal LNs according to histologic examination (pN2). The tumors were classified according to the Classification of Lung Cancer of the Japan Lung Cancer Society [13]. That classification scheme is identical to that of the International Union Against Cancer. Some patients had clinically positive hilar (cN1) and mediastinal (cN2) lymph nodes, as defined by LNs that measured ≥1 cm on a CT scan before surgery. 38 of 78 patients had clinical N2 (cN2) disease identified, and at the time of surgery, thirty-one of 78 patients had multiple-station enlargement of mediastinal (N2) lymph nodes identified. All patients were followed up for 10 years, and the mean survival was 3.46 years. Fiftty-five of 78 patients died, and deaths were attributable to cancer in 44 of 55 (80.0%) patients. The study was approved by our Institutional Review Board.

Immunohistochemistry

We performed IHC analysis of the specimens for the expression of survivin (nuclear and cytoplasmic) in 78 pN2-NSCLC patients. In addition, 61 out of 78 patients were evaluated for the expression of 6 cell cycle-related proteins (pRb, cyclin D1, $p16^{INK4A}$, p53, $p21^{Waf1}$, Ki-67), as shown in our previous work [4]. For survivin immunostaining, the IHC assay was carried out on 10% formalin-fixed, paraffin-embedded tissue sections that were cut to a thickness of 3 μm or 4 μm thickness and mounted on glass slides. All sections then were dewaxed in xylene, rehydrated through a graded alcohol series, and washed in Tris Buffered Saline with Tween 20; TBST (DakoCytomation, Carpinteria, CA). This buffer was used for all subsequent washes and for dilution of the antibodies. Antigen retrieval was achieved via heating in an autoclave at 121ºC for 15 minutes, after immersion of the tissue slides into Target Retrieval Solution (DakoCytomation, Carpinteria, CA). Then, all tissue sections were processed with the DAKO Catalyzed Signal

Amplification (CSA) System, Peroxidase (CSA System, HRP) [code K1500; DakoCytomation, Carpinteria, CA]. Mouse monoclonal antibody (DakoCytomation, Carpinteria, CA) raised against full-length recombinant survivin was used at dilutions of 1:400. The primary antibody was incubated overnight at 4ºC. 3,3′-diaminobenzidine was used as the final chromogen, and hematoxylin was used as the nuclear counterstain. Positive tissue controls were included in each experiment and consisted of tissues that previously had stained specifically for the target antigen after exposure to primary antibody. Each slide was examined independently by two observers (S.M. and K.H.) without knowledge of the patients' clinical data. Cytoplasmic survivin immunoreactivity was evaluated semiquantitatively based on the intensity of staining [11] and was scored as weak (+1), moderate (+2), and intense (+3). In tumors with heterogeneous immunostaining, the predominant pattern was considered for scoring. Specimens with no or weak staining were considered negative, whereas those with moderate and intense staining were considered positive [11]. For nuclear survivin, positive imunoreactivity was considered when more than 10% of tumor nuclei were positively stained [14].

Statistical Analysis

The associations between IHC parameters, as well as IHC/clinico-pathological ones, were analysed by the use of chi-square test and/or Fisher exact test. The IHC paramaers were; survivin (nuclear and cytoplasmic), and cell cycle-related proteins [4]. The clinico-pathological patients' parameters included; age, gender, histopathologic type, pathologic tumor (pT) status, clinical nodal (cN) status, and the number of mediastinal LN stations involvement. A univariate survival analysis of each prognostic variable was used to estimate overall survival according to the Kaplan-Meier method [15]. The prognostic variables for overall survival included the above-mentioned clinico-pathological parameters, as well as nuclear and cytoplasmic surviving. Overall survival was calculated from the date of surgery to the date of either death or the last follow-up. The terminal

event was death attributable to cancer or non-cancer causes. The significance of the differences in survival distribution among prognostic groups was evaluated with the log-rank test. A Cox proportional-hazards model was applied to the multivariate survival analysis [16]. Statistical analysis was carried out using the SPSS statistical software program package (SPSS version 12.0 for Windows, SPSS Inc., Chicago. IL). The criterion of significance chosen was $P < 0.05$, and all tests were two-tailed.

