Alisertib exerts KRAS allele-specific anticancer effects on colorectal cancer cell lines

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Abstract. The aim of the present study was to examine the effects of alisertib (ALS) on RAS signaling pathways against a panel of colorectal cancer (CRC) cell lines and engineered Flp-In stable cell lines expressing different Kirsten rat sarcoma virus (KRAS) mutants. The viability of Caco-2$^{KRAS\ wild\text{-}type}$, Colo-678$^{KRAS\ G12D}$, SK-CO-1$^{KRAS\ G12V}$, HCT116$^{KRAS\ G13D}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ cells was examined by Cell Titer-Glo assay, and that of stable cell lines was monitored by IncuCyte. The expression levels of phosphorylated (p-)Akt and p-Erk as RAS signal outputs were measured by western blotting. The results suggested that ALS exhibited different inhibitory effects on cell viability and different regulatory effects on guanosine triphosphate (GTP)-bound RAS in CRC cell lines. ALS also exhibited various regulatory effects on the PI3K/Akt and mitogen-activated protein kinase (MAPK) pathways, the two dominant RAS signaling pathways, and induced apoptosis and autophagy in a RAS allele-specific manner. Combined treatment with ALS and selumetinib enhanced the regulatory effects of ALS on apoptosis and autophagy in CRC cell lines in a RAS allele-specific manner. Notably, combined treatment exhibited a synergistic inhibitory effect on cell proliferation in Flp-In stable cell lines. The results of the present study suggested that ALS differentially regulates RAS signaling pathways. The combined approach of ALS and a MEK inhibitor may represent a new therapeutic strategy for precision therapy for CRC in a KRAS allele-specific manner; however, this effect requires further study *in vivo*.

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Introduction

Colorectal cancer (CRC) is one of the three most common cancer types worldwide, causing high morbidity and mortality (1-3). An estimated 151,030 new cases and 52,580 deaths occurred in the United States in 2022, rendering CRC one of the top three causes of cancer-related mortality (4). In China, CRC ranks third in incidence and is a common cause of cancer-related mortality (5), with 555,477 new CRC cases and 286,162 deaths in 2020 (6,7). Notably, these numbers are increasing. The overall 5-year survival rate in China is lower than that in the United States, but the number of patients with metastatic CRC in China is higher than that in the United States (8-10). An estimated 20-30% of patients are further diagnosed with unresectable metastatic CRC and 50-60% of patients develop metastatic CRC (11), which substantially jeopardizes therapeutic potential and impairs successful clinical outcomes. Therefore, the development of state-of-the-art screening approaches and new therapeutic strategies for CRC is required.

Oncogenic mutations often cause tumorigenesis and RAS mutations [Kirsten rat sarcoma virus (KRAS), HRas proto-oncogene, GTPase (HRAS), and NRAS proto-oncogene, GTPase NRAS)] are found in 20-30% of malignant human tumors and in ~45% of CRC cases (12).

RAS mutations cause tumor initiation and drive uncontrollable tumor cell proliferation (13,14); they are also associated with poor prognosis (15-17). RAS proteins are guanosine triphosphatases (GTPases) that function as binary switches cycling between inactive (guanosine diphosphate-bound) and active [guanosine-5'-triphosphate (GTP)-bound] states (18,19). Activated RAS proteins can bind to numerous downstream effectors, such as RAF and PI3K, which regulate critical cellular processes, including metabolism, proliferation and survival (20). RAS proteins are subject to a number of regulatory factors and this regulation is often tightly controlled in cells; however, oncogenic mutations in RAS proteins alter this tightly regulated process, leading to the constitutive activation of RAS proteins. Consequently, RAS mutations cause the aberrant activation of the RAS signaling network, including the two dominant pathways: The RAF/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway and the PI3K/Akt pathway. In CRC, RAS is a prognostic and predictive biomarker; however, therapeutic strategies targeting RAS-driven CRC are still lacking.

