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Synaptonemal complex protein 3 as a novel prognostic marker in early stage non-small cell lung cancer

Joon-Yong Chung, PhD[a,†], **Haruhisa Kitano, MD, PhD**[a,b,†], **Mikiko Takikita, MD, PhD**[a,c], **Hanbyoul Cho, MD**[a,d], **Kyung Hee Noh, PhD**[e], **Tae Woo Kim, PhD**[e], **Kris Ylaya, BSc**[a], **Jun Hanaoka, MD, PhD**[b], **Junya Fukuoka, MD, PhD**[f], and **Stephen M. Hewitt, MD, PhD**[a,*]

[a]Tissue Array Research Program & Applied Molecular Pathology Laboratory, Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

[b]Department of Thoracic Surgery, Shiga University of Medical Science, Otsu, Japan

[c]Division of Diagnostic Pathology, Shiga University of Medical Science, Otsu, Japan

[d]Department of Obstetrics and Gynecology, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Korea

[e]Department of Biomedical Sciences, Graduate School of Medicine, Korea University, Seoul, South Korea

[f]Laboratory of Pathology, Toyama University Hospital, Toyama, Japan

Summary

Synaptonemal complex protein 3 (SCP3) is a marker for cell transformation that has prognostic significance in various cancers. However, the prognostic significance of SCP3 has not been studied in non-small cell lung cancer (NSCLC). To investigate the potential correlation between SCP3 and various clinicopathologic parameters, we assessed the expression of SCP3 in archival tumor tissues from 258 NSCLC patients by immunohistochemical staining. By immunofluorescence, SCP3 was detected in both the cytoplasmic and nuclear fractions of NCI-H1299 cell. In tumor samples, SCP3 is detected as cytoplasmic expression pattern and observed in 50 (19.4%) clinical samples by immunohistochemical staining. SCP3 expression was correlated with T status (P=0.008), lymph node metastasis (P=0.010), tumor types (P=0.019) and pleural invasion (P=0.005). In multivariate analysis of patients with early stage disease, increased SCP3 expression predicted worse overall survival in early stage (stage I–II) with pT1 status (P=0.041). These results suggest that positive SCP3 expression is a portent of poor outcome, and may be a potential biomarker in the early stages of the NSCLC for survival and may provide clues in the identification of patients for adjuvant therapy.

Keywords

Immunohistochemistry; Non-small cell lung cancer; Prognostic marker; Synaptonemal complex protein 3; Tissue microarray

Correspondence: Dr. Stephen M. Hewitt, TARP & AMPL, Advanced Technology Center, MSC 4605, Bethesda, MD, 20892-4605 USA, genejock@helix.nih.gov, Fax: +1-301-402-6152.
[†]These authors contributed equally to this work

1. Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, with non-small cell lung cancer (NSCLC) being the most common type [1]. NSCLC accounts for more than 80% of all lung cancers. Most patients are diagnosed with advanced disease stage, and prognosis remains poor [2]. Current guidelines recommend first-line chemotherapy with platinum-based agents. However, most patients relapse and are evaluated for second-line therapy after the initial disease control with chemotherapy. Despite major advances in surgical techniques and new strategies with neoadjuvant treatment, the majority of patients still succumb to the disease within a year. The high mortality is mainly ascribed to disease recurrence after lung resection and the lack of effective treatment for advanced disease. There is a substantial need to develop biomarkers which allow better clinical outcome of patients with NSCLC. In the current clinical setting, many evaluated, though their utility is controversial due to their low sensitivity and specificity [3–5]. However, none of them have yet been incorporated to the clinical practice. Most of the recent efforts have addressed biomarkers that attempt to predict response to tyrosine kinas inhibitors.

Synaptonemal complex protein 3 (SCP3), the protein encoded by *SYCP3*, is a DNA-binding protein and a structural component of the synaptonemal complex, which mediates the synapsis or homologous pairing of chromosomes during human spermatogenesis [6–8]. Since SCP3 has an essential meiotic function and is involved in genetic stability, the expression of SCP3 in cells other than germ cells can be associated with a genetic instability such as aneuploidy. Consistently, SCP3 is preferentially expressed in multiple cancer cells. For instance, human acute lymphatic leukemias show frequent expression of SCP3 (47%) [9]. Recently, we also have demonstrated that SCP3 is expressed in human cervical cancer cells [10]. However, SCP3's role in NSCLC has not been studied. In the current study, SCP3 protein expression levels were evaluated by immunohistochemistry (IHC), with a tissue microarray (TMA) of surgically resected samples of NSCLC. The status of SCP3 expression was then analyzed for an association with clinicopathologic parameters/survival of NSCLC.