Results

Nuclear and cytoplasmic survivin immunoreactivity in pN2-NSCLCs

Survivin immunoreactivity was detected in 68 of 78 tumors examined (87.2%).[Fig1] Nuclear immunoreactivity was observed in 47/78 cases (60.3%) [Fig 1,A]. Cytoplasmic immunoreactivity was detected in 48 of 78 tumors (61.5%), with an intensity that was usually homogeneous and uniform within each case. [Fig 1,B] Indeed, twenty-seven tumors (34.6%) showed both cytoplasmic and nuclear immunoreactivity, and in ten cases (12.8%) neither cytoplasmic nor nuclear immunostaining was observed. Interestingly, there was no significant relation between nuclear and cytoplasmic survivin expressions (p=0.367).

Clinico-pathlogic features and survivin expression in relation to overall survival

The 5-year survival rate for the included patients was 25.6%. Univariate survival analysis revealed that only the number of mediastinal LN stations and nuclear survivin expression significantly influenced survival. [Table 1]

Table 1: Clinico-pathological features and survivin expression in relation to overall survival (Univariate analysis)†

Characteristic	Patients No. (Total =78)	Median survival (Months)	*P**
Mean age			
< 62.8 years	38	40.83	0.061
> 62.8 years	40	22.57	
Gender			
Female	20	37.77	0.103
Male	58	24.20	
Histopathology			
Non-squamous	55	33.50	0.563
Squamous	23	25.77	
pT status			
T1-2	58	31.07	0.815
T3	20	25.77	
cN status			
N0-1	40	37.77	0.224
N2	38	22.50	
MLN stations			
Single	47	41.13	0.004
Multiple	31	22.57	
Nuclear survivin			
Negative	31	45.27	0.006
Positive	47	19.23	
Cytoplasmic survivin			
Negative	30	31.07	0.846
Positive	48	26.47	

† <, less than; >, more than; pT, pathological tumor; cN, clinical nodal; MLN, mediastinal lymph nodes; Non-squamous, adenocarcinoma + large cell carcinoma ; squamous, squamous cell carcinoma
*P** Log-rank test

Patients with multiple and single mediastinal nodal stations had a median survival time of 22.57 and 41.13 months, respectively (p=0.004). [Fig 2] Moreover, patients with nuclear survivin overexpression had poorer survival than those with negative expression, with a median survival of 19.23 and 45.27 months, respectively (p=0.006). [Fig 3] According to the results of univariate analysis; multivariate analysis was performed and indicated that the number of mediastinal LN

stations and nuclear survivin expression were the independent prognostic factors in this series of pN2-NSCLCs. The calculated relative risk of death for patients with multiple nodal stations was 2.232 (95% confidence interval, 0.260-0.772; p=0.004), and that for patients with nuclear survivin overexpression was 2.208 (95% confidence interval, 0.257-0.797; p=0.006). [Table 2]

Table 2: Multivariate Cox Regression Analysis of overall survival in pN2-NSCLC patients[†]

Characteristic	RR of death	95% confidence interval	*P*
MLN stations			
Single	1	-	0.004
Multiple	2.232	0.260 – 0.772	
Nuclear survivin			
Negative	1	-	0.006
Positive	2.208	0.257 – 0.797	

[†] MLN, mediastinal lymph nodes; RR, relative risk

Immunohistochemical-clinical combinations

Based on the results of multivariate survival analysis, we divided the patients with pN2-NSCLC into 4 groups. These groups were; group (A) survivin negative/single station (n=19); group (B) survivin negative/multiple stations (n=12); group (C) survivin positive/single station (n=28); and group (D) survivin positive/multiple stations (n=19).

Survival analysis of these 4 combinations revealed that group (A) patients had the most favorable overall survival; a median survival time of 61.23 months, compared to that of group (D) patients, with a median survival time of 14.57 months (p=0.0008). Morerover, when we examined for possible relation(s) between these combinations and the clinico-pathologic patients' features, we

found an interesting relation with the clinical nodal (cN) status; where 84.2%, 16/19 patients in group (A) had cN0-1 stage, whereas 3/19, 15.8% patients had cN2 (p=0.040). [Table 3]

Table 3: Nuclear survivin/Mediastinal LN stations combinations in relation to survival and clinical nodal status