Notably, RAS signaling pathways can be modulated by Aurora kinases A, B and C (AURKA/B/C), which may lead to the development of a promising therapeutic strategy for RAS-driven cancer. Aurora kinases regulate cell mitosis, including centrosome duplication, spindle assembly, chromosome alignment, chromosome segregation and the fidelity-monitoring spindle checkpoint (21,22). Accumulating evidence shows an association between aberrant AURKA expression and cancer development, including breast, pancreatic, ovarian and gastric cancer (23), which can be ascribed to the development of aneuploidy, supernumerary centrosomes, defective mitotic spindles and resistance to apoptosis. Notably, it has been shown that aberrant AURKA expression is associated with poor prognosis and chemotherapy response in CRC (24,25). Aurora kinases are considered a promising cancer target and several Aurora kinase inhibitors, including alisertib (ALS) and barasertib, have been developed and evaluated at various preclinical and clinical stages (26). Furthermore, the co-operation between Aurora kinases and RAS proteins has been reported in numerous types of cancer (27), and targeting Aurora kinases and RAS signaling alone or combined results in various responses (28-33); suggesting that further analysis of this co-operation is needed. In particular, RAS allele specificity in cancer treatment should be considered.

In a previous study by our team, the regulatory effects of ALS, a selective inhibitor of AURKA, on cell proliferation, migration, apoptosis and autophagy were evaluated in KRAS wild-type (WT) and BRAF V600E-mutant CRC cell lines using stable isotope labeling by amino acids in a cell culture-based approach (34). However, comparison of the effects of AURKA inhibition was not conducted with regard to different RAS mutants in CRC, such as KRAS G12D, G12V, G13D and A146T. In the present study, the regulatory effects of ALS on PI3K/Akt and mitogen-activated protein kinase (MAPK) signaling pathways, apoptosis, autophagy and cell proliferation were assessed against a panel of human CRC cell lines and engineered Flp-In T-REx stable cell lines.

Materials and methods

Chemicals and reagents. ALS and selumetinib (Sel) were purchased from Selleck Chemicals and stored at 100 mM in DMSO at -20˚C. DMSO, FBS, ammonium persulfate for western blotting, protease and phosphatase inhibitor cocktails, doxycycline (DOX) and Dulbecco's PBS were purchased from MilliporeSigma. All required cell culture media, including Eagle's Minimum Essential Medium for Caco-2 and SK-CO-1 cells, McCoy's 5A for HT29 cells, Roswell Park Memorial Institute (RPMI)1640 for Colo-678 and CCCL-18 cells, and Dulbecco's Modified Eagle Medium for HCT116 cells were obtained from Corning, Inc. A CellTiter-Glo™ luminescent cell viability assay kit was purchased from Promega Corporation. Pierce™ bicinchoninic acid (BCA) protein assay kit and radioimmunoprecipitation assay (RIPA) buffer were sourced from Thermo Fisher Scientific, Inc. Western blotting substrate (20X LumiGLO® Reagent and 20X Peroxide; cat. no. 7003) was purchased from Cell Signaling Technology, Inc. Skimmed milk and nitrocellulose membrane were purchased from Bio-Rad Laboratories, Inc. Primary antibodies for cleaved poly ADP-ribose polymerase [PARP, (cat. no. 5625S)], phosphorylated (p-)Akt (Ser473) (cat. no. 4060S), Akt (cat. no. 9272S), p-Erk1/2 (Thr202/Tyr204) (cat. no. 4370S), Erk1/2 (cat. no. 4695S), RAS (cat. no. 3339S) and LC3B-I/II (cat. no. 3868S), and secondary antibodies for rabbit (cat. no. 7074S) and mouse (cat. no. 7076S) were purchased from Cell Signaling Technology, Inc., and β-actin (cat. no. sc-47778) was purchased from Santa Cruz Biotechnology, Inc. All primary antibodies were diluted at 1:1,000 and secondary antibodies were diluted at 1:4,000.

