Title 論文題目	MCL1 inhibition enhances the therapeutic effect of MEK inhibitors in KRAS-mutant lung adenocarcinoma cells. （MCL1 阻害は KRAS 変異陽性肺腺癌における MEK 阻害薬の治療効果を高める）
Author(s) 著　者	多田, 周
Degree number 学位記番号	甲第 3071 号
Degree name 学位の種別	博士（医学）
Issue Date 学位取得年月日	2019-09-30
Original Article 原著論文	Lung Cancer. 2019; 133: 88-95.
Doc URL	
DOI	
Resource Version	Publisher Version

Lung Cancer 133 (2019) 88–95

Contents lists available at ScienceDirect

Lung Cancer

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

MCL1 inhibition enhances the therapeutic effect of MEK inhibitors in *KRAS*-mutant lung adenocarcinoma cells

Makoto Tada[a,b], Toshiyuki Sumi[a,c], Yusuke Tanaka[a,c], Sachie Hirai[a], Miki Yamaguchi[a], Masahiro Miyajima[b], Toshiro Niki[d], Hiroki Takahashi[c], Atsushi Watanabe[b], Yuji Sakuma[a,*]

[a] *Department of Molecular Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, South 1, West 17, Chuo-ku, Sapporo 060-8556, Japan*
[b] *Department of Thoracic Surgery, Sapporo Medical University School of Medicine, South 1, West 17, Chuo-ku, Sapporo 060-8556, Japan*
[c] *Department of Respiratory Medicine and Allergology, Sapporo Medical University School of Medicine, South 1, West 17, Chuo-ku, Sapporo 060-8556, Japan*
[d] *Division of Integrative Pathology, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan*

ARTICLE INFO

Keywords:
KRAS-mutant lung adenocarcinoma
MCL1
Bcl-xL
Trametinib
Apoptosis

ABSTRACT

Objectives: MCL1 is an anti-apoptotic BCL2 family member that is highly expressed in various malignant tumors. However, little is known about the role of MCL1 in *KRAS*-mutant lung adenocarcinomas. In this study, we aimed to clarify whether MCL1 could be a therapeutic target in *KRAS*-mutant lung adenocarcinomas for which no effective molecular targeted drugs are available.

Materials and methods: We examined to what extent MCL1 knockdown either alone or in combination with MEK inhibitor trametinib suppressed growth or induced apoptosis in the *KRAS*-mutant lung adenocarcinoma cell line H441 and *EGFR*-mutant lung adenocarcinoma cell line H1975. Furthermore, we investigated the therapeutic effects of dual inhibition of MCL1 and Bcl-xL, another anti-apoptotic BCL2 family member, in these two cell lines.

Results: MCL1 knockdown alone did not induce apoptosis in H441 or H1975 cells. However, MCL1-depleted H441 and H1975 cells underwent apoptosis and decreased in number in the presence of trametinib. We also confirmed that combined therapy by MCL1 knockdown and trametinib almost completely suppressed the growth of H441 cells *in vivo*. Moreover, dual knockdown of MCL1 and Bcl-xL induced extensive apoptosis in H441 and H1975 cells.

Conclusion: These findings suggest that combined treatments of MCL1 knockdown and trametinib or dual inhibition of MCL1 and Bcl-xL would be effective therapies for lung adenocarcinomas including the *KRAS*-mutant subtype.

1. Introduction

Lung cancer is the leading cause of cancer deaths worldwide with a 5-year survival rate of 16% among patients with all stages of the disease [1]. The majority of lung cancers are classified as non-small cell lung cancer (NSCLC), and adenocarcinoma is the most common histological subtype of NSCLC. Certain subtypes of lung adenocarcinomas are treatable by 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]. However, there are no effective treatments for *KRAS*-mutant lung adenocarcinomas, which account for one third of lung adenocarcinomas [3]. Constitutively activated mutant KRAS protein is technically difficult to suppress by small molecule inhibitors [4]. Since mutant KRAS constantly activates MEK (MAPK/ERK kinase), it has been regarded as an alternative therapeutic target for *KRAS*-mutant tumors [5,6]. However, even the MEK inhibition strategy is rather difficult, because second line treatment with trametinib, a MEK inhibitor, did not significantly improve progression-free survival compared with docetaxel alone in a clinical trial for *KRAS*-mutant lung cancer [7]. However, we have recently reported that trametinib suppresses the expression of survivin and induces senescence in *KRAS*-mutant lung adenocarcinoma cells *in vitro* [8]. These findings raise the possibility that treatment with trametinib might have a certain efficacy against *KRAS*-mutant lung adenocarcinomas.

* Corresponding author at: Department of Molecular Medicine, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, South 1, West 17, Chuo-ku, Sapporo 060-8556, Japan.
E-mail address: sakuma@sapmed.ac.jp (Y. Sakuma).

https://doi.org/10.1016/j.lungcan.2019.05.014
Received 13 December 2018; Received in revised form 21 March 2019; Accepted 13 May 2019

Apoptosis can be induced via two main pathways, the cell extrinsic pathway and cell intrinsic pathway [9]. The cell intrinsic pathway is regulated by the BCL2 family of proteins, which shares a common BH (BCL2 homology) 3 domain. The "BH3-only" family of proteins, including BID, BAD, BIM, BMF, PUMA, and NOXA, possesses only the BH3 domain and promotes apoptosis induction, while the anti-apoptotic proteins, such as BCL2, Bcl-xL, BCL-W, A1/Bfl1, and MCL1, have additional BH1 and BH2 domains. Upon activation of BH3-only proteins, another proapoptotic family of proteins, the "BAX" family (BAK, BAK, and BOK), forms pores in mitochondria, resulting in cytochrome C release and apoptosis induction of the cell. Of note, BIM plays a particularly important role in apoptosis induction of lung adenocarcinoma cells [10,11]. The anti-apoptotic proteins bind to BH3-only proteins or BAX family members and prevent induction of apoptosis [9]. The balance between proapoptotic and anti-apoptotic members of the BCL2 family of proteins determines whether apoptosis occurs [12].

Several BH3-only protein mimetics (hereafter called BH3 mimetics) have been developed to promote apoptosis of cancer cells, and one of the representatives is ABT-263 (also known as navitoclax). The efficacies of ABT-263 alone or combined with other molecular targeted drugs against NSCLC have been investigated in preclinical studies [13,14]. Interestingly, ABT-263 inhibits the anti-apoptotic functions of BCL2 and Bcl-xL, but is ineffective against those of MCL1. Although there were no BH3 mimetics against MCL1 until recently, S63845 has been developed and considered promising [15]. However, its efficacy against lung cancer has not been elucidated.

Although the importance of Bcl-xL in the survival of *KRAS*-mutant lung adenocarcinoma cells has already been recognized [16], the extent to which MCL1 contributes to their survival has not been fully clarified. In this study, we demonstrate that MCL1 may be a new therapeutic target in well-differentiated *KRAS*-mutant lung adenocarcinomas.

2. Materials and methods

2.1. Kaplan-Meier plotter database

We used the Kaplan-Meier Plotter online service (http://kmplot.com/analysis/index.php?p=background) to clarify whether high *MCL1* mRNA expression was associated with an unfavorable outcome of patients with lung adenocarcinomas [17].

