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# *SPOCD1* Enhances Cancer Cell Activities and Serves as a Prognosticator in Esophageal Squamous Cell Carcinoma

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## Abstract

*Background/Aim:* Comprehensive transcriptome analysis has revealed SPOC Domain Containing 1 (SPOCD1) as a potential biomarker for esophageal squamous cell carcinoma (ESCC). However, the expression and oncological roles of SPOCD1 in ESCC remains underexplored. We aimed to evaluate the role of SPOCD1 in oncogenesis and prognosis of ESCC *in vitro* and *in vivo*.

*Materials and Methods:* The Cancer Cell Line Encyclopedia (CCLE) database was utilized to evaluate correlations between SPOCD1 expression and oncogenes in ESCC. mRNA and protein levels were measured by qRT-PCR and Simple Western assays, respectively. siRNA-mediated knockdown and overexpression experiments assessed the effects of SPOCD1 expression on proliferation, migration, and invasion of ESCC cell lines. *In vivo*, siRNA knockdown effects on tumor growth were tested in mouse xenograft models. SPOCD1 mRNA levels in 164 resected tissues were correlated with clinicopathological parameters and survival, while a cohort of 177 patients was analyzed for protein expression and survival.

*Results:* SPOCD1 mRNA expression varied widely among ESCC cell lines and correlated with epithelial-mesenchymal transition-related genes. Knockdown significantly suppressed proliferation, migration, and invasion ($p<0.001$), while overexpression increased proliferation ($p<0.001$). *In vivo*, siRNA knockdown reduced tumor growth compared to both si-control ($p=0.005$) and untransfected groups ($p<0.001$). High SPOCD1 mRNA expression was linked to poor disease-specific survival ($p=0.009$, HR=1.965, 95% CI=1.187-3.252) and disease-free survival ($p=0.047$, HR=1.602, 95% CI=1.007-2.549). Similarly, elevated protein levels were associated with unfavorable disease-specific ($p=0.013$, HR=1.860, 95% CI=1.137-3.041) and disease-free survival ($p=0.032$, HR=1.618, 95% CI=1.042-2.513).

*Conclusion:* SPOCD1 expression correlates with the aggressiveness of ESCC cells, and its expression levels in tumor tissues may serve as a prognostic factor for ESCC patients.

**Keywords:** Esophageal cancer, squamous cell carcinoma, SPOCD1, prognostic biomarker.

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## Introduction

Esophageal cancer is the eleventh most common cancer and seventh leading cause of worldwide cancer mortality (1). Esophageal cancer is histopathologically classified into the subtypes as follows: adenocarcinoma and squamous cell carcinoma (ESCC), which is the predominant histopathological type in the Asian population and developing countries (2). Despite recent advances in multimodal therapy (3, 4) comprising surgery with chemotherapy and radiotherapy for locally advanced disease and chemotherapy with immunotherapy or radiotherapy for metastatic disease, the overall prognosis of ESCC is dismal (5-7). Therefore, identification of novel biomarkers for ESCC will enhance development of improved strategies for risk stratification, individualized treatment, and follow-up.

Squamous cell cancer antigen (SCC), carcinoembryonic antigen (CEA) and cytokeratin-19-fragment (CYFRA21-1) are commonly used in clinical practice for ESCC. However, those biomarkers provide only limited accuracy for detection and risk stratification of ESCC (8-10). Although numerous researchers are actively seeking novel biomarkers, the majority of published studies remain constrained by several limitations, such as the use of single-institution cohorts, reliance on mRNA or protein expression levels for survival analysis, and the absence of *in vivo* experiments to substantiate their findings (11-14).

We therefore reasoned that assessing mRNA and protein expression levels in multiple cohorts with functional analysis using *in vitro* and *in vivo* studies may contribute to the discovery of potential prognostic biomarkers for ESCC with improved validity. For this purpose, we conducted comprehensive transcriptome analysis of tissues from patients with simultaneous distant metastasis, which revealed that SPOC domain containing 1 (*SPOCD1*) may serve as a potential candidate. *SPOCD1* encodes a member of the transcription factor S-II (TFIIS) family and acts as an essential executor of pi-RNA mediated transposon methylation (15). Furthermore, *SPOCD1* is associated with healing of the human gingiva after surgery (16). Recent studies show that SPOCD1 contributes to the malignant behaviors of gastric cancer, osteosarcoma, clear cell renal cell carcinoma glioma, bladder cancer, and ovarian cancer (17-23). Furthermore, bioinformatic data (24) show that ESCC expresses *SPOCD1;* however, in-depth expression analysis and data on the oncological functions of *SPOCD1* are lacking.

Building on previous knowledge, we aimed to further elucidate the role of SPOCD1 in ESCC oncogenesis, investigate its involvement in potential signaling pathways, and evaluate its impact on prognosis. Therefore, we assessed effects of *SPOCD1* expression on cellular functions, of ESCC cells using siRNA-mediated knockdown assays *in vitro* and *in vivo*, as well as enforced expression experiments *in vitro*. Furthermore, we evaluated the prognostic validity of *SPOCD1* expression at the mRNA and protein levels through an analysis of clinical samples from two different ESCC patient cohorts that correlated with clinical outcomes.

## Materials and Methods

*Ethics approval and consent to participate.* The present study conformed to the ethical guidelines outlined in the World Medical Association Declaration of Helsinki for Medical Research Involving Human Subjects. Approval for the research was obtained from the Institutional Review Board of Nagoya University, Japan (approval number 2014-0044) as well as from the Ethics Committee of Akita University School of Medicine (number 1943). Written informed consent for the use of clinical samples and data was acquired from patients prior to the study, either directly or through the appropriate database. Animal experiments were performed according to the ARRIVE guidelines (25) and were approved (approval number M240274-005) by the Animal Research Committee of Nagoya University.

*Transcriptome analysis.* We performed transcriptome analysis of tissues from 8 patients with metastatic ESCC. For this purpose, we used the HiSeq platform (Illumina,

Table I. *Sequences of primers and siRNAs.*

| | Experiment | Primer sequence (5'→3') | Product size | Annealing temperature |
|---|---|---|---|---|
| *SPOCD1* | RT-qPCR | F: CGT TCC ATG CAG GAG GTA CT<br>R: AAC AGC AGG CTG CGA TAC TT | 164 bp | 60˚C |
| | si-RNA | si-SPOCD1-1: CACUAAGAACCUGAAGAAATT<br>si-SPOCD1-2: GCAUGACCACCACUUCUUATT<br>si-SPOCD1-3: AGGUGGAGAAGAGAUACUATT | | |
| Control | si-RNA | si-Control: GCAAACAUCCCAGAGGUAU | | |
| *GAPDH* | RT-qPCR | F: GAAGGTGAAGGTCGGAGTC<br>Probe: CAAGCTTCCCGTTCTCAGCC<br>R: GAAGATGGTGATGGGATTTC | 221 bp | 60˚C |

SPOCD1, SPOC Domain Containing 1; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; RT qPCR, quantitative real time reverse transcription polymerase chain reaction; si-RNA, small interfering RNA; F, forward; R, reverse.

Inc., San Diego, CA, USA) to compare the mRNA expression levels of 57,749 genes in primary ESCC tissues with those of the corresponding normal esophageal mucosa.

*Cell lines and clinical samples.* The human ESCC cell lines KYSE30, KYSE70, KYSE140, KYSE150, KYSE180, KYSE270, KYSE450, KYSE510, KYSE590, KYSE890, KYSE1170, KYSE1260, and KYSE1440 were obtained from the Japanese Collection of Research Bio Resources Cell Bank (Osaka, Japan) (26). TE2, TE3, TT, Het1A, and TTn were obtained from the American Type Culture Collection (Manassas, VA, USA). NUEC2 and WSSC cell lines were established at Nagoya University. (27) Cell culture was performed using RPMI-1640 medium with 10% fetal bovine serum, and the cell lines were maintained in an atmosphere containing 5% $CO_2$ at 37˚C (28). Cell lines were authenticated by the Japanese Collection of Research Bio Resources Cell Bank using the short tandem repeat PCR method.

Primary ESCC tissue and corresponding normal mucosa were acquired from 164 patients who underwent esophageal resection at Nagoya University Hospital between October 2001 and January 2016. According to standard guidelines, fluorouracil combined with platinum-based neoadjuvant chemotherapy (NAC) was recommended since 2006 for patients with clinical stages II to III ESCC, unless contraindicated (4, 29). Upon resection, the tissue samples were promptly frozen in liquid nitrogen and preserved at –80˚C until RNA extraction (30). Specimens were confirmed as ESCC through histological examination according to the 8th edition of the Union for International Cancer Control (UICC) staging system for esophageal cancer (31).

The second cohort comprised 177 primary tissue samples collected from patients with ESCC who underwent radical esophageal resection at Akita University Hospital between February 2000 and July 2011. Before curative surgery, patients were untreated. A tissue microarray (TMA) using paraffin embedded ESCC tissues was constructed at the Pathology Institute (Toyama, Japan). Triplicate samples were punched and arranged on the TMA (32).

*Determination of SPOCD1 mRNA expression levels.* Total RNA (10 μg per sample) was extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) from 20 cell lines and 164 ESCC tissues, along with the corresponding adjacent noncancerous tissues. After verifying RNA quality by measuring optical density (absorbance ratio 260/280 nm between 1.8 and 2.0), complementary DNAs (cDNAs) were synthesized. Expression levels of *SPOCD1* mRNA were determined using qRT-PCR as previously described (33, 34). Each sample was tested in triplicate, and negative

controls without templates were included in each PCR plate. Specific primers are listed in Table I. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA, which served as an internal standard (35) was used to calculate the relative *SPOCD1* level in each sample.

*Pathway analysis. SPOCD1* mRNA expression data for 25 ESCC cell lines were obtained from the CCLE database (https://sites.broadinstitute.org/ccle/) and correlated with the expression levels of well-characterized genes related to cancer signal transduction pathways including those associated with the epithelial-mesenchymal transition (EMT) as well as the p53, apoptosis, and VEGF pathways. A heatmap illustrating *SPOCD1* expression and its correlation with cancer-related genes were designed using the Heatmapper tool (http://www.heatmapper.ca/).

*Small interfering RNA (siRNA)-mediated knockdown and enforced expression of SPOCD1.* Three *SPOCD1*-specific siRNAs were designed using siDirect (http://sidirect2.rnai.jp/) and i-Score Designer (https://www.med.nagoya-u.ac.jp/neurogenetics/i_Score/i_score.html), and a mixture of these three siRNAs was used (36). KYSE590 and KYSE70 cells were each seeded at $2\times10^5$ cells/ml into 6-well plates each and incubated overnight. The cells were then transiently transfected using LipoTrust EX Oligo (Hokkaido System Science, Sapporo, Japan) and 100 nM each siRNA (three *SPOCD*-specific siRNAs or a control siRNA). The sequences are listed in Table I. Subsequent to transfection, cells for conducting functional assays were incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), but without antibiotics, for 48 h.