Group	Patients No. (Total=78)	Median survivial (Months)	*P**	Clinical N status cN0-1	cN2	*P*†
(A) Survivin – /Single station	19	61.23	**0.0008**	16	3	**0.040**
(B) Survivin – /Multiple stations	12	34.80		4	8	
(C) Survivin + /Single station	28	20.73		11	17	
(D) Survivin + /Multiple stations	19	14.57		9	10	

*Log-rank test †Chi-square test

Relationship between survivin and cell cycle proteins

We searched for the possible relation(s) between survivin and cell cycle proteins (6) among 61 out of 78 patients with pN2-NSCLC. Among this panel of 6 cell cycle-related markers, only Ki-67 LI was significantly related to nuclear survivin expression. Twenty-eight of thirty-six (77.8%) cases with high Ki-67 labelling indices had concurrent survivin overexpression, compared with 8/36, 22.2% of cases with high KI-67 indices and negative survivin expression, respectively (p=0.003). On the contrary, we did not report a significant relation between cytoplasmic survivin expression and that of any of the 6 cell cycle markers. Also, based on this survivin/Ki-67 association, we divided pN2-NSCLC patients into 4 prognostic groups, as follows: group (1) survivin negative/low Ki-67 LI (n=15); group (2) survivin negative/high Ki-67 LI (n=8); group (3) survivin positive/low Ki-67 LI (n=10); and group (4) survivin positive/high Ki-67 LI (n=28). Again, survival analysis of these combinations revealed that group (1) patients had the most

favorable overall survival, compared to that of group (4) patients, with median survival times of 50.53 and 14.17 months, respectively (p=0.009). Finally, we found a significant association between these combinations and the histopathologic types; where 86.7%, 13/15; and 13.3%, 2/15 patients in group (1) had non-squamous, and squamous histopathology, respectively (p=0.027). [Table 4]

Table 4: Nuclear survivin/Ki-67 LI combinations in relation to survival and histopathology

Group	Patients No. (Total=61)	Median survivial (Months)	*P**	Histopathology‡ Non-squamous	Squamous	*P*†
(1) Survivin – /Low Ki-67 LI	15	50.53	**0.009**	13	2	**0.027**
(2) Survivin – /High Ki-67 LI	8	29.03		4	4	
(3) Survivin + /Low Ki-67 LI	10	37.13		9	1	
(4) Survivin + /High Ki-67 LI	28	14.17		15	13	

*Log-rank test † Fisher's exact test
‡ Non-squamous, adenocarcinoma+large cell carcinoma; Squamous, squamous cell carcinoma

Discussion

The current study results had revealed that nuclear survivin, as well as the number of affected mediastinal LN stations are independent prognostic factors in patients with N2-NSCLC. Moreover, nuclear survivin and its relations to the nodal factor and proliferative activity in those patients might help their selection into certain therapeutic strategies.

The burden of disease, hence the prognosis, in stage IIIA-N2 NSCLC varies from microscopic, single-station, mediastinal nodal involvement to bulky, multistation, fixed, mediastinal nodal disease [2,3]. While there is consensus to treat patients with bulky-N2 in the same group as locally advanced IIIB disease, and to treat with primary surgical resection patients with incidental or minimal N2 involvement, still there is no agreement about the best approach to patients with ipsilateral mediastinal LN metastasis diagnosed preoperatively, though considered technically potentially respectable [17]. Furthermore, studies comparing surgical intervention alone with preoperative chemotherapy followed by surgical resection, for patients with clinically evident N2 disease, have shown conflicting results [18]. Molecularly-based stratification of N2 NSCLC patients into homogeneous subgroups can help selection of those patients who might benefit from certain therapeutic strategies, thus improving their outcome [4]. The rationale for investigating survivin as a prognostic marker in malignancy is based on its ability to inhibit apoptosis, promote proliferation and enhance angiogenesis [5,6,8]. Because of its involvement in these processes, survivin is likely to be causally involved in tumor progression and consequently, increased levels would be expected to predict aggressive disease [5]. Indeed, several reports have shown, that high tumor levels of survivin are associated with adverse outcome in patients with different types of cancer [5,6], including NSCLC [10,11].