Cell lines. The well-recognized and commonly used CRC cell lines Caco-2$^{KRAS\ WT}$, Colo-678$^{KRAS\ G12D}$, SK-CO-1$^{KRAS\ G12V}$, HCT116$^{KRAS\ G13D}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ were purchased from American Type Culture Collection (ATCC). Cells were cultured in ATCC-recommended complete medium supplemented with 10% FBS and maintained in a humidified incubator at 37˚C with 5% CO_2. The Flp-In T-REx 293 cell line (cat. no. R78007) with the Flp-In system (cat. no K601001) was purchased from Thermo Fisher Scientific, Inc., maintained in DMEM with zeocin (100 μg/ml) and blasticidin (15 μg/ml) and stored at 37˚C with 5% CO_2 as instructed by the manufacturer. This cell line is designed for efficient generation of stable cell lines that ensures homogenous expression of the protein of interest (KRAS). Stable cell lines expressing KRAS WT, G12D and A146T were generated according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). Briefly, co-transfection of the Flp-In cell line with a Flp-In expression vector and the Flp recombinase vector results in targeted integration of the expression vector to the same locus in every cell, ensuring homogeneous levels of gene expression. In the present study, the KRAS mutation was generated using the PfuUltra II Hotstart PCR Master Mix (cat. no. 600850; Agilent Technologies, Inc.) following the manufacturer's instructions. The in-house constructs containing mCherry-H2B-P2A-linked KRAS WT, KRAS G12D and A146T were co-transfected with pOG44 into Flp-In T-REx 293 cells using Lipofectamine™ 3000 Transfection Reagent (cat. no. L3000001; Invitrogen; Thermo Fisher Scientific, Inc.), followed by 2 weeks of

hygromycin (100 μg/ml) and blasticidin (15 μg/ml) selection in DMEM supplemented with 10% Tet-free FBS. Subsequently, the selected clones were further seeded into 96-well plates at a density of 50 cells/plate to achieve a monoclonal cell population for 4-6 weeks. Doxycycline (2 ng) was used to induce KRAS protein expression. Protein expression was verified by western blotting and imaging. Mycoplasma Plus PCR Primer Set (Agilent Technologies, Inc.) was used to detect mycoplasma in all cell lines. Cells were treated with ALS or Sel with 0.05% DMSO.

Cell viability and proliferation assessment. To evaluate the different inhibitory effects of ALS on the cell viability of CRC cell lines, the CellTiter-Glo assay was performed according to the manufacturer's instructions. Briefly, a panel of CRC cell lines, including Caco-2, Colo-678, SK-CO-1, HCT116, CCCL-18 and HT29, were plated and treated with ALS for 48 and 96 h at concentrations of 0.001, 0.01, 0.1, 1 and 10 μM in clear-bottomed, white 96-well plates. The CellTiter-Glo substrate (1:1) was added prior to the assay. The luminescence was measured using a BioTek Synergy NEO plate reader instrument (BioTek Instruments, Inc.) equipped with upper EM 620/665 LUM and lower EX 330 LUM filters.

To assess the effects of AURKA and MEK inhibitors on cell proliferation, engineered Flp-In T-REx 293 stable cell lines expressing KRAS WT, G12D and A146T ($1x10^3$ cells/well) were cultured in the presence of 2 ng DOX upon exposure to ALS at 0.001, 0.01, 0.1, 1 and 10 μM and Sel at 0.001, 0.01, 0.1, 1 and 10 μM. Cell proliferation was monitored using an IncuCyte® live-cell analysis system, and images were captured every 4 h in three fields per well in clear-bottomed, black 96-well plates. To determine the synergistic effect of ALS and Sel, the combination index (CI) was calculated via the Chou-Talalay method using CompuSyn (35). CI<1 was considered to indicate synergism. Data were analyzed and plotted using Prism 9 (GraphPad Software; Dotmatics).

RAS-GTP pull-down. Caco-2, Colo-678, SK-CO-1, HCT116, CCCL-18 and HT29 CRC cell lines were grown in 10% FBS in 60-mm dishes and treated with ALS at 0.1, 1 and 5 μM for 48 h. Then, cells were washed in cold PBS twice and harvested in SDS-free lysis buffer (25 mM Tris-HCl, pH 7.2; 150 mM NaCl; 5 mM $MgCl_2$; 1% NP-40; 5% glycerol; and 1% protease inhibitor cocktails) to assess the RAS-GTP level using the active RAS detection kit (cat. no. 8821; Cell Signaling Technology, Inc.) according to the manufacturer's instructions. Briefly, protein samples were incubated with RAF1 RAS-binding domain (RAF1-RBD) for 4 h in a cold room at 4˚C in the presence of glutathione resin. Subsequently, the samples were pelleted by centrifugation at 6,000 x g for 15 sec and washed with lysis buffer twice before western blotting.