To achieve enforced expression of *SPOCD1*, the *SPOCD1* Human-Tagged Open Reading Frame Clone sequences were inserted into pLenti-C-Myc-DDK-P2A-Puro (product ID, RC218945L3V; OriGene, Rockville, MD, USA), and transduction was performed according to the manufacturer's protocol. We seeded $5\times10^4$ KYSE1440 cells into a 24 well plate, and the cells were transfected with lentiviral vectors at a multiplicity of infection (MOI) 2 in the presence of polybrene (8 μg/ml) for 16 h. A control vector purchased from GenScript (Piscataway, NJ, USA), and 2,500 ng was introduced into the cells using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA). Functional analyses were performed 72 h after transfection or transduction. To assess knockdown and knock-in efficacies of *SPOCD1*, RT-qPCR analysis was performed as described (37).

*Simple Western assays.* To detect knockdown effects of siRNA on protein expression level and to verify findings from the CCLE database analysis, Simple Western assays (ProteinSimple, San Jose, CA, USA) were performed using the Jess Protein Normalization Module (ProteinSimple) according to the manufacturer's protocol.(38) Gels were loaded with 5 μg of total protein and probed with the antibodies as follows (obtained from Cell Signaling Technologies, unless otherwise indicated): polyclonal rabbit anti-*SPOCD1* antibody (1:50, PAB22580; Abnova, Taipei, Taiwan, ROC), rabbit anti-Vimentin (#5741), rabbit anti-N-Cadherin (#13116), rabbit anti-E-Cadherin (#3195), anti-ZO1 (#8193), anti-Beta-catenin (#8480), and mouse anti-β-actin (ab6276, Abcam, Cambridge, UK). Protein expression levels were normalized to those of total protein, and the data were evaluated using the Compass for Simple Western software (ProteinSimple).

*In vitro functional analyses.* The WST-8 cell proliferation kit was employed (Cell Counting Kit-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). KYSE590 and KYSE70 cancer cell lines ($2\times10^3$ cells/well each) were seeded into 96-well plates in RPMI-1640 medium containing 5% or 2% FBS, respectively. The optical density (OD) of the solution (450 nm) in each well was measured at 450-nm 2 h after adding 10 μl of WST-8. We then calculated the fold-change in OD from the initial day of seeding (day 0). We used KYSE 590 and KYSE70 cell lines for cell proliferation assays to determine the effect of *SPOCD1* knockdown on high expression cells. Furthermore, we used low expression KYSE1440 cells to assess the effects of forced expression of *SPOCD1* on cell proliferation.

Migration (ibidi GmbH, Martinsried, Germany) and invasion (BioCoat Matrigel invasion chambers; BD Biosciences, Bedford, MA, USA) were evaluated as previously described (17, 18). We used a wound-healing assay to assess KYSE590 cell migration. The wound width was measured 20-times per well at 100-mm intervals. In the invasion assay, eight fields were randomly chosen from each well, and the numbers of invaded cells were manually counted. KYSE590 lacks the ability to penetrate the Matrigel membrane; therefore, the KYSE70 cell line was used for the invasion assay.

*In vivo subcutaneous xenograft model.* The Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines were followed for all animal experiments (25). The Animal Research Committee of Nagoya University approved (approval number M240274-005) experiments using animals. The experiments were carried out between October 30, 2024, and November 20, 2024. Six-week-old male non-obese diabetic mice with severe combined immunodeficiency (Nod-SCID) were obtained from Chubu Kagaku Shizai (Nagoya, Japan) and housed in groups of four per cage, with free access to water and food for at least one week before conducting experiments in temperature-controlled rooms. We used KYSE70 cells to compare subcutaneous tumor growth among untransfected, si-control, and si-*SPOCD1* groups. Cells in each group ($5\times10^6$ cells each) were resuspended in 100 µl of phosphate-buffered saline (PBS) and the entire 100 µl was subcutaneously injected into the right flanks of the mice ($n$=8/group). Transient knockdown of siRNA can last for 5-days, so we arranged our animal experiment in less time with frequent tumor measurements. Thus, tumor growth was measured on days 6, 8, 10, 12 and 14; and the mice were killed on day 14 after injection before the effect of transient knockdown on tumor growth ceased, and the size of si-*SPOCD1* group tumors reached the size of the other groups. A total of 8 mice from each group were euthanized in a designated area by exposure to 100% $CO_2$ at a controlled fill rate of 50% of the chamber volume per minute for 5 mins. Mice were closely monitored in a transparent chamber, and $CO_2$ flow was maintained for at least 1 min after the cessation of both cardiac and respiratory function and dilated pupils were observed. After these observations, cervical dislocations were performed as a secondary measure to ensure death. Tumor weight was compared after all mice were killed. Approximate tumor volumes ($mm^3$)=$d^2\times D/2$, where $d$ and $D$ are the shortest and longest diameters, respectively.

*Clinical significance of SPOCD1 mRNA expression levels.* Patients were classified into two groups according to the upper quartile value of *SPOCD1* mRNA expression of 164 patients in the transcript cohort. We evaluated the correlations between *SPOCD1* mRNA expression, clinicopathological parameters, and survival analysis that included disease-free survival (DFS), disease-specific survival (DSS), and recurrence pattern-specific survival. We used the Kaplan–Meier Plotter tool with ESCC datasets from TGCA to externally validate our institutional data (39).

*Immunohistochemical analysis.* TMA blocks of the second cohort were sectioned and incubated for 60 min at room temperature with a rabbit polyclonal antibody against SPOCD1 (HPA031714, MilliporeSigma, St. Louis, MO, USA) diluted 1:50 in ChemMate antibody diluent (Dako, Carpinteria, CA, USA). Two investigators, who were uninformed regarding the clinical data, performed semiquantification of SPOCD1 expression, which was scored as 3+ (intense cytoplasmic or nuclear staining), 2+ (moderate cytoplasmic or nuclear staining), 1+ (weak staining), or 0 (undetectable staining). Triplicates of each sample were individually assessed, and an average score ≥2 from the triplicate was categorized as high SPOCD1 expression.

*Statistical analysis.* An independent-samples $t$-test was performed to compare the expression levels of genes between normal and cancer tissues. Comparisons in the experimental and clinical analysis data between the two groups were analyzed using the Mann–Whitney or $\chi^2$

Table II. *Overview of transcriptome data. Genes significantly overexpressed (p<0.05) in primary cancerous tissues compared to normal adjacent tissues from patients (n=8) with metastatic esophageal cancer. The prognostic relevance of these genes was assessed using the Kaplan-Meier plotter tool, based on data from the TCGA database.*

| Function | Symbol | Full name | Localization | Cancer/Normal | | KM plotter 5-year OS | | |
|---|---|---|---|---|---|---|---|---|
| | | | | $Log_2$ | *p*-Value | HR | 95% CI | *p*-Value |
| Transcription factor | *SPOCD1* | SPOC Domain Containing 1 | Nucleus | 3.60 | <0.0001 | 3.37 | 1.26-8.96 | 0.0114 |
| | *GSC* | Goosecoid Homeobox | Nucleus | 4.50 | <0.0001 | 2.24 | 0.96-5.23 | 0.0566 |
| | *DPF1* | Double PHD Fingers 1 | Nucleus | 4.34 | <0.0001 | 1.98 | 0.89-4.41 | 0.0900 |
| Enzymes | *CST4* | Cystatin S | Extracellular | 5.08 | <0.0001 | 2.37 | 0.99-5.63 | 0.0452 |
| | *CST2* | Cystatin SA | Extracellular | 3.60 | 0.0044 | 2.01 | 0.89-4.56 | 0.0868 |
| | *AGBL4* | ATP/GTP Binding Protein Like 4 | Golgi apparatus | 3.15 | 0.0175 | 1.86 | 0.84-4.11 | 0.1207 |
| Cellular adhesin | *CDHR2* | Cadherin Related Family Member 2 | Plasma membrane | 3.37 | 0.0093 | 2.07 | 0.86-5.01 | 0.0977 |
| Metabolic proteins | *SLC5A12* | Solute Carrier Family 5 Member 12 | Plasma membrane | 4.34 | <0.0001 | 2.69 | 1.15-6.29 | 0.0182 |
| | *APOC1* | Apolipoprotein C1 | Extracellular | 3.62 | <0.0001 | 2.96 | 1.27-6.88 | 0.0089 |
| | *MUCL1* | Mucin Like 1 | Golgi apparatus, Plasma membrane | 3.47 | 0.0003 | 3.53 | 1.20-10.35 | 0.0147 |
| Keratin-associated protein | *KRTAP4-1* | Keratin-Associated Protein 4-1 | Cytosol | 4.81 | <0.0001 | 3.55 | 1.52-8.32 | 0.0018 |
| Neuron related proteins | *NECAB2* | N-Terminal EF-Hand Calcium Binding Protein 2 | Plasma membrane | 3.57 | 0.0001 | 2.18 | 0.93-5.12 | 0.0670 |
| | *KIAA0319* | KIAA0319 | Plasma membrane | 3.03 | 0.0107 | 2.02 | 0.90-4.56 | 0.0835 |
| | *CALCB* | Calcitonin Related Polypeptide Beta | Extracellular | 3.05 | 0.0221 | 1.81 | 0.82-3.99 | 0.1348 |
| Others | *SPANXA1* | Sperm Protein Associated With The Nucleus, X-Linked, Family Member A1 | Nucleus | 3.03 | 0.0249 | 2.11 | 0.96-4.67 | 0.0584 |
| | *C19orf81* | Chromosome 19 Open Reading Frame 81 | Cytoskeleton | 3.14 | 0.0003 | 3.70 | 1.41-9.73 | 0.0052 |
| | *VWA5B2* | Von Willebrand Factor A Domain Containing 5B2 | Nucleus | 4.65 | <0.0001 | 2.12 | 0.96-4.66 | 0.0570 |

Normal, corresponding adjacent normal esophageal tissue; Cancer, primary cancer tissue; KM, Kaplan Meier; OS, overall survival; HR, hazard ratio.

tests. Gene correlations were determined using the Pearson Correlation method. The Kaplan–Meier method was employed to generate DFS, DSS curves. The hazard ratio and *p*-value calculation were performed using the Cox proportional hazards model. Univariable and multivariable Cox proportional hazards models were used to evaluate the prognostic factors of ESCC. Variables which were statistically significant in univariable analysis were included in the multivariable model. Statistical analyses were performed using SPSS software (SAS Institute Inc., Cary, NC, USA), and *p*-values of less than 0.05 were considered statistically significant.

