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IJC
International Journal of Cancer

High expression of stratifin is a universal abnormality during the course of malignant progression of early-stage lung adenocarcinoma

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Adenocarcinoma *in situ* (AIS) of the lung has an extremely favorable prognosis, with a 5-year survival rate of 100%. However, early invasive adenocarcinoma (EIA) often has a fatal outcome. In this study, we compared the expression profiles of AIS with those of EIA showing lymph node metastasis or a fatal outcome, and screened the differentially expressed genes by cDNA microarray. From the genes selected, we focused on Stratifin (SFN, 14-3-3 σ), which showed significantly higher expression in EIA than in AIS. Immunohistochemistry for SFN revealed that more than 95% of EIAs were immunopositive for SFN, in comparison to only 13% of AISs ($p < 0.05$). Interestingly, positivity was detected not only in the invasive region but also in the *in situ* spreading component of EIA. Functionally, SFN facilitates the cell proliferation capacity of lung adenocarcinoma. These results indicate that SFN overexpression is a universal abnormality during the stepwise progression from *in situ* to invasive adenocarcinoma of the lung.

Introduction

Lung carcinoma is the leading cause of cancer death in developed countries.[1] Histologically it is divided into four major subtypes: squamous cell carcinoma, adenocarcinoma, small cell carcinoma and large cell carcinoma. Among them, adenocarcinoma is the most common histologic subtype, and is increasing in frequency, accounting for almost half of all non-small cell lung carcinomas. The 5-year survival rate is generally less than 40%, and the high mortality rate is due primarily to difficulty in the diagnosis of early-stage lung cancer and rapid progression of the disease in the advanced stage. Accordingly, early detection and treatment of the disease are essential.

On the basis of clinicopathological features, Noguchi *et al.* showed that small lung adenocarcinomas (2 cm in diameter or less) can be divided into two groups, each of which can be further subdivided into three types.[2] One of these two groups is the replacement growth type, which includes a lepidic growth (bronchioalveolar) component and constitutes a major group of peripheral pulmonary adenocarcinomas. This group includes localized bronchioloalveolar carcinoma (LBAC) without active fibroblastic proliferation (type A; pure bronchioloalveolar carcinoma, type B; pure bronchioloalveolar carcinoma with collapse) and LBAC with active fibroblastic proliferation (type C; mixed type adenocarcinoma with a predominant lepidic pattern). The other group is the nonreplacement growth type, which shows destruction of the original alveolar structure and does not include a lepidic growth (bronchioalveolar) component (type D; solid adenocarcinoma with mucin, type E; acinar adenocarcinoma, type F; papillary adenocarcinoma). Patients with types A and B have an extremely favorable prognosis, with a 5-year survival rate of 100%. Therefore, types A and B tumors correspond to *in situ* adenocarcinoma biologically. On the other hand, some type C tumors are associated with lymph node metastasis,[2] and the 5-year survival rate is lower than for types A and B (5-year survival rate for type C: 74.8%). Suzuki *et al.* and Sakurai *et al.* have also indicated the importance of fibrosis and stromal invasion for the prognosis of replacement growth-type adenocarcinomas.[3,4] These clinicopathological studies have indicated that types A and B tumors sequentially progress to type C tumors.

In this study, we carried out cDNA microarray analysis of five cases of type A from patients who survived for more than 5 years and five cases of type C from patients who had lymph node metastasis or died, to find universal factors that are biologically related to early progression of lung adenocarcinoma.

Key words: lung cancer, tumor promotion and progression, stratifin, adenocarcinoma

Additional Supporting Information may be found in the online version of this article.

DOI: 10.1002/ijc.25907

History: Received 30 Jul 2010; Accepted 10 Dec 2010; Online 4 Jan 2011

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Material and Methods

Patients and tissue specimens

For subtraction analysis and quantitative RT-PCR, 41 frozen specimens of human lung adenocarcinoma were obtained from patients who had undergone surgical resection at the Department of Thoracic Surgery, Tsukuba University Hospital (Ibaraki, Japan), Tsukuba Medical Center Hospital (Ibaraki, Japan), and Ibaraki-higashi National Hospital (Ibaraki, Japan). A small amount of each specimen was embedded directly in Tissue-Tek OCT Compound (Sakura Finetek Japan, Tokyo, Japan) and frozen immediately in acetone and dry ice. The specimens were then stored at −80°C until analysis. Among the 41 specimens, we used five cases of type A from patients who survived, and five cases of type C from patients who had suffered lymph node metastasis or died, for cDNA microarray analysis, and the others were used for real-time RT-PCR. The backgrounds of these 10 patients are summarized in Supporting Information Table 1. For clinicopathological analysis using immunohistochemistry, 106 adenocarcinomas were also obtained from patients who had undergone surgical resection. All specimens were fixed with 10% formalin and embedded in paraffin.

Cell lines and culture conditions

Cell lines A549, PC-14, RERF-LC-KJ, and LC-2/ad were purchased from RIKEN Cell Bank (Ibaraki, Japan), and NCI-H23 and Calu-3 from the American Type Culture Collection. PL16T and PL16B were established in our laboratory from human lung AAH (atypical adenomatous hyperplasia) and human alveolar epithelium.[5] A549 was maintained in D-MEM/F12 (Life Technologies Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), and 100 U/ml penicillin and streptomycin (Sigma-Aldrich). NCI-H23, PC-14, and RERF-LC-KJ were maintained in RPMI 1640 (Life Technologies) supplemented with 10% FBS, and 100 U/ml penicillin and streptomycin. Calu-3 was maintained in MEM (Sigma-Aldrich) supplemented with 10% FBS, and 100 U/ml penicillin and streptomycin. LC-2/ad was maintained in RPMI 1640/F12 (Sigma-Aldrich) supplemented with 15% FBS, 100 U/ml penicillin and streptomycin. PL16T and PL16B were maintained in MCDB153HAA (Wako, Osaka, Japan) supplemented with 2% FBS, 100 U/ml penicillin and streptomycin, 100 μg/ml EGF (Toyobo, Tokyo, Japan), 2.5 mg/ml Insulin (Wako), 600 μl/ml hydrocortisone (Wako), 10 mg/ml transferrin (Sigma-Aldrich), and 20 μg/ml sodium selenate (Sigma-Aldrich). All cells were cultured in a 5% CO_2 incubator at 37°C.