Western blotting. The effects of ALS and Sel on protein expression and the RAS signaling pathway were examined using western blotting. Caco-2, Colo-678, SK-CO-1, HCT116, CCCL-18 and HT29 CRC cell lines were seeded into 6-well plates at $2x10^5$ cells/well. The following day, cells were treated with either ALS alone (0.1, 1 and 5 μM) for 48 h or first treated with Sel (0.1 μM) for 24 h followed by ALS (0.1, 1 and 5 μM) for another 24 h. Subsequently, cells were washed once with ice-cold PBS and cell protein samples were collected and processed in RIPA buffer containing phosphatase and protease inhibitor cocktails. The protein concentration was measured via Pierce BCA protein assay and 20 μg protein/lane were separated by SDS-PAGE on a 10% gel containing ammonium persulfate (10%) and transferred using Trans-Blot Turbo Mini 0.2 μm Nitrocellulose Transfer Packs (cat. no. 1704158; Bio-Rad Laboratories, Inc.). Subsequently, the membrane was blocked with 5% skim milk in TBS-Tween (1%) for 1 h at room temperature and incubated with primary antibody (1:1,000) in a cold room (4˚C) overnight. The next day, the membrane was incubated with secondary antibodies at room temperature for 1 h before film development in a dark room. Protein expression levels were normalized to the densitometric value of the internal control, β-actin, using Image J 1.54b (National Institutes of Health).

Statistical analysis. Data are presented as the mean ± standard deviation. Multiple comparisons were assessed through a one-way ANOVA followed by Tukey's multiple comparison post hoc test using GraphPad Prism 9. $P<0.05$ was considered to indicate a statistically significant difference. All assays were repeated a minimum of three times ($n\geq3$).

Results

ALS modulates CRC cell proliferation and the active form of RAS in a KRAS allele-specific manner. In order to determine the effects of AURKA inhibition on RAS signal output, the effects of ALS on cell viability were examined against a panel of CRC cell lines bearing KRAS WT, G12D, G12V, G13D, A146T and BRAF V600E mutations. The KRAS G13D-expressing cell line, HCT116, was the most susceptible to ALS treatment over 48 and 96 h (Fig. 1A and B). Colo-$678^{KRAS\ G12D}$, SK-CO-$1^{KRAS\ G12V}$ and CCCL-$18^{KRAS\ A146T}$ were also susceptible but to a lesser extent. KRAS WT and BRAF V600E-expressing cell lines exhibited a moderate response to ALS treatment (Fig. 1A and B). Subsequently, the RAS-GTP level was evaluated following the treatment of cells with ALS. RAF1-RBD was used to pull down the active form of RAS from Caco-$2^{KRAS\ WT}$, Colo-$678^{KRAS\ G12D}$, SK-CO-$1^{KRAS\ G12V}$, HCT$116^{KRAS\ G13D}$, CCCL-$18^{KRAS\ A146T}$ and HT$29^{BRAF\ V600E}$ cells. In KRAS WT-expressing Caco-2 cells, ALS increased the level of RAS-GTP in a concentration-dependent manner (Fig. 2). In KRAS mutant-expressing cells, ALS increased RAS-GTP level in SK-CO-$1^{KRAS\ G12V}$, HCT$116^{KRAS\ G13D}$ and CCCL-$18^{KRAS\ A146T}$ cells, whereas ALS decreased the RAS-GTP level in Colo-$678^{KRAS\ G12D}$ cells. Notably, in HT29 cells expressing BRAF V600E, a constitutively active BRAF mutant commonly found in CRC that does not rely on RAS activation to activate the RAS-RAF-MEK-ERK signal, ALS decreased the level of the active form of RAS. In combination, these results suggested that the inhibition of AURKA exerts different inhibitory effects on cell proliferation and regulates the active form of RAS in a KRAS allele-specific manner.

