Pathology - Research and Practice 265 (2025) 155721

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Expression of SPRED2 in the lung adenocarcinoma

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ARTICLE INFO

Keywords:
SPRED2
ERK1/2
Ki67
Lung adenocarcinoma

ABSTRACT

SPRED2 (Sprouty-related, EVH1 domain-containing protein 2), a negative regulator of the ERK1/2 pathway, is downregulated in several cancers; however, the significance of SPRED2 expression in lung adenocarcinoma (LUAD) remains unclear. Here, we investigated the pathological expression of SPRED2 and its relationship with ERK1/2 activation (ERK1/2 phosphorylation), Ki67 index and clinicopathological features in 77 LUAD tissues from clinical patients. Immunohistochemically, SPRED2 expression was decreased in invasive adenocarcinoma (IA) compared to adenocarcinoma in situ (AIS). There was a negative correlation between SPRED2 expression and pERK1/2 levels and a positive correlation between SPRED2 expression and Ki67 index. In the database analysis, the survival probability was higher in patients with higher SPRED2 expression than in those with lower expression. In vitro, SPRED2 deletion increased cell proliferation, migration and invasion of three LUAD cell lines (A549:KRAS mutation, H1993:METamplification, and HCC4006:EGFR mutation), whereas SPRED2 overexpression decreased these responses. Thus, SPRED2 appears to be a regulator of LUAD progression and a potential target for the treatment of LUAD.

1. Introduction

Lung cancer remains the leading cause of cancer-related death regardless of gender (21.4 % of all cancer deaths in the U.S.) [1]. There are two main types of lung cancer: non-small cell lung cancer (NSCLC, 85 % of all diagnoses) and small cell lung cancer (SCLC, 15 % of all diagnoses). Adenocarcinoma is the most common NSCLC worldwide, including Japan, and its incidence has been increasing [2,3]. Recent efforts have revealed genetic abnormalities in NSCLC. Among them, the epidermal growth factor receptor (EGFR)-RAS-RAF signaling pathway is frequently mutated in lung adenocarcinoma (LUAD) [4,5]. EGFR overexpression is common and often associated with EGFR mutations [6]. RAS mutations are the most common oncogenic mutations in human cancers, and KRAS is most highly muted within the RAS family in NSCLC, which is associated with poor prognosis [2,7]. BRAF mutations are found in 4 % of NSCLC, particularly in LUAD [2].

EGFR/RAS/RAF overexpression/mutations lead to constitutive activation of kinase pathways, including their downstream ERK1/2, resulting in uncontrolled cancer cell growth and proliferation [8]. Striking therapeutic responses are often achieved when aberrant activation of these pathways is successfully inhibited. Gefitinib and erlotinib, tyrosine kinase inhibitors of EGFR, have produced substantial clinical responses in NSCLC patients with somatic mutations within the EGFR kinase domain [9,10]. KRAS inhibitors, sotorasib and adagrasib, have been approved for advanced NSCLC and other inhibitors are currently in clinical trials [11]. The Food and Drug Administration (FDA) has expanded the uses of dabrafenib (BRAF inhibitor) and trametinib (MEK inhibitor) to include the treatment of patients with metastatic NSCLC harboring BRAF V600E mutations [12]. Thus, EGFR-RAS-RAF is a major driver of NSCLC, including LUAD.

SPRED2 (Sprouty-related, EVH1 domain-containing protein 2) is a member of the SPRED protein family and negatively regulates Ras-dependent ERK1/2 by suppressing phosphorylation and activation of RAF [13]. There are three isoforms of SPRED proteins, of which SPRED2 is ubiquitously expressed in all human tissues including the lung [14,15]. The expression of SPRED2 is frequently downregulated in cancers,

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https://doi.org/10.1016/j.prp.2024.155721
Received 10 April 2024; Received in revised form 9 November 2024; Accepted 11 November 2024
Available online 19 November 2024

such as hepatocellular carcinoma [16,17], prostate cancer [18], urothelial cancer [19], and colorectal cancer [20].

Since the EGFR-RAS-RAF pathway is over-activated in LUAD, the level of endogenous SPRED2 expression may influence LUAD pathology. In the present study, we investigated the level of SPRED2 protein expression in clinical LUAD. Using cell lines, potential functions of SPRED2 were investigated by deleting or overexpressing SPRED2 in LUAD cell lines. Our results suggest that downregulated SPRED2 expression in LUAD promotes its progression via upregulation of the ERK1/2 signaling pathway.

2. Materials and methods

2.1. Clinical samples

A total of 77 LUAD specimens surgically resected from 2012 to 2016 were retrieved from the pathology records of Okayama University Hospital. None of the patients received preoperative chemotherapy, radiotherapy, or immunotherapy. All hematoxylin and eosin (HE) stained sections used in this study were blindly reviewed by two pathologists. One representative tumor slide was selected from each case and the corresponding paraffin block was used for immunohistochemistry. The protocol of this study was reviewed and approved by the ethics committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital (1805–021). Informed consent was obtained via an opt-out form on our website. Those who refused were excluded. This consent procedure complied with the amended Ethical Guidelines for Clinical Studies of the Ministry of Health, Labor and Welfare of Japan (May 31, 2015).