Extraction and amplification of RNA

For subtraction analysis, we used 10 frozen cryostat sections (8 μm thick) of types A and C. The tumor cells in each section were collected selectively using a laser capture microdissection system, LM-2000 (Arcturus Engineering, Mountain View, CA). Total RNA was isolated using a RNeasy Mini Plus Kit (Qiagen, Düsseldorf, Germany) in accordance with the manufacturer's protocol. The quality of the isolated total RNA was assessed by detection of 28S and 18S ribosomal RNA with an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Using 10 μl of sample buffer, T7 RNA polymerase promoter-attached, adaptor ligation-mediated, and PCR amplification followed by the *in vitro* T7-transcription (TALPAT) method was performed to amplify mRNA while maintaining the original gene expression ratio.[6]

cDNA microarray

Using the purified TALPAT product (ds cDNA) as a template, *in vitro* transcription was performed with biotin-labeled UTP, followed by conversion to biotin-labeled cRNA. The cRNA was purified using an RNeasy Mini Plus Kit (Qiagen) and the quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies) as described above. Fragmentation of cRNA, hybridization to a Human Whole Genome Bioarray (Applied Bioarrays, Tempe, AZ) including 54,841 probes, and washing of the array were performed in accordance with the manufacturer's protocols for the CodeLink™ iExpress Assay Reagent Kit (Applied Bioarrays). The signals on the arrays were detected with arrayWoRx[e] (GE Healthcare UK, Little Chalfont, England). The data analysis was performed with Gene Spring GX7.3 (Agilent Technologies). MIAME-compliant data have been deposited in the MIAME Express database (Accession No. E-MEXP-2825).

Quantitative real time RT-PCR

Expression of genes that showed relative overexpression in type C tumors was evaluated again by the quantitative real-time RT-PCR method. Total RNA was prepared from another 31 frozen lung adenocarcinoma specimens using an RNeasy Mini Plus Kit (Qiagen) and the quality was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies) as described above. One microgram of total RNA per 20 μl of reaction mixture was converted to cDNA using a High-capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Quantitative real-time PCR was performed with SYBR® Premix Ex Taq™ (Perfect Real Time; Takara Bio, Tokyo, Japan) on a GeneAmp® 7300 Sequence Detection System (Life Technologies) in accordance with the manufacturer's protocol. Primers used in this study are listed in Supporting Information Table 2.

Immunohistochemistry

Four-micrometer-thick sections were cut from 10% formalin-fixed, paraffin-embedded blocks. The deparaffinized and rehydrated sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 121°C for 10 min for antigen retrieval, then incubated with monoclonal anti-SFN antibody diluted 1:40 (Immuno-Biological Laboratories, Gunma, Japan) for 30 min at room temperature. Subsequently, the sections were incubated with peroxidase-labeled polymer conjugated to goat anti-mouse IgG (DAKO, Carpinteria, CA) for 30 min at

room temperature. Immunoreactivity was detected with a DAB substrate kit (Dako Japan, Kyoto, Japan), and the sections were counterstained with hematoxylin. The immunoreactivity was evaluated using a three-tier grading: negative (not stained), borderline (partially stained), positive (diffusely positive).

Pathway analysis

The PI3K-specific inhibitor LY294002 was purchased from Sigma (St. Louis, MO). The MEK1/2-specific inhibitor U0126 was from Merck (Whitehouse Station, NJ), and the JNK1/2-specific inhibitor SP600125 was from Biomol Research Laboratories. (Plymouth Meeting, MA). A549 cells carrying wild-type p53 and NCI-H23 cells harboring a p53 point mutation were cultured in 60-mm dishes (5×10^5 cells/dish). Serum-starved cells were pretreated or untreated with LY294002, U0126, or SP600125 for 1 h. The phosphorylation states of PI3K, ERK and JNK were determined by Western blotting with phosphate-specific antibodies. Then, SFN expression was also determined by Western blotting.

Western blotting

Total cell lysates were prepared on ice with M-PER™ Mammalian Protein Extraction Reagent (Pierce) containing protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Sigma). The lysates were centrifuged for 10 min at 4°C, and the insoluble fraction was discarded. The total protein in the soluble lysates was measured using a BCA protein assay kit (Pierce). Total protein aliquots (20 μg) were mixed with Laemmli sample buffer, denatured at 95°C for 5 min, and electrophoresed on 10% Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred to polyvinylidene difluoride membranes using an iBlot™ gel transfer system (Life Technologies Corporation). The blots were blocked and probed with the various antibodies. Antibodies used for Western blotting were obtained from various commercial sources as follows: SFN, GeneTex, (San Antonio, TX); phosphor-Akt (Ser473), Akt, phosphor-ERK1/2 (Thr202/Tyr204), and ERK1/2, Cell Signaling Technology, (Beverly, MA); phosphor-JNK1/2(Thr183/Tyr185) and JNK1/2, R&D Systems; β-actin, Pierce (Rockford, IL). After extensive washing, immunoreactivity was detected with specific secondary antibodies conjugated to horseradish peroxidase. Protein bands were visualized using SuperSignal West Dura® extended duration substrate (Pierce) and X-ray film (BioMax XAR film; Kodak, Rochester, NY).

Transfection using small interfering RNA (siRNA)

A549 cells were seeded at a density of $1.0–1.5 \times 10^6$/mL and incubated overnight in antibiotics-free D-MEM/F12 supplemented with 10% FBS. On the following day, the cells were washed with phosphate-buffered saline (PBS), and then OPTI-MEM® reduced serum medium (Life Technologies) was added to the cells. SFN-specific siRNA (forward, 5′-ACCACGUU CUUAUAGGCUACUGAGA-3′; reverse,5′-UCUCAGUAGC CUAUAAGAACGUGGU-3′; Life Technologies) and a nucleic acid transferring agent, lipofectamine RNAiMAX (Life Technologies), were incubated together in OPTI-MEM® reduced serum medium for 15 min at room temperature to form an siRNA-lipofectamine complex. The medium containing the siRNA-lipofectamine complex was added to the cells to give a final siRNA concentration of 5 nM. Six hours later, the complex-containing medium was exchanged for antibiotics-free D-MEM/F12 supplemented with 10% FBS. The cells were incubated at 37°C in a CO_2 incubator for 24–120 h, and then further analysis was carried out.