2. Materials and methods

2.1. Patients and tumor samples

This study was approved by the ethics committee at Toyama University, and a signed consent form was obtained from each patient. A total of 258 NSCLC cases were selected from the pathology case archive of Toyama University Hospital based on the histological diagnosis, and the quality of the available tissue on the paraffin blocks. None of the patients received neoadjuvant treatments, and they all underwent complete resections between 1988 and 2004. Smoking histories of 98 cases (38%) were unknown, and survival time and outcome was available for all patients. The tumors were staged according to the International Union against Cancer's tumor-node-metastasis (TNM) classification and histologically classified and graded according to 2004 World Health Organization (WHO) guidelines [11]. The follow-up period ranged from 10 to 153 months. No patients received neoadjuvant therapy. The patients who had relapsed were treated with standard chemotherapies [12].

2.2. Western blotting

To observe the cellular localization of SCP3 in H1299 cells, cells were subjected to fractionation using commercial kits (Nuclear/Cytosol Fractionation Kit, Thermo scientific, Rockford, IL). Equal amounts of nuclear protein and cytosol protein were solubilized in Laemmli buffer (62.5mM Tris/HCL pH 6.8, 10% glycerol, 2% SDS, 5% mercaptoethanol and 0.00625% bromophenol blue), boiled for 5 min, and then separated by 12% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The

membranes were blocked with 5% nonfat dry milk in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5) for 1 h at room temperature, and subsequently incubated with anti-SCP3 (diluted 1:500, BD Bioscience, San Diego, CA), anti-β-actin (diluted 1:2000, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-H2B (diluted 1:1000, Santa Cruz) in TBST containing 5% BSA at 4°C overnight, followed by 3 washes in TBST, 5 min per wash. The membranes were incubated with the appropriate secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Elpis Biotech, Seoul, Korea) reaction.

2.3. Plasmid construction

cDNA encoding the entire coding region of human SCP3 was amplified by PCR with forward primer 5′-GCTCGAGATGGTGTCCTCCGGAAAAAAG-3′, including a *Xho*I restriction site, and reverse primer 5′-GAGAATTCAGAATAACATGGATTGAAGAG-3′ with *Eco*RI site. The PCR product was digested and subcloned into plasmid EGFP-N1 (Clontech, Palo Alto, CA). The fidelity of expression was confirmed by sequencing. The nucleotide sequences were determined using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Biosystems, Foster City, CA) and an ABI PRISM 377 DNA sequencer (Perkin Elmer Biosystems).

2.4. Immunofluorescence

In order to examine the cellular localization of EGFP-SCP3 chimeric molecule, H1299cells were cultured on 2-well Laboratory-Tek tissue culture chamber slides (BD Falcon, Bedford, MA) and transfected with 0.4 μg of pEGFP-SCP3 using Lipofectamine™2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol and incubated for 24 h. The transfected cells were fixed and permeable with Cytofix/Cytoperm (BD Biosciences) for 20 min at 4°C. After a wash with PBS following counterstained nuclear with DAPI (100 ng/ml) for 30 min at room temperature, the localization of SCP3 was observed under a confocal laser scanning microscope (Carl Zeiss, Thornwood, NJ).

2.5. Tissue microarray construction

TMA was constructed using a TMA arrayer (Pathology Devices, Westminster, MD) as previously described [13]. For each case, areas with the most representative histology were selected from review of hematoxylin-eosin (H&E) stained slides. The cylindrical tissue samples (0.6 mm) were cored from the above described areas in the donor blocks and extruded into recipient blocks. Multiple 5-μm thick sections were cut with a microtome and H&E staining of TMA slides were examined at every 50th section to confirm the presence of tumor cells.