2.2. Cell culture and drug treatments

Two *KRAS*-mutant lung adenocarcinoma cell lines, NCI-H441 (G12 V) and NCI-H460 (Q61 H), and the *EGFR*-mutant lung adenocarcinoma cell line NCI-H1975 (L858R + T790 M) were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained at 37 °C in a humidified incubator with 5% CO_2. The cells were cultured in RPMI-1640 (Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum and antibiotics. Two MEK inhibitors, trametinib (AdooQ BioScience, Irvine, CA, USA) and selumetinib (AdooQ BioScience), and three BH3 mimetics, ABT-263 (AdooQ BioScience), S63845 (Cayman Chemical, Ann Arbor, MI, USA), and WEHI-539 (AdooQ BioScience) were also used in this study.

2.3. Immunofluorescence staining

Immunofluorescence staining was conducted as previously described for the expression of MCL1 in H441 and H1975 cells [18,19]. Briefly, cells grown on 35-mm glass bottom, collagen-coated dishes (D11134 H; Matsunami Glass, Osaka, Japan) were fixed with acetone/methanol (1:1) at −20 °C for 10 min. After the cells were rinsed with Tris-buffered saline (TBS), they were blocked with 3% BSA/TBS for 60 min at room temperature. The samples were then incubated overnight at 4 °C with the primary antibody against MCL1 (sc-819; 1:200: Santa Cruz Biotechnology, Dallas, TX, USA). The samples were subsequently incubated with a secondary antibody, Alexa Fluor 488-conjugated goat anti-rabbit IgG (A-11008; 1:400; Thermo Fisher Scientific Japan, Yokohama, Japan). The cells were finally observed under an inverted microscope (IX-71; Olympus, Tokyo, Japan) and photographed by a camera (DP80; Olympus).

2.4. RNA interference assay

Cells (3×10^6) were plated in 94-mm culture dishes and transfected with negative control (NC) siRNA duplexes (1027281; Qiagen, Valencia, CA, USA) or siRNA duplexes targeting MCL1 and Bcl-xL using Lipofectamine RNAiMAX reagent and OPTI-MEM I (Thermo Fisher Scientific), as described previously [18–20]. Two types of siRNA duplexes were used for transient MCL1 (encoded by the *MCL1* gene) or Bcl-xL (encoded by the *BCL2L1* gene) knockdown: Silencer Select Validated siRNA (Ambion #s8583, termed MCL1 siRNA #1 in this study; Thermo Fisher Scientific), Silencer Select Pre-designed siRNA (Ambion #s8585, termed MCL1 siRNA #2; Thermo Fisher Scientific), and Silencer Select Validated siRNAs (Ambion #s1920 and #s1921, termed Bcl-xL siRNA #1 and #2, respectively; Thermo Fisher Scientific). The final concentration of the siRNA used in each *in vitro* experiment was 10 nM. Downregulation of targeted gene expression was verified by western blot analysis.

2.5. 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. The luminescence of viable cells was measured by the Infinite 200 microplate reader (Tecan Japan, Kawasaki, Japan). All results are presented as the means ± SD. Apoptosis was assessed by western blot analysis of cleaved poly (ADP-ribose) polymerase 1 (PARP-1) or cleaved caspase 3, as described previously [19,20].

2.6. Western blot analysis

Western blot analysis of thyroid transcription factor 1 (TTF-1), PARP-1, Bim, total ERK1/2, phospho-ERK1/2, and β-actin was performed as described previously [18–20]. Additional primary antibodies used in the present study were anti-MCL1 (sc-819; 1:1,000; Santa Cruz Biotechnology), anti-Bcl-xL (#2764; 54H6; 1:1,000; Cell Signaling Technology Japan, Tokyo, Japan), anti-BCL2 (sc-509; 1:500; Santa Cruz Biotechnology), and anti-cleaved caspase 3 (#9661; Asp175; 1:1,000; Cell Signaling Technology Japan). The cells were lysed in NuPAGE LDS Sample Buffer (Thermo Fisher Scientific). The cell lysates (15 μg total protein in each well) were separated by SDS-PAGE (SuperSep Ace, 5–20%, 13 wells; Wako Pure Chemicals, Osaka, Japan) and then transferred onto polyvinylidene difluoride membranes. The membranes were blocked by incubation with 3% non-fat dry milk in TBS for 1 h at room temperature and then incubated overnight at 4 °C with one of the abovementioned primary antibodies. The membranes were washed three times and then incubated for 1 h at room temperature with a species-specific horseradish peroxidase-conjugated secondary antibody (NA931 or NA934; GE Healthcare, Buckinghamshire, UK). Blots were visualized using Supersignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

2.7. Crystal violet staining

Cell culture plates were placed on ice, and the cells were washed twice with cold PBS and then fixed in ice-cold 100% methanol for 10 min. Following fixation, the cells were stained with a 0.5% crystal violet solution (Tokyo Chemical Industry, Tokyo, Japan) diluted in 25% methanol for 10 min at room temperature. Plates containing stained cells were then photographed using a digital camera (D610; Nikon,

