

Survivin knockdown induces senescence in TTF-1-expressing, KRAS-mutant lung adenocarcinomas

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Abstract. Survivin plays a key role in regulating the cell cycle and apoptosis, and is highly expressed in the majority of malignant tumors. However, little is known about the roles of survivin in *KRAS*-mutant lung adenocarcinomas. In the present study, we examined 28 *KRAS*-mutant lung adenocarcinoma tissues and two *KRAS*-mutant lung adenocarcinoma cell lines, H358 and H441, in order to elucidate the potential of survivin as a therapeutic target. We found that 19 (68%) of the 28 *KRAS*-mutant lung adenocarcinomas were differentiated tumors expressing thyroid transcription factor-1 (TTF-1) and E-cadherin. Patients with tumors immunohistochemically positive for survivin (n=18) had poorer outcomes than those with survivin-negative tumors (n=10). In the H358 and H441 cells, which expressed TTF-1 and E-cadherin, survivin knockdown alone induced senescence, not apoptosis. However, in monolayer culture, the H358 cells and H441 cells in which survivin was silenced, underwent significant apoptosis following combined treatment with ABT-263, a Bcl-2 inhibitor, and trametinib, a MEK inhibitor. Importantly, the triple combination of survivin knockdown with ABT-263 and trametinib treatment, clearly induced cell death in a three-dimensional cell culture model and in an *in vivo* tumor xenograft model. We also observed that the growth of the H358 and H441 cells was slightly, yet significantly suppressed *in vitro* when TTF-1 was silenced. These findings collectively suggest that the triple combination of survivin knockdown with

ABT-263 and trametinib treatment, may be a potential strategy for the treatment of *KRAS*-mutant lung adenocarcinoma. Furthermore, our findings indicate that the well-differentiated type of *KRAS*-mutant lung tumors depends, at least in part, on TTF-1 for growth.

Introduction

Lung cancer is one of the most severe forms of cancer, with a 5-year survival rate of only 15% among patients with all stages of the disease (1). Certain subtypes of lung adenocarcinomas are treatable with effective molecular targeted drugs. For example, tyrosine kinase inhibitors against epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) are highly effective against *EGFR*-mutant and *ALK* fusion-positive lung adenocarcinomas, respectively (2). Effective therapies for rare lung adenocarcinomas have also been developed. For example, crizotinib for ROS proto-oncogene 1, receptor tyrosine kinase (*ROS-1*) fusion-positive tumors, and vandetanib for *RET* fusion-positive tumors (3,4). However, there are still no effective treatment strategies for *KRAS*-mutant lung adenocarcinomas, which account for ~8.5% and 20-25% of lung adenocarcinoma cases in the Japanese and Caucasian (European and North American) populations, respectively (5-7). As a result, systemic chemotherapy is still preferentially used to treat the majority of patients with *KRAS*-mutant lung adenocarcinomas. Constitutively activated mutant *KRAS* protein has been proven to be notoriously difficult to target with small molecule inhibitors, and this has led to the use of trametinib and selumetinib, two MEK (MAPK/ERK kinase) inhibitors, as promising candidates in the treatment of *KRAS*-mutant cancer (5,6). Clinical trials have been conducted for *KRAS*-mutant lung cancer using second line treatment with trametinib alone, or combination therapy with selumetinib and docetaxel; however, these treatments have failed to significantly improve progression-free survival (8,9). Other effective therapeutic options are currently available, including treatment with anti-vascular endothelial growth factor (VEGF) antibody

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(bevacizumab), anti-VEGFR antibody (ramucirumab) and anti-PD1 antibodies (nivolumab and pembrolizumab) (10-13); however, each of these drugs has shown only limited efficacy against *KRAS*-mutant lung adenocarcinomas.

Survivin, which is encoded by the baculoviral IAP repeat containing 5 (*BIRC5*) gene, is not expressed in normal adult tissues, apart from the thymus, placenta, CD34-positive hematopoietic stem cells and the colorectal mucosa. However, the protein is clearly expressed in the majority of malignant tumors, and is involved in carcinogenesis and therapeutic resistance (14,15). Survivin is a member of the inhibitor of apoptosis family of proteins, and it functions to suppress the activity of caspase 3/7 (14). Moreover, it plays an important role in chromosomal segregation and cytokinesis, and its expression level increases mostly at the G2/M phase of the cell cycle (15). The overexpression of survivin has been reported to be associated with a poor outcome in brain tumors, colorectal cancers, breast cancers and non-small-cell lung cancers (16-19). Furthermore, its expression levels are particularly high in tumors in which carcinoma cells are known to evade apoptosis, despite treatment with radiation or cytotoxic agents, including taxane and platinum (20-22). Indeed, the inhibition of survivin by treating cells with small-interfering RNAs (siRNAs) or ribozymes has been shown to enhance the sensitivity of cancer cells to these treatments (23). Thus, it has been proposed that inhibiting the expression or function of survivin may be a promising approach for overcoming resistance to therapy. However, to date, to the best of our knowledge, studies on the role(s) of survivin in *KRAS*-mutant lung adenocarcinomas are limited (24). In this study, through clinicopathological and molecular pathological analyses, we demonstrate that survivin may be a potential therapeutic target in *KRAS*-mutant lung adenocarcinomas.

The majority of invasive mucinous adenocarcinomas of the lung bear *KRAS* mutations and are negative for thyroid transcription factor 1 (TTF-1), an essential transcription factor for lung development encoded by the NK2 homeobox 1 (*NKX2-1*) gene. TTF-1 is expressed and is oncogenic in virtually all *EGFR*-mutant lung adenocarcinomas (25); however, the expression rate and role(s) of TTF-1 in *KRAS*-mutant lung adenocarcinomas remain to be elucidated. In the present study, we aimed to determine the role of survivin in *KRAS*-mutant lung adenocarcinomas, as well as the role of TTF-1. Our data indicate that the majority of *KRAS*-mutant lung adenocarcinomas analyzed were positive for TTF-1; thus, TTF-1 may play a role in the proliferation of *KRAS*-mutant cancer cells.

Materials and methods

Primary *KRAS*-mutant lung adenocarcinoma tissues and immunohistochemistry. All the experimental procedures involving human samples were approved by the Institutional Review Board at Sapporo Medical University. The samples used in this study were obtained from patients (n=208) who were operated on for lung adenocarcinoma at the Sapporo Medical University Hospital, Sapporo, Japan between January, 2005 and December, 2009. From these patients, we selected 28 patients (13%) whose tumors had a *KRAS* mutation (at codon 12 or 13) detected by the loop-hybrid mobility shift assay (26). These patients included 20 males and 8 females,

with a median age of 63 years (range, 31-85 years; Table I). We did not obtain written informed consent from the patients for conducting this retrospective study. Instead, the patients were informed of the outline of this study through the website of Sapporo Medical University so that they could 'opt out' from the study if they wished. All pathological slides were reviewed and evaluated by two of the authors (T.S. and Y.S.). Hematoxylin and eosin (H&E) staining was performed using Tissue-Tek Hematoxylyn 3G (8657; Sakura Finetek Japan, Tokyo, Japan) and Tissue-Tek Eosin (8660; Sakura FineTek Japan) according to the manufacturer's instructions. Immunohistochemical analysis for the expression of survivin, TTF-1 and E-cadherin was also carried out on formalin-fixed, paraffin-embedded tissue sections of the cancer specimens. Whole-tissue sections were retrieved using Novocastra Epitope Retrieval Solution 1 (pH 6.0) for E-cadherin expression or Solution 2 (pH 9.0) (both from Leica Biosystems, Nussloch, Germany) for the other antigens at 100°C for 20 min. The primary antibodies used were anti-survivin (sc-17779; D-8; 1:200; Santa Cruz Biotechnology, Dallas, TX, USA), anti-TTF-1 (N1635; 8G7G3/1; pre-diluted; Dako Japan, Tokyo, Japan) and anti-E-cadherin (#14472; 4A2; 1:100; Cell Signaling Technology Japan, Tokyo, Japan). Immunohistochemical staining was conducted using the Leica BOND-MAX (Leica Biosystems). In this study, the cancer tissues were judged as positive for survivin and TTF-1 expression when $\geq 10\%$ and $\geq 50\%$ of the cancer cells exhibited positive nuclear staining, respectively. As for E-cadherin, the tissues were judged as positive when membranous staining was observed in $\geq 80\%$ of the cancer cells. In addition, all the cancer tissues were classified as terminal respiratory unit (TRU) type or non-TRU type based on the definition in the literature (27).

Kaplan-Meier Plotter database. We used the Kaplan-Meier Plotter online service (<http://kmplot.com/analysis/index.php?p=background>) to clarify whether a high *BIRC5* mRNA expression was associated with an unfavorable outcome in patients with lung adenocarcinomas (28).