2.2. Immunohistochemistry

SPRED2 immunostaining was performed using the Polink-2 plus HRP rabbit with DAB kit (Golden Bridge International, Bothell, WA, USA) according to the manufacturer's instructions. Briefly, Section (4-μm thick) were deparaffinized, rehydrated, and treated with 0.3 % H_2O_2 in methanol for 10 minutes at room temperature. After antigen retrieval by microwave in 0.1 M citric acid buffer (pH 6.0), sections were blocked with DAKO Protein Block Serum-Free (Dako, Carpinteria, CA, USA) and then incubated with anti-human SPRED2 polyclonal antibody (Proteintech, Rosemont, IL, USA) for 90 minutes at room temperature. After washing, sections were incubated with rabbit antibody specific enhancer (Golden Bridge International) for 15 minutes at room temperature, followed by incubation with polymer HRP for rabbit IgG (Golden Bridge International) for 30 minutes at room temperature and the antigen-antibody interaction was visualized with diaminobenzidine (Dako). Nuclear counterstaining was performed with hematoxylin. Immunostaining for phospho-44/42 MAPK (pERK1/2) (clone D13.14.4E, Cell Signaling Technology, Danvers, MA, USA), phospho-Akt (pAkt, Cell Signaling), and Ki67 (clone MIB-1, Dako) was performed on a Ventana Discovery XT automated stainer (Ventana, Tucson, AZ, USA) using the iVIEW DAB Detection Kit (Ventana).

2.3. Evaluation of immunohistochemical staining

Immunohistochemically stained specimens were scored using the H-score, a semi-quantitative scoring system [21]. Ten fields were randomly selected at ×400 magnification and the staining intensity was scored as 0 (no staining), 1+ (weak staining), 2+ (moderate staining), or 3+ (strong staining). The total number of cells in each field and the number of positively stained cells at each intensity were counted. The H-score was calculated as follows: [(% of 1+ cells) ×1 + (% of 2+ cells) ×2 + (% of 3+ cells) ×3] within the target region, ranging from 0 (100 % of cells score 0) to 300 (100 % of cells score 3+).

2.4. Data collection

Gene chip-based data from lung tumors, normal and metastatic tissues were obtained from TNMplot (https://tnmplot.com/analysis/). SPRED2 mRNA expression data in LUAD were obtained from The Cancer Genome Atlas (TCGA) dataset and analyzed using the University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN, https://ualcan.path.uab.edu). The prognostic value of SPRED2 mRNA expression in LUAD was evaluated using the data from the Kaplan-Meier database (http://www.kmplot.com) and UALCAN. A log-rank p-value <0.05 was considered statistically significant.

2.5. Cell culture

LUAD cell lines, A549 (KRAS mutation), H1993 (MET amplification) and HCC4006 (EGFR mutation), were obtained from the JCRB Cell Bank, Osaka, Japan, and cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10 % fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

2.6. Transfection

Cells were seeded in 6-well plates and incubated in a 5 % CO_2 incubator at 37°C until the cells reached 70–80 % confluence. For loss-of-function analysis, cells were transfected with 2 μg of SPRED2-specific or non-specific siRNAs (Thermo Fisher Scientific, MA, USA) using Lipofectamine RNAiMax (Thermo Fisher Scientific). For gain-of-function analysis, cells were transfected with SPRED2 expression plasmid (kindly provided by Dr. Masakiyo Sakaguchi, Okayama University, Japan) using Lipofectamine 3000 (Thermo Fisher Scientific). Transfection was performed for 24 hours according to the manufacturer's instructions.

2.7. Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from cultured cells using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany). First-strand cDNA was synthesized from 2 μg total RNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). RT-qPCR was performed using a StepOnePlus system (Thermo Fisher Scientific). Taqman gene expression assay kits (Thermo Fisher Scientific) were used (SPRED2: Hs00986220_ml, GAPDH: Hs02758991_gl). The expression level of SPRED2 gene was normalized to the expression level of GAPDH.

2.8. Western blotting

Cells were lysed using lysis buffer (Cell Signaling Technology). Protein concentration in the lysates was measured by protein-dye binding assay (Bio-Rad, Hercules. CA, USA). Samples (15 μg each) were loaded on gels, and proteins were fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Thermo Fisher Scientific) and transferred to nitrocellulose membranes. After blocking, the membranes were incubated overnight with a primary antibody, followed by a horseradish peroxidase-conjugated secondary antibody. The antibodies used in this study were anti-SPRED2 (Proteintech), anti-44/42 MAPK (ERK1/2), anti-phospho-44/42 MAPK (pERK1/2), anti-CyclinD1, and anti-GAPDH (Cell Signaling Technology). The target proteins were visualized using ImmunoStar LD (Wako, Osaka, Japan), and the membranes were scanned using a C-DiGit blot scanner (LI-COR Biotechnology, Lincoln, NE). The blot images were semi-quantitated using Image Studio software.