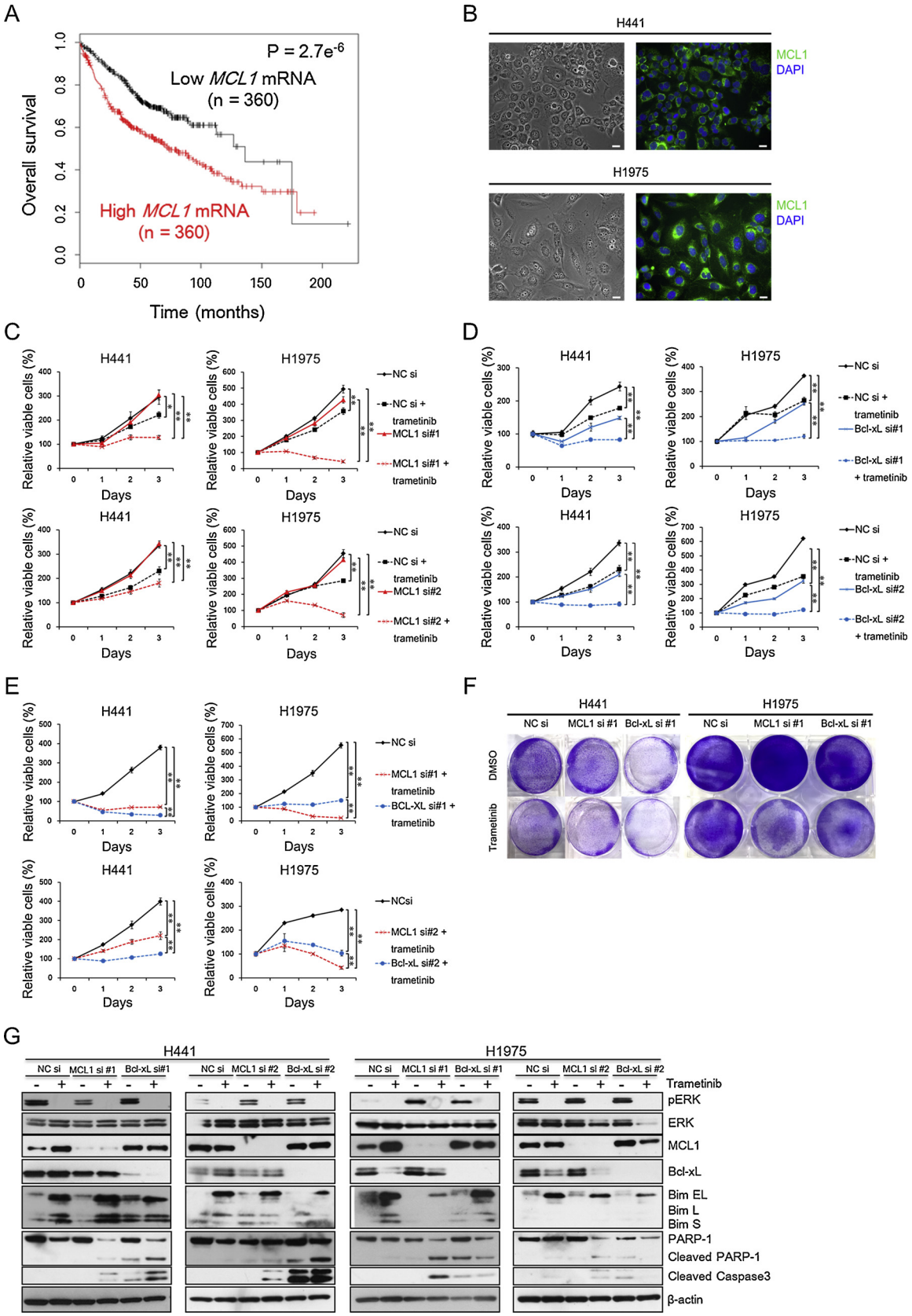

Fig. 1. Combined therapy by MCL1 knockdown and trametinib suppresses proliferation and induces apoptosis in H441 and H1975 cells. (A) Kaplan-Meier curves for overall survival according to mRNA expression levels of *MCL1* in patients with lung adenocarcinomas (n = 720). Data were obtained from the Kaplan-Meier Plotter database [17]. (B) Immunofluorescence of MCL1 in H441 and H1975 cells. Scale bars, 20 μm. (C–E) Effects of the combined therapy by MCL1 or Bcl-xL knockdown and trametinib on the viability of H441 and H1975 cells. Cells were transfected with NC siRNA, MCL1 siRNA (#1 or #2), or Bcl-xL siRNA (#1 or #2) (10 nM each) as indicated and then cultured for 48 h. Cells were then untreated or treated with trametinib (10 nM) for another 72 h. Cell viability was assessed in triplicate. Results are shown as means ± SD. *P < 0.05, **P < 0.01. (F) Crystal violet staining of viable cells. Cells were treated as described in (C–E). (G) Western blot analyses of the effects of the combined therapy in H441 and H1975 cells. Cells were treated as described in (C–E).

Tokyo, Japan).

2.8. RT-PCR

Total RNA extraction, and quantitative or conventional RT-PCR analyses of mRNA expression were carried out as described previously [19–21]. The following PCR primers were purchased from Qiagen (QuantiTect Primer Assay): β-actin, encoded by the *ACTB* gene (Hs_ACTB_1_SG); BCL2 (Hs_BCL2_1_SG); MCL1 (Hs_MCL1_1_SG); Bcl-xL, encoded by the *BCL2L1* gene (Hs_BCL2L1_1_SG); TTF-1, encoded by the *NKX2-1* gene (Hs_NKX2-1_1_SG). The amount of β-actin mRNA in each sample was used to standardize the quantity of target mRNAs. Relative mRNA expression levels were calculated by the comparative ΔΔCT method and are presented as the averages of triplicate experiments.

2.9. Mouse tumor xenograft model

All animal experimentation was conducted in accordance with a protocol approved by the Animal Committee at Sapporo Medical University. The experiments were performed using 6–8-week-old female mice (n = 20) (KSN/Slc nude mice; Hokudo, Sapporo, Japan). This study used a minimum of five mice per group. The mice were maintained under comfortable conditions (temperature: 20–26 °C; humidity: 40–60%) and had free access to food and water. The weights of mice upon purchase and sacrifice were 22 g and 24 g on average, respectively. At 6 h after NC siRNA (Ambion *In Vivo* Negative Control siRNA #1; Thermo Fisher Scientific) or MCL1 siRNA #1 transfection, 5×10^6 H441 cells were collected in 50 μl RPMI-1640 and then mixed with 50 μl Matrigel (Corning, Corning, NY, USA). 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 H441 cells, and thus developed a single tumor nodule. At 12 and 22 days after the injection, we injected AteloGene (Koken, Tokyo, Japan) containing NC siRNA (n = 10) or MCL1 siRNA (n = 10) near the tumor nodules to administer the siRNA continuously. Mice were administered the vehicle (n = 10) or trametinib (0.6 mg/kg) (n = 10) orally once daily for 20 days (days 12–32 post-injection of the cells) [22,23]. Trametinib was dissolved in 10% Cremophor EL, 10% PEG400, and 80% dH_2O (Nacalai Tesque). The mice were monitored daily for body weight and general conditions. Tumors were measured twice weekly using a caliper, and their volume was calculated by 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 weighing and histology [hematoxylin and eosin (H&E) staining].

2.10. Statistical analysis

Differences in the viability of untreated and treated cells were evaluated by Dunnett's test. One-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test was performed to evaluate the effects of MCL1 knockdown and MCL1 depletion plus therapy with trametinib on cells and tumor xenografts. A P-value of less than 0.05 was considered significant. All statistical calculations were performed using JMP software (JMP for Windows version 7, SAS Institute Japan, Tokyo, Japan).

3. Results

3.1. Combined therapy by MCL1 knockdown and trametinib suppresses proliferation and induces apoptosis in KRAS-mutant lung adenocarcinoma cells

First, we searched the Kaplan-Meier Plotter for an association between the *MCL1* mRNA expression level and outcome of lung adenocarcinoma patients, and found that the overall survival of patients with high *MCL1* expression (n = 360) was markedly shorter than that of patients with low *MCL1* expression (n = 360) (hazard ratio, 1.76; P = 2.7e−6) as shown in Fig. 1A [17]. Similar effects of the *MCL1* expression on survival were observed even in patients with lung adenocarcinomas of stage I (Supplementary Fig. S1). We then found that MCL1 protein was diffusely expressed in the cytoplasm of the *KRAS*-mutant lung adenocarcinoma cell line H441 and *EGFR*-mutant lung adenocarcinoma cell line H1975 (Fig. 1B). MCL1 knockdown by RNAi alone did not affect proliferation (Fig. 1C, F) or induce apoptosis in H441 or H1975 cells (Fig. 1G). Trametinib treatment alone slightly suppressed the growth of cells, but did not induce apoptosis, although BIM was dephosphorylated and accumulated by the treatment (Fig. 1C, F, G). Combined therapy by trametinib and MCL1 depletion, however, significantly suppressed growth and induced apoptosis in H441 and H1975 cells (Fig. 1C, F, G). These findings suggest that the combined therapy by MCL1 knockdown and trametinib has a certain efficacy against lung adenocarcinoma cells regardless of differences in their driver mutation.