Transfection of expression vectors

An entry clone encoding SFN (Ultimate Human ORF CLONE, IOH2986) was purchased from Life Technologies (Carlsbad, CA). With the entry clone and pcDNA-DEST47 Gateway Vectors (Life Technologies Corporation), we cloned SFN destination vectors (pDEST-SFN) using Gateway LR Clonase II Enzyme Mix (Life Technologies) in accordance with the manufacturer's protocol. The plasmid DNA was purified using an EndoFree Plasmid Maxi kit (Qiagen).

The day before transfection, A549 or PL16T cells were trypsinized and counted, and plated at 2×10^5 cells per well into 6-well plates, so that they were 50–80% confluent on the day of transfection. Cells were plated in 2 mL of complete growth medium. For each well of cells to be transfected, 2.5 μg pDEST-SFN or pDEST-GFP DNA was diluted in 500 μL of OPTI-MEM® (Life Technologies) and incubated for 5 min at room temperature. Then, 6.25 μL of Lipofectamine LTX Reagent (Life Technologies) was added, followed by incubation for 30 min at room temperature. We added 0.5 mL of DNA-Lipofectamine LTX complex directly to each well, and performed gentle mixing by rocking the plate back and forth. The cells were incubated at 37°C in a CO_2 incubator for 24 h, after which we performed further analysis.

Cell proliferation assay

To analyze cell proliferation activity, a cell proliferation ELISA, BrdU (colorimetric) kit (Roche Diagnostics) was used in accordance with the manufacturer's protocol.

Invasion assay

Cell invasion potential was assessed using a cell invasion assay kit (Millipore) in accordance with the manufacturer's protocol. The kit had a system for evaluating the invasion of tumor cells through a basement membrane model. The assays were performed 48 h after siRNA transfection.

Results

cDNA microarray

From the cDNA microarray data, we first eliminated the genes whose quality flags were L (near background; poor signal quality) in arrayWoRx analysis. Then we calculated the expression ratio of type C and type A, using the expression average of each type. Finally we selected 25 genes whose

Table 1. Comprehensive differential gene expression analysis using cDNA microarray

	Gene symbol	Full name	Function	C/A
1	SCGB1A1	Secretoglobin, family 1A, member 1 (uteroglobin)	Protein, steroid binding	4.26
2	HTATIP2	HIV-1 Tat interactive protein 2, 30 kDa	Protein binding	2.99
3	IL1F7	Interleukin 1 family, member 7 (zeta)	Protein binding	2.75
4	SFN	Stratifin	Protein binding	2.38
5	HIST2H2AA	Histone 2, H2aa	Nucleic acid binding	2.23
6	CP	Ceruloplasmin (ferroxidase)	Ion binding	2.18

C/A: Expression ratio of type C (early invasive carcinoma) and type A (*in situ* carcinoma) tumors.

expression in type C tumors was more than two times higher than that in type A (type C/type A, >2, Supporting Information Table 3). These included some well-known cancer-related genes such as survivin, BIRC5, and MDK. These candidate genes were then classified by their functions, such as binding, transcriptional regulation, transport, and signal transduction. Among the functions of the various genes, most (12 genes) were classified as having a binding function. We therefore focused on those genes that played specific roles in the cell by binding to various molecules (*e.g.,* nucleic acids, protein, and ion). With the aim of finding genes that were expressed specifically in type C tumors, we selected six genes whose expression in all five type A cases was lower than the expression average in type C cases. The final candidates were SCGB1A1, HTATIP2, IL1F7, SFN, HIST2H2AA and CP, as shown in Table 1.

Quantitative real-time RT-PCR

To confirm previously identified differences in the expression levels of the genes selected by cDNA microarray, quantitative real-time PCR assays were carried out using the samples that had been used for microarray analysis (TALPAT products). These assays confirmed that all of the candidate genes were expressed at a higher level in type C tumors than in type A.

Moreover, quantitative real-time RT-PCR assays with 20 cases of lung adenocarcinoma mixed subtype with BAC and 11 cases of type A were performed to validate the difference in expression of these genes on a large scale. As most of the patients with type C tumors survived, we examined mixed-subtype adenocarcinomas with BAC, which were thought to be type C tumors at an advanced stage, in the assays. Among the six genes, SFN and CP showed significantly higher expression in adenocarcinoma mixed subtype than in type A (Fig. 1*a*) but the expression of the others was not significantly higher. CP is a copper-containing acute-phase protein primarily of hepatic origin, and has important antioxidant properties that protect lipids from peroxidation.[7] We speculated that the high level of CP might reflect its property as an acute-phase reactant rather than a cancer-related function. Therefore, in this study, we focused on SFN and subjected it to further analysis.

Expression of SFN in lung adenocarcinoma cell lines and resected materials

First, we examined SFN expression in six lung adenocarcinoma cell lines and two immortalized cell lines by real time RT-PCR and Western blotting (Fig. 1*b*). A549 and PC14 cells showed high expression of SFN, but all of the six lung adenocarcinoma cell lines showed SFN expression to some extent. Conversely, we were unable to detect SFN expression in PL16T and PL16B, which were immortalized cell lines from AAH and normal bronchial epithelium. Secondly, we examined SFN expression in 106 small lung adenocarcinomas (<2 cm) by immunohistochemistry. Since SFN is localized mainly in the cytoplasm, we evaluated cytoplasmic immunoreactivity (Fig. 2). Normal bronchial tissue and lung parenchyma were completely negative for SFN. Among adenocarcinomas, 98% of type C, D, E and F cases showed positive staining in the cytoplasm, whereas only 14% of type A and B cases did so (Table 2, $p < 0.001$). It was of particular interest that *in situ* spreading cells of type C tumors were also immunopositive for SFN, similarly to the tumor cells in invasive areas (Fig. 2*c*).