Our results revealed that the majority of tumor samples (87.2%) have shown survivin immunoreactivity, and survival analysis could reveal important relevances. Univariate survival

analysis has shown that only the number of affected mediastinal LN stations and nuclear survivin affected survival. Moreover, multivariate analysis revealed that those two factors were independent prognostic factors for N2-NSCLC patients. The so-called N2-bulky (multi-station) disease is a well known factor to be associated with an inverse prognostic outcome in N2-NSCLC patients [2,3].

Because of the large difference in expression between cancer and corresponding normal tissue, and being a multifunctional protein that play vital roles in various cancer-related aspects, it is not surprising that survivin expression had prognostic significance in various cancer types [5]. Our results confirm this prognostic significance of survivin in NSCLC [5,11]. On the other hand, the prognostic relevances of the subcellular pools of survivin have been a matter of debate in many cancers [9], including NSCLC [10,11]. These controversies could be explained, in immunohistochemical studies, on the bases of using antibodies of different specificities or concentrations, different cut-off points employed, and different approaches for storing and processing tissues [5,9]. With this regards, our data revealed that nuclear survivin can be utilized as a prognostic marker for N2-NSCLCs. On the contrary, these data cofirm that cytoplasmic survivin expression had neither biological nor clinical value in our N2-NSCLCs series. Combining these two prognostic factors, we could stratify pN2-NSCLC patients into prognostic subgroups. Patients with combined single station mediastinal LN involvement and negative nuclear survivin had the best overall survival. Indeed, these combinations were significantly related to the clinical nodal status of pN2-NSCLC patients. These results are consistent with those reported that survivin was a marker of LN metastasis [19,20]. The link between survivin expression and the potential for LN metastasis could be explained in two ways. First, survivin is being an apoptosis inhibitor, the proportion of cancerous cells in a tissue; that would otherwise be removed by apoptosis; increases with continued growth and increased potential for invasion and metastasis [19]. Second, survivin is being related to microvessel density and enhanced angiogenesis [6], both of which are strongly associated with high

potential of LN metastasis. Taking into consideration that metastasis to the ipsilateral mediastinal LNs (N2 disease) is the most important prognostic factor in completely resectable NSCLC [2,21], together with this relevance of survivin to LN metastasis, our results could have important implications for preoperative planning of N2-NSCLC patients. Thus, patients with combined single station mediastinal LN/negative nuclear survivin; especially those with clinical N0-1 stage; are expected to have favorable postoperative prognosis; and could be candidates for primary resection.

Finding a possible relationship between survivin expression and that of the cell cycle-related proteins, would be of interest. Therefore, we examined for such relationship among 61/78 patients with pN2-NSCLC. Among a panel of 6 cell cycle-related markers [4], nuclear survivin was related only to Ki-67 (proliferative) labeling indices. This finding is in agreement with many studies [5-7, 22] reported a strong link between survivin expression and increased tumor proliferative activivity. Survivin has been implicated in a dual role connecting suppression of apoptosis to regulation of chromosomal segregation and cell division [8]. Targeting experiments using antisense survivin or dominant-negative mutants resulted in spontaneous apoptosis, increased caspase activity, and inhibition of cell proliferation [23]. The protective anti-apoptotic effect of survivin in proliferating malignant cells may be a mechanism to stabilize tumor cells with chromosomal abnormalities favoring the survival of these cells and the progression of the tumors [7]. Even more, Ikeguchi *et al*, concluded that survivin gene expression may control cell proliferation rather than apoptosis, in esophageal cancer [22]. Remarkably, our data support the fact that, in genetically normal proliferating cells, Ki-67 and survivin should be completely linearly related on the basis that Ki-67 is present during 75% of the length of the S-phase and survivin only during a 2-3 hours period of G2-M [24]. However, although Ki-67 in not overexpressed as an oncogene, it could be that surviving is, thus justifying a concomitant study of both factors. Should uncoupling of both Ki-67 and survivin be considered as an aberrant proliferation; needs to be further evaluated.