We then compared the therapeutic effects of Bcl-xL knockdown with those of MCL1 silencing. As mentioned above, MCL1 knockdown alone had no effect on proliferation or apoptosis of H441 and H1975 cells, whereas Bcl-xL knockdown alone induced apoptosis, resulting in decreased viability of H441 cells (Fig. 1D, F, G). Similar, but less prominent, effects of Bcl-xL silencing on viability and apoptosis of H1975 cells were also observed (Fig. 1D, F, G). As expected, the combined treatment with Bcl-xL knockdown and trametinib almost completely suppressed proliferation and induced apoptosis in the two examined cell lines (Fig. 1D, F, G). Interestingly, combined therapy by trametinib and Bcl-xL knockdown was more efficacious against H441 cells than combined therapy by trametinib and MCL1 knockdown, whereas H1975 cells were more sensitive to the latter (Fig. 1E–G). We were able to substitute selumetinib, another MEK inhibitor, for trametinib in the treatment of the two cell lines (Supplementary Fig. S2A–C). In addition, S63845, the selective inhibitor of MCL1, was able to replace MCL1 knockdown, while ABT-263, the BCL2/Bcl-xL inhibitor, was a substitute for Bcl-xL knockdown (Supplementary Fig. S3A, B). These findings collectively suggest that MCL1 and Bcl-xL play certain roles in the survival of lung adenocarcinoma cells, and that each cell line depends on MCL1 and Bcl-xL for survival to varying extents.

3.2. Combined therapy by MCL1 knockdown and trametinib effectively suppresses the growth of H441 cells in vivo

To confirm the efficacy of the combined therapy by MCL1 knockdown and trametinib *in vivo*, we implanted NC siRNA- or *MCL1* siRNA-transfected H441 cells into subcutaneous tissues of mice. Since the efficacy of pretransfected siRNA was transient, we administered siRNA to the tumor nodules using Atelocollagen at the start of trametinib therapy (day 0) and 10 days later (day 10). As expected, based on *in vitro* analyses, the *MCL1* siRNA-transfected cells grew steadily and comparably to NC siRNA-transfected cells, whereas MCL1-depleted nodules hardly increased in size in the presence of trametinib (Fig. 2A–C). For NC siRNA-transfected H441 cells, although trametinib suppressed the growth of these cells to some extent, there were no statistically significant differences in the tumor volume (P = 0.17) or weight (P = 0.09) (NC si alone versus NC si + trametinib) (Fig. 2A–C). In addition, representative pathological findings seem to support the therapeutic effects of the four groups (Fig. 2D). These results clearly demonstrate that MCL1 knockdown enhances the efficacy of trametinib in *KRAS*-mutant H441 cells *in vivo*.

3.3. Well-differentiated lung adenocarcinoma cells undergo extensive apoptosis after silencing both MCL1 and Bcl-xL

Next, we focused on the role of the three major anti-apoptotic BCL2

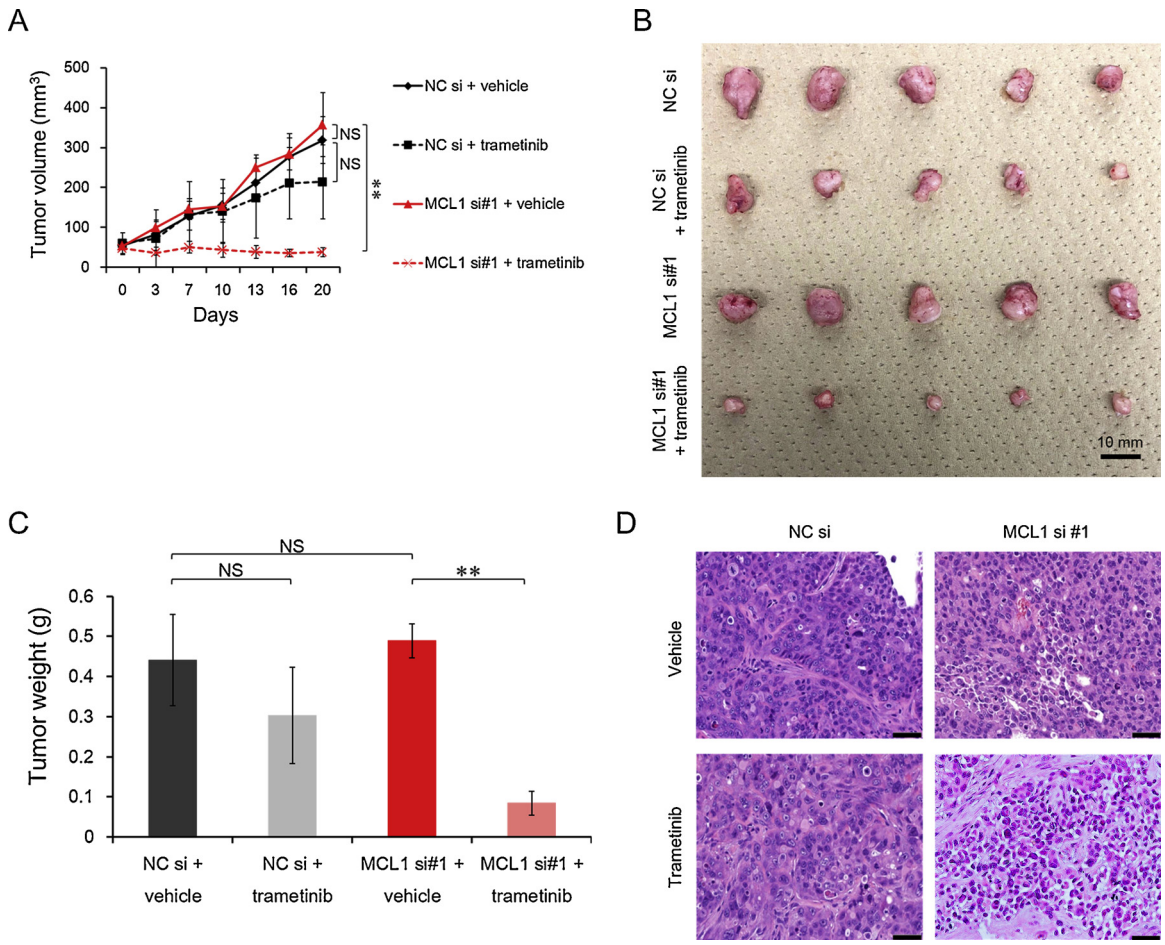

Fig. 2. Combined therapy by MCL1 knockdown and trametinib prevents H441 cells from growing *in vivo*. (A) Effects of the combined therapy on xenografts of H441 cells. Mice with NC siRNA-transfected xenografts (n = 10) and those with MCL1 siRNA-transfected xenografts (n = 10) were untreated (n = 5) or treated (n = 5) with trametinib (0.6 mg/kg) for 20 days. Drugs were administered once daily by oral gavage. Tumor volumes (means ± SD) were measured after the initiation of trametinib treatment (day 0). 'Day 0' corresponds to day 12 after cell implantation, when AteloGene was also administered. **P < 0.01. (B) Effects of the combined therapy on xenografts derived from H441 cells. Macroscopic images of tumors resected from mice are shown. The 20 tumor nodules presented were derived from 20 treated mice. (C) Effect of the combined therapy on xenografts of H441 cells. The weight of each tumor, shown in (B), was measured. Columns, mean (n = 5); bars, ± SD. **P < 0.01. (D) Pathological examination of xenografts. Representative images (H&E stained) are shown. Scale bars, 50 μm.