Function analysis of SFN

Proliferation assay and invasion assay were performed using A549 and PL16T cells after transfection with siRNA-SFN or SFN expression vectors. We found that transfection with siRNA-SFN significantly decreased the proliferation of A549 cells continuously for 120 h, although there was no apparent change in cell invasiveness (Figs. 3*a* and 3*b*). On the other hand, transfection with the SFN expression vector led to a significant increase in the proliferation of both A549 and PL16T cells (Fig. 3*c*).

Pathway analysis

Whereas SFN (14-3-3 sigma) is known to regulate cell cycle progression negatively as a p53 downstream factor, overexpression of SFN has been reported in several malignancies (colon and breast),[8,9] where the PI3K/Akt pathway has been shown to be one of the representative SFN upstream factors. To validate whether the PI3K/Akt pathway is related to SFN expression also in lung adenocarcinoma, we examined the signaling pathway of SFN in lung adenocarcinoma using pharmacological inhibitors specific to the pathways

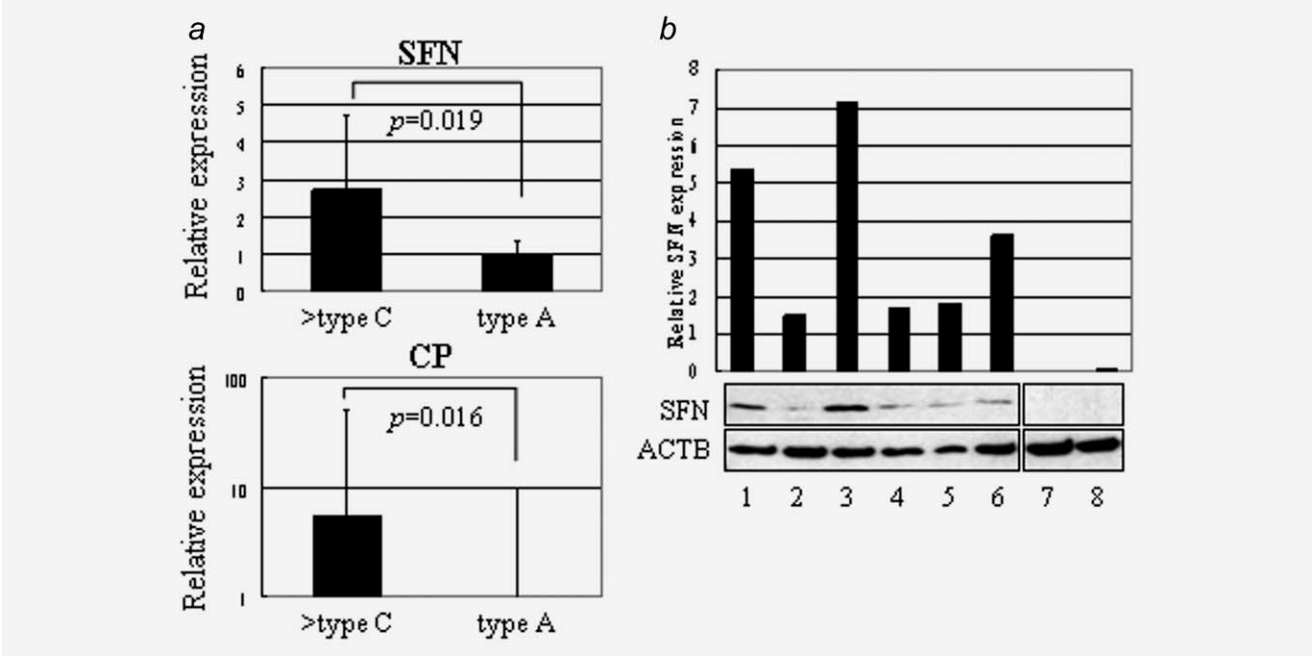

Figure 1. Expression analysis of SFN and CP. (*a*) Real-time RT-PCR of SFN and CP using 20 cases of type C tumors and 11 cases of type A. Both SFN and CP showed significantly higher expression in type C than in type A. type C: early invasive adenocarcinoma, type A: *in situ* adenocarcinoma. See Table 1 for gene symbols. (*b*) Real-time RT-PCR and Western blotting using mRNA or protein of 6 human lung adenocarcinoma cell lines and 2 immortalized cell lines. 1: A549, 2: NCI-H23, 3: PC-14, 4: Calu-3. 5: RERF-LC-KJ, 6: LC-2/ad, 7: PL16T, 8: PL16B.

(LY294002). As SFN has been reported to be a downstream factor of the p53 gene, we used A549 that bears wild-type p53 and NCI-H23 that harbors a p53 point mutation. Serum-starved A549 or NCI-H23 cells were treated with the inhibitors (final concentration 25, 50 or 100 μM) and without them (DMSO at a final concentration of 100 μM as a negative control) for 1 h. As shown in Figure 4, LY294002 inhibited p-Akt, and we found that the PI3K inhibitor LY294002 decreased the expression of SFN in a dose-dependent manner in both cell lines (Fig. 4). However, neither U0126 (a MAPK pathway inhibitor) nor SP600125 (a JNK1/2 pathway inhibitor) affected SFN expression (data not shown). Therefore, the expression of SFN in A549 and NCI-H23 cells was shown to be mediated mainly *via* the PI3K/Akt pathway.

Discussion

The molecular mechanism involved in malignant transformation from *in situ* carcinoma to invasive adenocarcinoma during the course of lung adenocarcinogenesis is still unknown. Here we performed comprehensive differential gene expression analysis using a cDNA microarray with type A (*in situ* adenocarcinoma) and C (early invasive adenocarcinoma) tumors, and identified 25 genes that were differentially and highly expressed in type C tumors in comparison with type A tumors. Although these genes included some that are well known to be cancer-related (survivin, BIRC5 and MDK), we finally selected SFN, which showed a significant difference in expression between type A and type C tumors.