Cell culture and drugs used. Two *KRAS*-mutant lung adenocarcinoma cell lines, NCI-H358 (G12C) and NCI-H441 (G12V), were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained at 37°C in a humidified incubator with 5% CO₂. The cells were cultured in RPMI-1640 (Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum and antibiotics. The MEK inhibitor, trametinib (AdooQ BioScience, Irvine, CA, USA) and the Bcl-2 inhibitor (also known as a BH3 mimetic drug), ABT-263 (AdooQ BioScience), were also used in this study.

Immunofluorescence staining of the cells. Immunofluorescence staining was conducted as previously described for the expression of survivin (sc-17779; D-8; 1:200; Santa Cruz Biotechnology) and cytokeratin 7 (#4465; D1E4; 1:400; Cell Signaling Technology) in the H441 cells (29,30). Briefly, cells grown on 35-mm glass bottom, collagen-coated dishes (D11134H; Matsunami Glass, Osaka, Japan) were fixed with 4% paraformaldehyde at room temperature for 15 min followed by permeabilization with 0.5% Triton X-100 for 2 min. After the cells were rinsed with tris-buffered saline

Table I. Clinicopathological findings of the 28 patients with *KRAS*-mutant lung adenocarcinoma.

Pt	Age/ sex	Smoking history (pack year)	Dominant history	TRU or non-TRU type	Staging	Survivin	TTF-1	E-cadherin	OS (months)	Deceased or alive	DFS (months)	Recurrence
1	74/M	37.5	Pap	TRU	1B	+	+	+	117	Alive	117	-
2	68/M	34.5	MIA	TRU	1A	+	+	+	55	Alive	55	-
3	62/M	82	MIA	TRU	1A	-	+	+	127	Alive	127	-
4	81/F	50	Pap	TRU	2A	+	+	+	25	Deceased	25	-
5	60/M	80	Pap	TRU	2A	+	+	+	120	Alive	120	-
6	58/M	40	Pap	TRU	1B	+	-	+	45	Deceased	28	+
7	64/M	0	MIA	TRU	1A	-	+	+	115	Alive	115	-
8	60/M	69	AIS	TRU	1A	-	+	+	51	Alive	51	-
9	49/F	14	Pap	Non-TRU	2A	-	-	+	97	Alive	97	-
10	65/M	44	AIS	TRU	1A	-	+	+	106	Alive	106	-
11	73/F	50	Pap	Non-TRU	2A	+	-	+	76	Alive	76	-
12	31/F	0	IMA	Non-TRU	4	+	-	+	7	Deceased	1	+
13	73/M	53	Pap	Non-TRU	3A	+	-	+	11	Deceased	11	+
14	33/F	0	IMA	Non-TRU	2B	-	-	+	71	Alive	71	-
15	68/M	48	Pap	Non-TRU	1B	+	-	+	86	Alive	86	-
16	67/M	0	Acinar	TRU	2A	+	+	+	10	Deceased	9	+
17	59/M	39	Pap	TRU	1A	+	+	+	104	Alive	104	-
18	50/M	40	IMA	Non-TRU	2B	+	+	+	25	Alive	25	-
19	61/F	0	IMA	Non-TRU	1A	+	-	+	82	Alive	82	-
20	40/M	13.5	AIS	TRU	1A	-	+	+	99	Alive	99	-
21	62/M	30	Pap	TRU	1A	+	+	+	96	Alive	96	-
22	79/M	0	Pap	TRU	1A	+	+	+	53	Deceased	9	+
23	55/M	37	Pap	TRU	1A	-	+	+	91	Alive	91	-
24	71/F	0	Pap	TRU	1A	-	+	+	120	Alive	120	-
25	59/M	38	Solid	Non-TRU	1A	+	+	+	42	Deceased	43	-
26	85/F	0	IMA	Non-TRU	2B	+	-	+	26	Deceased	14	+
27	73/M	40	MIA	TRU	1A	+	+	+	57	Deceased	57	-
28	78/M	0	Pap	TRU	3A	-	+	+	64	Deceased	64	-

Pt, patient; Pap, papillary predominant adenocarcinoma; MIA, minimally invasive adenocarcinoma; AIS, adenocarcinoma *in situ*; IMA, invasive mucinous adenocarcinoma; acinar, acinar-predominant adenocarcinoma; solid, solid adenocarcinoma; TRU, terminal respiratory unit; TTF-1, thyroid transcription factor 1; OS, overall survival, DFS, disease-free survival.

(TBS), they were blocked using 3% BSA/TBS for 60 min at room temperature. The samples were then incubated overnight at 4°C with the two primary antibodies mentioned above. The samples were subsequently incubated with two secondary antibodies, Alexia Fluor 488-conjugated donkey anti-mouse IgG (A-21202; 1:400; Thermo Fisher Scientific Japan, Yokohama, Japan) and Alexia Fluor 594-conjugated goat anti-rabbit IgG (A-11012; 1:400; Thermo Fisher Scientific). The cells were finally observed under an inverted microscope (IX-71; Olympus, Tokyo, Japan) and photographed (DP80; Olympus).

RNA interference assay. The cells (3x10⁶) were plated in 94-mm culture dishes and transfected with negative control (NC) siRNA duplexes (1027281; Qiagen, Valencia, CA, USA) or siRNA duplexes targeting *BIRC5* and *NKX2-1* using Lipofectamine RNAiMAX reagent and OPTI-MEM I

(Thermo Fisher Scientific), as previously described (28-30). Two types of siRNA duplexes were used for transient survivin (encoded by the *BIRC5* gene) or TTF-1 (encoded by the *NKX2-1* gene) knockdown: Silencer Select Validated siRNA (Ambion #s1457 and #s1458, termed *BIRC5* siRNA #1 and #2, respectively, in this study; Thermo Fisher Scientific) and Silencer Select Pre-designed siRNA (Ambion #s14152 and #s14153, termed *NKX2-1* siRNA #1 and #2, respectively; Thermo Fisher Scientific). The final concentration of the siRNA used in each *in vitro* experiment was 10 nM. The downregulation of the expression of the targeted genes was verified by western blot analysis.

Assessment of cell viability and apoptosis. The number of viable cells was estimated using a CellTiter Glo 3D Cell Viability assay (Promega, Madison, WI, USA) according

to the manufacturer's instructions. Apoptosis was assessed by western blot analysis of cleaved poly(ADP-ribose) polymerase 1 (PARP-1) or Caspase-Glo 3/7 assay (Promega), as previously described (30,31). The luminescence of viable or apoptotic cells was measured with the Infinite 200 microplate reader (Tecan Japan, Kawasaki, Japan). All results are presented as the means \pm SD.

Cell cycle analysis by flow cytometry. The cells were reverse transfected with NC siRNA or *BIRC5* siRNA, and then grown for 48 h. The cells were then harvested and fixed in 75% methanol at 4°C overnight. The cells were washed twice with cold phosphate-buffered saline (PBS) and stained with propidium iodide (PI) (0.5 mg/ml RNase, and 0.1 mg/ml PI in PBS) for 30 min at room temperature. The stained cells were characterized using a flow cytometer (Beckman Coulter Corp., Tokyo, Japan), and the DNA content was analyzed using FlowJo software.

Senescence associated β -galactosidase staining and the counting of multinuclear cells. The cells (5×10^5 cells) were plated in 60-mm culture dishes before staining. Cellular senescence was detected using the Cellular Senescence kit (OZ Biosciences, San Diego, CA, USA), according to the manufacturer's instructions. Briefly, the cells were washed twice with PBS and then fixed in the fixation buffer provided in the kit at room temperature for 15 min. The cells were then washed twice with PBS, and stained using the β -galactosidase staining solution provided with the kit for 18 h at 37°C in a humidified incubator. The cells were visualized under a light microscope (IX-71; Olympus) and photographed (DP80; Olympus). Granules stained blue within the cytoplasm were considered positive for β -galactosidase staining. We also assessed the proportion of multinuclear cells, which are cells with two or more nuclei in the cytoplasm, by counting the number of multinuclear cells in at least 3 high-power fields under the same microscope mentioned above.