2.6. Immunohistochemistry and scoring

The tissue sections were deparaffinized in xylene and rehydrated through a graded alcohol series to distilled water. After the deparaffinization and rehydration, antigen retrieval was performed using a pressure chamber (Pascal, Dako, Carpinteria, CA) with pH 9 Target Retrieval Solution (Dako). These slides were blocked with hydrogen peroxide/methanol. After rinsing, the slides were incubated with the anti-SCP3 antibodies overnight at 4°C (BD Bioscience; dilution 1:50). Target signals were detected with Envision$^+$ kit (Dako). The stain was visualized using DAB$^+$ kit (diaminobenzidine; Dako) and then lightly counterstained with hematoxylin, dehydrated in ethanol, and cleared in xylene. The slides were covered and observed under a light microscope (Axioplot, Carl Zeiss, Jena, Germany).

The SCP3 staining results were scored based on (a) intensity [categorized as 0 (absent), 1 (weak), 2 (moderate), or 3 (strong)] and (b) the percentage of positively stained epithelial

cells [scored as 0 (0% positive), 1 (1–25%), 2 (26–50%), 3 (51–75%), or 4 (>75%)]. An overall protein expression score was calculated by multiplying the intensity and positivity scores (overall score range, 0–12). This overall score for each patient was further simplified by dichotomizing it to negative (overall score of 0, 1) or positive (score of ≥ 2) [14]. The rationale for the choice of cutoff for SCP3 protein expression was arbitrary but based on simple criteria that considered visually discernible differences both the intensity and the percentage of positive cells. To exclude cores with faint or non-specific staining, we considered less than 25% of cells stained (the percentage of positivity score of 0 or 1) with absent or weak staining (the intensity score of 0 or 1) should be negative. Thus the overall score, the product of these two scores, had to be ≥ 2 to be scored positive.

2.7. Statistical analysis

Statistical analysis was performed using the SPSS for Window (19.0) package (SPSS, Chicago, IL). We performed survival analysis for all cases. Kaplan–Meier survival analysis was used to determine the univariate relationship of SCP3 expression with overall survival times and the survival curves were compared with the log-rank test. Subsequently, we used a multivariate proportional Cox models and adjusted for the following clinical/pathologic variables: age at diagnosis, sex, tumor type, differentiation, lymph node metastasis and pleural invasion. *P* values were considered significant when they were less than 0.05.

3. Results

3.1. Clinicopathologic features of patients

Clinical and pathologic features of the patients analyzed are summarized in Table 1. The overall mean age was 65.4 ± 9.0 years for all NSCLC patients and almost 70% of the patients were male. Tumor staging was available for all 258 cases, and 151 cases were stage I, 52 cases were stage II, 52 cases were stage III and 3 cases were stage IV. The following histological types were assigned: 158 adenocarcinomas (61.2%), 93 squamous cell carcinomas (36.0%) and 7 other types (including 5 adenosquamous and 2 neuroendocrine carcinomas) (2.7%). Smoking history was available on a small subset of patients (n=160).

3.2. Localization of SCP3 in lung cancer cell

To verify the specificity and capability of the anti-SCP3 antibody for immunohistochemical staining, we first examined the SCP3 expression pattern using H1299 cells. Nuclear and cytosolic proteins from H1299 cells were fractionated, and subsequently applied to western blotting using anti-SCP3 antibody. As shown in Fig. 1A, SCP3 was detected as doublet bands (30 kDa and 33 kDa) which have been reported in mammals [15]. The lower SCP3 band was constantly detected in the both nuclear and cytosolic fractions whereas the higher band was mainly detected in the cytosolic fraction. To confirm the nuclear/cytosolic localization of SCP3, the H1299 cells were transfected with SCP3/EGFP DNA, and, in turn, examined under a confocal laser scanning microscope after nuclear counterstaining with DAPI. As shown in Fig. 1B, we observed the dominant localization of SCP3/EGFP in the perinuclear region of cytoplasm even though the expression of SCP3/EGFP was examined over all of the H1299 cells. We also observed a similar localization pattern of endogenous SCP3 after immunofluorescence staining, using a SCP3-specific antibody (data not shown). These results support the finding of SCP3 in the cytosol, although SCP3 was originally identified as a nuclear protein in meiotic cells.