Similarly, we could group N2-NSCLC patients into prognostic subsets, according to nuclear survivin/Ki-67 labeling indices combinations. Patients' group with concurrent low proliferative indices and negative nuclear survivin had the best overall survival. The demonstration that survivin-negative and low Ki-67 LI patients had the best survival might be expected; but is adequately demonstrated in the present data. Interestingly, 86.7% of this group had non-squamous histopathology. Both survivin overexpression and high Ki-67 labelling indices, were related to squamous histopathology in esophageal cancer [25]and NSCLC [26], respectively. Thus, N2-NSCLC patients with concurrent negative nuclear survivin/low Ki-67 indices, particularly those with non-squamous histopathology, are expected to have favorable postoperative prognosis, and could be candidates for primary resection.

Again, the clinical utility of our results can be highlighted by the use of the real-time endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA), as a minimally-invasive and accurate tool for preoperative staging of NSCLC patients [27]. Therefore, we expect the clinical usefulness of preoperative evaluation of N2-NSCLC patients, using survivin and/or Ki-67 expression, in EBUS-TBNA obtained mediastinal nodal tissue samples [28,29]. Indeed, from the therapeutic point of view, survivin is being a promising marker for anti-cancer therapy [5, 30], it is anticipated to be of value for molecular-targeted therapy of N2-NSCLC in the near future. This study could have one limitation, that is being retrospective. Therefore, further prospective studies evaluating molecular markers in N2-NSCLC are warranted.

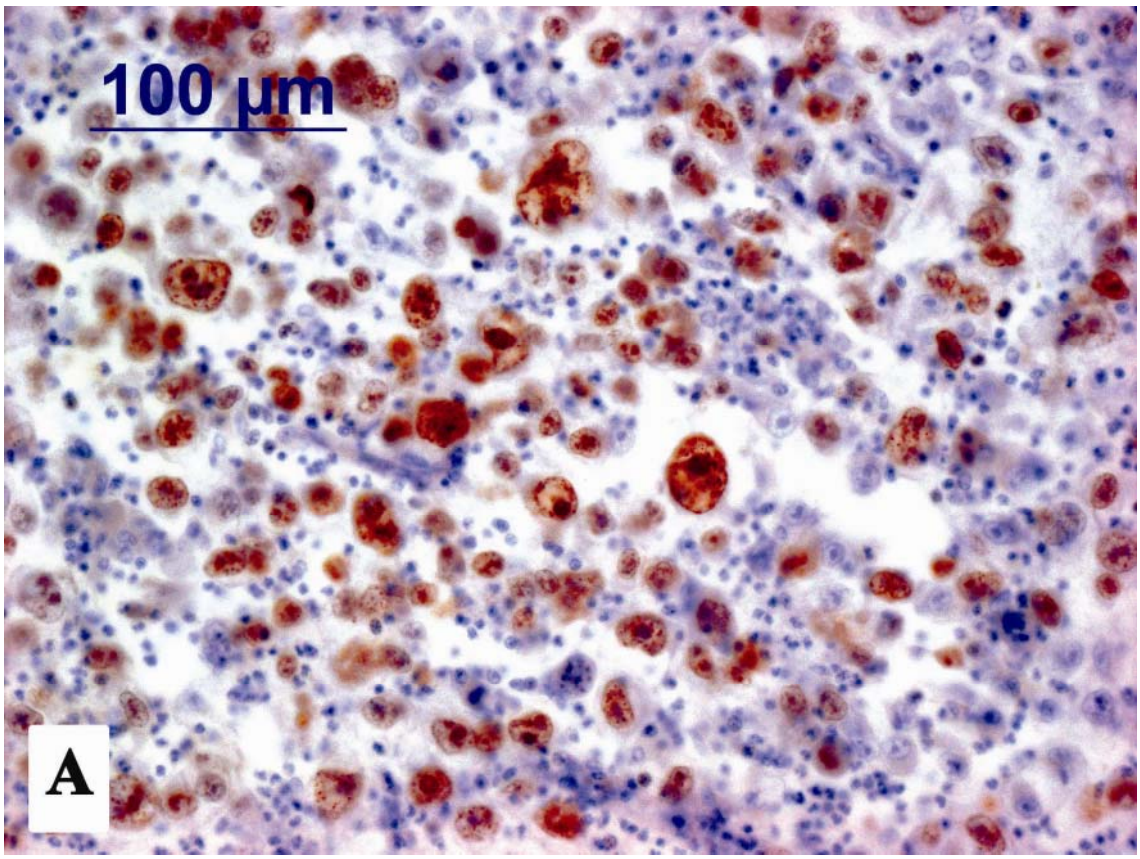
Conclusion

Our results suggest that nuclear survivin and number of affected mediastinal LN stations are two independent prognostic factors in pN2-NSCLC. Nuclear survivin is strongly related to LN metastasis and proliferative potentials in N2-NSCLC patients. Preoperative N2-NSCLC patients with combined negative nuclear survivin and single mediastinal LN station, or low proliferative

indices, particularly in clinical N0-1 disease and non-squamous histopathology, respectively, are expected to have a favorable postoperative prognosis and may be candidates for primary resection.