Western blot analysis. Western blot analysis for the expression of E-cadherin, vimentin, TTF-1, PARP-1, γ H2AX, Bim, total AKT, phospho-AKT, total ERK1/2, phospho-ERK1/2 and β -actin was performed as previously described (29-31). Additional primary antibodies used in the present study were anti-survivin (#2808; 71G4B7; 1:1,000 dilution; Cell Signaling Technology) and anti-p21 (#2947; 12D1; 1:1,000 dilution; Cell Signaling Technology). The cells were lysed in NuPAGE LDS Sample Buffer (Thermo Fisher Scientific). The cell lysates (15 μ g of total protein in each well) were separated by SDS-PAGE (SuperSep Ace, 5-20%, 13 wells; Wako Pure Chemicals, Osaka, Japan) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked by incubation with 3% non-fat dry milk in TBS for 1 h at room temperature and incubated overnight at 4°C with the primary antibodies mentioned above. The membranes were then washed 3 times and then incubated for 1 h at room temperature with species-specific horseradish peroxidase-conjugated secondary antibodies (NA931 or NA934; GE Healthcare, Buckinghamshire, UK). Blots were visualized using Supersignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Band intensity levels on

X-ray films were normalized to β -actin using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Crystal violet staining. The NC siRNA- or *BIRC5* siRNA-transfected H358 cells and H441 cells were cultured for 48 h. The cells were then plated at a density of 5×10^5 cells into the wells of a 6-well plate and treated with ABT-263 (1 μ M) alone, trametinib (25 nM) alone, both, or neither for 72 h. The plate was placed on ice, and the cells were washed twice with cold PBS, and then were fixed in ice-cold 100% methanol for 10 min. Following fixation, the cells were moved to room temperature and stained with 0.5% crystal violet solution (Tokyo Chemical Industry, Tokyo, Japan) diluted in 25% methanol for 10 min. Plates containing stained cells were then photographed using a digital camera (D610; Nikon, Tokyo, Japan).

Three-dimensional (3D) 'on-top' culture. The cells were cultured above a thin layer of 100% Matrigel (Corning, Corning, NY, USA) in RPMI-1640 medium, termed '3D on-top culture', as previously described, with minor modifications (32,33). Briefly, the Corning 96-well Flat Clear White Polystyrene TC-Treated Microplates were coated with 30 μ l/well of Matrigel and incubated at 37°C for 30 min to allow the Matrigel to solidify. Subsequently, each microspheroid [consisting of 50 cells in suspension for 24 h using PrimeSurface 96U plate (Sumitomo Bakelite, Tokyo, Japan)] was placed on the top of the Matrigel in each well, and grown for 72 h in 100 μ l medium.

Mouse tumor xenograft model. All animal experimentation was conducted in accordance with the protocol approved by the Animal Committee at Sapporo Medical University. The experiments were performed using 6-8-week-old female mice (n=15) (KSN/Slc nude mice; Hokudo, Sapporo, Japan). This study used a minimum of 5 mice per group. The mice were kept throughout the experiment in pleasant conditions (temperature, 20-26 °C; humidity, 40-60%) and were able to freely access food and water. The weights of the mice upon purchase and upon sacrifice were 23 g and 20 g on average, respectively. At 6 h following NC siRNA or *BIRC5* siRNA #1 transfection, 5×10^6 H358 cells were collected in 50 μ l of RPMI-1640 and then mixed with 50 μ l of Matrigel. The cell/Matrigel mixture was subcutaneously injected into the right flanks of mice anesthetized by inhalation of isoflurane (3-5%; Pfizer, New York, NY, USA). Each mouse received a single injection of H358 cells, and thus developed a single tumor nodule later. At 10 days after the injection, we injected AteloGene (Koken, Tokyo, Japan) containing NC siRNA (n=5) or *BIRC5* siRNA (n=10) near the tumor nodules to administer the siRNA continuously. Mice that were injected with *BIRC5* siRNA-transfected cells (n=10) were administered the vehicle (n=5) or a combination of ATB-263 (50 mg/kg) and trametinib (0.6 mg/kg) (n=5) orally once daily for 22 days (days 10-32 post-injection of the cells) (34-36). ABT-263 was dissolved in 10% ethanol, 30% polyethylene glycol 400, and 60% Phosal 50 PG; trametinib was dissolved in 10% Cremophor EL, 10% PEG400, and 80% dH₂O (Nacalai Tesque). The mice were monitored daily for body weight and general condition. Following any sign of deterioration in any mouse during the ABT-263 and trametinib therapy, the administration to the mouse was terminated immediately. Tumors were measured twice weekly using a caliper,

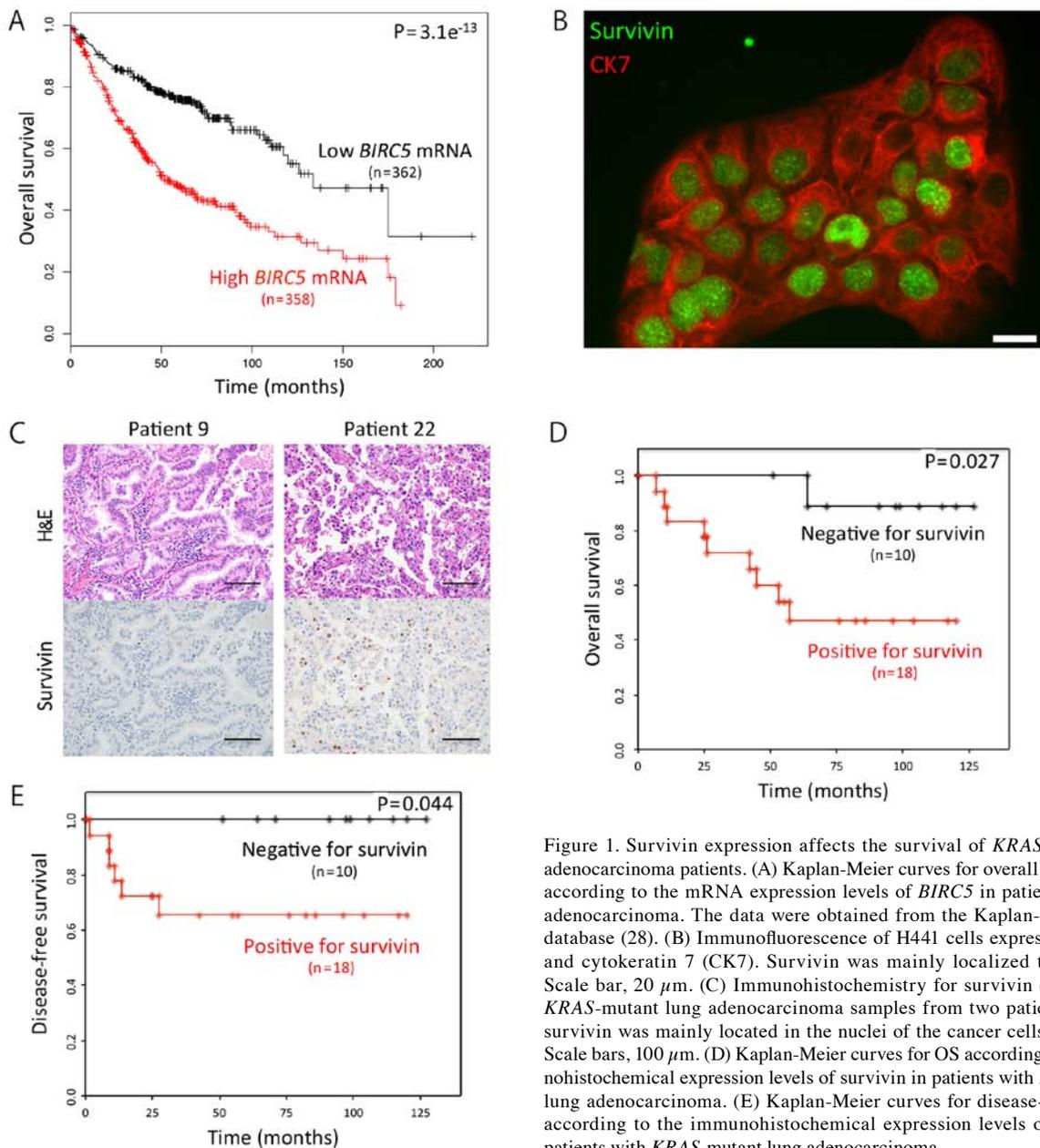


Figure 1. Survivin expression affects the survival of *KRAS*-mutant lung adenocarcinoma patients. (A) Kaplan-Meier curves for overall survival (OS) according to the mRNA expression levels of *BIRC5* in patients with lung adenocarcinoma. The data were obtained from the Kaplan-Meier Plotter database (28). (B) Immunofluorescence of H441 cells expressing survivin and cytokeratin 7 (CK7). Survivin was mainly localized to the nuclei. Scale bar, 20 μ m. (C) Immunohistochemistry for survivin expression in *KRAS*-mutant lung adenocarcinoma samples from two patients. Of note, survivin was mainly located in the nuclei of the cancer cells (patient 22). Scale bars, 100 μ m. (D) Kaplan-Meier curves for OS according to the immunohistochemical expression levels of survivin in patients with *KRAS*-mutant lung adenocarcinoma. (E) Kaplan-Meier curves for disease-free survival according to the immunohistochemical expression levels of survivin in patients with *KRAS*-mutant lung adenocarcinoma.

and the volume was calculated using the following formula: $0.5 \times (\text{width})^2 \times \text{length}$. After the observation, mice were anesthetized by inhalation of isoflurane and then sacrificed by cervical dislocation. The tumor nodules were subsequently removed for weight and histology.