## Results

*Identification of SPOCD1 from transcriptome data.* Transcriptome analysis detected 17 genes that were significantly overexpressed in primary cancer tissue

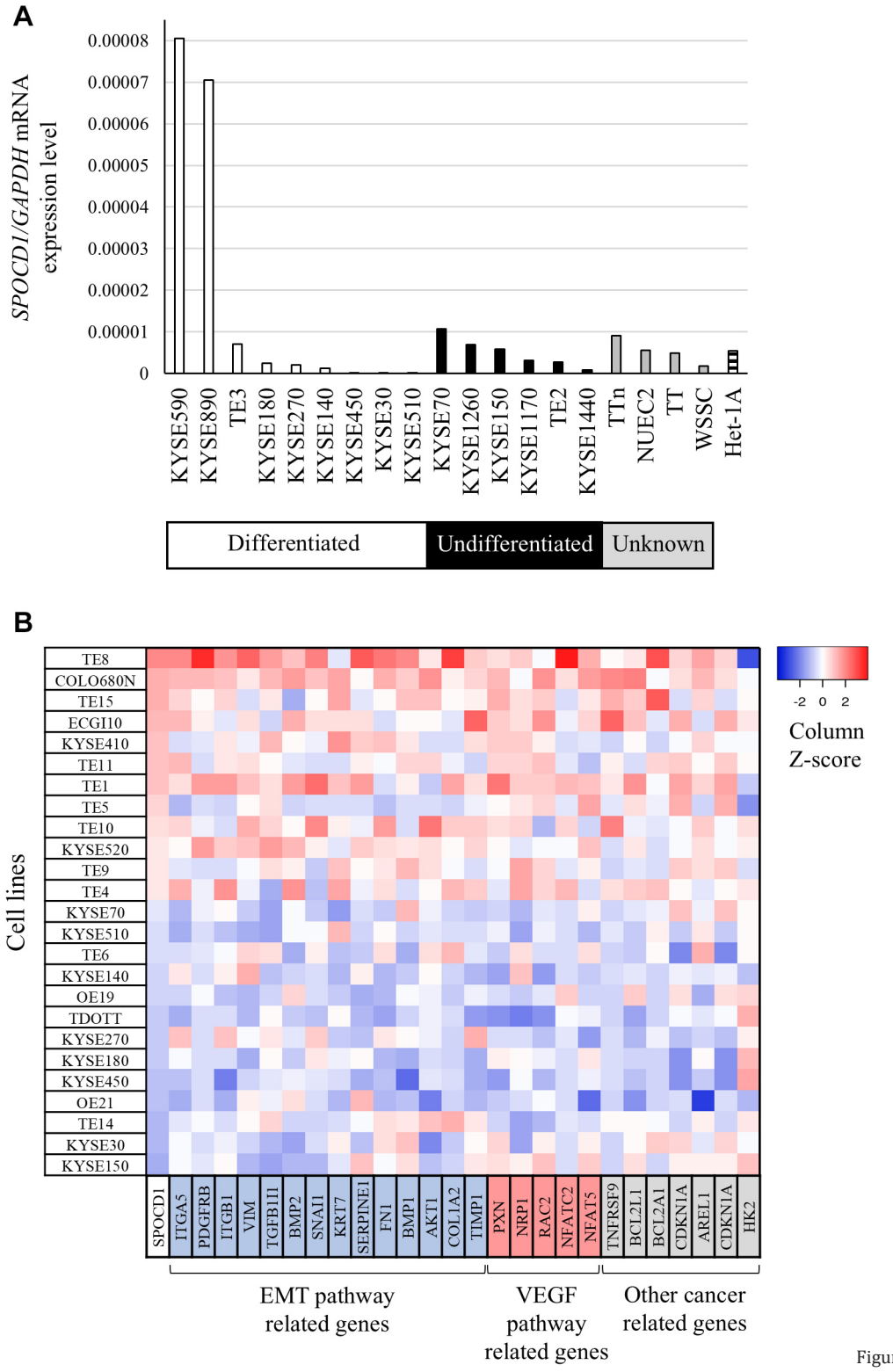


Figure 1. *Continued*

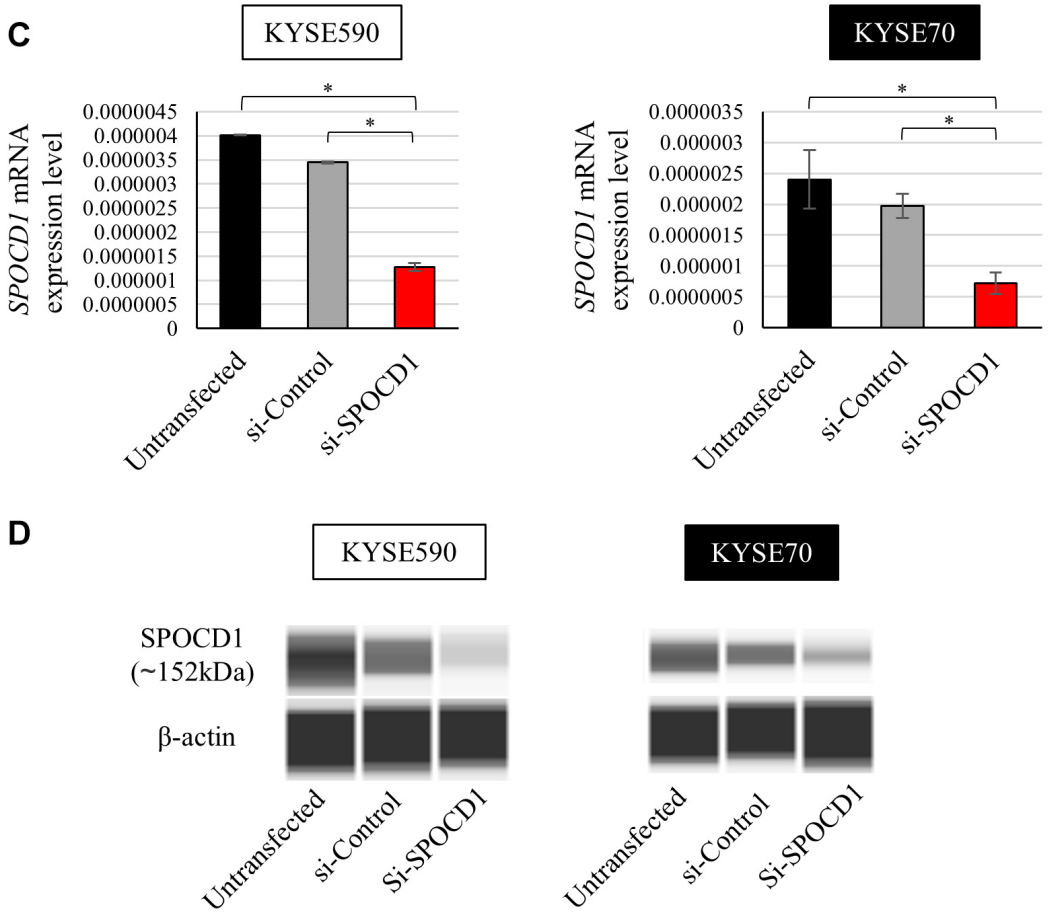


Figure 1. *SPOCD1 mRNA expression and the effects of siRNA-mediated SPOCD1 knockdown in ESCC cells. (A) SPOCD1 mRNA levels in 20 ESCC cell lines. (B) Heatmap from CCLE data illustrating expression of genes highly correlated with SPOCD1 and their related pathways in 25 ESCC lines (C) siRNA-mediated knockdown effect of SPOCD1 in KYSE590 and KYSE70 cells on SPOCD1 expression. (D) Simple Western images illustrating detection of SPOCD1 and siRNA-mediated knockdown efficiency of SPOCD1 in KYSE590 and KYSE70 cells. Error bars indicate the standard deviation. *p<0.05, **p<0.01, ***p<0.001.*

compared to the normal mucosa (Table II). After conducting a literature review, we decided to study *SPOCD1* in detail, despite the findings of a previous report, for the following reasons: (i) *SPOCD1* functions as a transcription factor, consistent with our research interests. (ii) The nucleotide sequence of *SPOCD1* is available. (iii) *SPOCD1* achieved good prognostic capability according to our analysis of the TGCA dataset [$p$=0.0114, hazard ratio (HR) 3.37]. (iv) The role of *SPOCD1* in esophageal cancer is insufficiently studied.

*SPOCD1 mRNA expression in ESCC cell lines and pathway analysis. SPOCD1* expression levels in 20 ESCC cell lines (Figure 1A) varied widely. The mRNA expression levels of *SPOCD1* and oncogenes in 25 cancer cell lines from the CCLE database are represented as a heatmap in Figure 1B. *SPOCD1* expression closely correlated with that of established EMT markers such as VIM and SNAI1 as well as other EMT-related genes including *ITGA5, PDGFRB, ITGB1, TGFB1I1, BMP2, KRT7, SERPINE1, FN1, BMP1, COL1A2* and *TIMP1*. Furthermore, expression levels of VEGF pathway-related genes (*PXN, NRP1, RAC2, NFATC2*, and *NFAT*) correlated with those of *SPOCD1*.

Consistent with these results, "Simple Western" expression analysis of EMT-related markers in SPOCD1