family members, MCL1, Bcl-xL, and BCL2, in the survival of lung adenocarcinoma cells. Although it has been reported that NSCLC cell lines rarely express BCL2 protein, *KRAS*-mutant H460 cells are an exception [24]. H441 and H1975 cells were relatively well-differentiated lung adenocarcinoma cells expressing TTF-1 (encoded by the *NKX2-1* gene), a representative alveolar epithelial marker, at mRNA and protein levels, whereas it was hardly detectable in H460 cells (Supplementary Fig. S4A, B). The lack of TTF-1 expression in H460 cells was consistent with the phenotype of "large cell carcinoma" [25,26]. We then examined the expression levels of BCL2, Bcl-xL (encoded by the *BCL2L1* gene), and MCL1 at protein and mRNA levels in these cells. Fig. 3A illustrates that both H441 and H1975 cells clearly expressed MCL1 and Bcl-xL, but not BCL2, at protein levels, which was supported by RT-PCR analyses (Fig. 3B). We then investigated whether dual knockdown of MCL1 and Bcl-xL through RNAi induced massive apoptosis in H460, H441, and H1975 cells. Although H460 cells were resistant to the dual knockdown therapy, H441 and H1975 cells underwent extensive apoptosis, as expected, so that viability was markedly decreased in the two cell lines (Fig. 3C–E).

3.4. Verification of MCL1 and Bcl-xL-dependent survival of differentiated lung adenocarcinoma cells by BH3 mimetics

We next verified these findings using three BH3 mimetics, S63845, ABT-263, and WEHI-539 (a selective inhibitor of Bcl-xL). BH3 mimetic monotherapy did not induce apoptosis in the three examined cell lines (Fig. 4A, B). However, all cell lines clearly underwent apoptosis after combined therapy by S63845 and ABT-263 (inhibition of MCL1, BCL2, and Bcl-xL) (Fig. 4A–D). However, combined treatment with S63845 and WEHI-539 (inhibition of MCL1 and Bcl-xL) induced extensive apoptosis in H441 and H1975 cells, but not H460 cells. This interesting phenomenon appeared to be caused by the difference in the expression levels of BCL2 in the three cell lines (Fig. 3A, B). It is likely that H441 and H1975 cells depend critically on MCL1 and Bcl-xL for survival, whereas H460 cells rely on BCL2 as well as MCL1 and Bcl-xL for survival. Taken together, these results suggest that the survival of differentiated lung adenocarcinoma cells is highly dependent on MCL1 and Bcl-xL, and that the dual blockade strategy of the two anti-apoptotic molecules might be generally efficacious for treatment of differentiated lung adenocarcinomas irrespective of differences in their driver mutation.

4. Discussion

We have shown that high expression levels of *MCL1* mRNA are associated with unfavorable outcome in lung adenocarcinoma patients, and that combined therapy by a MEK inhibitor and MCL1 knockdown (or inhibitor) is an effective treatment for not only *KRAS*-mutant H441

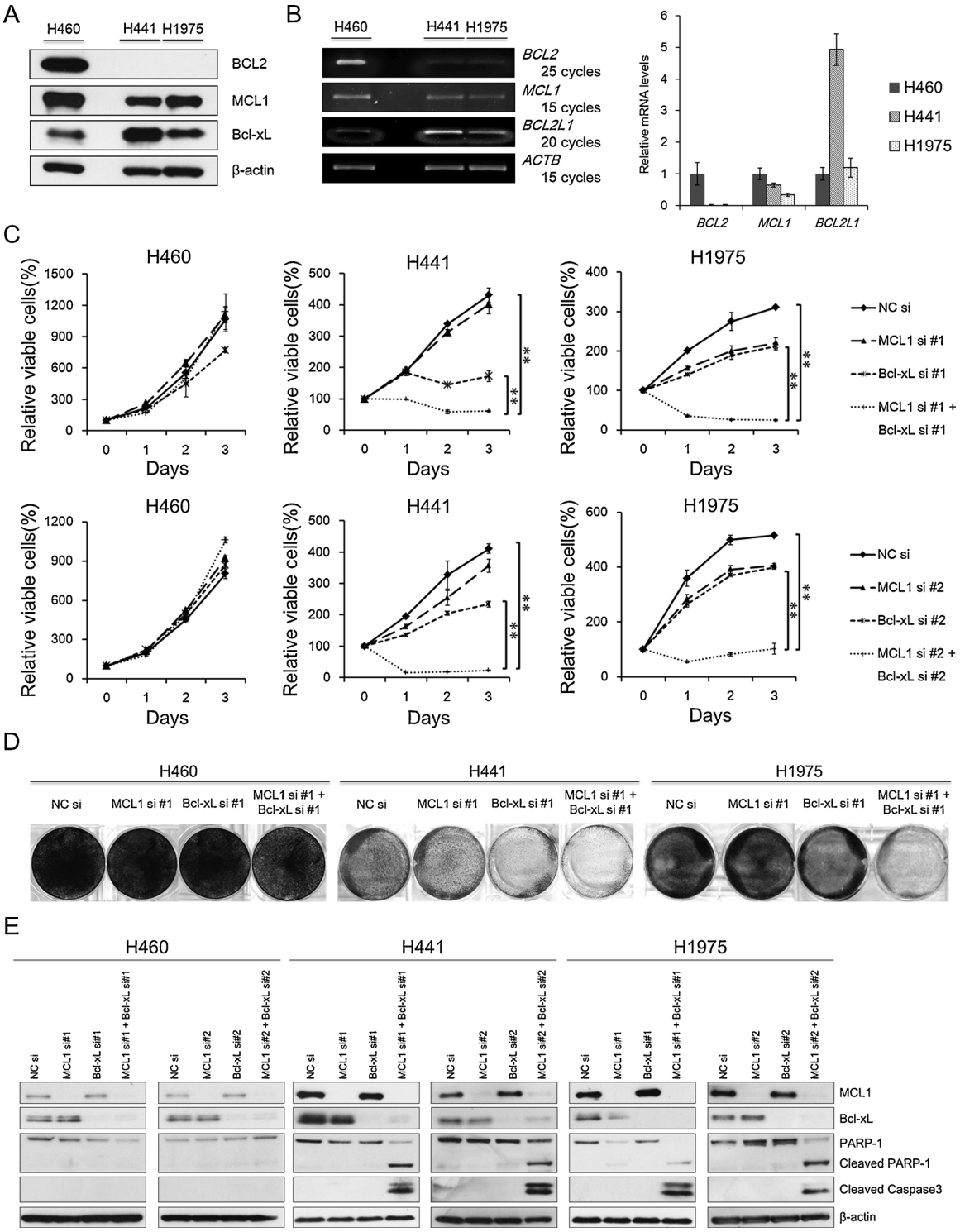

Fig. 3. Dual knockdown of MCL1 and Bcl-xL induces massive apoptosis in H441 and H1975 cells. (A) Western blot analyses of anti-apoptotic BCL2 family proteins in H460, H441, and H1975 cells. (B) RT-PCR analyses of *BCL2*, *MCL1*, and *BCL2L1* mRNAs in H460, H441, and H1975 cells. (C) Effects of dual inhibition of MCL1 and Bcl-xL on H460, H441, and H1975 cells. Cells were transfected with siRNAs as indicated and then cultured for 48 h. Cell viability was then assessed for another 72 h in triplicate. Results are expressed as means ± SD. **P < 0.01. (D) Crystal violet staining of viable cells. Cells were treated as described in (C). (E) Western blot analyses of the effects of dual knockdown of MCL1 and Bcl-xL in H460, H441, and H1975 cells. Cells were treated as described in (C).