ALS affects RAS signaling in a KRAS allele-specific manner. Following the examination of RAS-GTP level, the phosphorylation levels of Akt and Erk were assessed in CRC cell lines. The PI3K/Akt and MAPK signaling pathways are the two dominant downstream pathways of RAS. The phosphorylation

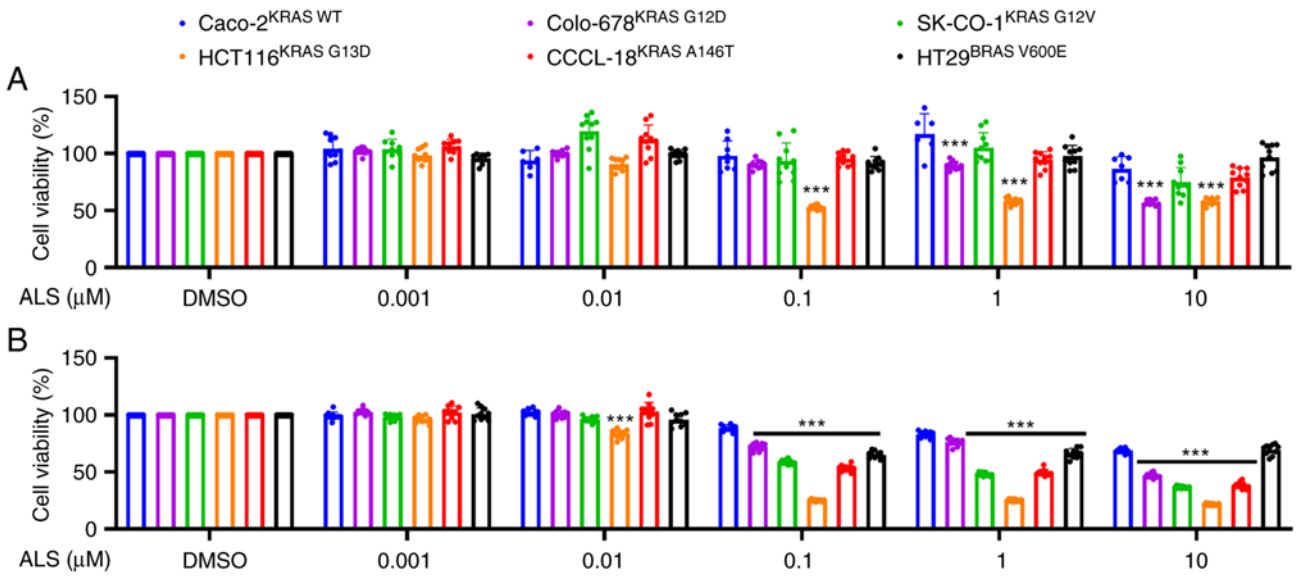

Figure 1. ALS differentially inhibits the proliferation of CRC cells. A panel of CRC cell lines, including Caco-2$^{KRAS\ WT}$, Colo-678K$^{RAS\ G12D}$, SK-CO-1$^{KRAS\ G12V}$, HCT116$^{KRAS\ G13D}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$, were treated with ALS for (A) 48 and (B) 96 h. Cell viability was measured using CellTiter-Glo assay. Data are expressed as the mean ± standard deviation. ***P<0.001 compared with Caco-2KRAS WT under the same treatment condition (n=6). ALS, alisertib; CRC, colorectal cancer; WT, wild-type.