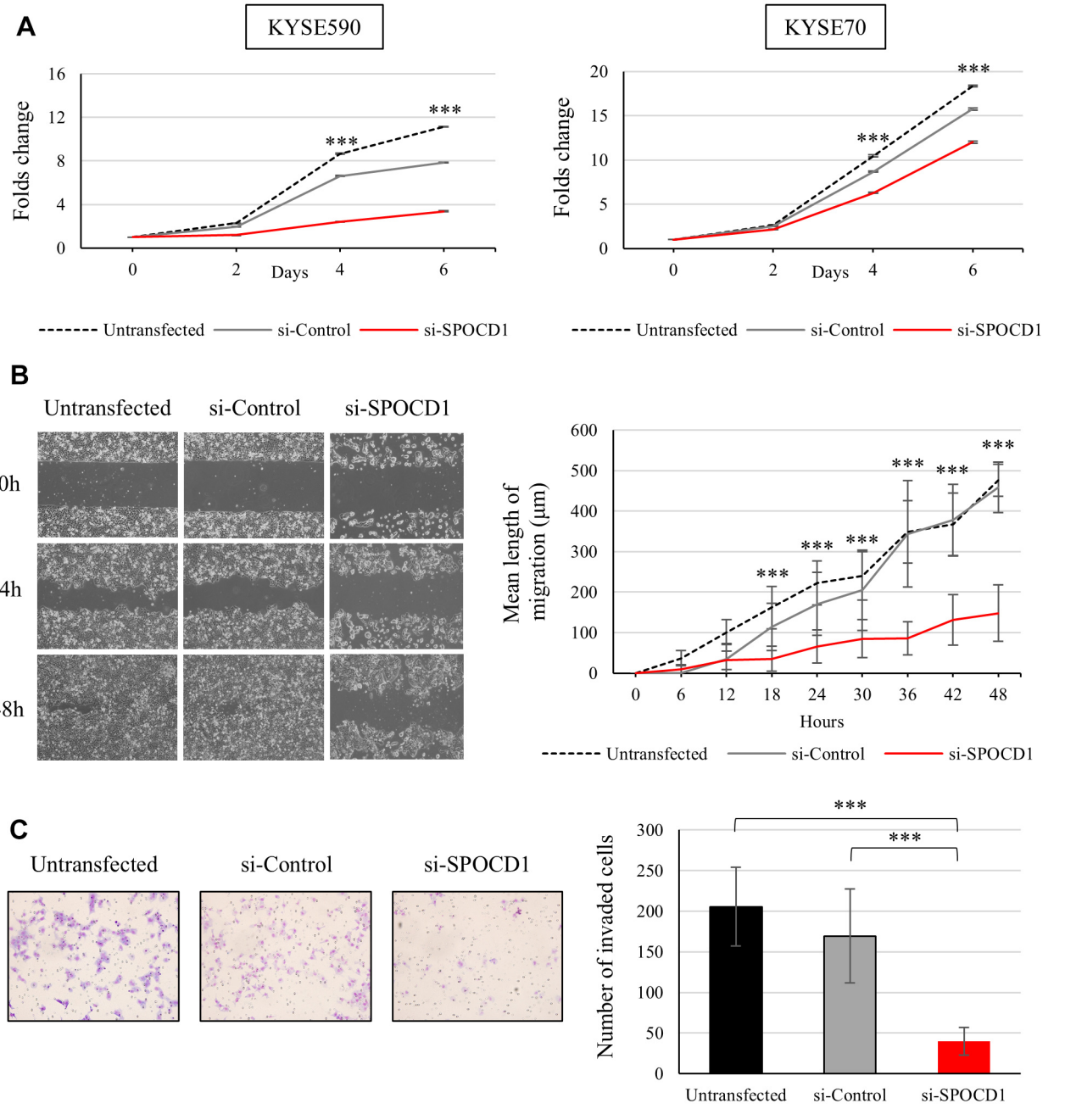


Figure 2. *Continued*

knockdown KYSE590 cells revealed that the expression of the established mesenchymal marker VIM and N-cadherin (CDH2) was reduced in the si-SPOCD1 knockdown cells (Figure S1). These results suggest that *SPOCD1* contributes to cancer aggressiveness through the EMT signaling pathway in ESCC.

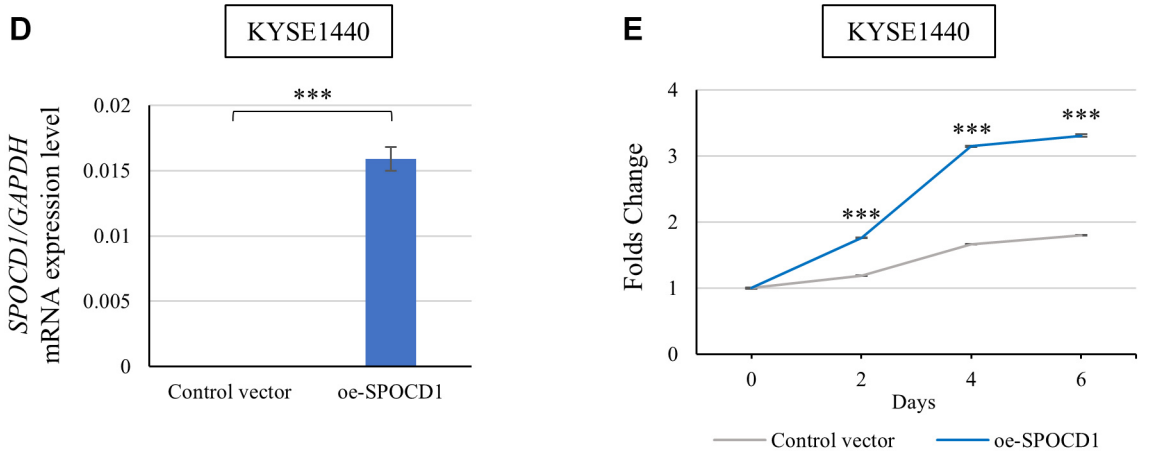


Figure 2. *Effects of siRNA-mediated SPOCD1 knockdown on ESCC cell function in vitro. (A) Proliferation of KYSE590 and KYSE70 cells in which SPOCD1 expression was inhibited using an SPOCD1-specific siRNA. (B) Migration wound healing assay in which SPOCD1 expression was inhibited in KYSE590 cells treated with an SPOCD1-specific siRNA. The left panel shows representative images of invasion, and the right panel shows the mean length of migration. (C) Cell invasion assay of KYSE70 cells in which SPOCD1 expression was inhibited by an SPOCD-siRNA. The left panels show representative images of stained invading KYSE70 cells (×200 magnification). The right graph shows the mean numbers of invaded cells in eight randomly selected fields. (D) Efficiency of enforced expression of SPOCD1 in KYSE1440 compared with that in cells transduced with the control vector. (E) Analysis of cell proliferation in KYSE1440 cells transduced with a control vector and si-SPOCD1. Error bars indicate the standard deviation. *p<0.05, **p<0.01, ***p<0.001. Asterisk refers to the comparison of si-SPOCD1 group to both untransfected and si-control groups in plots A and B.*

*Functional analysis.* The siRNA-mediated knockdown of *SPOCD1* was attempted in the top two differentiated cell lines, KYSE890 and KYSE590 as well as the top undifferentiated cell line, KYSE70. Sufficient knockdown efficiency of >60% was observed in KYSE590 and KYSE70 (Figure 1C), and consequently these cell lines were subjected to subsequent experiments. Simple Western analysis demonstrated a decrease in SPOCD1 expression in the siRNA-knockdown group compared to that in the si-control and untransfected cell groups (Figure 1D). Enforced expression of *SPOCD1* was induced in the low expression KYSE1440 cell line, and knock-in efficiency was confirmed using qPCR (Figure 2D).

*SPOCD1* knockdown significantly decreased the proliferation of KYSE590 and KYSE70 cells (Figure 2A) and inhibited the migration and invasion capabilities of ESCC cells (Figure 2B, C). Enforced expression of *SPOCD1* significantly accelerated the proliferation rate of KYSE1440 cells compared to that of the cells transduced with the control vector (Figure 2E). In subcutaneous xenograft mouse models, we were unable to acquire reliable data on days 4 and 6 owing to a lack of measurable tumor growth and injection site edema. In contrast, on days 8, 10, 12 and 14 the average tumor volume was significantly lower in the siRNA-mediated *SPOCD1*-knockdown group compared to the parental KYSE70 and si-control groups ($p<0.001$) (Figure 3A). The average weight of subcutaneous tumors in the si-*SPOCD1* group was significantly lower compared to the other groups by day 14 ($p<0.001$ and $p=0.005$, for comparison of si-SPOCD1 group to untransfected and si-control groups, respectively) (Figure 3B, C).

*Correlation between SPOCD1 mRNA expression levels, clinicopathological factors, and prognosis.* Among the total cohort of 164 patients with ESCC, 38 were women, the median age was 65 years (range=44-83 years), 140 patients were diagnosed with differentiated ESCC, and the remaining 24 patients had undifferentiated ESCC. The distribution of patients across pathological stages I-IV (8th edition UICC classification) was 27, 39, 88, and

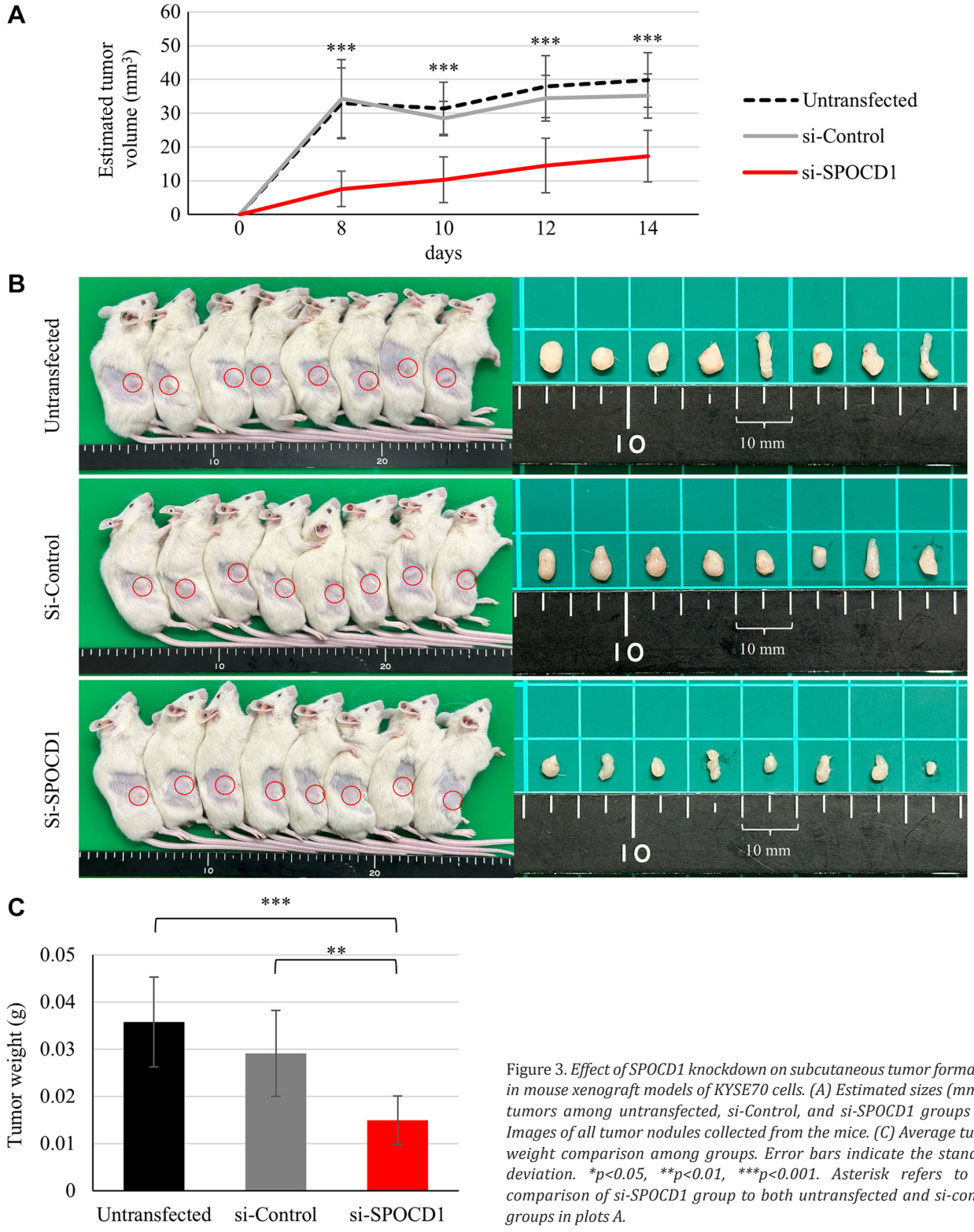


Figure 3. *Effect of SPOCD1 knockdown on subcutaneous tumor formation in mouse xenograft models of KYSE70 cells. (A) Estimated sizes (mm) of tumors among untransfected, si-Control, and si-SPOCD1 groups (B). Images of all tumor nodules collected from the mice. (C) Average tumor weight comparison among groups. Error bars indicate the standard deviation. *p<0.05, **p<0.01, ***p<0.001. Asterisk refers to the comparison of si-SPOCD1 group to both untransfected and si-control groups in plots A.*