3.3. SCP3 protein expression

The expressions of SCP3 in relation to clinicopathologic characteristics were evaluated (Table 2). SCP3 expressions were evident in 50 (19.4%) out of 258 NSCLC cases and observed in 27.1% and 13.9% of pT1 and pT2–4 NSCLC, respectively. The expression was

also detected in 10.9% and 24.1% of NSCLC with and without lymph node metastasis, respectively. The positivity of SCP3 expression was 20.2% and 16.4% of NSCLC at the early stage (stage I, II) and the advanced stage (stage III, IV), respectively. SCP3 expression was correlated with features associated with advanced disease and poor outcome, including and T status (P=0.008), lymph node metastases (P=0.010), tumor type (P=0.019), and pleural invasion (P=0.005). All SCP3 staining was observed in the cytoplasm of tumor cells (Fig. 2). To further validate the SCP3 expression in NSCLC, subgroup analysis was conducted for various stages and pT status of NSCLC patients (Table 3). The results were similar between the stages and pT status according to disease severity, as the earlier tumors showed the higher SCP3 positive rates.

3.4. Prognostic significance of SCP3 expression

We analyzed the prognostic significance of SCP3 expression within subgroups according to disease severity. Kaplan–Meier estimates of survival for patients with different SCP3 expression are shown in Fig. 3. Multivariate analysis of all the patients failed to identify SCP3 as an independent predictor of outcome. However, when patients are stratified into early stage (I or II) and tumor size (pT1), SCP3 expression was significantly associated with worse prognosis (P=0.014) with an even stronger association in stage IA patients (P=0.007). In early stage with pT1 subgroup, a Cox univariate proportional hazards analysis showed that SCP3 positivity, sex, tumor type, and tumor differentiation were related to poor overall survival. On multivariate analysis, SCP3 positivity (hazard ratio = 2.54 (95% CI, 1.03–6.25), P=0.041) was an independent predictor of poor prognosis (Table 4).

4. Discussion

In this study, we validated the use of SCP3, for the first time, as a potentially relevant NSCLC prognostic marker. Our findings demonstrated that SCP3 expression seems to be associated with poorer prognosis and is an independent prognostic factor for survival in patients with NSCLC. Although SCP3 is suspected to be involved in stabilizing chromosomes during spermatogenesis, the exact role of SCP3 in cancer cells has not been proven [6, 8]. Ectopic expression of SCP3 can be a cause of genetic instability of cancer cells, leading to abnormality such as aneuploidy. It has been reported that patients who benefit from a surgical resection for NSCLC with aneuploid DNA content prove to have a higher risk of death [16]. In this context, the role of SCP3 in genetic instability of NSCLC needs to be explored. In addition to the possibility of SCP3 as a factor disrupting genetic stabilization, SCP3 can be an apoptosis-associated multiple resistant factor for various cancer therapeutic agents. In a previous study, we demonstrated that the activation of PI3K/AKT mediated by overexpression of SCP3 in tumor cells leads to the upregulation of antiapoptotic proteins, resulting in the immune resistant phenotype [10]. SCP3-mediating AKT activation also appears to be highly associated with resistance of tumor cells to cancer drugs [17]. These data suggest that SCP3 could be a novel prognostic marker in NSCLC.

SCP3 is a meiosis-specific protein and identified as a nuclear protein in meiotic germ cells [18]. However, we observed SCP3 expression in the cytoplasm of the lung cancer cells by IHC. In addition, we demonstrated by western blot analysis that SCP3 subcellular localization is altered in cancer cells (Fig. 1). The altered subcellular localization could be a cancer-specific phenomenon. Jiao *et al* suggest that aberrant nucleocytoplasmic localization of the retinoblastoma (RB) tumor suppressor protein, disrupt normal cell differentiation programs and accelerate the cancer progress via aberrant nucleocytoplasmic transport, related with tumor progression [19]. In addition, it is well known that the function of tumor suppressor proteins (APC, p53, Smad4, and $p27^{kip1}$) can be inactivated by mislocalization [20–23]. Therefore, the ectopic expression of SCP3 in NSCLC is not a surprising observation and may be linked to the cancer progression by an unknown mechanism.