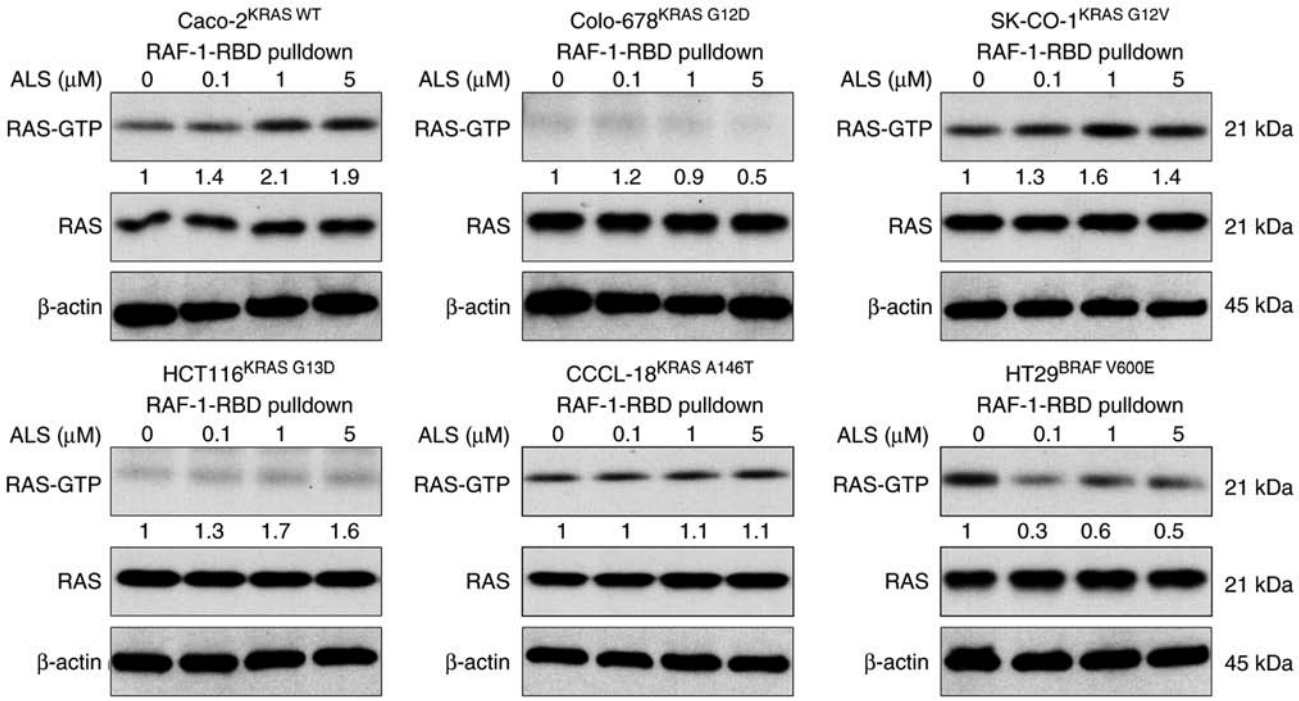

Figure 2. ALS modulates RAS-GTP level in a RAS allele-specific manner in colorectal cancer cell lines. Caco-2$^{KRAS\ WT}$, Colo-678$^{KRAS\ G12D}$, SK-CO-1$^{KRAS\ G12V}$, HCT116$^{KRAS\ G13D}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ cells were treated with ALS and protein samples were subject to pulldown using RAF-RBD. The levels of RAS-GTP were detected by western blotting (n=3). Fold change was calculated in comparison to the control group treated with DMSO. ALS, alisertib; WT, wild-type.

levels of Akt and Erk are the two major indicators for RAS activation. CRC cells were treated with 0.1, 1 and 5 μM ALS, and the levels of p-Akt and p-Erk were analyzed. ALS inhibited the phosphorylation of Akt but enhanced the phosphorylation of Erk in KRAS WT-expressing Caco-2 cells (Figs. 3 and S1). There was no marked change in the levels of p-Akt and p-Erk in Colo-678$^{KRAS\ G12D}$ and SK-CO-1$^{KRAS\ G12V}$ cells. In KRAS G13D-expressing HCT116 cells, ALS suppressed the phosphorylation of Akt and Erk. Similarly, ALS suppressed the activation of Akt and Erk in BRAF V600E-expressing HT29 cells, although the effect on Erk phosphorylation was not significant. However, ALS enhanced the phosphorylation of Akt and Erk in CCCL-18$^{KRAS\ A146T}$ cells. These results suggested that the inhibition of AURKA by ALS leads to a RAS allele-specific modulation in the PI3K/Akt and MAPK signaling pathways.