2.9. MTT assay

Cells were seeded at 2000 cells/well (100 μl of cell suspension) in a

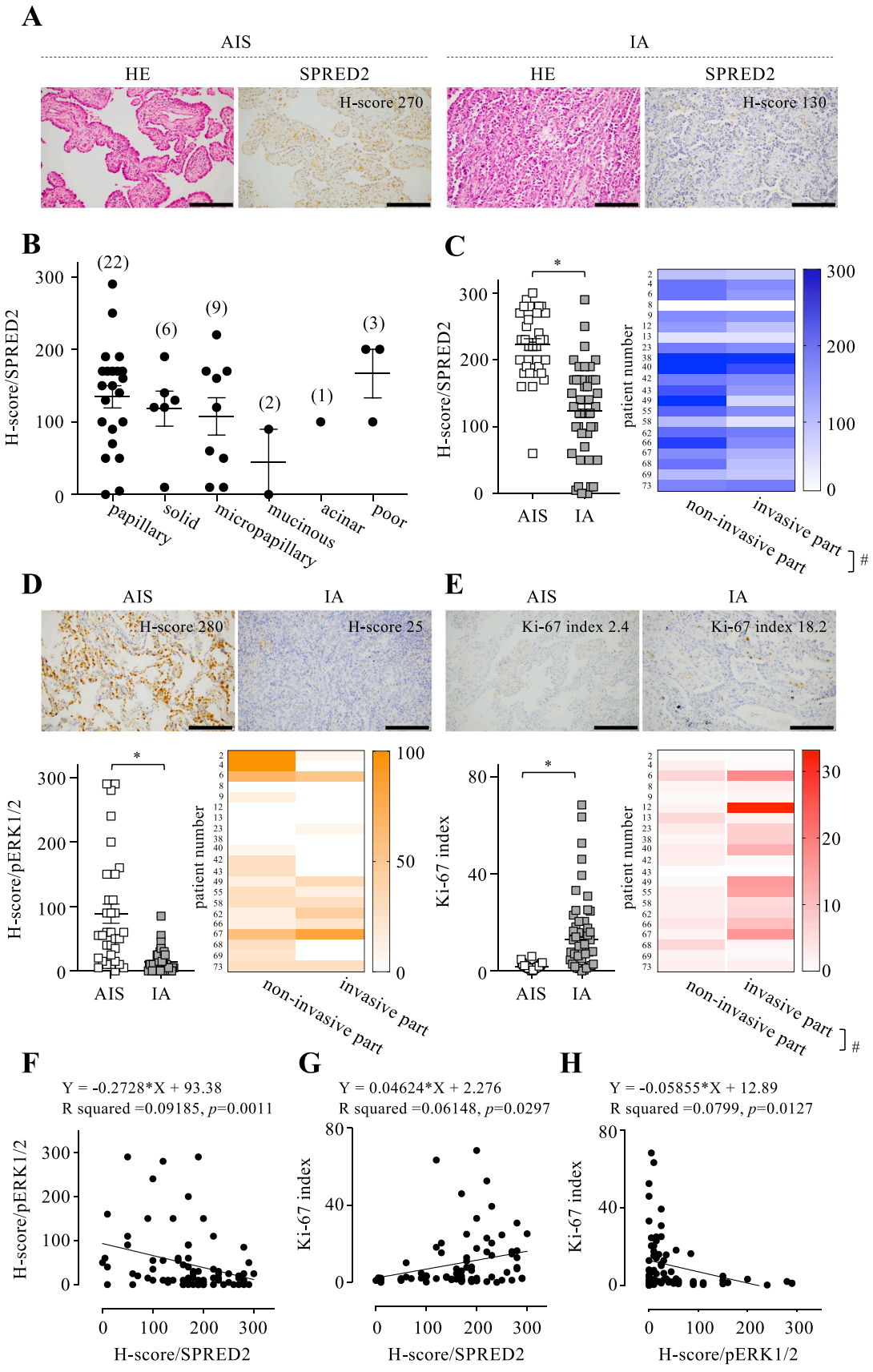

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Fig. 1. Expression of SPRED2, pERK1/2 and Ki67 in LUAD tissues. (A) Representative photographs of HE-staining (original magnification 200×) and SPRED2 immunohistochemistry (original magnification 200×) of adenocarcinoma in situ (AIS) and invasive adenocarcinoma (IA) tissues are shown. Immunohistochemically stained specimens were scored using the H-score. (B) H-scores of SPRED2 among the different histologic types of IA are shown (papillary; n=22, solid; n=6, micropapillary; n=9, mucinous; n=2, acinar; n=1, poor; n=3). (C) Left, H-scores of SPRED2 in AIS (n=34) and IA (n=43) are shown. Right, H-scores of SPRED2 in non-invasive part and invasive part of each IA (n=21) are shown. (D) Upper, Representative photographs of pERK1/2 immunohistochemistry (original magnification 200×) of AIS and IA tissues are shown. Lower left, H-scores of pERK1/2 in AIS (n=34) and IA (n=43) are shown. Lower right, H-scores of pERK1/2 in non-invasive part and invasive part of each IA (n=21) are shown. (E) Upper, Representative photographs of Ki67 immunohistochemistry (original magnification 200×) of AIS and IA tissues are shown. Lower left, Ki67 indexes in AIS (n=34) and IA (n=43) are shown. Lower right, Ki67 indexes in non-invasive part and invasive part of each IA (n=21) are shown. (F-H). The relationships between H-score of SPRED2 and pERK1/2 (F), H-score of SPRED2 and Ki67 index (G), and H-score of pERK1/2 and Ki67 index (H) are shown. $^{\#}p<0.05$, $^{*}p<0.0001$ (two-tailed unpaired t-test).