In the current study, we showed, through IHC, that SCP3 protein expression is significantly associated with T status (P=0.008), lymph node metastases (P=0.010), tumor type (P=0.019), and pleural invasion (P=0.004). Although the percentage of patients expressing SCP3 in NSCLC is not high (19.6% of all cases), it is expressed in an increased percentage of early stage patients (30.2% in stage IA patients). Within this group of early stage disease (stage IA, pT1), multivariate analysis suggests SCP3 has the best discriminative value with reference to survival.. Early diagnosis of NSCLC is thought to be important as surgical treatment, in early stage disease, could provide patients the best chance of cure. In this circumstance, early biomarkers are urgently needed to allow better clinical management of patients with NSCLC.

Some of biomarkers have been reported to indicate poor prognosis of early stage NSCLC patients. In retrospective study of 411 patients with surgically resected NSCLC, Yoo *et al.* showed that polymorphisms in the CASPASE genes had poor survival in early-stage NSCLC [24]. In another recent study, Dhillon *et al.* reported that mTOR staining provided a new biomarker for poor outcome in early stage NSCLC [25]. In current study, the patients with SCP3 positive cases have a worse prognosis and therefore are better candidates for adjuvant chemotherapy. SCP3 may play a role as an indicator that specialized treatment is necessary for early stage NSCLC patients.

In conclusion, the current study has demonstrated the potential value of SCP3 as a useful prognostic marker for NSCLC, especially when it is applied to early stage disease. Although SCP3 cannot provide all of the necessary information for optimal NSCLC prognosis, our results suggest an association between SCP3 expression and NSCLC. Therefore, with further research assessing its potential clinical usefulness, SCP3 may play a role as an indicator of adjuvant chemotherapy and lead to a new strategy for early stage NSCLC patients.

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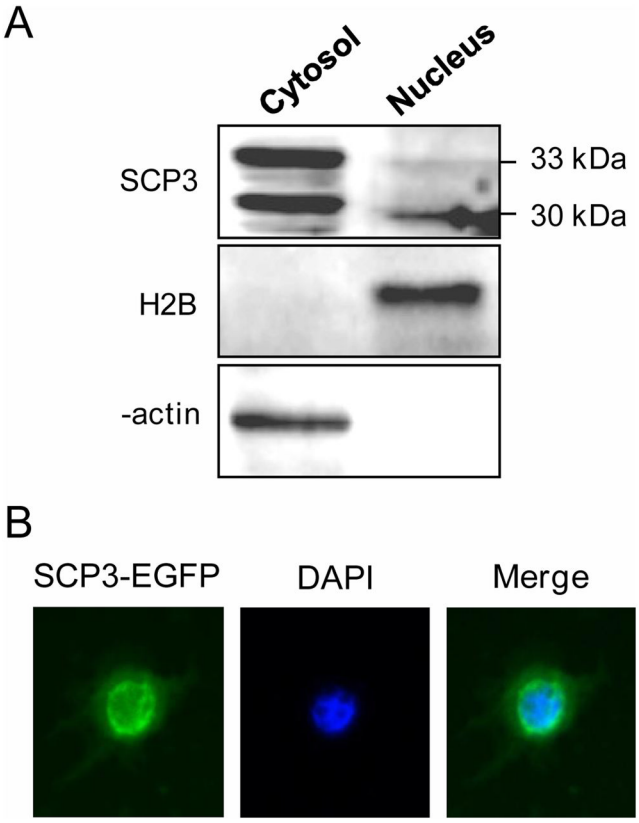

Fig. 1.

Synaptonemal complex protein 3 (SCP3) localization in a lung cancer cell line. (A) Nuclear and cytoplasmic fractions from H1299 cells were analyzed by western blot analysis using a SCP3 antibody. H2B and beta-actin were used as indexes for nuclear and cytoplasmic fractions, respectively. (B) Confocal fluorescent microscopy was used to further evaluate the distribution of SCP3 in H1299 cells 24 h after transfection of SCP3/EGFP DNA.