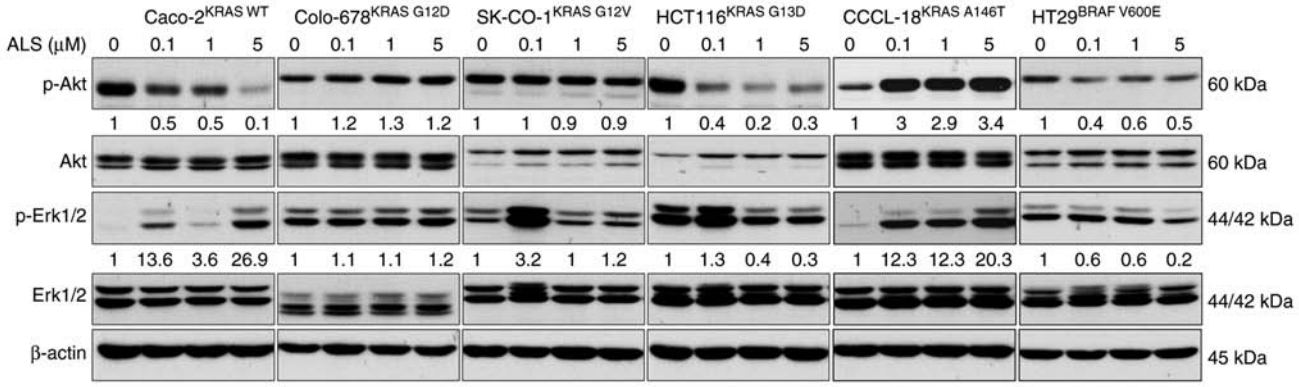

Figure 3. Inhibition of Aurora kinase A affects PI3K/AKT and mitogen-activated protein kinase signaling in a RAS allele-specific manner in colorectal cancer cell lines. Caco-2$^{KRAS\ WT}$, Colo-678$^{KRAS\ G12D}$, SK-CO-1$^{KRAS\ G12V}$, HCT116$^{KRAS\ G13D}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ cells were treated with ALS and proteins were subject to western blotting for the assessment of Erk and Akt phosphorylation (n=3). Fold change was calculated in comparison to the control group. ALS, alisertib; p, phosphorylated; WT, wild-type.

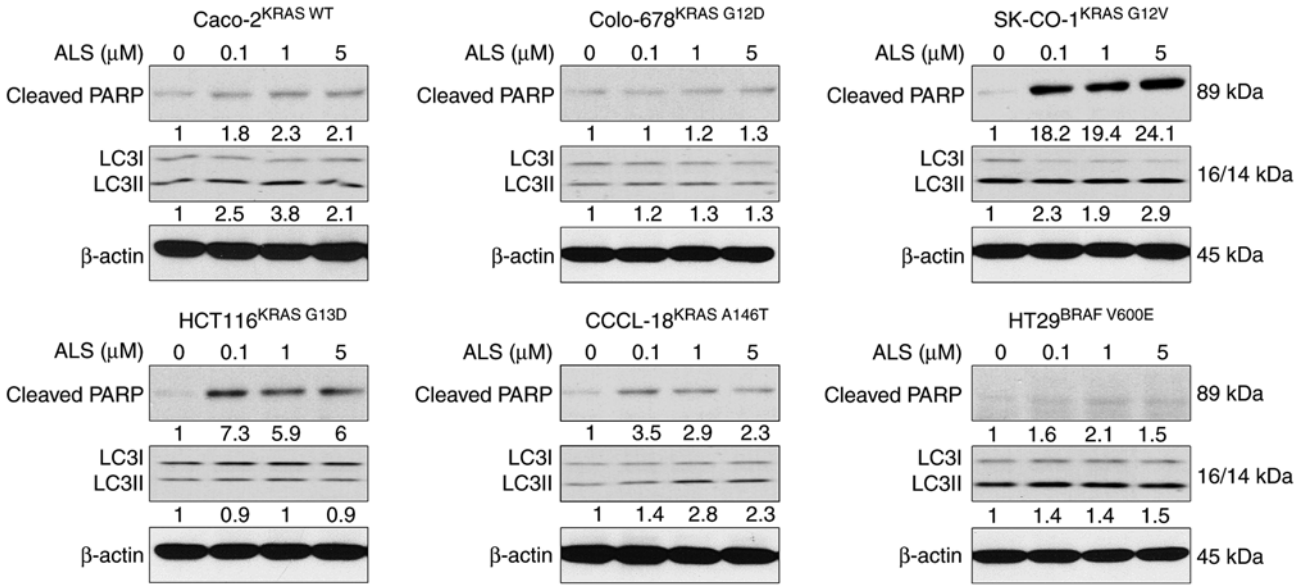

Figure 4. ALS manipulates apoptosis and autophagy in a RAS allele-specific manner in colorectal cancer cell lines. Caco-2$^{KRAS\ WT}$, Colo-678$^{KRAS\ G12D}$, SK-CO-1$^{KRAS\ G12V}$, HCT116$^{KRAS\ G13D}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ cells were treated with ALS, and apoptosis and autophagy were examined as indicated by cleaved-PARP and LC3I and II by western blotting (n=3). Fold change was calculated in comparison to the control group treated with DMSO. ALS, alisertib; PARP, poly ADP-ribose polymerase; WT, wild-type.