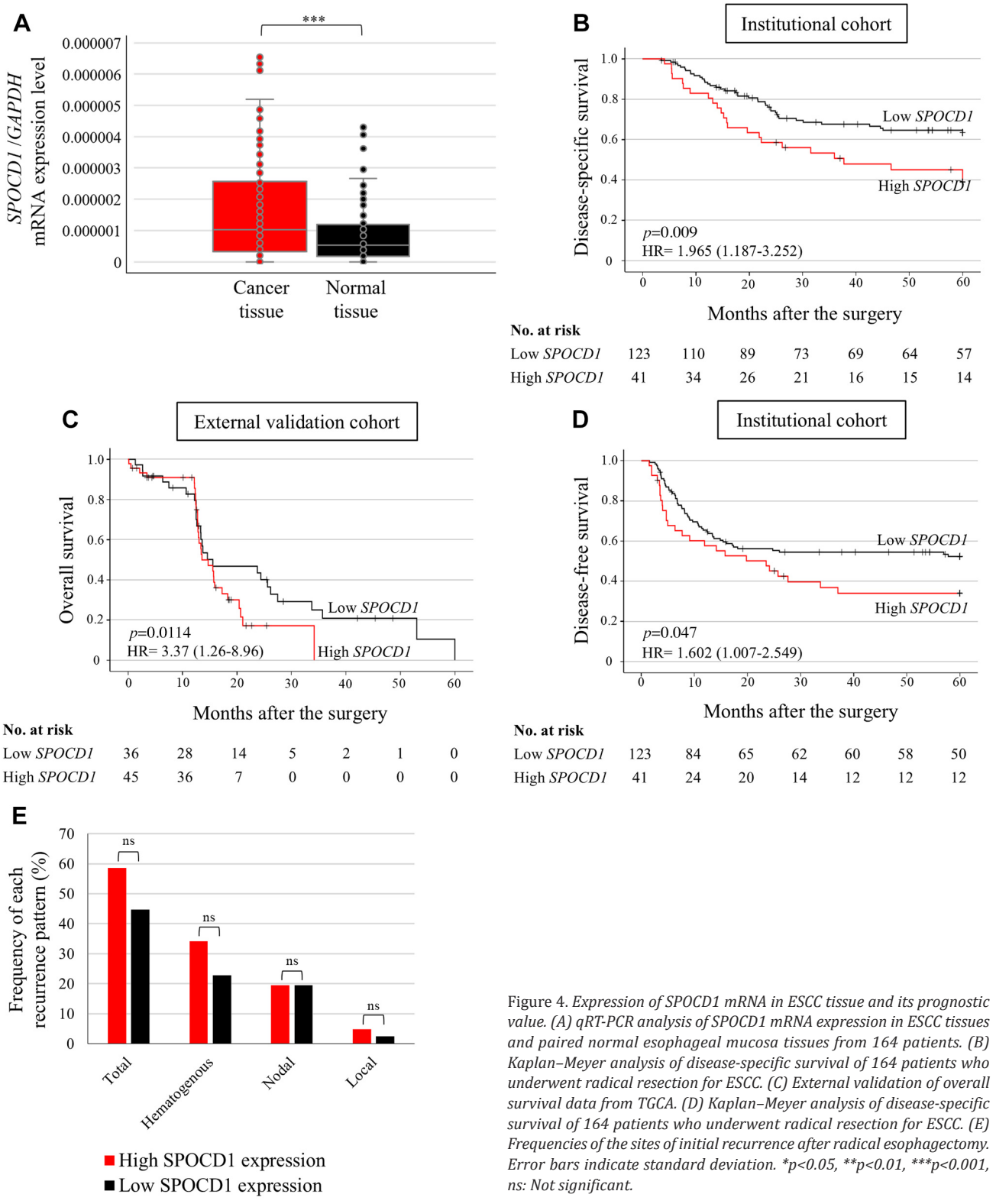


Figure 4. *Expression of SPOCD1 mRNA in ESCC tissue and its prognostic value. (A) qRT-PCR analysis of SPOCD1 mRNA expression in ESCC tissues and paired normal esophageal mucosa tissues from 164 patients. (B) Kaplan–Meyer analysis of disease-specific survival of 164 patients who underwent radical resection for ESCC. (C) External validation of overall survival data from TGCA. (D) Kaplan–Meyer analysis of disease-specific survival of 164 patients who underwent radical resection for ESCC. (E) Frequencies of the sites of initial recurrence after radical esophagectomy. Error bars indicate standard deviation. *p<0.05, **p<0.01, ***p<0.001, ns: Not significant.*

Table III. *Association between expression level of SPOCD1 mRNA and clinicopathological parameters in 164 patients with esophageal squamous cell carcinoma.*

| Variables | | High *SPOCD1* expression | Low *SPOCD1* expression | *p*-Value |
|---|---|---|---|---|
| Age (year) | <65 | 25 (61%) | 58 (47%) | 0.125 |
| | ≥65 | 16 (39%) | 65 (53%) | |
| Sex | Male | 32 (78%) | 94 (76%) | 0.831 |
| | Female | 9 (22%) | 29 (24%) | |
| Smoking history | Present | 29 (83%) | 89 (79%) | 0.598 |
| | Absent | 6 (17%) | 24 (21%) | |
| Tumor location | Ce,Ut,Mt | 27 (66%) | 73 (59%) | 0.46 |
| | Lt,Ae | 14 (34%) | 50 (41%) | |
| Double cancer | Present | 8 (20%) | 21 (17%) | 0.674 |
| | Absent | 32 (80%) | 102 (83%) | |
| Tumor size (mm) | <50 | 29 (73%) | 89 (72%) | 0.986 |
| | ≥50 | 11 (27%) | 34 (28%) | |
| CEA (ng/ml) | ≤5 | 25 (81%) | 69 (88%) | 0.285 |
| | >5 | 6 (19%) | 9 (12%) | |
| SCC (IU/ml) | ≤1.5 | 23 (77%) | 52 (68%) | 0.354 |
| | >1.5 | 7 (23%) | 25 (32%) | |
| Tumor multiplicity | Present | 5 (12%) | 14 (11%) | 0.888 |
| | Absent | 36 (88%) | 109 (89%) | |
| cT | T1/T2 | 12 (29%) | 32 (26%) | 0.684 |
| | T3/T4 | 29 (71%) | 91 (74%) | |
| cN | Present | 31 (76%) | 89 (72%) | 0.684 |
| | Absent | 10 (24%) | 34 (28%) | |
| pT | T1/T2 | 15 (37%) | 39 (32%) | 0.565 |
| | T3/T4 | 26 (63%) | 84 (68%) | |
| pN | Present | 29 (71%) | 77 (63%) | 0.346 |
| | Absent | 12 (29%) | 46 (37%) | |
| Differentiation | Differentiated | 33 (81%) | 107 (87%) | 0.308 |
| | Undifferentiated | 8 (19%) | 16 (13%) | |
| Lymphatic invasion | Present | 30 (73%) | 92 (75%) | 0.836 |
| | Absent | 11 (27%) | 31 (25%) | |
| Vascular invasion | Present | 13 (32%) | 52 (42%) | 0.231 |
| | Absent | 28 (68%) | 71 (58%) | |
| Intramural metastasis | Present | 5 (12%) | 8 (7%) | 0.243 |
| | Absent | 36 (88%) | 115 (93%) | |
| Clinical UICC stage | I/II | 16 (39%) | 46 (37%) | 0.852 |
| | III/IV | 25 (61%) | 77 (63%) | |
| Neoadjuvant therapy | Present | 20 (49%) | 69 (56%) | 0.415 |
| | Absent | 21 (51%) | 54 (44%) | |
| Pathological UICC stage | I/II | 15 (37%) | 51 (42%) | 0.581 |
| | III/IV | 26 (63%) | 72 (58%) | |

CEA, Carcinoembryonic antigen; SCC, squamous cell carcinoma-related antigen; *SPOCD1*, SPOC Domain Containing 1.

10, respectively. Neoadjuvant therapy was administered to 89 patients (54.3%). During follow-up (median=45.83 months), 83 patients (50.6%) experienced recurrence, and 64 patients (39%) died because of ESCC.

*SPOCD1* mRNA levels in 102/164 (62%) patients, determined using qPCR, were elevated in ESCC tissues compared to the adjacent normal esophageal tissues. Significantly higher expression of *SPOCD1* mRNA was detected in ESCC tissues compared to that observed in the normal adjacent tissues ($p<0.001$) (Figure 4A). Correlations between *SPOCD1* mRNA expression levels and clinicopathological factors among 164 patients with

Table IV. *Prognostic factors for disease-specific survival.*

| | Univariable analysis | | | Multivariable analysis | | |
|---|---|---|---|---|---|---|
| | Hazard ratio | 95% CI | *p*-Value | Hazard ratio | 95% CI | *p*-Value |
| Age (≥65 years) | 0.88 | 0.54-1.44 | 0.621 | | | |
| Sex (male) | 0.91 | 0.51-1.63 | 0.769 | | | |
| Smoking | 0.64 | 0.36-1.16 | 0.144 | | | |
| Tumor location (lower) | 0.99 | 0.60-1.64 | 0.989 | | | |
| Double cancer | 1.18 | 0.63-2.21 | 0.604 | | | |
| Tumor multiplicity | 1.27 | 0.63-2.57 | 0.498 | | | |
| Tumor size (≥50 mm) | 1.06 | 0.62-1.84 | 0.810 | | | |
| CEA (>5 ng/ml) | 1.28 | 0.57-2.87 | 0.548 | | | |
| SCC (>1.5 IU/ml) | 1.22 | 0.63-2.36 | 0.548 | | | |
| Tumor depth (pT3-4) | 1.53 | 0.90-2.63 | 0.115 | | | |
| Lymph node metastasis | 3.70 | 1.93-7.09 | <0.001 | 2.26 | 1.15-4.44 | 0.018 |
| Tumor differentiation (undifferentiated) | 0.67 | 0.35-1.28 | 0.230 | | | |
| Lymphatic invasion | 5.69 | 2.28-14.2 | <0.001 | 4.27 | 1.65-11.0 | 0.003 |
| Vascular invasion | 1.52 | 0.93-2.48 | 0.09 | | | |
| Intramural metastasis | 2.54 | 1.29-5.01 | 0.07 | 1.80 | 0.91-3.58 | 0.090 |
| High *SPOCD1* expression | 1.96 | 1.18-3.25 | 0.009 | 1.92 | 1.18-3.27 | 0.009 |

CEA, Carcinoembryonic antigen; SCC, squamous cell carcinoma-related antigen; CI, confidence interval; SPOCD1, SPOC Domain Containing 1.