Table 1
Relationship between SPRED2 expression and clinicopathological features.

Parameters	Category	H-score/SPRED2				*p* value[a]
		0	1–100	101–200	201–300	
Age	≤70 (n=39)	1	8	17	13	0.9212
	>70 (n=38)	1	8	19	10	
Sex	F (n=46)	2	9	22	13	0.6694
	M (n=31)	0	7	14	10	
Size	≤3 cm (n=64)	0	12	30	22	0.0043
	>3 cm (n=13)	2	4	6	1	
Lymph node metastasis	No (n=70)	2	13	32	23	0.2137
	Yes (n=7)	0	3	4	0	
TNM stage	0-I (n=64)	0	11	31	22	0.0018
	II-III (n=13)	2	5	5	1	
EGFR mutation	No (n=45)	2	7	20	16	0.2468
	Yes (n=32)[b]	0	9	16	7	

[a] Chi-square test
[b] Gene mutations: L858R (n=17), del19 (n=12), G719A (n=2), L861Q (n=1), G719C (n=1)

96-well plate. Cell growth was determined using the MTT assay (Roche, Mannheim, Germany). Optical density (OD) values at 570 nm were determined using a microplate reader. A higher absorbance value indicates an increase in cell proliferation. Each assay was performed in triplicate.

2.10. Cell migration and invasion assays

Cell migration and invasion were evaluated by a Boyden chamber-based assay using an uncoated (for migration) or Matrigel-coated (for invasion) Transwell membrane filter (insert pore size, 8 μm) in a 24-well plate (BD Biosciences, Franklin Lakes, NJ). After 24 hours of starvation, cells (2×10^5) were cultured with serum-free (migration assay) or 1 % FBS-containing (invasion assay) medium in the upper chamber, and the lower chamber was filled with medium containing 10 % FBS. After incubation for 48 hours, cells passing through the filter were counted after staining with 0.01 % crystal violet in 25 % methanol. Three low-power fields (20× magnification) were randomly selected from each membrane to count migrated cells. Experiments were performed in triplicate.

2.11. Statistics

Data from the database were analyzed using Dunn's multiple comparison test. Survival prognosis was evaluated using the log-rank test. Data from clinical samples and in vitro studies were analyzed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). After data were normalized, statistical significance was analyzed using the parametric two-tailed unpaired t-test. Contingency table analysis was performed using chi-squared and Fisher's exact test. Data are expressed as mean ± SEM. A value of $p<0.05$ was considered statistically significant.

3. Result

3.1. SPRED2 expression in LUAD tissues

We first examined the expression of SPRED2 protein in 77 LUAD tissues from clinical patients. SPRED2 staining was evaluated by H-score based on the staining intensity and the percentage of stained cells (Fig. 1A). In all cases, SPRED2 was weakly stained in the lung epithelial cells in the non-cancer area (not shown). The relationship between SPRED2 expression and clinicopathological features in each category was evaluated. SPRED2 was highly expressed in LUAD less than 3 cm in size and in early TNM stages (stage 0-I) (Table 1). No statistical significance was found between SPRED2 expression and age, gender, with or without lymph node metastasis, and EGFR mutations (Table 1). There was no difference in SPRED2 expression between the different histological types of LUAD (Fig. 1B). Notably, SPRED2 expression was significantly lower in invasive adenocarcinoma (IA, n=43) than in adenocarcinoma in situ (AIS, n=34) (Fig. 1C, left). Among 43 cases of IA, 21 cases were accompanied by a non-invasive part, and SPRED2 expression in the invasive part was lower than that in the non-invasive part (Fig. 1C, right). Thus, SPRED2 expression was higher in AIS compared with IA and in the non-invasive part in IA.

3.2. Expression of pERK1/2 and Ki67 in LUAD tissues

Cancer progression depends on the proliferative capacity of cells, and ERK1/2-MAPK plays a critical role in cancer development and progression [22]. We examined the protein expression of pERK1/2 and Ki67, an indicator of cell proliferation marker, in LUAD tissues. There was no positive staining for either pERK1/2 or Ki67 in the lung epithelial cells in the non-cancer area (not shown). The expression of pERK1/2 was high in AIS, but significantly decreased in IA (Fig. 1D, upper and lower left). In IA, there was a trend towards lower pERK1/2 expression in the invasive part (p=0.14) compared to the non-invasive part (Fig. 1D, lower right). The Ki67 index was significantly higher in IA than in AIS (Fig. 1E, upper and lower left), and the Ki67 index in the invasive part was higher than that in the non-invasive part (Fig. 1E, lower right).