Stratifin (SFN, also known as 14-3-3 sigma) belongs to the 14-3-3 family of abundant, widely expressed 28-33-kDa acidic polypeptides that spontaneously self-assemble as homodimers or heterodimers. There are seven closely related genes, encoding beta, epsilon, eta, gamma, tau, zeta, and sigma isoforms, that are conserved across mammalian species. They can bind to >100 functionally diverse cellular proteins and thereby play important roles in various cellular processes such as signal transduction, cell cycle regulation, apoptosis, cytoskeleton organization, and malignant transformation.[10,11] 14-3-3 proteins regulate enzyme activity and may act as localization anchors, controlling the subcellular localization of proteins. In addition, 14-3-3 proteins can function as adaptors or scaffolds, stimulating protein–protein interaction.[12] 14-3-3 sigma (SFN) was originally identified as a p53-inducible gene that is responsive to DNA-damaging agents.[13] SFN sequesters the mitotic initiation complex, cdc2-cyclin B1, and prevents it from entering the nucleus, thus preventing initiation of mitosis.[14] In this manner, SFN induces G2 arrest and allows the repair of damaged DNA. Additionally, SFN can bind cyclin-dependent kinase-2 and cyclin-dependent kinase-4 (CDK2, CDK4) and thereby block the transition of the eukaryotic cell cycle.[15] These findings define SFN as a negative regulator of cell cycle progression. Furthermore, in primary human epidermal keratinocytes, down-regulation of SFN allows escape from senescence.[16] Therefore, functional inactivation of SFN may be linked to carcinogenesis, a hypothesis that is supported by the discovery of SFN down-regulation in various

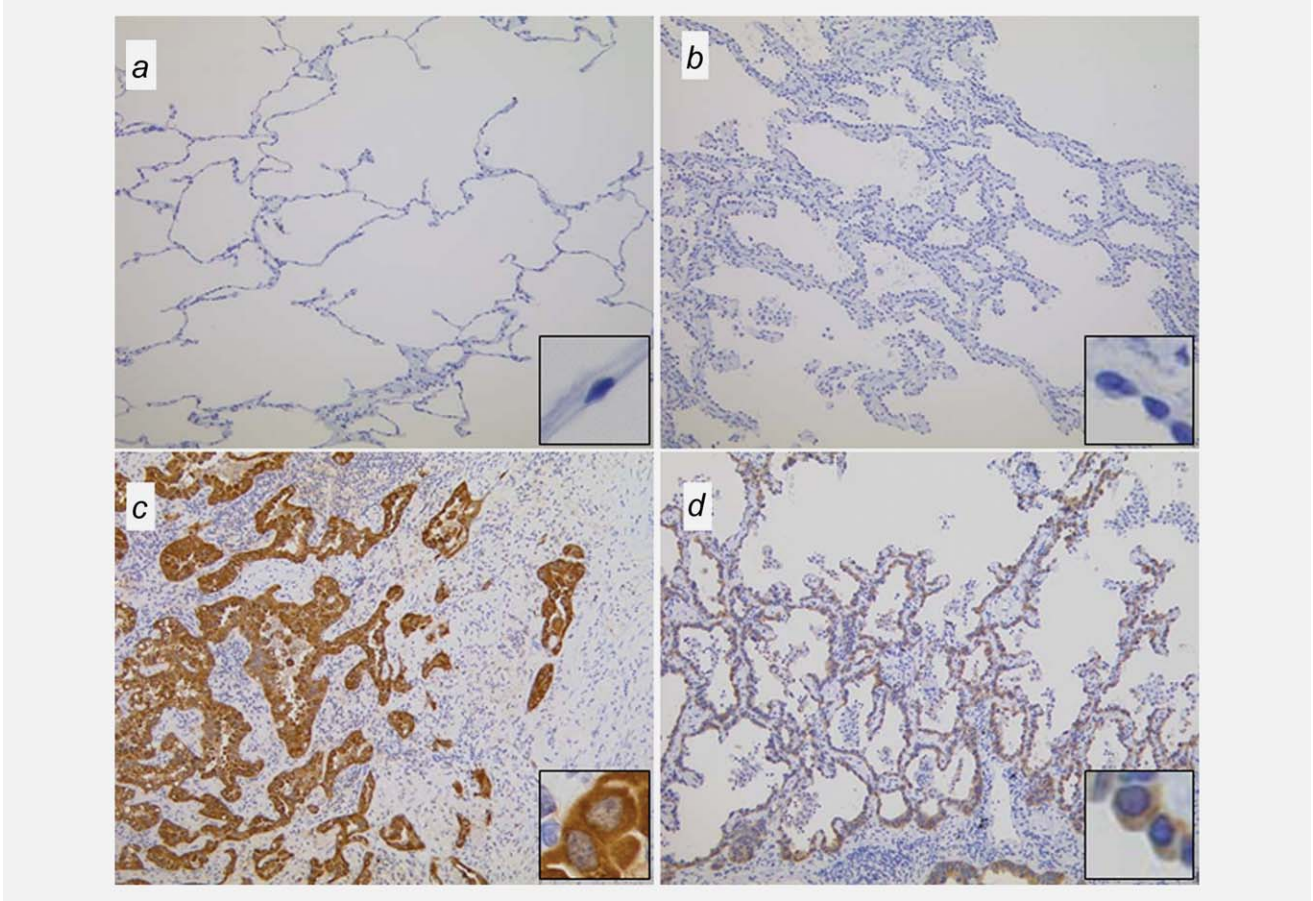

Figure 2. SFN immunohistochemistry with formalin-fixed paraffin-embedded (FFPE) specimens. SFN was detected mainly in the cytoplasm of tumor cells (C in let). (*a*) normal lung parenchyma is negative for SFN. (*b*) type A tumor is negative for SFN. (*c*) invasive component (acinar adenocarcinoma) of type C tumor is positive for SFN. (*d*) peripheral *in situ* spreading component of type C tumor is also positive for SFN. SFN: stratifin. Type A: *in situ* adenocarcinoma. Type C: early invasive adenocarcinoma.