Figure legends

Figure 1 : Immunoreactivity patterns of survivin expression in a representative adenocarcinoma specimen of pN2-NSCLC, showing (A) Positively stained nuclei of neoplastic cells (nuclear survivin) (B) Intense cytoplasmic staining of malignant cells (cytoplasmic survivin). [Both figures have a 100µ internal scale]



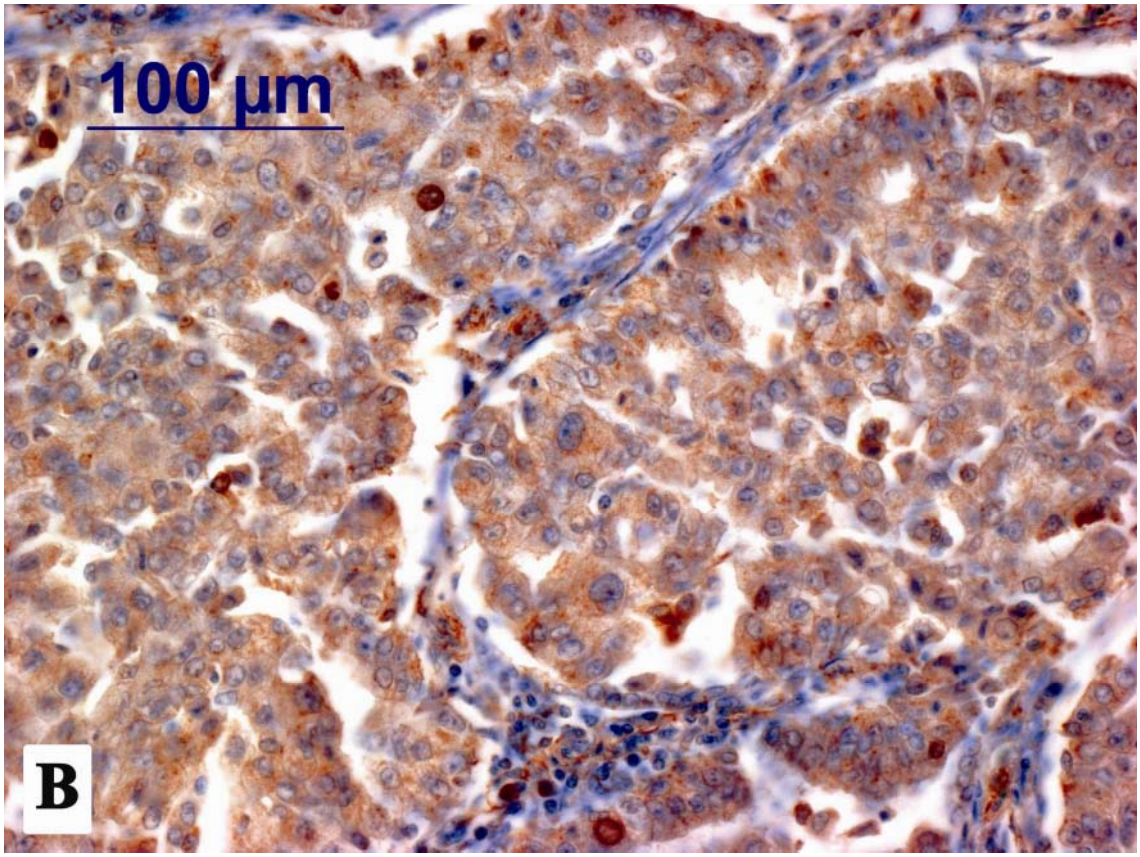


Figure 2 : Kaplan-Meier survival curve in patients with pN2-NSCLC shows that multi-station mediastinal lymph node (MLN) involvement is a bad prognostic factor.