EFGR mutations, detected in 32 out of 77 LUAD cases, could differently affect the expression of SPRED2, pERK1/2 and Ki67. However, there were no statistically significant differences in the expression of these three molecules between patients with or without EGFR mutations in either AIS or IA (Supplementary Fig. 1). Thus, IA appeared to express lower levels of pERK and higher levels of Ki67 than in AIS, regardless of EGFR mutations. The relationship between SPRED2, pERK1/2 and Ki67 expression was next examined. There was a negative correlation between SPRED2 and pERK1/2 expression (Fig. 1F), a positive correlation between SPRED2 and Ki67 expression (Fig. 1G), and finally a negative correlation between pERK1/2 and Ki67 expression in LUAD (Fig. 1H).

3.3. Database analyses of SPRED2 expression and survival prognosis

We then examined SPRED2 expression in the cancer database TNMplot. SPRED2 expression was significantly lower in tumor and

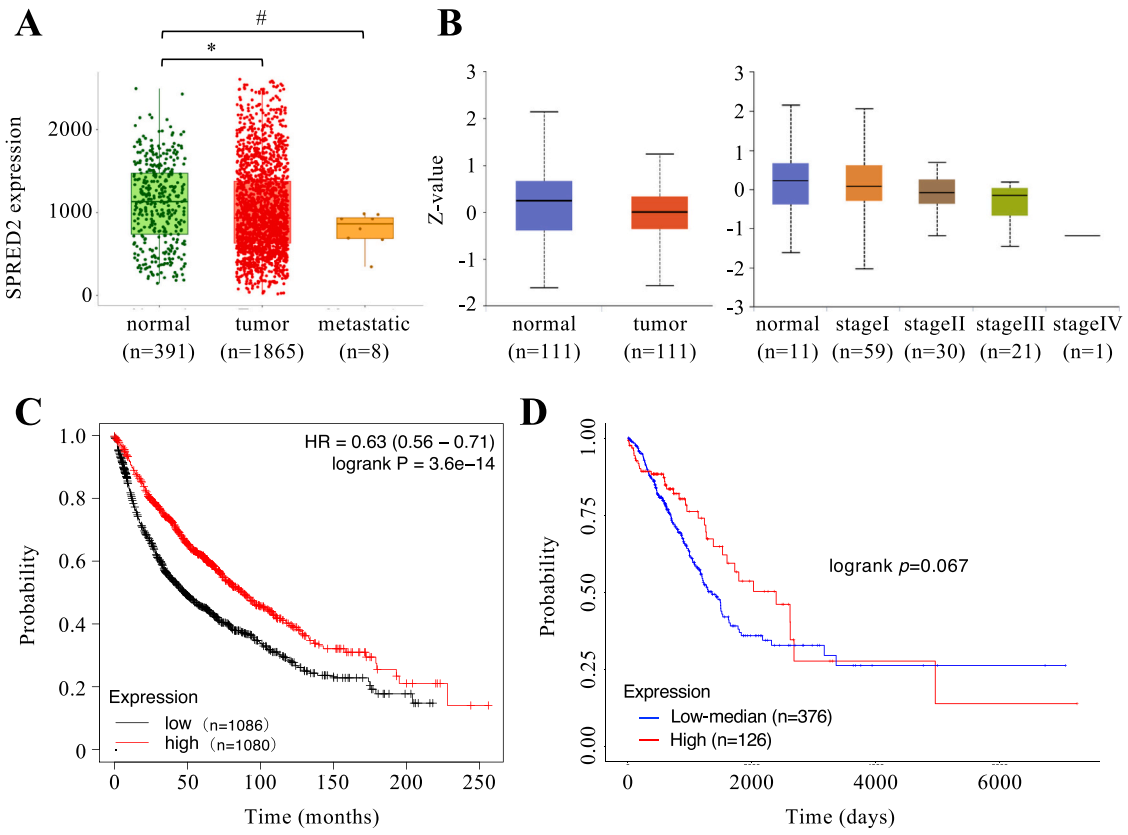

Fig. 2. Database analysis of SPRED2 expression and survival prognosis. (A) SPRED2 expression data in lung tumor, normal and metastatic tissues were collected from TNMplot. $^{\#}p<0.05$, $^{*}p<0.0001$ (Dunn's multiple comparison test). (B) SPRED2 mRNA expression data in lung adenocarcinoma from The Cancer Genome Atlas (TCGA) dataset were analyzed using the University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN). (C, D) The prognostic value of SPRED2 mRNA expression in lung adenocarcinoma was evaluated using data from the Kaplan-Meier database (C) and UALCAN (D). A log-rank p value <0.05 was considered to indicate a statistically significant difference.

metastatic samples than in normal lung samples (Fig. 2A). In the TCGA dataset, SPRED2 expression was lower in LUAD than in normal tissue, although it was not statistically significant (p=0.190, Fig. 2B, left). SPRED2 expression tended to decrease with TNM stage progression (Fig. 2B, right). We then evaluated the prognostic value of SPRED2 in LUAD patients using the Kaplan-Meier plot. The probability of survival for 250 months was higher in patients with higher SPRED2 mRNA levels (Fig. 2C). Median survival in the high SPRED2 expression cohort (88 months) was 1.8 times longer than in the low expression cohort (48 months). Patients with high expression of SPRED2 tended to have a better prognosis than those with low and median expression in the TGCA database derived prognosis (Fig. 2D). Thus, the expression level of SPRED2 may serve as a marker for lung cancer progression.