Table 2. SFN immunoreactivity in small lung adenocarcinomas (less than 2 cm in diameter)

	Negative (%)	Borderline (%)	Positive (%)[1]	Total
Normal lung tissue	106 (100)	0	0	106
Type A	10 (72)	2 (14)	2 (14)	14
Type B	10 (59)	5 (29)	2 (12)	17
Type C	1 (2)	0	50 (98)	51
Type D, E, F	0	0	24 (100)	24

Types A and B: *in situ* adenocarcinoma.
Type C: early invasive adenocarcinoma.
Types D, E, and F: invasive adenocarcinoma (See Ref. 2).
Negative: not stained.
Borderline: partially stained.
Positive: diffusely positive.
[1] χ^2 test, $p < 0.05$.

human malignancies, including cancers of the breast, stomach, colon, liver, prostate, oral cavity, and vulva,[17–24] attributed to hypermethylation of the CpG island present in the promoter area of the gene.[25] In lung cancer, Osada *et al.* have also found that DNA hypermethylation occurs in small-cell lung cancer cell lines (69%) and non-small cell lung cancer cell lines (6%), and subsequent silencing of SFN has also been observed.[26] On the other hand, many reports have indicated up-regulation of SFN in cancers of the head and neck,[27] stomach,[28] pancreas,[29–31] and colorectum.[8] Moreover, Moreira *et al.* have reported that down-regulation of SFN is a sporadic event in breast cancers,[25] and a similar study of colorectal cancers has demonstrated that hypermethylation of the SFN promoter area is a rare event.[32] These studies suggest that SFN might be a context-dependent gene, and that its functions may vary among organs or tissues.

Our study indicated that SFN was abnormally overexpressed not only in human lung adenocarcinoma cell lines but also in resected invasive adenocarcinomas. However, SFN expression was not detected in immortalized AAH and normal bronchial cell lines and normal lung tissues. Most of the *in situ* carcinomas were also negative for SFN, although a few (13%) did show SFN expression. Functionally, SFN did not facilitate invasiveness of A549 cells, but induced their proliferation. These findings indicate that SFN plays a

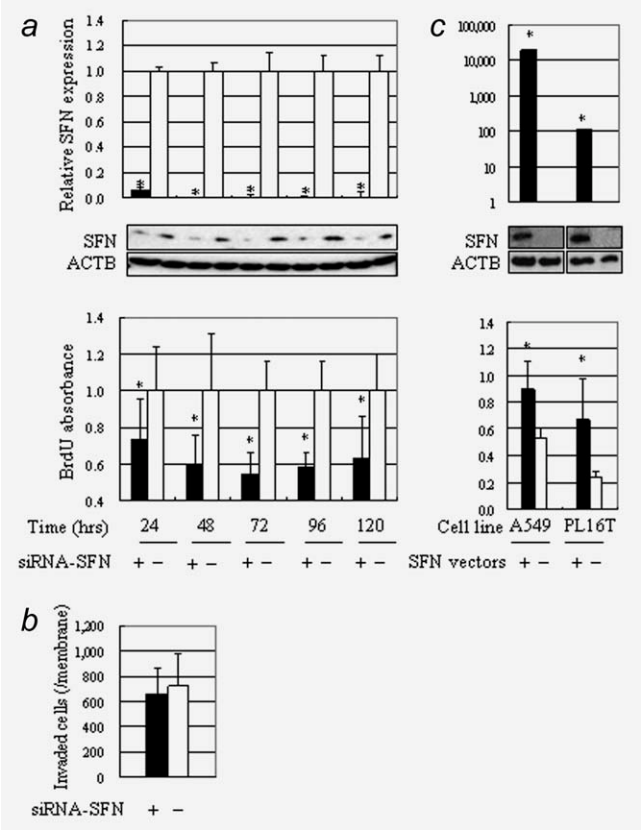

Figure 3. Functional analysis of SFN using the A549 line. (*a*) Transfection with siRNA-SFN and cell proliferation assay for the A549 line. A549 cells were transfected with siRNA-SFN and sampled every 24 h for 120 h. Expression of SFN mRNA and protein was determined by quantitative real-time RT-PCR and Western blotting, respectively. Scrambled siRNA was used as a control. Cell proliferation was found to decrease continuously for 120 h in A549 transfected with siRNA-SFN (bottom panel). (*b*) Invasion assay of A549 cells transfected with siRNA-SFN and scrambled siRNA. The assays were performed 48 h after siRNA transfection. Invasiveness remained unchanged by siRNA-SFN transfection. (*c*) Transfection of A549 and PL16T cells with SFN expression vectors and cell proliferation assay. A549 or PL16T cells were transfected with SFN expression vectors and sampled after 24 h. Expression of SFN mRNA and protein was determined by quantitative real-time RT-PCR and Western blotting, respectively. GFP expression vectors were used as controls. Cell proliferation activity was increased in both cell lines after transfection with the SFN expression vector.

common key role in tumor progression from *in situ* adenocarcinoma (types A and B) to invasive adenocarcinoma (types CF) by enhancing tumor cell proliferation. Interestingly, SFN-positive *in situ* carcinomas and negative cases could not be differentiated histologically. We speculated that the former may have the potential for progression to invasive adenocarcinoma, or may already be early invasive adenocarcinoma functionally. Clinically, with regard to the choice of therapeutic strategy for