Table V. *Pre-operatively determined prognostic factors for disease-specific survival.*

| | Univariable analysis | | | Multivariable analysis | | |
|---|---|---|---|---|---|---|
| | Hazard ratio | 95% CI | *p*-Value | Hazard ratio | 95% CI | *p*-Value |
| Age (≥65 years) | 0.88 | 0.54-1.44 | 0.621 | | | |
| Sex (male) | 0.91 | 0.51-1.63 | 0.769 | | | |
| Smoking | 0.64 | 0.36-1.16 | 0.144 | | | |
| Tumor location (lower) | 0.99 | 0.60-1.64 | 0.989 | | | |
| Double cancer | 1.18 | 0.63-2.21 | 0.604 | | | |
| Tumor multiplicity | 1.27 | 0.63-2.57 | 0.498 | | | |
| Tumor size (≥50 mm) | 1.06 | 0.62-1.84 | 0.810 | | | |
| CEA (>5 ng/ml) | 1.28 | 0.57-2.87 | 0.548 | | | |
| SCC (>1.5 IU/ml) | 1.22 | 0.63-2.36 | 0.548 | | | |
| Tumor differentiation (undifferentiated) | 0.67 | 0.35-1.28 | 0.230 | | | |
| Tumor depth (cT3-4) | 1.24 | 0.70-2.19 | 0.443 | | | |
| Lymph node metastasis (cN+) | 1.90 | 1.01-3.56 | 0.044 | 1.89 | 1.01-3.54 | 0.046 |
| High *SPOCD1* expression | 1.96 | 1.18-3.25 | 0.009 | 1.96 | 1.18-3.24 | 0.009 |

CEA, Carcinoembryonic antigen; SCC, squamous cell carcinoma-related antigen; CI, confidence interval; *SPOCD1*, SPOC Domain Containing 1.

ESCC are shown in Table III. DSS (Figure 4B) was significantly lower in the high *SPOCD1* expression group compared to the low *SPOCD1* expression group [*p*=0.009, HR=1.965, 95% confidence interval (CI)=1.187-3.252].

Analysis of the extra validation dataset (n=81 patients) (Figure 4C) revealed that high *SPOCD1* expression correlated with a significant decline in overall survival (OS) (*p*=0.0114, HR=3.37, 95% CI=1.26-8.96). The 5-year DFS rate (Figure 4D) was significantly lower for patients with high tumor expression of *SPOCD1* (*p*=0.047, HR=1.602, 95% CI=1.007-2.549). Recurrence pattern analysis revealed that the high *SPOCD1* group

exhibited higher overall recurrence rates and hematogenous recurrence (Figure 4E), although the difference was not significantly different. Multivariable Cox proportional hazard model analysis showed that high tumor-specific *SPOCD1* mRNA expression served as an independent prognostic factor for DSS after radical resection ($p$=0.009, HR=1.92, 95% CI=1.18-3.27) (Table IV). When assessed as one of the preoperatively determined prognostic factors, including clinical TNM stage variables, for DSS of ESCC, *SPOCD1* expression served as an independent prognostic factor, capable of preoperatively predicting outcomes ($p$=0.009, HR=1.96, 95% CI=1.18-3.24) (Table V).

*Correlation between* SPOCD1 *expression levels, clinico-pathological factors*, *and prognosis.* Among 177 patients in the TMA cohort, 24 were female and 153 were male (median age 66 years, range=38-82). One hundred twenty and 57 patients were diagnosed with differentiated or undifferentiated ESCC, respectively. According to the 8th edition of the UICC classification, the numbers of patients with pathological stages I-IV were 10, 44, 105, and 18 respectively. Immunohistochemical analysis of SPOCD1 was assessed using a semiquantitative scoring method that evaluates the intensity and extent of protein expression. Representative images of specimens with different staining intensities of the low and high SPOCD1 groups are presented in Figure 5A. Among 177 patient samples, in 7 (4%), staining was undetectable and weak in 101 (57%). In contrast, 60 (34%) and 9 (5%) samples exhibited moderate or strong staining, respectively.

Patients were classified into high (average scores 2, or 2+; n=69) and low SPOCD1 (average scores 0, or 1; n=108) groups (Figure 5A). Kaplan–Meier survival analysis indicated that 5-year DSS and DFS were significantly lower in the group with high tumor expression of SPOCD1 compared with those in the group with low expression ($p$=0.013, HR=1.860, 95% CI=1.137-3.041; and $p$=0.032, HR=1.618, 95% CI=1.042-2.513, respectively) (Figure 5B).

## Discussion

In the present study, transcriptome analysis identified *SPOCD1* as a potential biomarker for ESCC. Based on a recent bioinformatics analysis (24) that suggested oncogenic function of SPOCD1 in ESCC and correlated *SPOCD1* overexpression with the poorer prognosis of the ESCC, here we aimed to conduct a more in-depth investigation of *SPOCD1* function through *in vitro* and *in vivo* studies employing two independent clinical cohorts of patients with ESCC accompanied by studies of ESCC-derived cell lines.

*SPOCD1* belongs to the transcription factor S-II (TFIIS) family (40). Transcription factors, which are potential drug targets that mediate abnormal levels of gene expression, have been comprehensively studied for their roles in oncogenesis (41). Interestingly, *SPOCD1* is up-regulated during human gingival wound healing (16) which involves cell proliferation and migration in healthy individuals. Up-regulation of *SPOCD1* occurs in gastric cancer, clear cell renal cancer, ovarian cancer, osteosarcoma, and glioma (15, 17, 19, 22).

The EMT signaling pathway contributes to development and healing as well as cancer progression and metastasis (42, 43). Our current study found that *SPOCD1* expression levels correlated with those of several EMT markers, including established markers such as VIM and SNAI1. For example, transient siRNA knockdown significantly reduced the levels of promesenchymal markers such as VIM and CDH2, although it had a minimal effect on those of CDH1, TJP1 and CTNNB1. Thus, *SPOCD1* suppression may cause partial reversal of the EMT in cancer cells.

Interestingly, a recent study by Yavari *et al*. employing enrichment analysis concluded that SPOCD1 expression correlates with the EMT pathway, with *VIM* exhibiting a high correlation (23); our findings are consistent with these results. Furthermore, *SPOCD1* expression was significantly associated with several genes related to the VEGF signaling pathway, which plays a crucial role in cancer metastasis, primarily by promoting angiogenesis (44).

We demonstrate here that *SPOCD1* can be associated with the malignant phenotype of ESCC. *In vitro* knockdown

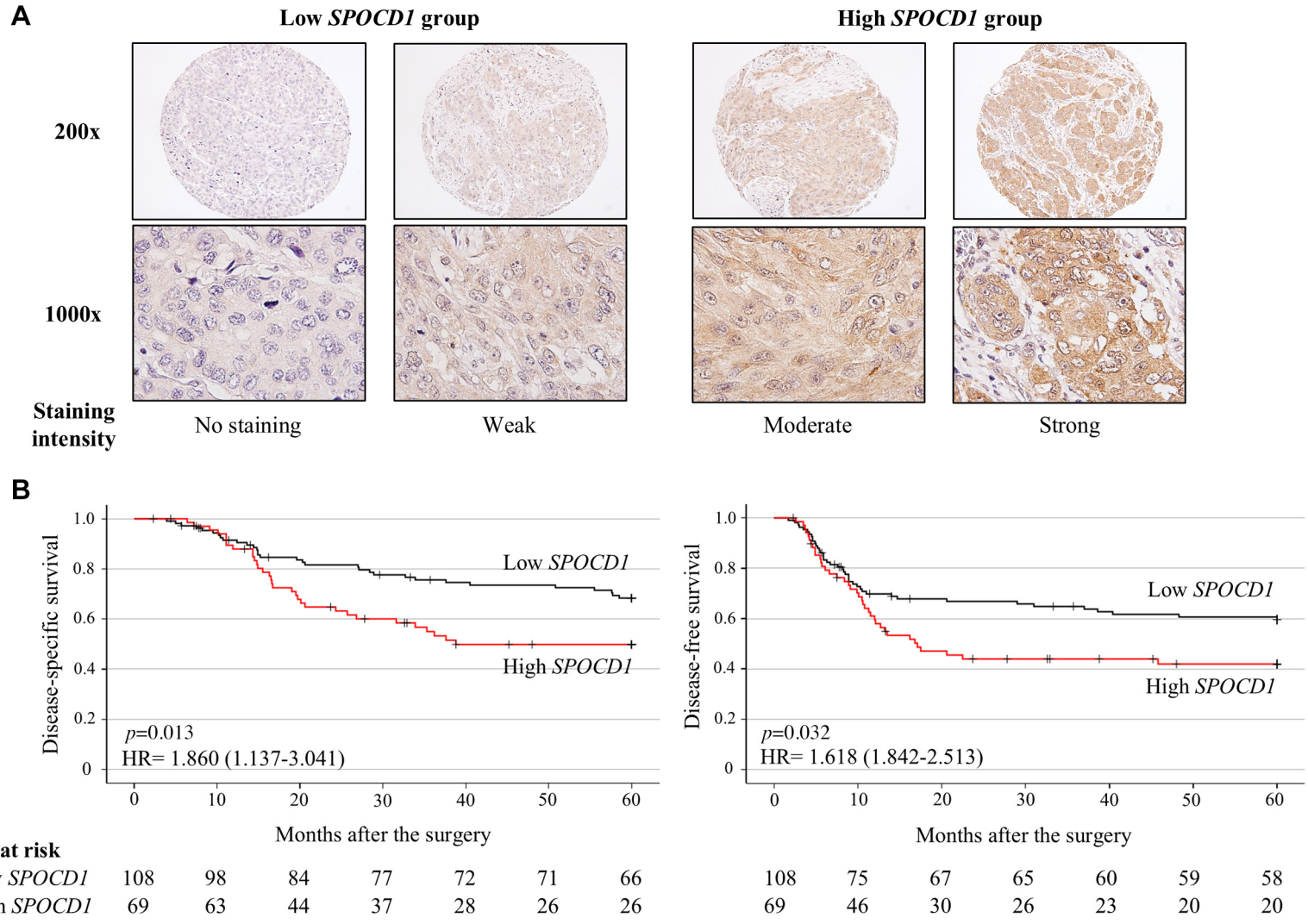


Figure 5. *Analysis of SPOCD1 expression in ESCC tissues and its prognostic value. (A) Representative images of immunohistochemical detection in a TMA. Examples of negative (0) and positive (1+, 2+, and 3+) expression levels are shown (200× and 1,000× magnification). (B) Kaplan–Meyer analysis of disease-specific and disease-free survival of 177 patients stages I-III ESCC who underwent radical resection of the esophagus.*

of *SPOCD1* expression significantly decreased the proliferation, migration, and invasion of ESCC cells, while its enforced expression significantly increased the cell proliferation rate. Proliferation, invasion, and migration of ESCC cells are the hallmark of metastatic potential, because they mediate the detachment of cancer cells and their migration from the primary site as well as penetration of the extracellular matrix to reach adjacent lymphatic or blood vessels, leading to their proliferation in the target organ (45).