3.4. *SPRED2 negatively regulates ERK1/2 activation in LUAD cells*

To understand the molecular role of SPRED2 in LUAD, in vitro studies were performed using three LUAD cell lines (A549, H1993, HCC4006) with different genetic features; A549 with a KRAS mutation, H1993 with MET amplification and HCC4006 with an EGFR mutation. All three cell lines expressed detectable levels of endogenous SPRED2, but the expression level was different among them, highest in A549 and lowest in HCC4006 (Fig. 3A,B). To confirm the function of SPRED2 in ERK1/2 activation, SPRED2-knockdown (KD) or overexpression (OE) cells were generated by transfection with SPRED2-specific siRNA or SPRED2 expression plasmid, respectively. The efficiency of transfection was determined by RT-qPCR (Fig. 3C). Under these conditions, the ratio of pERK1/2 to total ERK1/2 (tERK1/2) was increased in the SPRED2-KD group in all cell lines and decreased by SPRED2-OE in H1993 and HCC4006 cells, but not in A549 cells (Fig. 3D). These results indicated that SPRED2 can downregulate the ERK1/2 signaling pathway independent of the genetic mutations or amplifications.

3.5. *SPRED2 downregulates cell proliferation, migration and invasiveness in LUAD cells*

Altered SPRED2 expression might affect cancer cell behavior. In all three cell lines, cell proliferation was higher in SPRED2-KD cells and lower in SPRED2-OE cells than in control cells (Fig. 4A). The expression of the cell cycle marker cyclin D1 was increased in SPRED2-KD cells and decreased in SPRED2-OE cells (Fig. 4B). Cell migration (Fig. 4C) and invasive capacity (Fig. 4D) were higher in SPRED2-KD cells and lower in SPRED2-OE cells than in control cells. Taken together, these results suggest that SPRED2 downregulates cell proliferation, migration, and invasion in LUAD cells regardless of the different genetic abnormalities.

4. Discussion

We and others have shown that SPRED2 downregulates proliferation, migration, epithelial-mesenchymal transition (EMT) and stemness in cancer cells by inhibiting the ERK1/2 pathway [17,20,23,24]. However, the role of SPRED2 in lung cancer remains largely unexplored. In this study, we showed that SPRED2 was highly expressed in LUAD less than 3 cm in size and in early TNM stages (stage 0-I). As in cancers of other organs, SPRED2 expression was significantly decreased in IA compared to AIS. When examined in IA tissues, SPRED2 was expressed