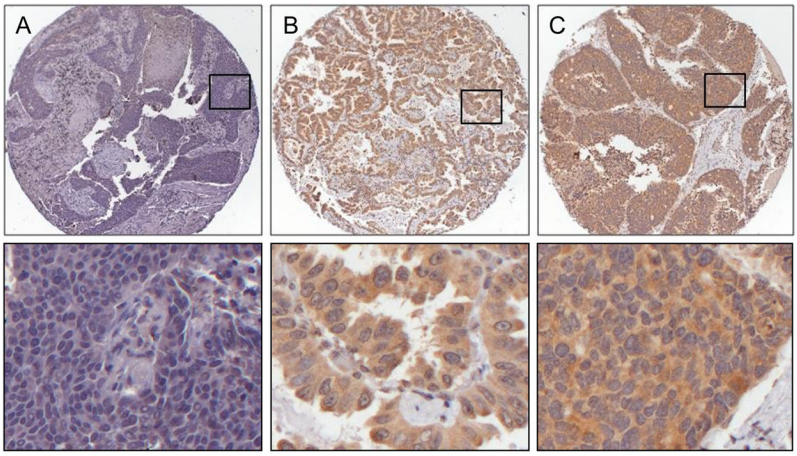

Fig. 2.

Representative images of immunohistochemistry for synaptonemal complex protein 3 (SCP3). Negative - demonstrating a lack of SCP3 expression (A); Positive - cytoplasmic staining in adenocarcinoma (B) and squamous cell carcinoma (C). Boxed regions are displayed at high magnification in the bottom panel. All photomicrographs ×100.

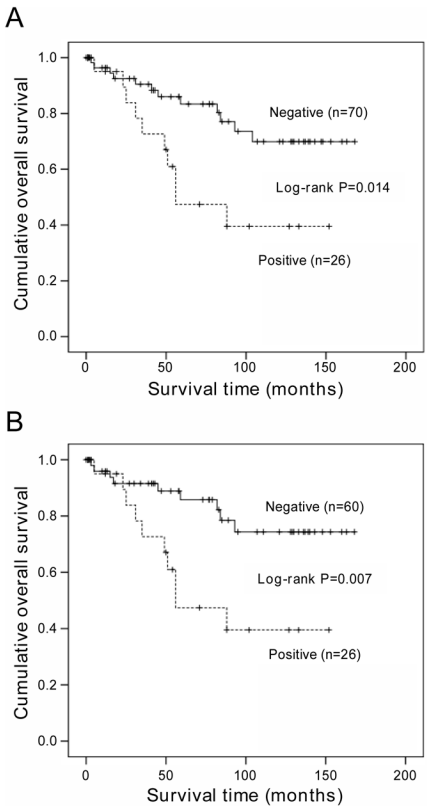

Fig. 3.

Kaplan-Meier survival analysis ofNSCLC according to SCP3 expression. (A) In early stage (≤ stage II) with pT1 group, patients with positive SCP3 expression (median survival, 87 months) showed significantly worse survival than those with negative SCP3 expression (median survival, 134 months). (B) Stage IA group with positive SCP3 expression (median survival, 87 months) had a significantly worse patients' survival time than those with negative SCP3 expression (median survival, 138 months).

Table 1

Clinicopathologic characteristics of 258 patients with non-small cell lung cancer

Category	No. of cases	% of total
Sex		
Male	180	69.8
Female	78	30.2
Age at time of diagnosis (years)		
Average (mean ± SD)	65.4 ± 9.0	
< 50	15	5.8
50 – 59	45	17.4
60 – 69	106	41.1
≥ 70	92	35.7
Smoking status		
Never	85	32.9
Ever	75	29.1
Unknown	98	38.0
Stage		
IA	86	33.3
IB	65	25.2
IIA	9	3.5
IIB	43	16.7
IIIA	48	18.6
IIIB	4	1.5
IV	3	1.2
T status		
pT1	107	41.5
pT2	121	46.9
pT3	27	10.4
pT4	3	1.2
Lymph node metastasis		
pN0	166	64.3
pN1	47	18.2
pN2	44	17.1
pN3	1	0.4
Tumor type		
Adenoca.	158	61.2
SCC	93	36.0
Adenosquamous	5	1.9
Neuroendocrine	2	0.8
Differentiation		
Well	108	41.8
Moderate	97	37.6