Statistical analysis. The Kaplan-Meier method was used to estimate overall survival and disease-free survival of the 28 patients with *KRAS*-mutant lung adenocarcinoma. Differences in the survival curves between patients with survivin-positive and -negative tumors were compared using the log-rank test. Differences in caspase activity or cell viability between the untreated and treated cells were evaluated by Dunnett's test, a multiple comparison test as were differences in the number of multinuclear cells or senescence-associated β -galactosidase-positive cells between untreated and treated cells. One-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test was

performed to evaluate the effects of survivin knockdown, and survivin depletion plus combined therapy with ATB-263 and trametinib, on the cells or tumor xenografts. A P-value <0.05 were considered significant. All statistical calculations were performed using JMP software (JMP for Windows version 7, SAS Institute Japan, Tokyo, Japan).

Results

Patients with survivin-positive tumors have poorer outcomes. First, we searched the Kaplan-Meier Plotter for an association between the mRNA expression levels of the *BIRC5* and the outcome of patients with lung adenocarcinoma. We found that the overall survival (OS) of the patients with a high *BIRC5* expression (n=358) was markedly shorter than that of patients with a low *BIRC5* expression (n=362) (hazard ratio, 2.41; $P=3.1e^{-13}$) (Fig. 1A) by utilizing the Kaplan-Meier Plotter (28). We then focused on survivin (*BIRC5*) protein, not its mRNA,

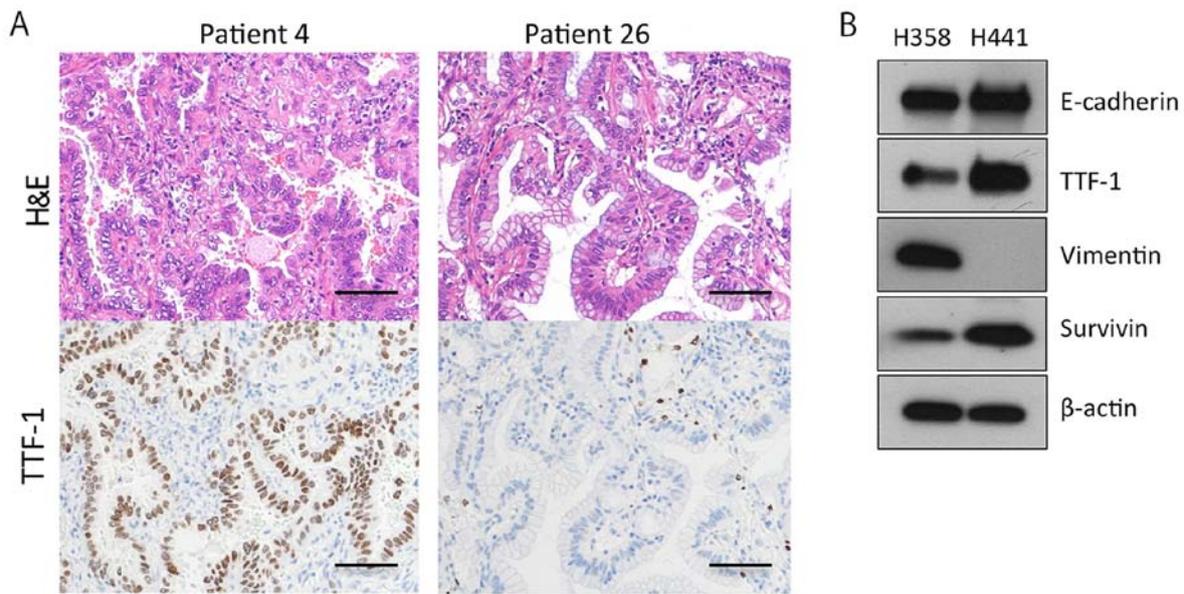


Figure 2. There are *KRAS*-mutant lung adenocarcinomas expressing thyroid transcription factor 1 (TTF-1). (A) Immunohistochemistry for TTF-1 expression in *KRAS*-mutant lung adenocarcinoma samples from two patients. The tumor cells of invasive mucinous adenocarcinoma (patient 26) were completely negative for TTF-1. Scale bars, 100 μ m. (B) Representative western blots of untreated H358 and H441 cells. Both cell lines were positive for TTF-1.

using immunocytochemistry, and found that it was mainly localized to the nuclei of the cells (Fig. 1B). Subsequently, we examined 28 *KRAS*-mutant lung adenocarcinoma samples for survivin expression using immunohistochemistry (Fig. 1C), and found that the OS rates of the patients with survivin-positive adenocarcinoma ($n=18$) was significantly shorter than that of patients with survivin-negative tumors ($n=10$) ($P=0.027$) (Fig. 1D). As expected, we obtained a similar result in the disease-free survival data analyzed (Fig. 1E).

KRAS-mutant lung adenocarcinomas are differentiated and positive for TTF-1 and E-cadherin. The majority of invasive mucinous adenocarcinomas of the lung bear the *KRAS* mutation, and do not express TTF-1 (25). Furthermore, more than half of *KRAS*-mutant lung adenocarcinoma cell lines (41/75, 55%) are negative for E-cadherin and positive for vimentin, indicating that the cell lines are more mesenchymal (36). In this study, however, the *KRAS*-mutant lung adenocarcinoma tissues were mostly positive for TTF-1 (19/28, 68%) and E-cadherin (28/28, 100%) (Table I). In addition, more than half of the tumors (18/28, 64%) were classified as TRU-type, and 7 out of the 28 (25%) tumors were diagnosed as adenocarcinoma *in situ* (AIS) or minimally invasive adenocarcinoma (MIA) (Table I; Fig. 2A). These findings suggested that the majority of the operable *KRAS*-mutant lung adenocarcinomas in Japan are of a relatively well-differentiated type. We further confirmed that 4 out of the 5 invasive mucinous adenocarcinomas stained negative for TTF-1 (Table I; Fig. 2A). Based on these findings, we then sought to analyze two *KRAS*-mutant lung adenocarcinoma cell lines, H358 cells and H441 cells, expressing TTF-1, E-cadherin and survivin (Fig. 2B).

Survivin knockdown impairs cell division, and induces senescence in KRAS-mutant lung adenocarcinoma cells. We knocked down survivin expression through RNA interference in the H358 cells and H441 cells in order to elucidate whether

and to what degree survivin affects the survival and/or proliferation of these cells. Survivin knockdown clearly decreased the proliferation of the two cell lines examined (Fig. 3A); however, it is unlikely that this was a result of apoptosis in terms of PARP-1 cleavage analyses (Fig. 3B). In addition, survivin knockdown considerably upregulated the phosphorylation of ERK1/2 (the main proliferative signal downstream of *KRAS*); however, the knockdown of survivin had a lesser effect on the PI3K-AKT pathway (Fig. 3B).

Survivin knockdown significantly increased the ratio of swelled to flattened cells, some of which had several nuclei (Fig. 3C and D). Cell cycle analyses using flow cytometry revealed an increase in the peak of tetraploid cells, reflecting cell cycle arrest and an increase in the number of binuclear cells. Furthermore, we observed a peak in the number of octoploid cells, suggesting that the binuclear cells were unable to divide under these conditions (Fig. 3E). The expression of p21, a senescence-associated protein, increased gradually with time in the cells in which survivin was knocked down (Fig. 3B), and senescence-associated β -galactosidase activity was also elevated (Fig. 3F and G). These findings collectively suggest that survivin knockdown inhibits cell division, and induces senescence in *KRAS*-mutant lung adenocarcinoma cells.