cells, but also *EGFR*-mutant H1975 cells. For H441 cells, the combined therapy by trametinib and MCL1 knockdown appeared to be slightly less efficacious than the combined therapy by trametinib and Bcl-xL knockdown. However, we believe that MCL1 is still a potential therapeutic target for the treatment of *KRAS*-mutant lung adenocarcinomas, because Bcl-xL inhibition by ABT-263 caused thrombocytopenia in a dose-dependent manner in clinical trials [27,28]. During the preparation of this article, another study has highlighted the efficacy of combined inhibition of MEK and MCL1 against *KRAS*-mutant lung adenocarcinoma cells [29].

Overexpression of the *MCL1* gene has been reported in various hematopoietic and solid tumors [30]. Experimental findings in our study

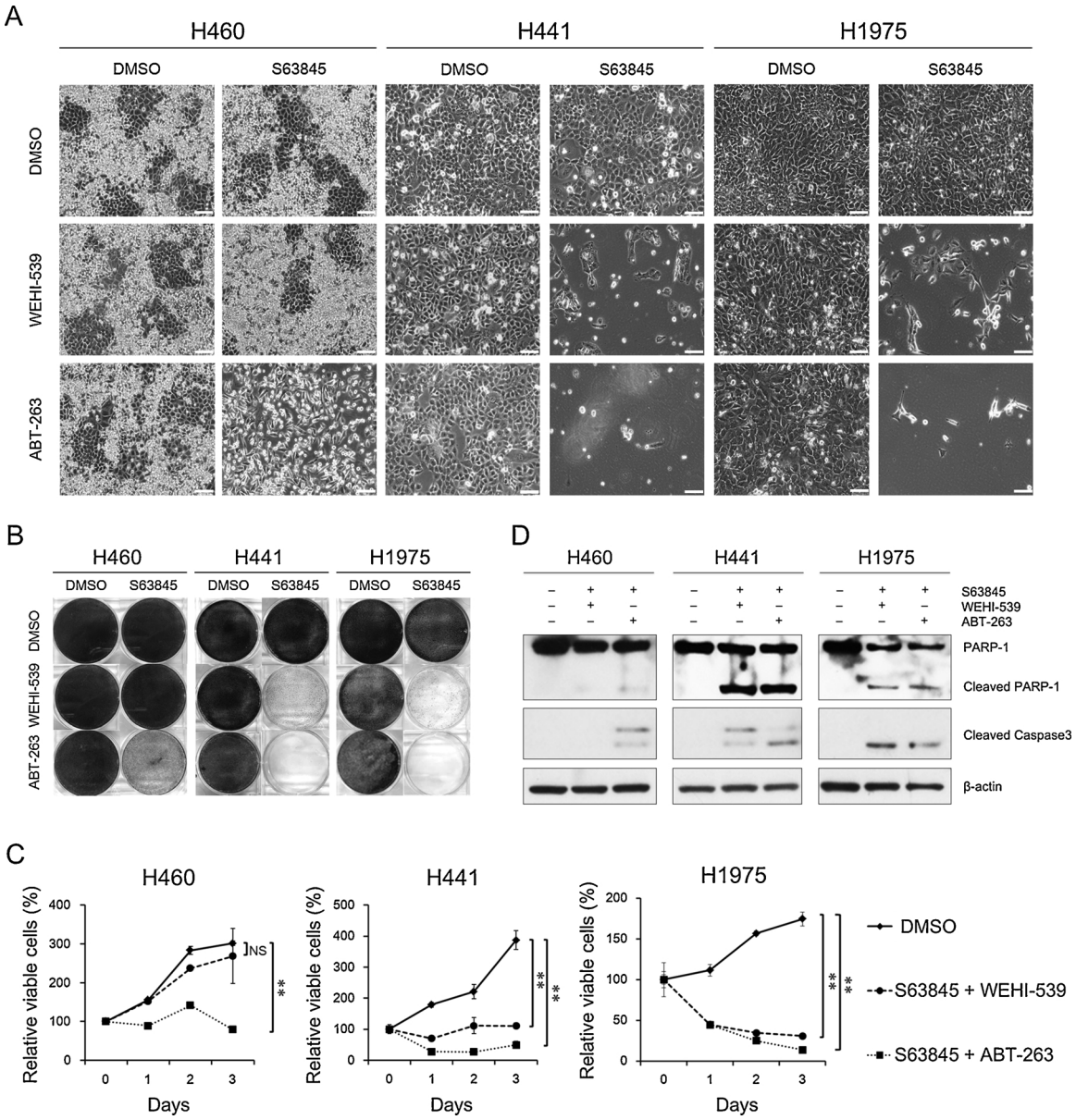

Fig. 4. Verification of MCL1 and Bcl-xL-dependent survival of differentiated lung adenocarcinoma cells by BH3 mimetics. (A) Effects of the combined therapy by BH3 mimetics on the viability of H460, H441, and H1975 cells. A total of 5×10^5 cells were seeded in 6-well plates and then treated with S63845 (0.1 μM) alone, WEHI-539 (2 μM) alone, ABT-263 (1 μM) alone, the combination of S63845 and WEHI-539 or S63845 and ABT-263, or neither for 72 h. Scale bars, 100 μm. (B) Crystal violet staining of viable cells. Cells were treated as described in (A). (C) Effects of the combined therapy by S63845 and WEHI-539 or S63845 and ABT-263 on the viability of H460, H441, and H1975 cells. Cells were treated as described in (A). Cell viability was assessed for 72 h in triplicate. Results are shown as means ± SD. **P < 0.01. (d) Western blot analyses of the effects of the combined therapies in H460, H441, and H1975 cells. Cells were treated as described in (A).

suggested that MCL1 plays a certain role in the survival of lung adenocarcinoma cells. Interestingly, H1975 cells were more sensitive to the combined therapy by MCL1 knockdown and trametinib than H441 cells. It has been reported that MCL1-dependent NSCLC cells generally express more MCL1 than Bcl-xL at protein levels, and that the ratio of *MCL1* to *BCL2L1* mRNA expression in MCL1-dependent cells is two to 10 times higher than that in MCL1-independent cells [31]. This finding may be applicable to H441 and H1975 cells extensively analyzed in this study. However, a simpler method would be required to use BH3 mimetics such as S63845 in the clinic.