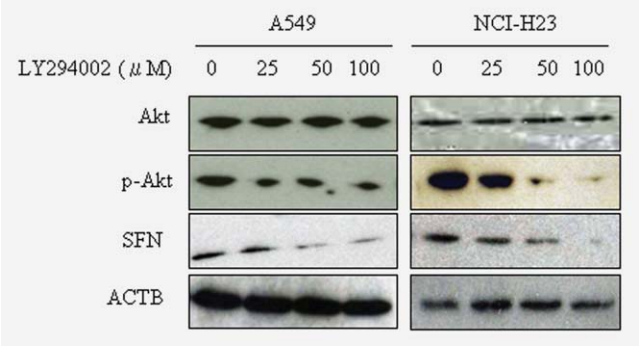

Figure 4. Pathway analysis of SFN. A549 or NCI-H23 cells were cultured in 60-mm dishes (5×10^5 cells/dish). Serum-starved cells were pretreated or untreated with LY294002 for 1 h. The phosphorylation states of PI3K were determined by Western blotting with phosphate-specific antibodies. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

lung adenocarcinoma, the Noguchi classification is quite important because it directly reflects prognosis, and types A and B tumors are targets for limited surgery. However, histological differentiation between type B and type C tumors is sometimes difficult. Therefore, histological differentiation is critical for therapeutic decision-making. In this study, we showed that SFN was characteristically expressed in most type C tumors, whereas more than 80% of type A and B tumors were negative for it. Even the *in situ* spreading areas of type C tumors were positive, similarly to the invasive areas. Therefore, SFN IHC and immunocytochemistry using biopsy and cytology materials are very valuable tools for differentiating between type C and type B tumors. In this study, early invasive adenocarcinoma was included among type C tumors, but we did not try to differentiate them from pure invasive adenocarcinoma, since no concrete histological classification of early invasive adenocarcinoma has yet emerged. Hereafter, it would be very informative to examine the expression of SFN in early invasive adenocarcinoma. On the other hand, type A (pure bronchioloalveolar carcinoma) and type B (pure bronchioloalveolar carcinoma with collapse) tumors are both adenocarcinoma *in situ*, but are also sometimes difficult to differentiate histologically. In this study, the rate of SFN positivity in type A (14%) was similar to that in type B (12%) (Table 2). These results suggest that the two types resemble each other, not only histologically but also biologically.

Zhang *et al.* showed that SFN mediates cell cycle progression *via* the PI3K/Akt pathway in a p53-independent manner.[9] We also found that the PI3K/Akt signaling pathway plays a critical role in stimulating SFN gene expression in a p53-independent manner, as PI3K inhibitor blocked SFN expression in both A549 cells carrying wild-type p53 and NCI-H23 cells harboring a p53 mutation. No similar responses were seen for MAPK inhibitor (U0126) or JNK1/2 inhibitor (SP600125). The aggressive behavior of cancer cells is determined by a complex array of signaling pathways that regulate key functions, such as growth, survival, migration,

and invasion. The PI3K/Akt signaling pathway has been causally linked to all four of these responses.[33–36] Further evidence of the importance of PI3K/Akt signaling in cancer comes from studies that have detected overexpression and hyperactivation of PI3K/Akt in a wide range of human tumors, including lung cancer, and this is often linked with poor prognosis.[37] Also in lung adenocarcinoma, tumor progression is reportedly facilitated through that pathway.[37] Taken together, the available data suggest that SFN has the potential to facilitate lung adenocarcinoma cell proliferation *via* the PI3K/Akt pathway.