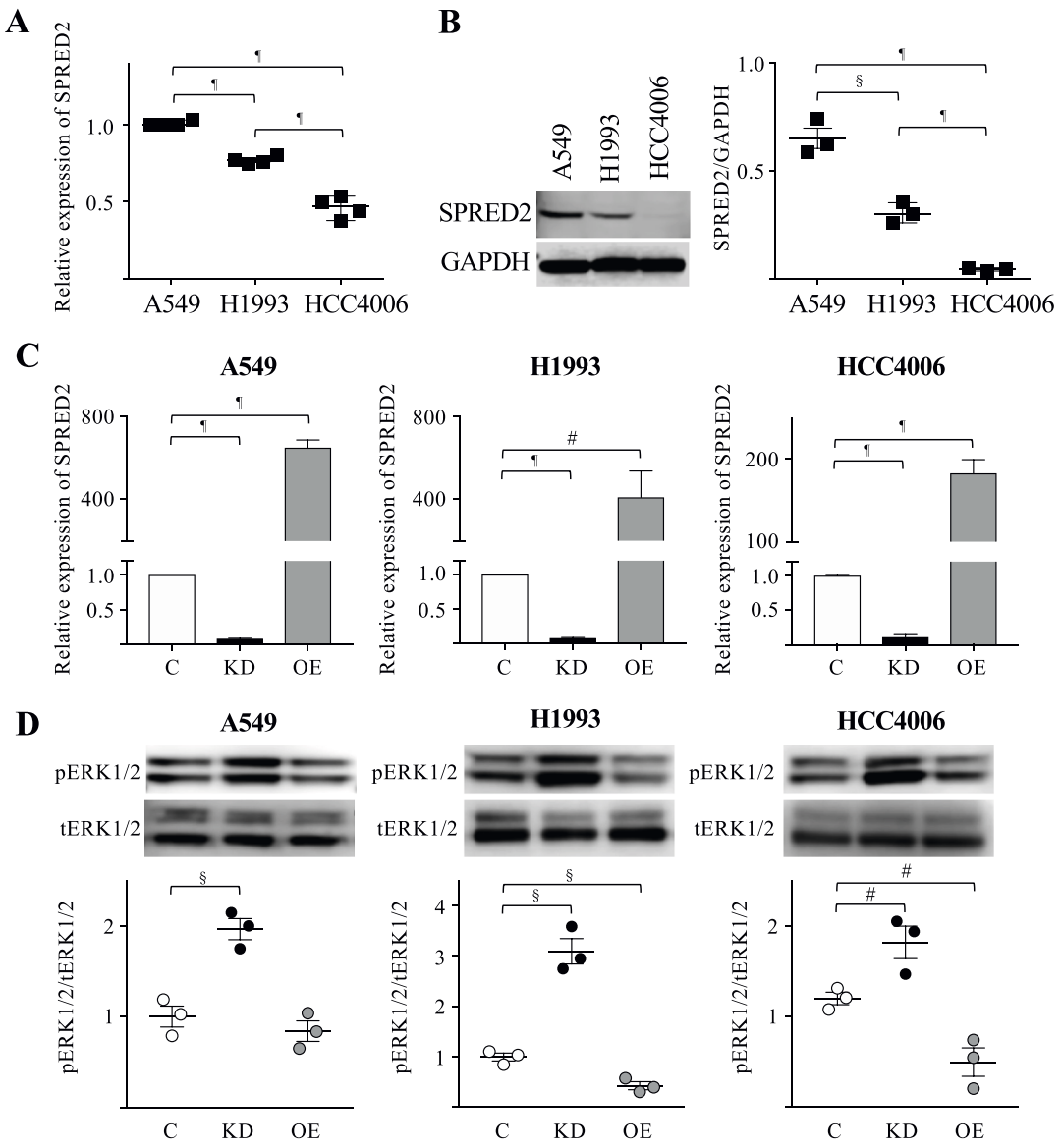

Fig. 3. SPRED2 negatively regulates ERK1/2 activation in LUAD cells. (A, B) The expression level of SPRED2 in A549, H1993 and HCC4006 cells was evaluated by RT-qPCR (A) and Western blotting (B). (A) The level in A549 was considered as 1. (B) Band densities were digitized and semi-quantitated (n=3, each). (C) SPRED2 expression levels in A549, H1993, and HCC 4006 were evaluated by RT-qPCR after knockdown (KD) or overexpression (OE) of SPRED2. The level in the control of each cell type was considered as 1 (n=3, each). (D) ERK1/2 phosphorylation in A549, H1993, and HCC4006 after knockdown (KD) or overexpression (OE) of SPRED2 was evaluated by Western blotting (n=3, each).

at lower levels in the invasive part compared to the non-invasive part. The expression of pERK was also lower in IA than in AIS; however, there was a negative correlation between SPRED2 and pERK1/2 expression. In the database analysis, the survival probability was higher in patients with higher SPRED2 mRNA expression compared to those with lower expression. In vitro studies clearly demonstrated that SPRED2 reduced cancer cell proliferation, migration and invasiveness through down-regulation of the ERK1/2 pathway. Taken together, our results support the hypothesis that SPRED2 functions as a negative regulator of LUAD progression.

Comprehensive studies using whole exome sequencing (WES) and/or transcriptome sequencing (RNA-seq) have identified alterations in the RTK/RAS/RAF signaling in 70–80 % of LUAD [4], which may lead to upregulation of the downstream ERK1/2 pathway. There was a strong statistical correlation between pERK staining and advanced stages [25]. High ERK phosphorylation was associated with tumor aggressiveness and poor prognosis [26]. In contrast, our study showed that pERK1/2 expression was significantly lower in IA than in AIS, regardless of EGFR mutations. We selected untreated patients to avoid the influence of treatment. Therefore, most tumors were small in size (≤3 cm, 83 %) and the percentage of stage II/III (17 %) was low. Differences in clinical case background may account for this difference; however, it remains unclear why pERK1/2 expression was lower in IA in our study.

Ki67, a nuclear protein involved in the regulation of cell proliferation, has been reported to predict survival in many cancers, such as breast cancer [27] and prostate cancer [28]. Our present results showed that the Ki67 index was significantly increased in IA compared to AIS in our clinical samples. High expression of Ki67 is clinically relevant in terms of prognostic and clinicopathologic features of lung cancer [29]. Unexpectedly, there was a positive correlation between SPRED2 and Ki67 index. This could be a feedback mechanism where the expression of SPRED2 is upregulated to suppress high mitotic activity. Another possibility is that the expression of Ki67 is regulated by other signaling pathways, such as the PI3K-Akt pathway, as the ERK1/2 and PI3K-Akt pathways have been reported to stimulate or inhibit each other [30–32]. Therefore, we examined pAkt expression and its relationship