In a mouse subcutaneous xenograft model employed here, the average weight of injected tumors was significantly lower in the group where *SPOCD1* expression was inhibited compared to that in the control groups. Although tumor measurements were conducted beyond the effective period of siRNA, typically 5-7 days, and may have been influenced by the partial return of gene function, a significant difference in tumor size was still observed on days 8, 10, 12 and 14. This result is consistent with decreased cell growth, migration, and invasiveness exhibited by *SPOCD1* knockout cells *in vitro*, supporting the role of *SPOCD1* in the tumorigenicity of ESCC cells.

In the present study, high *SPOCD1* mRNA levels in ESCC tissue were significantly associated with poorer prognosis, consistent with published data (24) on the prognostic value of *SPOCD1* in ESCC. Furthermore, our second cohort data analysis indicated that the expression levels of SPOCD1 in ESCC predicted poor clinical outcomes, further extending our knowledge on the oncogenic role of *SPOCD1* in ESCC.

Despite its strong association with poor prognosis, high expression of *SPOCD1* was not correlated with well-established prognostic factors such as lymphatic invasion, elevated levels of tumor markers such as CEA or SCC, or UICC staging. Although our knowledge of the molecular profiling of cancer has rapidly developed, our clinical decision-making for the management of ESCC is mainly based on the clinical tumor-node-metastasis (cTNM) staging system. For example, not all patients diagnosed with ESCC at the same TNM stage exhibit identical clinical outcomes (46, 47). Thus, our current strategies for risk stratification, particularly in the pretreatment setting, can be improved through implementation of more individualized strategies based on the molecular characteristics of cancers.

Here, elevated *SPOCD1* expression levels were identified as an independent preoperatively detected risk factor for ESCC. Therefore, its detection from endoscopic biopsy samples could be used as a pretreatment risk stratification tool for ESCC. As a novel prognostic factor independent of other established risk factors, *SPOCD1* overexpression may contribute to the pool of potential biomarkers, which can be integrated into clinical decision-making.

Our IHC and TMA data suggest that SPOCD1 expression analysis has potential for application to clinical practice because of its simplicity and effectiveness compared with mRNA expression analysis. High *SPOCD1* expression may serve as a reference measure to predict treatment outcomes in ESCC. Furthermore, high SPOCD1 expression detected in biopsy samples may indicate more aggressive treatment options, including NAC and aggressive chemotherapy combined with detailed follow-up. Patients that expressed high levels of SPOCD1 in surgical specimens may benefit as well from additional adjuvant treatment and careful follow-up. Clinical data analysis results suggest that overexpression of *SPOCD1* predicts higher rates of overall recurrence and hematogenous recurrence. Therefore, multimodal imaging surveillance, including Gd-EOB-DTPA-enhanced magnetic resonance imaging may enhance the early detection of liver metastasis. Furthermore, positron emission tomography may be employed to detect recurrence in lungs, lymph nodes, bone and other possible metastatic sites may be required for patients with advanced *SPOCD1*-associated disease. Further research may indicate that *SPOCD1* will potentially serve as a therapeutic target for small molecules, nucleic acid drugs, or both (34, 41).

Correct interpretation of the present findings is subject to several limitations. First, we conducted a retrospective clinical analysis. Second, our *in vivo* analysis included siRNA-mediated knockdown. To validate the *in vivo* effect of *SPOCD1,* analyses including shRNA or CRISPR knockout methods should be employed in future studies. Third, prospective studies of a larger sample size are required to

validate the prognostic ability of *SPOCD1* expression levels, and an optimal cutoff value suitable for clinical application will be required.

## Conclusion

Our findings indicate the active contribution of *SPOCD1* to the malignant phenotypes of ESCC cells in both *in vitro* and *in vivo* settings. Its expression in tissues, not only at the transcript level, but also at the protein levels was correlated with poor clinical outcomes, implying that this biomarker has potential to serve as a useful prognostic biomarker for ESCC. By identifying the oncogenic role of SPOCD1 and its association with epithelial-mesenchymal transition-related pathways, this study highlights a molecular target for further research. Future studies should evaluate SPOCD1 as a potential therapeutic target to improve outcomes for ESCC patients.

## Supplementary Material

Figure S1 is available as supplementary material and can be accessed at https://figshare.com/s/97d5e7a34b9f5771dab9

## Availability of Data and Materials

RNA-sequencing data used for the present study have been registered to the DDBJ Sequence Read Archive (https://ddbj.nig.ac.jp/search/entry/bioproject/PRJDB18929). The reference sequence information used for mapping is available under accession numbers DRX589457-DRX589472.

## Conflicts of Interest

The Authors declare that they have no competing interests.

## Authors' Contributions

TB, HZ, MHH, and MK performed the experiments. FEG, YuI, TS, and MS contributed to the experiments and data analysis. TB, MK, YS, DS, SU, YoI, NH, MH, CT, GN, and YK collected cases and clinical data. TB and MK confirmed the authenticity of the raw data. TB and MK conceived and designed the study and prepared the initial draft of the manuscript. YK supervised the project. All Authors contributed to the final manuscript. All authors read and approved the final manuscript collected.

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## References

1 Ferlay J, Ervik M, Lam F, Laversanne M, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, Bray F: Global Cancer Observatory: Cancer Today. Lyon, France, International Agency for Research on Cancer, 2024. Available at: https://gco.iarc.who.int/today [Last accessed August 9, 2024]

2 Malhotra GK, Yanala U, Ravipati A, Follet M, Vijayakumar M, Are C: Global trends in esophageal cancer. J Surg Oncol 115(5): 564-579, 2017. DOI: 10.1002/jso.24592

3 Lordick F, Hölscher AH, Haustermans K, Wittekind C: Multimodal treatment of esophageal cancer. Langenbecks Arch Surg 398(2): 177-187, 2013. DOI: 10.1007/s00423-012-1001-1

4 Ando N, Kato H, Igaki H, Shinoda M, Ozawa S, Shimizu H, Nakamura T, Yabusaki H, Aoyama N, Kurita A, Ikeda K, Kanda T, Tsujinaka T, Nakamura K, Fukuda H: A randomized trial comparing postoperative adjuvant chemotherapy with cisplatin and 5-fluorouracil *versus* preoperative chemotherapy for localized advanced squamous cell carcinoma of the thoracic esophagus (JCOG9907). Ann Surg Oncol 19(1): 68-74, 2012. DOI: 10.1245/s10434-011-2049-9

5 Chapman BC, Weyant M, Hilton S, Hosokawa PW, McCarter MD, Gleisner A, Nader ND, Gajdos C: Analysis of the National Cancer Database esophageal squamous cell carcinoma in the United States. Ann Thorac Surg 108(5): 1535-1542, 2019. DOI: 10.1016/j.athoracsur.2019.05.053

6 Mariette C, Piessen G, Triboulet JP: Therapeutic strategies in oesophageal carcinoma: role of surgery and other modalities. Lancet Oncol 8(6): 545-553, 2007. DOI: 10.1016/S1470-2045(07)70172-9

7 Matsuda S, Takeuchi H, Kawakubo H, Ando N, Kitagawa Y: Current advancement in multidisciplinary treatment for resectable cStage II/III esophageal squamous cell carcinoma in Japan. Ann Thorac Cardiovasc Surg 22(5): 275-283, 2016. DOI: 10.5761/atcs.ra.16-00111

8 Yang Y, Huang X, Zhou L, Deng T, Ning T, Liu R, Zhang L, Bai M, Zhang H, Li H, Ba Y: Clinical use of tumor biomarkers in prediction for prognosis and chemotherapeutic effect in esophageal squamous cell carcinoma. BMC Cancer 19(1): 526, 2019. DOI: 10.1186/s12885-019-5755-5

9 Kawaguchi H, Ohno S, Miyazaki M, Hashimoto K, Egashira A, Saeki H, Watanabe M, Sugimachi K: CYFRA 21-1 determination in patients with esophageal squamous cell carcinoma. Cancer 89(7): 1413-1417, 2000. DOI: 10.1002/1097-0142(20001001)89:7<1413::aid-cncr1>3.0.co;2-i

10 Kanie Y, Okamura A, Maruyama S, Sakamoto K, Fujiwara D, Kanamori J, Imamura Y, Watanabe M: Clinical significance of serum squamous cell carcinoma antigen for patients with recurrent esophageal squamous cell carcinoma. Ann Surg Oncol 28(12): 7990-7996, 2021. DOI: 10.1245/s10434-021-09945-5

11 Baba H, Kanda M, Sato Y, Sawaki K, Shimizu D, Koike M, Motoyama S, Kodera Y, Fujii T: Expression and malignant potential of B4GALNT4 in esophageal squamous cell carcinoma. Ann Surg Oncol 27(9): 3247-3256, 2020. DOI: 10.1245/s10434-020-08431-8

12 Baba H, Kanda M, Sawaki K, Nakamura S, Ueda S, Shimizu D, Koike M, Kodera Y, Fujii T: SLC7A9 as a potential biomarker for lymph node metastasis of esophageal squamous cell carcinoma. Ann Surg Oncol 29(4): 2699-2709, 2022. DOI: 10.1245/s10434-021-11001-1

13 Baba H, Kanda M, Sawaki K, Shimizu D, Umeda S, Koike M, Kodera Y, Fujii T: *PRAME* expression as a potential biomarker for hematogenous recurrence of esophageal squamous cell carcinoma. Anticancer Res 39(11): 5943-5951, 2019. DOI: 10.21873/anticanres.13799

14 Nakamura S, Kanda M, Koike M, Shimizu D, Umeda S, Hattori N, Hayashi M, Tanaka C, Kobayashi D, Yamada S, Omae K, Kodera Y: KCNJ15 expression and malignant behavior of esophageal squamous cell carcinoma. Ann Surg Oncol 27(7): 2559-2568, 2020. DOI: 10.1245/s10434-019-08189-8

15 Zoch A, Auchynnikava T, Berrens RV, Kabayama Y, Schöpp T, Heep M, Vasiliauskaitė L, Pérez-Rico YA, Cook AG, Shkumatava A, Rappsilber J, Allshire RC, O'Carroll D: SPOCD1 is an essential executor of piRNA-directed de novo DNA methylation. Nature 584(7822): 635-639, 2020. DOI: 10.1038/s41586-020-2557-5

16 Wang Y, Tatakis DN: Human gingiva transcriptome during wound healing. J Clin Periodontol 44(4): 394-402, 2017. DOI: 10.1111/jcpe.12669