Figure 2

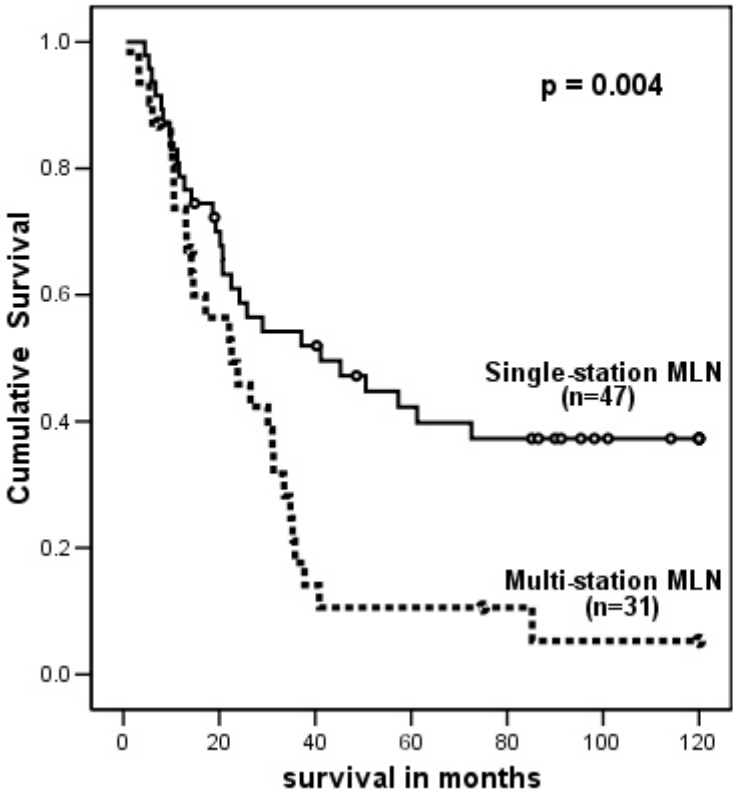
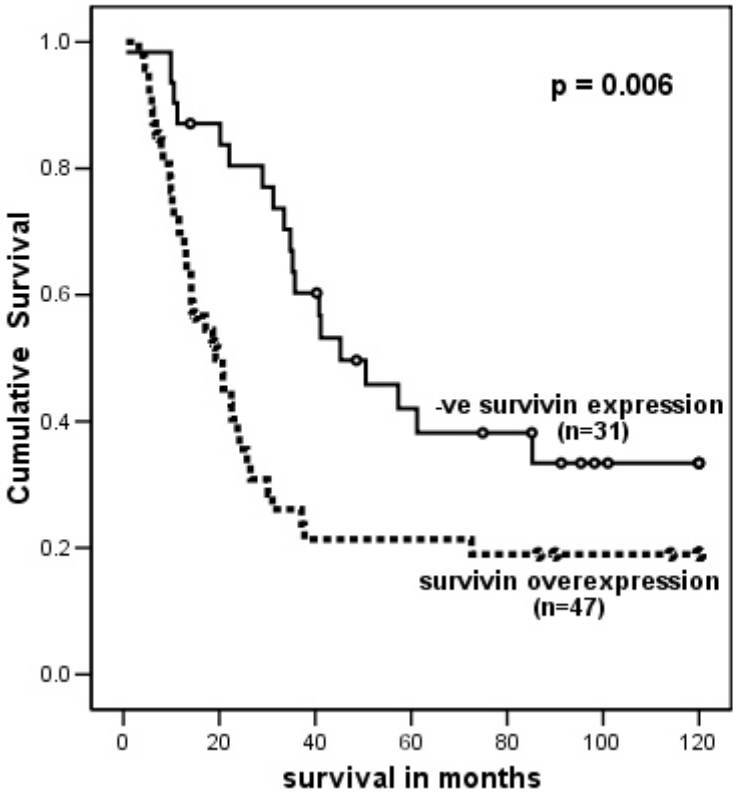


Figure 3 : N2-NSCLC patients with nuclear survivin overexpression have significantly lower overall survival than those with negative expression.

Figure 3



All authors do not have potential conflict of interest

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