In this study, we demonstrated that overexpression of SFN is a universal and key event during the course of malignant progression of lung adenocarcinoma. Overexpression of SFN in lung adenocarcinoma was associated with cell proliferation, and the overexpressed SFN was located downstream of the PI3K/Akt pathway, at least in the lung adenocarcinoma cell line we examined, A549. As the mechanism responsible for SFN overexpression remains unknown, genetic alteration of SFN and events downstream of the SFN pathway should be examined to clarify the molecular machinery of malignant transformation in lung adenocarcinoma.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin* 2009;59:225–49.
2. Noguchi M, Morikawa A, Kawasaki M, Matsuno Y, Yamada T, Hirohashi S, Kondo H, Shimosato Y. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. *Cancer* 1995; 75:2844–52.
3. Suzuki K, Yokose T, Yoshida J, Nishimura M, Takahashi K, Nagai K, Nishiwaki Y. Prognostic significance of the size of central fibrosis in peripheral adenocarcinoma of the lung. *Ann Thorac Surg* 2000;69:893–7.
4. Sakurai H, Maeshima A, Watanabe S, Suzuki K, Tsuchiya R, Maeshima AM, Matsuno Y, Asamura H. Grade of stromal invasion in small adenocarcinoma of the lung: histopathological minimal invasion and prognosis. *Am J Surg Pathol* 2004;28:198–206.
5. Shimada A, Kano J, Ishiyama T, Okubo C, Iijima T, Morishita Y, Minami Y, Inadome Y, Shu Y, Sugita S, Takeuchi T, Noguchi M. Establishment of an immortalized cell line from a precancerous lesion of lung adenocarcinoma, and genes highly expressed in the early stages of lung adenocarcinoma development. *Cancer Sci* 2005;96:668–75.
6. Aoyagi K, Tatsuta T, Nishigaki M, Akimoto S, Tanabe C, Omoto Y, Hayashi S, Sakamoto H, Sakamoto M, Yoshida T, Terada M, Sasaki H. A faithful method for PCR-mediated global mRNA amplification and its integration into microarray analysis on laser-captured cells. *Biochem Biophys Res Commun* 2003;300:915–20.
7. Esme H, Cemek M, Sezer M, Saglam H, Demir A, Melek H, Unlu M. High levels of oxidative stress in patients with advanced lung cancer. *Respirology* 2008;13:112–6.
8. Perathoner A, Pirkebner D, Brandacher G, Spizzo G, Stadlmann S, Obrist P, Margreiter R, Amberger A. 14-3-3sigma expression is an independent prognostic parameter for poor survival in colorectal carcinoma patients. *Clin Cancer Res* 2005; 11:3274–9.
9. Zhang Y, Karas M, Zhao H, Yakar S, LeRoith D. 14-3-3sigma mediation of cell cycle progression is p53-independent in response to insulin-like growth factor-I receptor activation. *J Biol Chem* 2004;279: 34353–60.
10. Dougherty MK, Morrison DK. Unlocking the code of 14-3-3. *J Cell Sci* 2004;117: 1875–84.
11. Fu H, Subramanian RR, Masters SC. 14-3-3 proteins: structure, function, and regulation. *Annu Rev Pharmacol Toxicol* 2000;40:617–47.
12. Hermeking H. The 14-3-3 cancer connection. *Nat Rev Cancer* 2003;3:931–43.
13. Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW, Vogelstein B. 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell* 1997;1:3–11.
14. Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* 1999;401: 616–20.
15. Laronga C, Yang HY, Neal C, Lee MH. Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression. *J Biol Chem* 2000;275: 23106–12.
16. Dellambra E, Golisano O, Bondanza S, Siviero E, Lacal P, Molinari M, D'Atri S, De Luca M. Downregulation of 14-3-3sigma prevents clonal evolution and leads to immortalization of primary human keratinocytes. *J Cell Biol* 2000;149:1117–30.
17. Umbricht CB, Evron E, Gabrielson E, Ferguson A, Marks J, Sukumar S. Hypermethylation of 14-3-3 sigma (stratifin) is an early event in breast cancer. *Oncogene* 2001;20:3348–53.
18. Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, Hermeking H, Marks JR, Lambers AR, Futreal PA, Stampfer MR, Sukumar S. High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. *Proc Natl Acad Sci USA* 2000;97: 6049–54.
19. Gasco M, Bell AK, Heath V, Sullivan A, Smith P, Hiller L, Yulug I, Numico G, Merlano M, Farrell PJ, Tavassoli M, Gusterson B, et al. Epigenetic inactivation of 14-3-3 sigma in oral carcinoma: association with p16(INK4a) silencing and human papillomavirus negativity. *Cancer Res* 2002;62:2072–6.
20. Iwata N, Yamamoto H, Sasaki S, Itoh F, Suzuki H, Kikuchi T, Kaneto H, Iku S, Ozeki I, Karino Y, Satoh T, Toyota J, et al. Frequent hypermethylation of CpG islands and loss of expression of the 14-3-3 sigma gene in human hepatocellular carcinoma. *Oncogene* 2000; 19:5298–302.
21. Lodygin D, Diebold J, Hermeking H. Prostate cancer is characterized by epigenetic silencing of 14-3-3sigma expression. *Oncogene* 2004;23:9034–41.
22. Suzuki H, Itoh F, Toyota M, Kikuchi T, Kakiuchi H, Imai K. Inactivation of the 14-3-3 sigma gene is associated with 5′ CpG island hypermethylation in human cancers. *Cancer Res* 2000;60:4353–7.
23. Urano T, Saito T, Tsukui T, Fujita M, Hosoi T, Muramatsu M, Ouchi Y, Inoue S. Efp targets 14-3-3 sigma for proteolysis and promotes breast tumour growth. *Nature* 2002;417:871–5.
24. Nakanishi K, Hashizume S, Kato M, Honjoh T, Setoguchi Y, Yasumoto K. Elevated expression levels of the 14-3-3 family of proteins in lung cancer tissues. *Hum Antibodies* 1997;8:189–94.
25. Moreira JM, Ohlsson G, Rank FE, Celis JE. Down-regulation of the tumor suppressor protein 14-3-3sigma is a sporadic event in cancer of the breast. *Mol Cell Proteomics* 2005;4:555–69.
26. Osada H, Tatematsu Y, Yatabe Y, Nakagawa T, Konishi H, Harano T, Tezel E, Takada M, Takahashi T. Frequent and histological type-specific inactivation of 14-3-3sigma in human lung cancers. *Oncogene* 2002;21:2418–24.
27. Villaret DB, Wang T, Dillon D, Xu J, Sivam D, Cheever MA, Reed SG. Identification of genes overexpressed in

head and neck squamous cell carcinoma using a combination of complementary DNA subtraction and microarray analysis. *Laryngoscope* 2000;110:374–81.
28. Shimomura K, Sakakura C, Fujita Y, Nakanishi M, Hagiwara A, Yamagishi H. [Overexpression of 14-3-3 in primary gastric cancers and clinical application for the detection of micrometastases in the peritoneal cavity]. *Nippon Geka Gakkai Zasshi* 2002;103:386.
29. Friess H, Ding J, Kleeff J, Fenkell L, Rosinski JA, Guweidhi A, Reidhaar-Olson JF, Korc M, Hammer J, Buchler MW. Microarray-based identification of differentially expressed growth- and metastasis-associated genes in pancreatic cancer. *Cell Mol Life Sci* 2003; 60:1180–99.
30. Iacobuzio-Donahue CA, Ashfaq R, Maitra A, Adsay NV, Shen-Ong GL, Berg K, Hollingsworth MA, Cameron JL, Yeo CJ, Kern SE, Goggins M, Hruban RH. Highly expressed genes in pancreatic ductal adenocarcinomas: a comprehensive characterization and comparison of the transcription profiles obtained from three major technologies. *Cancer Res* 2003;63: 8614–22.
31. Logsdon CD, Simeone DM, Binkley C, Arumugam T, Greenson JK, Giordano TJ, Misek DE, Kuick R, Hanash S. Molecular profiling of pancreatic adenocarcinoma and chronic pancreatitis identifies multiple genes differentially regulated in pancreatic cancer. *Cancer Res* 2003;63: 2649–57.
32. Ide M, Nakajima T, Asao T, Kuwano H. Inactivation of 14-3-3sigma by hypermethylation is a rare event in colorectal cancers and its expression may correlate with cell cycle maintenance at the invasion front. *Cancer Lett* 2004;207: 241–9.
33. Yoeli-Lerner M, Toker A. Akt/PKB signaling in cancer: a function in cell motility and invasion. *Cell Cycle* 2006;5: 603–5.
34. Cantley LC. The phosphoinositide 3-kinase pathway. *Science* 2002;296:1655–7.
35. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
36. Bouwknecht JA, Paylor R. Behavioral and physiological mouse assays for anxiety: a survey in nine mouse strains. *Behav Brain Res* 2002;136:489–501.
37. Chen CH, Lai JM, Chou TY, Chen CY, Su LJ, Lee YC, Cheng TS, Hong YR, Chou CK, Whang-Peng J, Wu YC, Huang CY. VEGFA upregulates FLJ10540 and modulates migration and invasion of lung cancer *via* PI3K/AKT pathway. *PLoS One* 2009;4:e5052.