Combination therapy with survivin knockdown, ABT-263 and trametinib, induces massive apoptosis of KRAS-mutant lung adenocarcinoma cells. Given that survivin knockdown induced senescence, not apoptosis of *KRAS*-mutant lung adenocarcinoma cells, we then wished to determine whether ABT-263, a Bcl-2 inhibitor, and trametinib, a MEK inhibitor, induced apoptosis of *KRAS*-mutant lung cancer cells, as others have demonstrated that ABT-263 preferentially induces apoptosis of senescent cells (37), and MEK inhibitors are particularly effective at inducing apoptosis of *KRAS*-mutant cells when combined with ABT-263 (38). Thus, in this study,

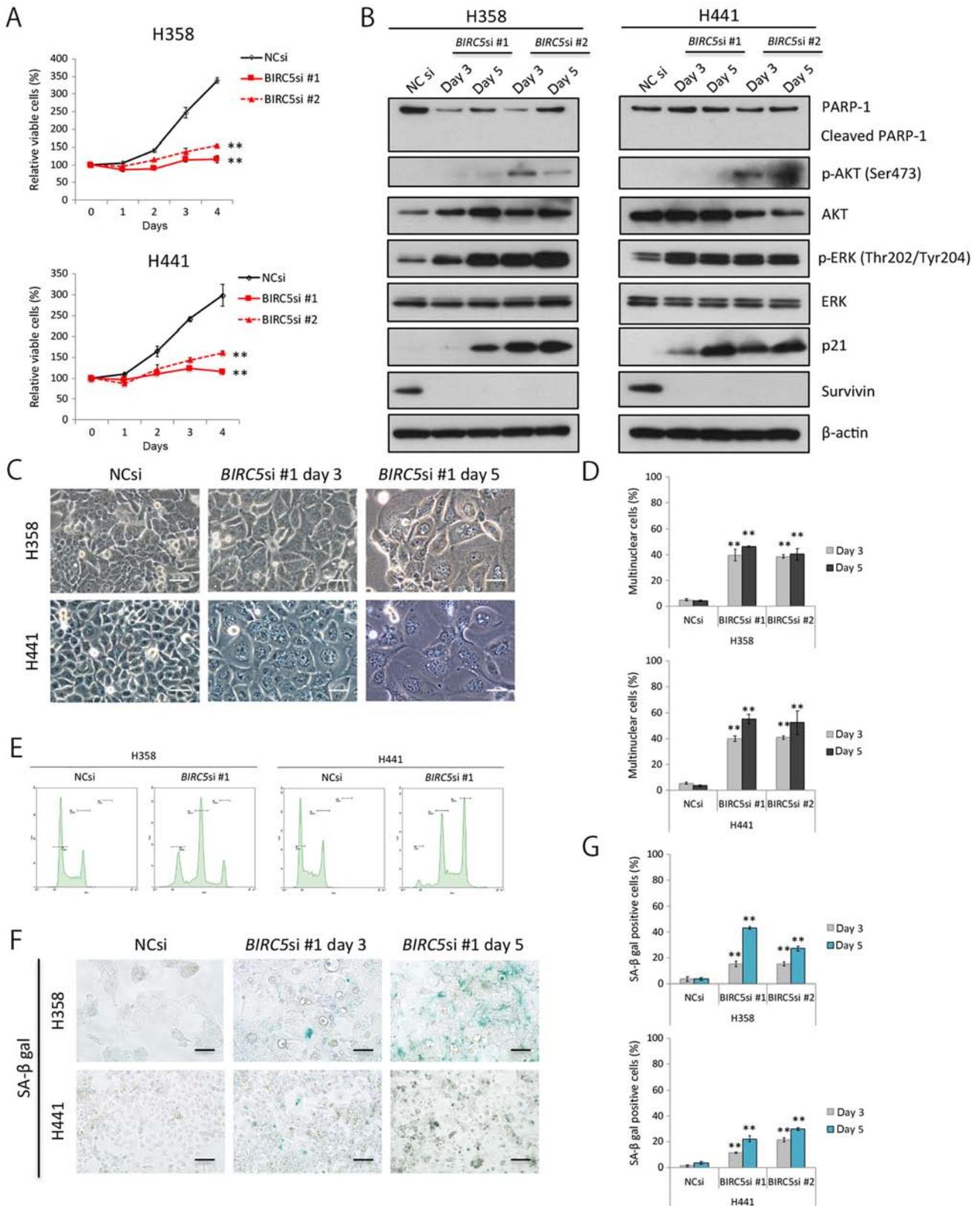


Figure 3. Survivin knockdown induces senescence, but not apoptosis of *KRAS*-mutant lung adenocarcinoma cells. (A) Effects of survivin knockdown on proliferation of H358 and H441 cells. Cells were transfected with NC siRNA or *BIRC5* siRNA (10 nM each) and cultured for 48 h. Cell viability was then assessed for a further 96 h in triplicate. Results are expressed as the means \pm SD; **P<0.01. (B) Western blot analysis of the effects of survivin knockdown in H358 and H441 cells. Cell were transfected with NC siRNA or *BIRC5* siRNA and then cultured for 72 or 120 h. (C and D) Induction of multinucleation in cells following survivin knockdown. The NC siRNA- or *BIRC5* siRNA (10 nM each)-transfected H358 and H441 cells were cultured for 72 or 120 h, and the number of nuclei in each cell line was then counted. Results are expressed as the means \pm SD; Scale bars, 50 μ m; **P<0.01. (E) Cell cycle analysis using a flow cytometer. Cells were transfected with NC siRNA or *BIRC5* siRNA (10 nM each) and then grown for 48 h. The number of tetraploids and octoploids markedly increased. (F and G) Induction of senescence-associated β -galactosidase-positive cells by survivin knockdown. The NC siRNA- or *BIRC5* siRNA (10 nM each)-transfected H358 and H441 cells were cultured for 72 or 120 h, and the number of stained cells was then evaluated. Results are shown as the means \pm SD; Scale bars, 50 μ m; ***P<0.01.