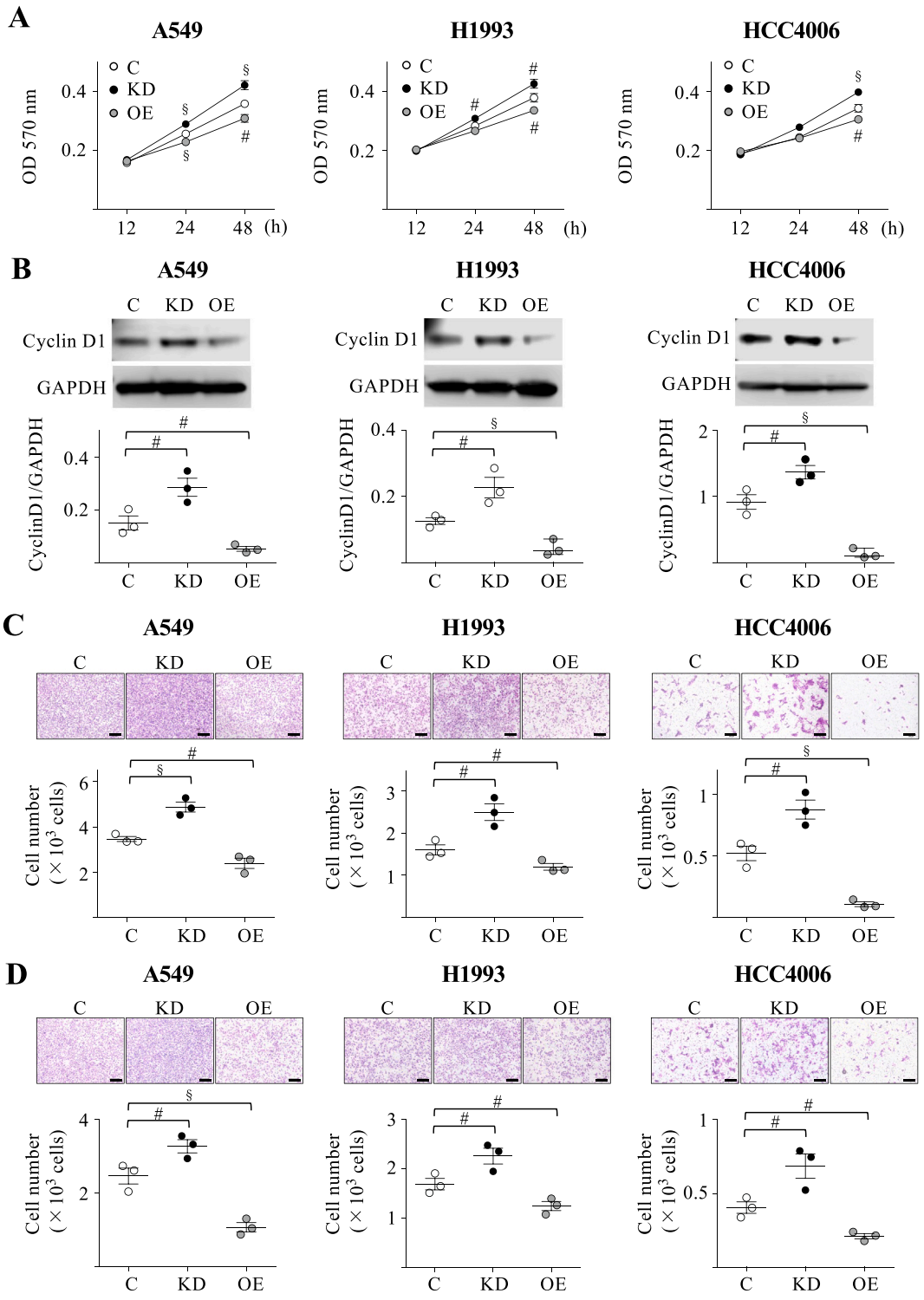

Fig. 4. SPRED2 downregulates cell proliferation, migration and invasiveness in LUAD cells. SPRED2 was knocked down (KD) or overexpressed (OE) in A549, H1993, and HCC4006 cells. (A) Proliferation of each cell line was assessed by MTT assay. (B) Cell lysates were prepared from control, SPRED2-KD and SPRED2-OE cells and the cyclin D1 expression was evaluated by Western blotting. Band densities were digitized and semi-quantitated (n=3, each). The expression level of cyclin D1 was normalized against the expression level of GAPDH. (C, D) Cell migration (D) and invasion assay (D). Upper, representative photos. Scale bars: 100 μm. Lower, three low-power fields (magnification, 10×) were randomly selected from each membrane for cell counting (n=3, each). $^{\#}p<0.05$, $^{\S}p<0.01$, two-tailed unpaired t-test.

with SPRED2 expression in our LUAD tissues, but there was no difference in pAkt expression between AIS and IA, regardless of EGFR mutations. No correlation was found between pAkt and SPRED2 expression (Supplementary Fig. S2). These results suggest that Ki67 expression is not regulated by the PI3K-Akt pathway. Further studies are needed to identify signaling factors involved in cancer growth in LUAD.

In conclusion, we demonstrated that SPRED2 expression is decreased in invasive LUAD tissues. Database analyses revealed that low expression of SPRED2 was significantly associated with poor prognosis in LUAD patients. In vitro studies suggest that SPRED2 inhibits cell proliferation, migration and invasion of LUAD cells via downregulation of the ERK1/2 signaling pathway. Our results suggest that SPRED2 is a

novel molecular target for the treatment of LUAD.

Funding

This work was supported in part by JSPS KAKENHI Grant number 25293095, 21H02988 and 16K15258.

CRediT authorship contribution statement

Teizo Yoshimura: Writing – review & editing, Investigation. **Shinichi Toyooka:** Investigation. **Akihiro Matsukawa:** Writing – review & editing, Funding acquisition, Formal analysis, Conceptualization. **Tong Gao:** Investigation, Data curation. **Yoko Ota:** Writing – original draft, Investigation, Data curation. **Sumardika I Wayan:** Investigation. **Masayoshi Fujisawa:** Investigation, Data curation. **Masakiyo Sakaguchi:** Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Mr. Hiroyuki Watanabe for excellent technical assistance.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2024.155721.

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