Category	No. of cases	% of total
Poor	49	19.0
Pleural invasion		
Negative	173	67.1
Positive	85	32.9

Table 2

The correlation between the status of SCP3 expression and clinicopathologic characteristics in NSCLC

Category	No. of case	%	SCP3 expression		*P* value
			Negative, *n* (%)	Positive, *n* (%)	
Total	258	100	208 (80.6)	50 (19.4)	
Sex					
Male	180	69.8	145 (80.6)	35 (19.4)	0.968
Female	78	30.2	63 (80.8)	15 (19.2)	
Age (years)					
< 60	60	23.3	51 (85.0)	9 (15.0)	0.327
≥ 60	198	76.7	157 (79.3)	41 (20.7)	
Smoking status					
Never	85	32.9	68 (80.0)	17 (20.0)	0.105
Ever	75	29.1	67 (89.3)	8 (10.7)	
Stage					
I – II	203	78.7	162 (79.8)	41 (20.2)	0.523
III – IV	55	21.3	46 (83.6)	9 (16.4)	
T status					
pT1	107	41.5	78 (72.9)	29 (27.1)	0.008*
pT2–4	151	58.5	132 (86.1)	20 (13.9)	
Lymph node metastasis					
Negative	166	64.3	126 (75.9)	40 (24.1)	0.010*
Positive	92	35.7	82 (89.1)	10 (10.9)	
Tumor type					
Adenoca.	158	61.2	119 (75.3)	39 (24.7)	0.019*
SCC	93	36.0	82 (88.2)	11 (11.8)	
Others	7	2.7	7(100)	0 (0)	
Differentiation					
Well	108	41.8	94 (87.0)	14 (13.0)	0.054
Moderate	97	37.6	76 (78.4)	21 (21.6)	
Poor	49	19.0	35 (71.4)	14 (28.6)	

Category	No. of case	%	SCP3 expression		*P* value
			Negative, *n* (%)	Positive, *n* (%)	
Pleural invasion					
Negative	173	67.1	131 (75.7)	42 (24.3)	0.005 *
Positive	85	32.9	77 (90.6)	8 (9.4)	

*Significant at the level of $p<0.05$

†Degree of differentiation for 2 adenocarcinomas and 2 other tumor types was not available

Adenoca.; adenocarcinoma, SCC; squamous cell carcinoma.

Table 3

SCP3 expression in various stages and pT status of NSCLC

	No. of cases	% of total	SCP3 expression	
			Negative	Positive
Stage IA	86	33.3	60 (69.8%)	26 (30.2%)
Stage IA + IB	151	58.5	114 (75.5%)	37 (24.5%)
Stage I+Stage II	203	78.7	162 (79.8%)	41 (20.2%)
Stage I+Stage II+Stage III	255	98.8	205 (80.4%)	50 (19.6%)
Stage I+Stage II+Stage III+Stage IV	258	100	208 (80.6%)	50 (19.4%)
pT1	107	41.5	78 (72.9%)	29 (27.1%)
pT1+pT2	228	88.4	181 (79.4%)	47 (20.6%)
pT1+pT2+pT3	255	98.8	205 (80.4%)	50 (19.6%)
pT1+pT2+pT3+pT4	258	100	208 (80.6%)	50 (19.4%)

Table 4

Survival analysis of the patients in early stage (stage I and II) with pT1 (n=96) by Cox proportional hazards

Variables	Univariate		Multivariate	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
SCP3				
Negative	1	0.014 *	1	0.041 *
Positive	2.90 (1.24 – 6.75)		2.54 (1.03 – 6.25)	
Sex			NS	
Female	1	0.027 *		
Male	3.39 (1.14 – 10.04)			
Tumor type			NS	
Adenoca.	1	0.005 *		
SCC	3.31 (1.43 – 7.66)			
Differentiation			NS	
Well	1	0.001 *		
Moderate - Poor	6.05 (2.04 – 17.96)			
Lymph node metastasis	NS		NS	
Pleural invasion	NS		NS	
Age (years)	NS		NS	

* Significant at the level of $p<0.05$

Adenoca.; adenocarcinoma, SCC; squamous cell carcinoma.

HR; hazard ratio, CI; confidence interval, NS; not significant.