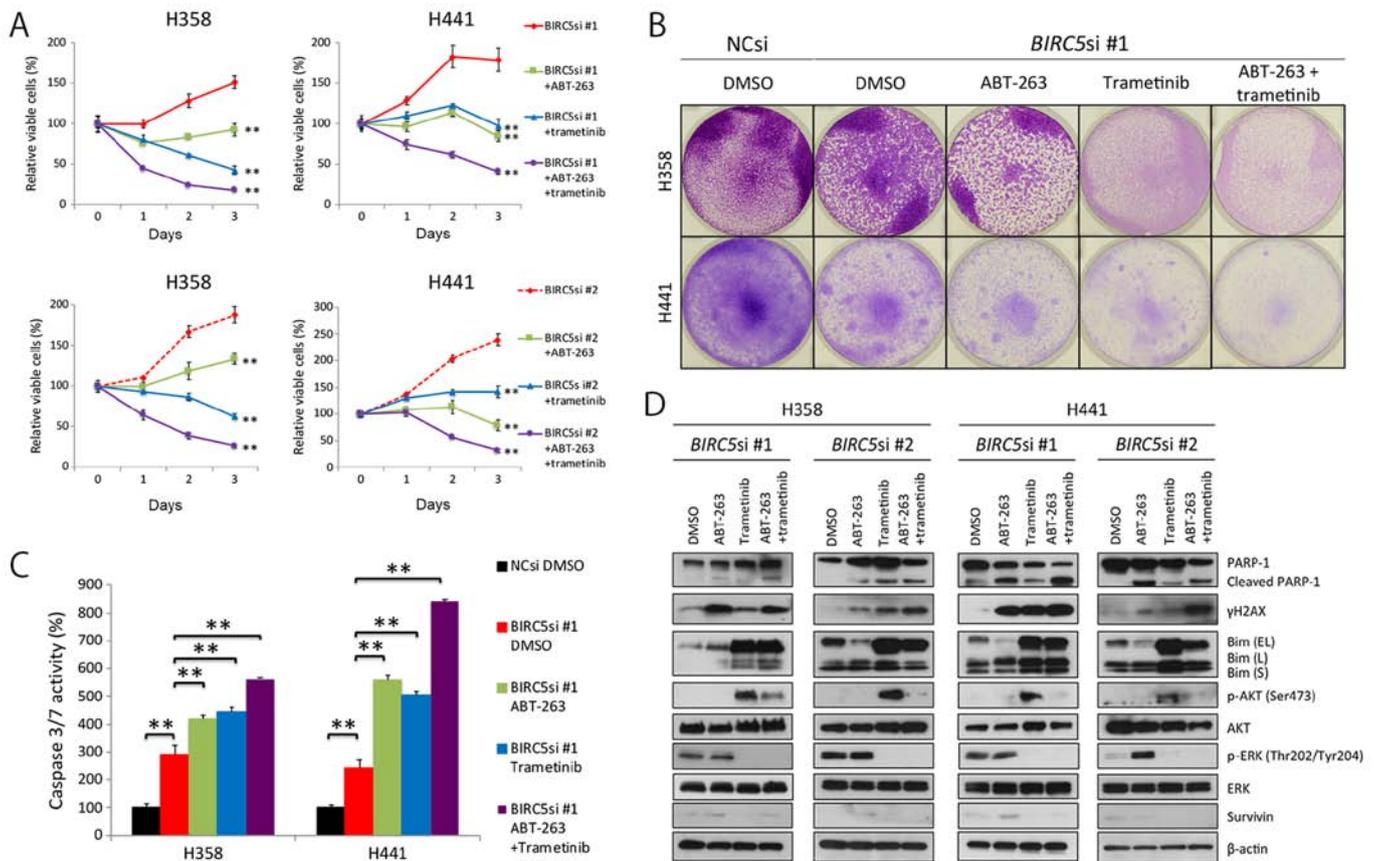


Figure 4. Combination of survivin knockdown with ABT-263 and trametinib effectively induces apoptosis of *KRAS*-mutant lung adenocarcinoma cells. (A) Effects of triple combination therapy of survivin knockdown, ABT-263 and trametinib on the viability of H358 and H441 cells. Cells were transfected with *BIRC5* siRNA #1 or #2 (10 nM each) and cultured for 48 h. Cells were then treated with the ABT-263 (1 μ M) alone, trametinib (25 nM) alone, both, or neither for a further 72 h. Results are shown as the means \pm SD; ** P <0.01. (B) Crystal violet staining of viable cells. NC siRNA or *BIRC5* siRNA #1 (10 nM each)-transfected cells were grown for 48 h. A total of 5×10^5 cells were then seeded in 6-well plates and treated with ABT-263 (1 μ M) alone, trametinib (25 nM) alone, both, or neither for a further 72 h. Cells were then fixed and stained with crystal violet. (C) The effects of triple combination therapy of survivin knockdown, ABT-263 and trametinib on caspase 3/7 activity in H358 and H441 cells. The NC siRNA- or *BIRC5* siRNA #1 (10 nM each)-transfected cells were cultured for 48 h, and the cells were then treated with ABT-263 (1 μ M) alone, trametinib (25 nM) alone, both, or neither for a further 24 h before evaluating caspase activity. Caspase 3/7 activity was normalized to 100 for the mean of three control (NCsi) dishes. Columns, mean (n=3); bars, SD; ** P <0.01. (D) Western blot analysis of the effects of the triple combination therapy on H358 and H441 cells. Cells were treated in the same manner as described in (A) or (B). Of note, Bim (EL) was dephosphorylated by trametinib treatment, and then accumulated in the cell. EL, extra-long; L, long; S, short.

we treated the H358 and H441 cells in which survivin was knocked down with ABT-263 alone, trametinib alone, or a combination of ABT-263 and trametinib (hereafter referred to as AT therapy). As expected, AT therapy clearly decreased cell viability (Fig. 4A and B), markedly elevated caspase 3/7 activation (Fig. 4C), and considerably increased the expression levels of γ H2AX and cleaved PARP-1 (Fig. 4D). Of note, selumetinib, another MEK inhibitor, replaced trametinib and had similar results (data not shown). Taken together, these findings suggest that the combination treatment of survivin knockdown, ABT-263 and trametinib, is an efficacious treatment for *KRAS*-mutant lung adenocarcinomas.

Triple combination therapy is also effective against 3D-cultured KRAS-mutant lung adenocarcinomas cells. Up to this point, our experiments were carried out using conventional, monolayer culture conditions, which do not truly reflect the *in vivo* environment (32,33). We thus employed a 3D on-top culture condition to recapitulate aspects of the *in vivo* environment (Fig. 5A), and then analyzed microspheroids comprising ~ 50 cells ($<100 \mu\text{m}$ in diameter), which we believe

mimic the micrometastases of lung adenocarcinoma. The NC siRNA-transfected microspheroids steadily increased in size over time, whereas the *BIRC5* siRNA-transfected microspheroids grew at a much slower rate (Fig. 5B). In addition, the microspheroids treated with the triple combination of survivin knockdown and AT therapy were markedly decreased in size (Fig. 5B), and this was confirmed by a viability assay (Fig. 5C). These findings suggest that the triple combination therapy could potentially eradicate the micrometastases of *KRAS*-mutant lung adenocarcinoma cells *in vivo*.

Triple combination therapy is also effective for subcutaneous KRAS-mutant lung adenocarcinomas in mice. To confirm the *in vivo* potency of the triple combination therapy, we subcutaneously implanted NC siRNA- or *BIRC5* siRNA-transfected H358 cells into mice. Since the efficacy of the pre-treated siRNAs was transient, we used the AteloGene to administer siRNAs to subcutaneous tumors. The NC siRNA-transfected cells grew steadily, whereas the *BIRC5* siRNA-transfected cells exhibited minimal proliferation. Moreover, AT therapy further reduced the size and weight of the tumor nodules derived from

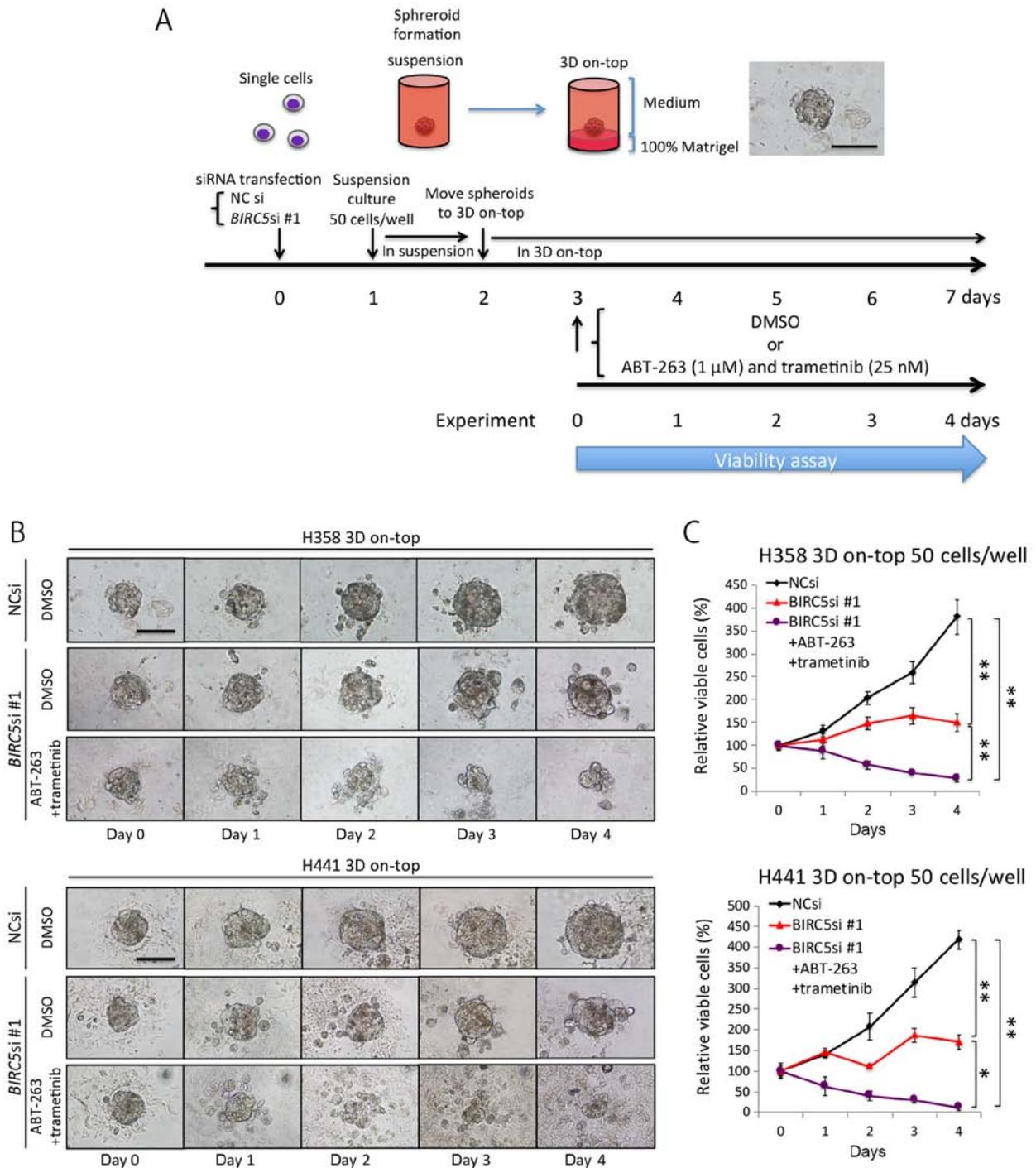


Figure 5. Triple combination therapy comprising of survivin knockdown, ABT-263 and trametinib is also effective against three-dimensionally (3D) cultured *KRAS*-mutant lung adenocarcinoma cells. (A) Schematic description of the experimental procedure and the representative image of a microspheroid in the 3D 'on-top' culture condition. (B) Representative images of microspheroids of 50 cells in 3D 'on-top' culture condition. The spheroids derived from NC siRNA- or *BIRC5* siRNA (10 nM each)-transfected cells were cultured in the absence or presence of ABT-263 (1 μ M) plus trametinib (25 nM) for 96 h. (C) Effects of the triple combination therapy on H358 and H441 spheroids. Viability was assessed as described in the schema in (A). Results are shown as the means \pm SD; * P <0.05, ** P <0.01.

BIRC5 siRNA-transfected cells, although the effects of the therapy did not reach statistical significance (when comparing the *BIRC5* siRNA group to the triple combination treatment group) (Fig. 6A-C). Pathological analyses demonstrated that not only the NC siRNA-transfected tumors, but also the *BIRC5* siRNA-transfected nodules thrived in the absence of AT therapy, with a positive survivin expression noted in the cancer cell nuclei of both tumor types (Fig. 6D). We surmised that the

BIRC5 siRNA in the AteloGene had already been consumed by day 11, as Fig. 6A indicates that the tumor nodules derived from the *BIRC5* siRNA-transfected cells slightly, but steadily increased in size after day 11. However, Fig. 6D demonstrates that the triple combination therapy did suppress the expression of survivin, and induced apoptosis to a certain extent in the cells, indicating the *in vivo* efficacy of the triple combination therapy.