We have also shown that BCL2 is hardly detected in H441 and H1975 cells, well-differentiated lung adenocarcinoma cell lines, and that these two cell lines depend critically on MCL1 and Bcl-xL for survival. It appears that cancer cells are generally dependent on several anti-apoptotic BCL2 family members, not a single one, for survival [32]. Our findings suggest that dual blockade of MCL1 and Bcl-xL might be effective for a wide range of cancers except for undifferentiated carcinomas, such as H460 lung cancer and PC-3 prostate cancer cells, which often express BCL2 [24,33]. Such dependency of differentiated carcinomas on MCL1 and Bcl-xL for survival is consistent with the findings in a previous report [34]. However, dual inhibition of MCL1 and Bcl-xL by BH3 mimetics *in vivo* might cause severe side effects, and the therapeutic window is yet to be determined [32,35,36]. Much remains to be studied before concurrent inhibition therapy is available in the clinic.

This study has several limitations. First, although *MCL1* mRNA expression levels were significantly correlated with unfavorable outcomes of lung adenocarcinoma patients, the correlation between MCL1 protein expression and OS is yet to be analyzed. Second, trametinib is available in the clinic, but BH3 mimetics S63845 and ABT-263 used in the present study are being investigated in clinical trials of hematologic

tumors and small cell lung cancer (clinicaltrials.gov: NCT02979366 for S63845; NCT00445198 for ABT-263). It has been indicated that ABT-263 is not effective as a single agent and causes the side effect of thrombocytopenia as mentioned above [27,28]. Third, we extensively analyzed only one *KRAS*-mutant differentiated lung adenocarcinoma cell line, H441, in this study, and many more *KRAS*-mutant cell lines need to be examined.

In summary, lung adenocarcinomas with high *MCL1* mRNA expression are associated with shorter survival, and combined therapy by MCL1 knockdown and trametinib substantially suppresses the growth of *KRAS*-mutant lung adenocarcinoma H441 cells *in vitro* and *in vivo*. We have also demonstrated that concurrent inhibition of MCL1 and Bcl-xL induces massive apoptosis in well-differentiated lung adenocarcinoma H441 and H1975 cells. These findings collectively suggest that MCL1 plays a certain role in the survival of lung adenocarcinoma cells and is a potential therapeutic target for the treatment of lung adenocarcinomas including the *KRAS*-mutant subtype.

Conflict of interest statement

None of the authors of the present study have a conflict of interest to declare.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science (JSPS) (Grant Numbers 15K08364 and 18H02634 to YS). We thank Dr. Mitchell Arico from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.05.014.