17 Zhu M, Yan C, Ren C, Huang X, Zhu X, Gu H, Wang M, Wang S, Gao Y, Ji Y, Miao X, Yang M, Chen J, Du J, Huang T, Jiang Y, Dai J, Ma H, Zhou J, Wang Z, Hu Z, Ji G, Zhang Z, Shen H, Shi Y, Jin G: Exome array analysis identifies variants in SPOCD1 and BTN3A2 that affect risk for gastric cancer. Gastroenterology 152(8): 2011-2021, 2017. DOI: 10.1053/j.gastro.2017.02.017

18 Sakaguchi T, Yoshino H, Sugita S, Miyamoto K, Yonemori M, Osako Y, Meguro-Horike M, Horike SI, Nakagawa M, Enokida H: Bromodomain protein BRD4 inhibitor JQ1 regulates potential prognostic molecules in advanced renal cell carcinoma. Oncotarget 9(33): 23003-23017, 2018. DOI: 10.18632/oncotarget.25190

19 Liang J, Zhao H, Hu J, Liu Y, Li Z: SPOCD1 promotes cell proliferation and inhibits cell apoptosis in human osteosarcoma. Mol Med Rep 17(2): 3218-3225, 2017. DOI: 10.3892/mmr.2017.8263

20 Liu D, Yang Y, Yan A, Yang Y: SPOCD1 accelerates ovarian cancer progression and inhibits cell apoptosis via the PI3K/AKT pathway. Onco Targets Ther 13: 351-359, 2020. DOI: 10.2147/OTT.S200317

21 van der Heijden AG, Mengual L, Lozano JJ, Ingelmo-Torres M, Ribal MJ, Fernández PL, Oosterwijk E, Schalken JA, Alcaraz A, Witjes JA: A five-gene expression signature to predict progression in T1G3 bladder cancer. Eur J Cancer 64: 127-136, 2016. DOI: 10.1016/j.ejca.2016.06.003

22 Liu Q, Wang XY, Qin YY, Yan XL, Chen HM, Huang QD, Chen JK, Zheng JM: SPOCD1 promotes the proliferation and metastasis of glioma cells by up-regulating PTX3. Am J Cancer Res 8(4): 624-635, 2018.

23 Yavari S, Naseroleslami M, Peymani M, Yekani F, Khayam Nekouei N: Investigation of SPOCD1 as a suitable diagnostic and prognostic biomarker in various common cancer types: bioinformatics and practical analysis. Cell J 26(5): 309-319, 2024. DOI: 10.22074/cellj.2024.2022401.1506

24 Lin Z, Chen L, Wu T, Zhang Y, Huang X, Chen Y, Chen J, Xu Y: Prognostic value of SPOCD1 in esophageal squamous cell carcinoma: a comprehensive study based on bioinformatics and validation. Front Genet 13: 872026, 2022. DOI: 10.3389/fgene.2022.872026

25 Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG: Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. Osteoarthritis Cartilage 20(4): 256-260, 2012. DOI: 10.1016/j.joca.2012.02.010

26 Tanaka H, Shimada Y, Imamura M, Shibagaki I, Ishizaki K: Multiple types of aberrations in the p16 (INK4a) and the p15(INK4b) genes in 30 esophageal squamous-cell-carcinoma cell lines. Int J Cancer 70(4): 437-442, 1997. DOI: 10.1002/(sici)1097-0215(19970207)70:4<437::aid-ijc11>3.0.co;2-c

27 Kanda M, Shimizu D, Nakamura S, Sawaki K, Umeda S, Miwa T, Tanaka H, Inokawa Y, Hattori N, Hayashi M, Tanaka C, Nakayama G, Iguchi Y, Katsuno M, Kodera Y: Blockade of CHRNB2 signaling with a therapeutic monoclonal antibody attenuates the aggressiveness of gastric cancer cells.

Oncogene 40(36): 5495-5504, 2021. DOI: 10.1038/s41388-021-01945-9

28 Ueda S, Kanda M, Sato Y, Baba H, Nakamura S, Sawaki K, Shimizu D, Motoyama S, Fujii T, Kodera Y, Nomoto S: Chromobox 2 expression predicts prognosis after curative resection of oesophageal squamous cell carcinoma. Cancer Genomics Proteomics 17(4): 391-400, 2020. DOI: 10.21873/cgp.20198

29 Kanda M, Shimizu D, Sawaki K, Nakamura S, Umeda S, Miwa T, Tanaka H, Tanaka C, Hayashi M, Iguchi Y, Yamada S, Katsuno M, Kodera Y: Therapeutic monoclonal antibody targeting of neuronal pentraxin receptor to control metastasis in gastric cancer. Mol Cancer 19(1): 131, 2020. DOI: 10.1186/s12943-020-01251-0

30 Sakata K, Kanda M, Shimizu D, Nakamura S, Inokawa Y, Hattori N, Hayashi M, Tanaka C, Nakayama G, Kodera Y: Expression of cellular retinoic acid binding protein 1 predicts peritoneal recurrence of gastric cancer. Int J Oncol 60(6): 63, 2022. DOI: 10.3892/ijo.2022.5353

31 Brierley J, Gospodarowicz MK, Wittekind C: TNM classification of malignant tumours. Eighth edition. Chichester, West Sussex, UK; Hoboken, NJ, USA, John Wiley & Sons, Inc., 2017.

32 Sato Y, Wakita A, Maeda E, Nagaki Y, Sasamori R, Kemuriyama K, Nozaki S, Ito S, Terata K, Imai K, Nanjo H, Nomura K, Minamiya Y: High TLR6 expression status predicts a more favorable prognosis after esophagectomy for locally advanced thoracic esophageal squamous cell carcinoma. Curr Oncol 30(5): 4724-4735, 2023. DOI: 10.3390/curroncol30050356

33 Kanda M, Shimizu D, Tanaka H, Tanaka C, Kobayashi D, Hayashi M, Iwata N, Niwa Y, Yamada S, Fujii T, Sugimoto H, Murotani K, Fujiwara M, Kodera Y: Significance of SYT8 for the detection, prediction, and treatment of peritoneal metastasis from gastric cancer. Ann Surg 267(3): 495-503, 2018. DOI: 10.1097/sla.0000000000002096

34 Kanda M, Kasahara Y, Shimizu D, Miwa T, Umeda S, Sawaki K, Nakamura S, Kodera Y, Obika S: Amido-bridged nucleic acid-modified antisense oligonucleotides targeting SYT13 to treat peritoneal metastasis of gastric cancer. Mol Ther Nucleic Acids 22: 791-802, 2020. DOI: 10.1016/j.omtn.2020.10.001

35 Sasahara M, Kanda M, Shimizu D, Tanaka C, Inokawa Y, Hattori N, Hayashi M, Nakayama G, Kodera Y: Tissue *RNFT2* expression levels are associated with peritoneal recurrence and poor prognosis in gastric cancer. Anticancer Res 41(2): 609-617, 2021. DOI: 10.21873/anticanres.14812

36 Sasahara M, Kanda M, Tanaka C, Shimizu D, Umeda S, Takami H, Inokawa Y, Hattori N, Hayashi M, Nakayama G, Kodera Y: Therapeutic antibody targeting natriuretic peptide receptor 1 inhibits gastric cancer growth *via*BCL-2-mediated intrinsic apoptosis. Int J Cancer 154(7): 1272-1284, 2024. DOI: 10.1002/ijc.34831

37 Kanda M, Shimizu D, Fujii T, Sueoka S, Tanaka Y, Ezaka K, Takami H, Tanaka H, Hashimoto R, Iwata N, Kobayashi D, Tanaka C, Yamada S, Nakayama G, Sugimoto H, Koike M, Fujiwara M, Kodera Y: Function and diagnostic value of Anosmin-1 in gastric cancer progression. Int J Cancer 138(3): 721-730, 2016. DOI: 10.1002/ijc.29803

38 O'Neill RA, Bhamidipati A, Bi X, Deb-Basu D, Cahill L, Ferrante J, Gentalen E, Glazer M, Gossett J, Hacker K, Kirby C, Knittle J, Loder R, Mastroieni C, Maclaren M, Mills T, Nguyen U, Parker N, Rice A, Roach D, Suich D, Voehringer D, Voss K, Yang J, Yang T, Vander Horn PB: Isoelectric focusing technology quantifies protein signaling in 25 cells. Proc Natl Acad Sci USA 103(44): 16153-16158, 2006. DOI: 10.1073/pnas.0607973103

39 Nagy Á, Munkácsy G, Győrffy B: Pancancer survival analysis of cancer hallmark genes. Sci Rep 11(1): 6047, 2021. DOI: 10.1038/s41598-021-84787-5

40 Kimura K, Wakamatsu A, Suzuki Y, Ota T, Nishikawa T, Yamashita R, Yamamoto J, Sekine M, Tsuritani K, Wakaguri H, Ishii S, Sugiyama T, Saito K, Isono Y, Irie R, Kushida N, Yoneyama T, Otsuka R, Kanda K, Yokoi T, Kondo H, Wagatsuma M, Murakawa K, Ishida S, Ishibashi T, Takahashi-Fujii A, Tanase T, Nagai K, Kikuchi H, Nakai K, Isogai T, Sugano S: Diversification of transcriptional modulation: large-scale identification and characterization of putative alternative promoters of human genes. Genome Res 16(1): 55-65, 2006. DOI: 10.1101/gr.4039406

41 Bushweller JH: Targeting transcription factors in cancer - from undruggable to reality. Nat Rev Cancer 19(11): 611-624, 2019. DOI: 10.1038/s41568-019-0196-7

42 Lamouille S, Xu J, Derynck R: Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 15(3): 178-196, 2014. DOI: 10.1038/nrm3758

43 Pastushenko I, Blanpain C: EMT transition states during tumor progression and metastasis. Trends Cell Biol 29(3): 212-226, 2019. DOI: 10.1016/j.tcb.2018.12.001

44 Yang Y, Cao Y: The impact of VEGF on cancer metastasis and systemic disease. Semin Cancer Biol 86(Pt 3): 251-261, 2022. DOI: 10.1016/j.semcancer.2022.03.011

45 Lambert AW, Pattabiraman DR, Weinberg RA: Emerging biological principles of metastasis. Cell 168(4): 670-691, 2017. DOI: 10.1016/j.cell.2016.11.037

46 Wang C, Wang J, Chen Z, Gao Y, He J: Immunohistochemical prognostic markers of esophageal squamous cell carcinoma: a systematic review. Chin J Cancer 36(1): 65, 2017. DOI: 10.1186/s40880-017-0232-5

47 Xiao Z, Jia Y, Jiang W, Wang Z, Zhang Z, Gao Y: FOXM1: A potential indicator to predict lymphatic metastatic recurrence in stage IIA esophageal squamous cell carcinoma. Thorac Cancer 9(8): 997-1004, 2018. DOI: 10.1111/1759-7714.12776