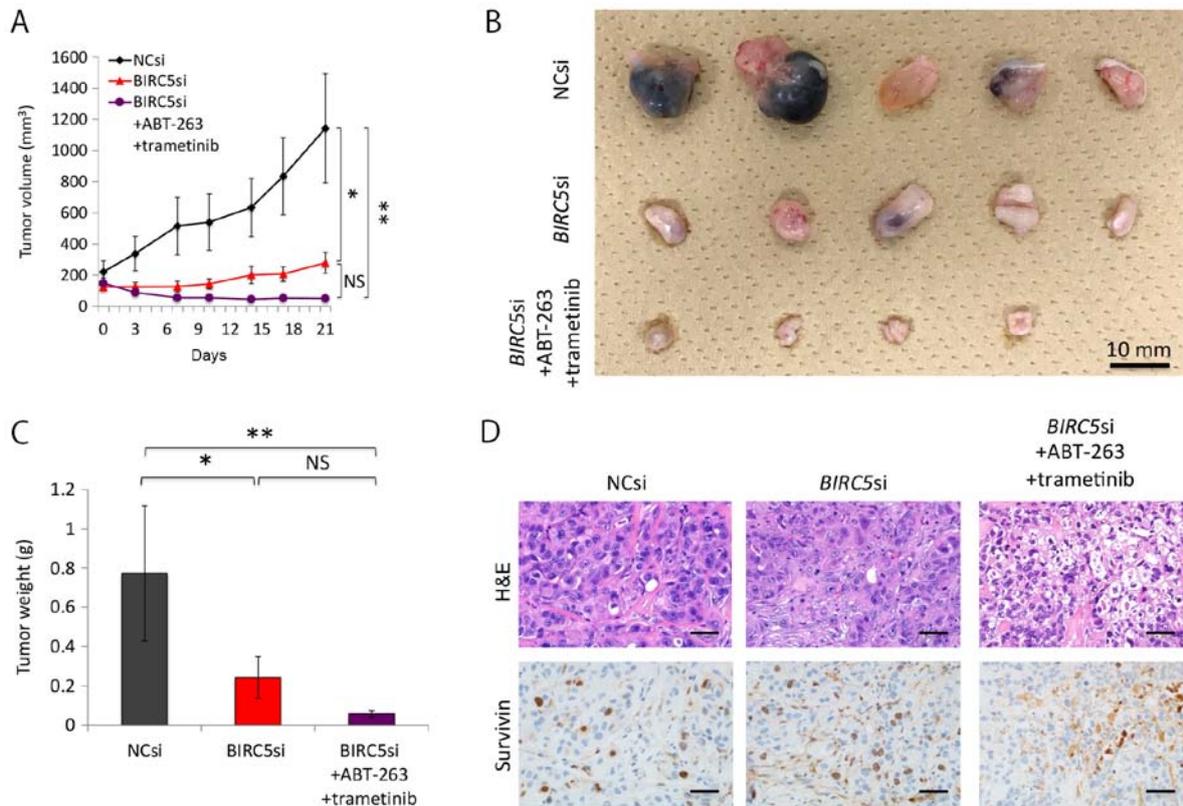


Figure 6. Triple combination of survivin knockdown, ABT-263, and trametinib decreases the size of xenografts of H358 cells. (A) Effects of the triple combination therapy on xenografts of H358 cells. Mice with tumor xenografts derived from *BIRC5* siRNA-transfected cells were untreated (n=5) or treated (n=5) with ABT-263 (50 mg/kg) and trametinib (0.6 mg/kg) for 22 days. Mice with NC siRNA-transfected xenografts (n=5) were treated with vehicle. Drugs were administered once daily by oral gavage. Tumor volumes (means \pm SD) were measured after the initiation of the AT treatment (day 1). ‘Day 1’ in this figure corresponds to the day 10 after implantation of the cells, when the AteloGene was also administered. Of note, one of the five mice with *BIRC5* siRNA-transfected xenografts receiving AT treatment died during the experiment. The death at day 13 was probably due to the side-effects of trametinib used, as only this mouse developed a severe skin rash from day 10, when the administration to the mouse was terminated. As previously shown in clinical practice, the use of trametinib can lead to the development of skin rash (55). * $P < 0.05$, ** $P < 0.01$. (B) Effects of the triple combination therapy on xenografts derived from H358 cells. Macroscopic images of the tumors resected from mice are represented. Fourteen tumor nodules presented herein were derived from the 14 mice that survived to the end of the therapy. (C) Effect of the triple combination therapy on xenografts of H358 cells. The weight of each tumor, shown in (B), was measured, and the results are presented as the means \pm SD. * $P < 0.05$, ** $P < 0.01$. (D) Pathological examination of xenografts. Hematoxylin and eosin (H&E) staining and immunohistochemistry for survivin of xenografts in each treatment condition. Of note, tumors derived from both the NC si-transfected and *BIRC5* si-transfected cells without AT therapy expressed survivin in the nuclei, whereas the staining observed in the tumors derived from *BIRC5* si-transfected cells with AT therapy were likely to reflect immunoglobulins derived from the mouse; i.e., non-specific staining.

TTF-1 knockdown suppresses the proliferation of *KRAS*-mutant lung adenocarcinoma cells and induces partial survivin knockdown. *TTF-1* knockdown using *NKX2-1* siRNA slightly, but significantly suppressed the proliferation of the H358 cells and H441 cells (Fig. 7A). Of note, the expression levels of survivin appeared to decrease slightly when *TTF-1* was knocked down (Fig. 7B and C). These observations suggest that *TTF-1* probably enhances the growth of *TTF-1*-positive, differentiated *KRAS*-mutant lung adenocarcinoma cells, and that the expression levels of survivin are possibly controlled, at least in part, by *TTF-1*.

Discussion

In this study, we demonstrated that in lung adenocarcinoma patients, the OS of patients with tumors expressing high mRNA levels of *BIRC5* (survivin) is markedly shorter than that of patients with low expression levels. Furthermore, the OS of patients with *KRAS*-mutant lung adenocarcinomas expressing survivin protein is also significantly shorter than

that of patients with survivin-negative tumors. Since the majority of the *KRAS*-mutant lung adenocarcinoma tissues we analyzed were well-differentiated and positive for *TTF-1* and E-cadherin, we selected and analyzed H358 and H441 cells, two *TTF-1*-positive, differentiated *KRAS*-mutant lung adenocarcinoma cell lines, to explore a treatment strategy that would be able to induce extensive apoptosis of these cancer cells. We found that the combination of survivin knockdown along with ABT-263 and trametinib treatment induced massive apoptosis of these cancer cells.

Previous studies have indicated that survivin-positive, small-sized lung adenocarcinomas and non-small cell lung carcinomas are significantly associated with an unfavorable outcome (39-42); however, little information is available as to the roles of survivin in *KRAS*-mutant lung adenocarcinoma. In this study, we demonstrated that survivin-positive, *KRAS*-mutant lung adenocarcinomas are more malignant than survivin-negative tumors in terms of survival data. Although the knockdown of survivin in cells reportedly inhibits cell division and causes multinucleation (43,44), we confirmed