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2017, CA Cancer J. Clin. 67 (2017) 7–30.
[2] R. Chalela, V. Curull, C. Enríquez, L. Pijuan, B. Bellosillo, J. Gea, Lung adenocarcinoma: from molecular basis to genome-guided therapy and immunotherapy, J. Thorac. Dis. 9 (2017) 2142–2158.
[3] Cancer Genome Atlas Research Network, Comprehensive molecular profiling of lung adenocarcinoma, Nature 511 (2014) 543–550.
[4] A. Young, J. Lyons, A.L. Miller, V.T. Phan, I.R. Alarcón, F. McCormick, Ras signaling and therapies, Adv. Cancer Res. 102 (2009) 1–17.
[5] M. Serizawa, Y. Koh, H. Kenmotsu, M. Isaka, H. Murakami, H. Akamatsu, K. Mori, M. Abe, I. Hayashi, T. Taira, et al., Assessment of mutational profile of Japanese lung adenocarcinoma patients by multitarget assays: a prospective, single-institute study, Cancer 120 (2014) 1471–1481.
[6] Y. Pylayeva-Gupta, E. Grabocka, D. Bar-Sagi, RAS oncogenes: weaving a tumorigenic web, Nat. Rev. Cancer 11 (2011) 761–774.
[7] G.R. Blumenschein Jr, E.F. Smit, D. Planchard, D.W. Kim, J. Cadranel, T. De Pas, F. Dunphy, K. Udud, M.J. Ahn, N.H. Hanna, et al., A randomized phase II study of the MEK1/MEK2 inhibitor trametinib (GSK1120212) compared with docetaxel in KRAS-mutant advanced non-small-cell lung cancer (NSCLC), Ann. Oncol. 26 (2015) 894–901.
[8] T. Sumi, S. Hirai, M. Yamaguchi, Y. Tanaka, M. Tada, G. Yamada, T. Hasegawa, Y. Miyagi, T. Niki, A. Watanabe, et al., Survivin knockdown induces senescence in TTF-1-expressing, KRAS-mutant lung adenocarcinomas, Int. J. Oncol. 53 (2018) 33–46.
[9] R. Li, T. Moudgil, H.J. Ross, H.M. Hu, Apoptosis of non-small-cell lung cancer cell lines after paclitaxel treatment involves the BH3-only proapoptotic protein Bim, Cell Death Differ. 12 (2005) 292–303.
[10] Y. Gong, R. Somwar, K. Politi, M. Balak, J. Chmielecki, X. Jiang, W. Pao, Induction of BIM is essential for apoptosis triggered by EGFR kinase inhibitors in mutant EGFR-dependent lung adenocarcinomas, PLoS Med. 4 (2007) e294.
[11] J. Meng, B. Fang, Y. Liao, C.M. Chresta, P.D. Smith, J.A. Roth, Apoptosis induction by MEK inhibition in human lung cancer cells is mediated by Bim, PLoS One 5 (2010) e13026.
[12] D. Mérino, P. Bouillet, The Bcl-2 family in autoimmune and degenerative disorders, Apoptosis 14 (2009) 570–583.
[13] W. Fan, Z. Tang, L. Yin, B. Morrison, S. Hafez-Khayyata, P. Fu, H. Huang, R. Bagai, S. Jiang, A. Kresak, et al., MET-independent lung cancer cells evading EGFR kinase inhibitors are therapeutically susceptible to BH3 mimetic agents, Cancer Res. 71 (2011) 4494–4505.
[14] A. Zeuner, F. Francescangeli, P. Contavalli, G. Zapparelli, T. Apuzzo, A. Eramo, M. Baiocchi, M.L. De Angelis, M. Biffoni, G. Sette, et al., Elimination of quiescent/slow-proliferating cancer stem cells by Bcl-XL inhibition in non-small cell lung cancer, Cell Death Differ. 21 (2014) 1877–1888.
[15] A. Kotschy, Z. Szlavik, J. Murray, J. Davidson, A.L. Maragno, G. Le Toumelin-Braizat, M. Chanrion, G.L. Kelly, J.N. Gong, D.M. Moujalled, et al., The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models, Nature 538 (2016) 477–482.
[16] R.B. Corcoran, K.A. Cheng, A.N. Hata, A.C. Faber, H. Ebi, E.M. Coffee, P. Greninger, R.D. Brown, J.T. Godfrey, T.J. Cohoon, et al., Synthetic lethal interaction of combined BCL-XL and MEK inhibition promotes tumor regressions in KRAS mutant cancer models, Cancer Cell 23 (2013) 121–128.
[17] B. Győrffy, P. Surowiak, J. Budczies, A. Lánczky, Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer, PLoS One 8 (2013) e82241.
[18] M. Yamaguchi, S. Hirai, Y. Tanaka, T. Sumi, M. Miyajima, T. Mishina, G. Yamada, M. Otsuka, T. Hasegawa, T. Kojima, et al., Fibroblastic foci, covered with alveolar epithelia exhibiting epithelial-mesenchymal transition, destroy alveolar septa by disrupting blood flow in idiopathic pulmonary fibrosis, Lab. Invest. 97 (2017) 232–242.
[19] Y. Sakuma, H. Nishikiori, S. Hirai, M. Yamaguchi, G. Yamada, A. Watanabe, T. Hasegawa, T. Kojima, T. Niki, H. Takahashi, Prolyl isomerase Pin1 promotes survival in EGFR-mutant lung adenocarcinoma cells with an epithelial-mesenchymal transition phenotype, Lab. Invest. 96 (2016) 391–398.
[20] Y. Sakuma, S. Matsukuma, Y. Nakamura, M. Yoshihara, S. Koizume, H. Sekiguchi, H. Saito, H. Nakayama, Y. Kameda, T. Yokose, et al., Enhanced autophagy is required for survival in EGFR-independent EGFR-mutant lung adenocarcinoma cells, Lab. Invest. 93 (2013) 1137–1146.
[21] Y. Tanaka, M. Yamaguchi, S. Hirai, T. Sumi, M. Tada, A. Saito, H. Chiba, T. Kojima, A. Watanabe, H. Takahashi, et al., Characterization of distal airway stem-like cells expressing N-terminally truncated p63 and thyroid transcription factor-1 in the human lung, Exp. Cell Res. 372 (2018) 141–149.
[22] T. Yamaguchi, R. Kakefuda, N. Tajima, Y. Sowa, T. Sakai, Antitumor activities of JTP-74057 (GSK1120212), a novel MEK1/2 inhibitor, on colorectal cancer cell lines in vitro and in vivo, Int. J. Oncol. 39 (2011) 23–31.
[23] H. Kitai, H. Ebi, S. Tomida, K.V. Floros, H. Kotani, Y. Adachi, S. Oizumi, M. Nishimura, A.C. Faber, S. Yano, Epithelial-to-mesenchymal transition defines feedback activation of receptor tyrosine kinase signaling induced by MEK inhibition in KRAS-mutant lung cancer, Cancer Discov. 6 (2016) 754–769.
[24] J. Li, J. Viallet, E.B. Haura, B. Győrffy, P. Surowiak, J. Budczies, A. Lánczky, A small molecule pan-Bcl-2 family inhibitor, GX15-070, induces apoptosis and enhances cisplatin-induced apoptosis in non-small cell lung cancer cells, Cancer Chemother. Pharmacol. 61 (2008) 525–534.
[25] H. Osada, Y. Tatematsu, Y. Yatabe, Y. Horio, T. Takahashi, ASH1 gene is a specific therapeutic target for lung cancers with neuroendocrine features, Cancer Res. 65 (2005) 10680–10685.
[26] M. Lee, Zia F. Drao, A. Gazdar, H. Oie, G. Bepler, F. Bellot, C. Tarr, R. Kris, T.W. Moody, Epidermal growth factor receptor monoclonal antibodies inhibit the growth of lung cancer cell lines, J. Natl. Cancer Inst. Monogr. 13 (1992) 117–123.
[27] L. Gandhi, D.R. Camidge, M. Ribeiro de Oliveira, P. Bonomi, D. Gandara, D. Khaira, C.L. Hann, E.M. McKeegan, E. Litvinovich, P.M. Hemken, et al., Phase I study of Navitoclax (ABT-263), a novel Bcl-2 family inhibitor, in patients with small-cell lung cancer and other solid tumors, J. Clin. Oncol. 29 (2011) 909–916.
[28] C.M. Rudin, C.L. Hann, E.B. Garon, M. Ribeiro de Oliveira, P.D. Bonomi, D.R. Camidge, Q. Chu, G. Giaccone, D. Khaira, S.S. Ramalingam, et al., Phase II study of single-agent navitoclax (ABT-263) and biomarker correlates in patients with relapsed small cell lung cancer, Clin. Cancer Res. 18 (2012) 3163–3169.
[29] V. Nangia, F.M. Siddiqui, S. Caenepeel, D. Timonina, S.J. Bilton, N. Phan, M. Gomez-Caraballo, H.L. Archibald, C. Li, C. Fraser, et al., Exploiting MCL1 dependency with combination MEK + MCL1 inhibitors leads to induction of apoptosis and tumor regression in KRAS-mutant non-small cell lung cancer, Cancer Discov. 8 (2018) 1598–1613.
[30] C. Akgul, Mcl-1 is a potential therapeutic target in multiple types of cancer, Cell. Mol. Life Sci. 66 (2009) 1326–1336.
[31] H. Zhang, S. Guttikonda, L. Roberts, T. Uziel, D. Semizarov, S.W. Elmore, J.D. Leverson, L.T. Lam, Mcl-1 is critical for survival in a subgroup of non-small-cell lung cancer cell lines, Oncogene 30 (2011) 1963–1968.
[32] C.E. Weeden, C. Ah-Cann, A.Z. Holik, J. Pasquet, J.M. Garnier, D. Merino, G. Lessene, M.L. Asselin-Labat, Dual inhibition of BCL-XL and MCL-1 is required to induce tumour regression in lung squamous cell carcinomas sensitive to FGFR inhibition, Oncogene 37 (2018) 4475–4488.
[33] S. Arai, O. Jonas, M.A. Whitman, E. Corey, S.P. Balk, S. Chen, Tyrosine kinase inhibitors increase MCL1 degradation and in combination with BCLXL/BCL2 inhibitors drive prostate cancer apoptosis, Clin. Cancer Res. 24 (2018) 5458–5470.
[34] R.S. Soderquist, L. Crawford, E. Liu, M. Lu, A. Agarwal, G.R. Anderson, K.H. Lin, P.S. Winter, M. Cakir, K.C. Wood, Systematic mapping of BCL-2 gene dependencies in cancer reveals molecular determinants of BH3 mimetic sensitivity, Nat. Commun. 9 (2018) 3513.
[35] G.R. Anderson, S.E. Wardell, M. Cakir, L. Crawford, J.C. Leeds, D.P. Nussbaum, P.S. Shankar, R.S. Soderquist, E.M. Stein, J.P. Tingley, et al., PIK3CA mutations enable targeting of a breast tumor dependency through mTOR-mediated MCL-1 translation, Sci. Transl. Med. 8 (2016) 369ra175.
[36] D. Merino, J.R. Whittle, F. Vaillant, A. Serrano, J.N. Gong, G. Giner, A.L. Maragno, M. Chanrion, E. Schneider, B. Pal, et al., Synergistic action of the MCL-1 inhibitor S63845 with current therapies in preclinical models of triple-negative and HER2-amplified breast cancer, Sci. Transl. Med. 9 (2017) 401.