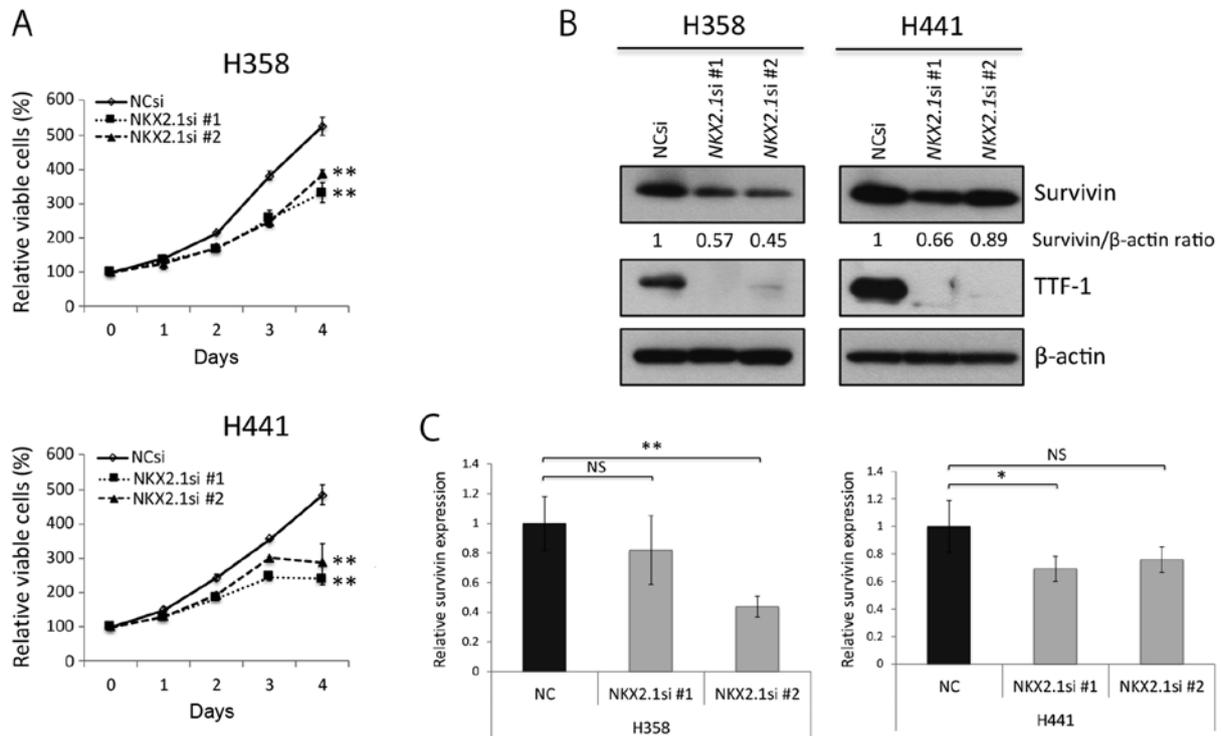


Figure 7. *KRAS*-mutant lung adenocarcinoma cells are slightly dependent on TTF-1 for proliferation. (A) Effects of TTF-1 knockdown on the proliferation of H358 and H441 cells. Cells were transfected with NC siRNA or *NKX2-1* siRNA (10 nM each) and cultured for 48 h. Viable cells were estimated for another 96 h in triplicate. Results are shown as the means \pm SD; ***P*<0.01. (B) Western blot analysis of the effects of TTF-1 knockdown in H358 and H441 cells. Cells were transfected with NC siRNA or *NKX2-1* siRNA (10 nM each) and then cultured for 72 h. (C) Quantification of survivin expression, as shown in (B), in NC siRNA- or *NKX2-1* siRNA (10 nM each)-transfected H358 and H441 cells. Columns, mean (n=3); bars, \pm SD; **P*<0.05, ***P*<0.01.

that survivin knockdown renders the H358 and H441 cells multinucleated owing to a cytokinesis block. Furthermore, we observed a gradual increase in p21 expression and an increase in senescence-associated β -galactosidase activity in the survivin-depleted cells. In other words, survivin knockdown alone was not able to trigger apoptosis of the H358 and H441 cells, but induced cellular senescence. However, the *KRAS*-mutant cells in which survivin was knocked down in monolayer underwent massive apoptosis following AT therapy, probably as trametinib dephosphorylates Bim, a pro-apoptotic Bcl-2 family member, and dephosphorylated Bim can avoid undergoing degradation through the ubiquitin-proteasome system (37,38). It has also been indicated that *KRAS*-mutant carcinomas, particularly ones exhibiting an epithelial phenotype, usually depend on Bcl-xL, an anti-apoptotic Bcl-2 family of proteins, for survival (38). Although we inhibited the anti-apoptotic function of Bcl-xL using ABT-263 in this study, a recent study demonstrated that not only Bcl-xL, but also Mcl-1, another anti-apoptotic Bcl-2 family member, plays a key role in the survival of *KRAS*-mutated lung cancers (45). More importantly, the triple combination of survivin knockdown, ABT-263 and trametinib, substantially decreased the size of the microspheroids of *KRAS*-mutant cancer cells in 3D on-top culture. Although the 3D 'on-top' culture model exploited in this study cannot fully recapitulate all aspects of the *in vivo* microenvironment, it does offer more benefits than a monolayer culture (32,33). Distant metastases following surgery in clinical practice are likely to result from the marked growth of pre-existing micrometastatic foci, which cannot be detected using current imaging diagnostics (46). Our triple

combination therapy clearly decreased the viability of the cells in the 3D-cultured microspheroids, each of which consisted of only 50 *KRAS*-mutant cancer cells as a model of micrometastasis. This suggests that the triple combination could eradicate micrometastatic foci, or at least prevent them from growing. We further confirmed the effectiveness of the triple combination therapy for H358 cells *in vivo*, and also found that the treatment induced extensive apoptosis of the *EGFR*-mutant H1975 cells in monolayer (data not shown), suggesting that the triple combination could be applicable to lung adenocarcinoma in general.

Almost all *EGFR*-mutant lung adenocarcinomas express TTF-1, whereas invasive mucinous adenocarcinomas of the lung, most of which harbor the *KRAS* mutation, do not express TTF-1 (25,27,47). Of note, the loss of TTF-1 expression in pulmonary *KRAS*-mutated mucinous tumors is probably due to an inactivating mutation and/or hypermethylation of the *NKX2-1* gene (48,49). TTF-1 serves as a lineage-specific oncogene in *EGFR*-mutant lung adenocarcinomas through the induction of ROR1, a tyrosine kinase (50). By contrast, the TTF-1-negative, *KRAS*-mutant lung adenocarcinoma cell line, A549 cells, are sensitized to cisplatin by forced expression of the *NKX2-1* gene, suggesting that *KRAS* mutation and TTF-1 expression have negative effect(s) on each other (51). However, the majority of the primary *KRAS*-mutant lung adenocarcinoma tissues analyzed in the present study were relatively differentiated, TTF-1-positive tumors. In addition, the H358 cells and H441 cells, exhibiting a similar phenotype to that of the cancer tissues examined, are partly dependent on TTF-1 for proliferation. This effect of TTF-1 on *KRAS*-mutant

cancer cells may be regulated by, at least in part, survivin. Collectively, these findings raise the possibility that TTF-1 serves as an oncogene in *KRAS*-mutant, well-differentiated lung adenocarcinomas through survivin induction and other unknown mechanism(s).

More than half of the *KRAS*-mutated lung adenocarcinomas analyzed in the present study were of the TRU-type, and one fourth of them were classified as AIS or MIA. Although these pathological findings may seem unexpected in that quite a few *KRAS*-mutant lung adenocarcinomas are less aggressive than might be expected, we would like to stress that the findings mentioned above are consistent with those of previous reports (27,52). This suggests that *KRAS*-mutant lung adenocarcinomas, at least operable ones, appear more differentiated and less aggressive than might be thought.

This study has several limitations. First, although the immunohistochemical expression of survivin in *KRAS*-mutant lung adenocarcinomas was linked to an unfavorable outcome, we analyzed the tissue samples from a limited number of patients. We thus need to confirm our findings with a larger selection of *KRAS*-mutant lung cancer tissue samples. Second, although trametinib is available in clinical practice, ABT-263 is currently undergoing clinical trials (<https://clinicaltrials.gov/ct2/show/NCT02079740>), and has been reported to cause thrombocytopenia (53,54). Finally, we limited our analysis of *KRAS*-mutant lung adenocarcinoma cell lines to two types, and therefore we cannot conclude that the majority of *KRAS*-mutant lung cancers will be sensitive to the triple combination therapy.

In conclusion, *KRAS*-mutant lung adenocarcinoma tissues examined in this study mostly express TTF-1, and the cancer cells depend, at least in part, on TTF-1 for growth. In addition, survivin-positive *KRAS*-mutant lung adenocarcinomas are more malignant than survivin-negative adenocarcinomas, and survivin knockdown induces senescence in cancer cells. The triple combination therapy of survivin-depletion, ABT-263, and trametinib can induce substantial apoptosis in *KRAS*-mutant cancer cells both *in vitro* and *in vivo*. It can thus be concluded that survivin is a promising target for the treatment of *KRAS*-mutant lung adenocarcinomas, with a triple combination therapy potentially a valuable approach for treating such cancers.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

TS conducted most of the experiments in this study with some assistance from YT and MT. SH, YM, and YS examined the lung cancer tissues for *KRAS* mutation. MY carried out cell cycle analyses. GY, TH, AW, and HT provided the tumor tissues and analyzed the clinical data. TN, AW and HT provided helpful discussions and critically reviewed the manuscript. YS designed and conceived the study. TS and YS analyzed all the data, and wrote the manuscript. All authors commented on and approved the manuscript.

Ethics approval and consent to participate

All the experimental procedures involving human samples were approved by the Institutional Review Board at Sapporo Medical University. Written informed consent was not obtained from the patients for conducting this retrospective study. Instead, the patients were informed of the outline of this study through the website of Sapporo Medical University so that they could 'opt out' from the study if they wished. All animal experimentation was conducted in accordance with the protocol approved by the Animal Committee at Sapporo Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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