ALS manipulates apoptosis and autophagy in a KRAS allele-specific manner. To test the influence of the RAS allele-specific regulatory effect of ALS on cell death, the expression levels of cleaved PARP and LC3I and II, as surrogate markers of apoptosis and autophagy, were examined. The panel of CRC cell lines was treated with ALS, and the expression levels of cleaved PARP and LC3I and II were measured. As shown in Fig. 4, KRAS WT-expressing Caco-2 cells underwent apoptosis and autophagy upon exposure to ALS, which was evident from the increase in the levels of cleaved PARP and the ratio of LC3II/I. Notably, apoptosis and autophagy-related proteins were only measured as an indicator instead of direct examination of apoptosis and autophagy, but the results are consistent with our previous study (34). There was no marked alteration in apoptosis and autophagy in Colo-678$^{KRAS\ G12D}$ cells following treatment with ALS. However, there was a marked increase in the expression levels of cleaved PARP and the ratio of LC3II/I in SK-CO-1$^{KRAS\ G12V}$ cells. Furthermore, in HCT116$^{KRAS\ G13D}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ cells, ALS markedly enhanced the expression levels of cleaved PARP and, to a lesser extent, also increased the ratio of LC3II/I in CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ cells. Collectively, the pharmacological inhibition of AURKA via ALS resulted in different regulatory effects on apoptosis and autophagy in a RAS allele-specific manner.

MEK inhibitor displays different regulatory effects on RAS signals in a KRAS allele-specific manner. As the present study observed the RAS allele-specific regulatory effects of ALS on RAS signaling pathways in CRC cell lines, it was subsequently examined whether a MEK inhibitor could have similar effects. To test this hypothesis, the effects of Sel, a MEK inhibitor, on PI3K/Akt and MAPK signaling pathways were first evaluated. Figs. 5 and S2 show that Sel inhibited the MAPK signaling

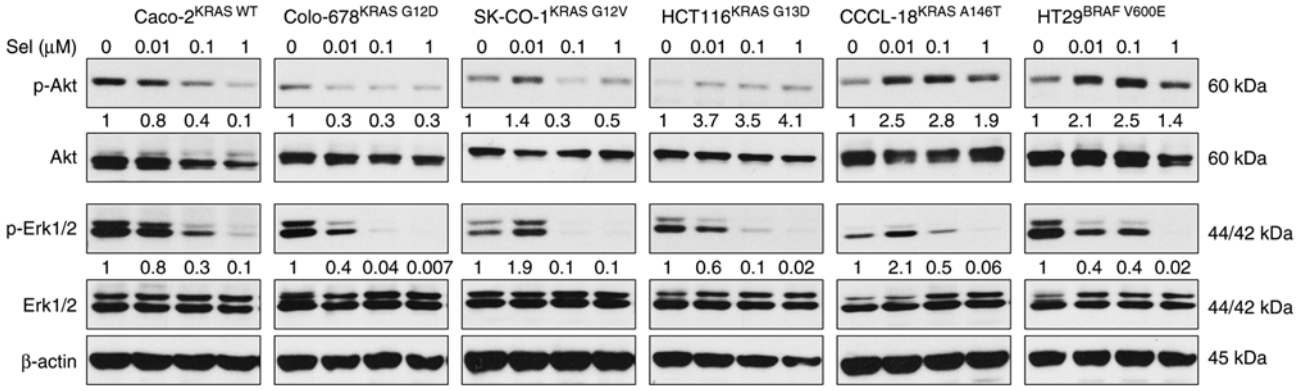

Figure 5. Inhibition of mitogen-activated protein kinase kinase leads to differential responses in RAS signaling in colorectal cancer cell lines. Caco-2$^{KRAS\ WT}$, Colo-678K$^{RAS\ G12D}$, SK-CO-1$^{KRAS\ G12V}$, HCT116$^{KRAS\ G13D}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ cells were treated with Sel and proteins were subject to western blotting for the assessment of Erk and Akt phosphorylation (n=3). Fold change was calculated in comparison to the control group treated with DMSO. Sel, selumetinib; p, phosphorylated; WT, wild-type.

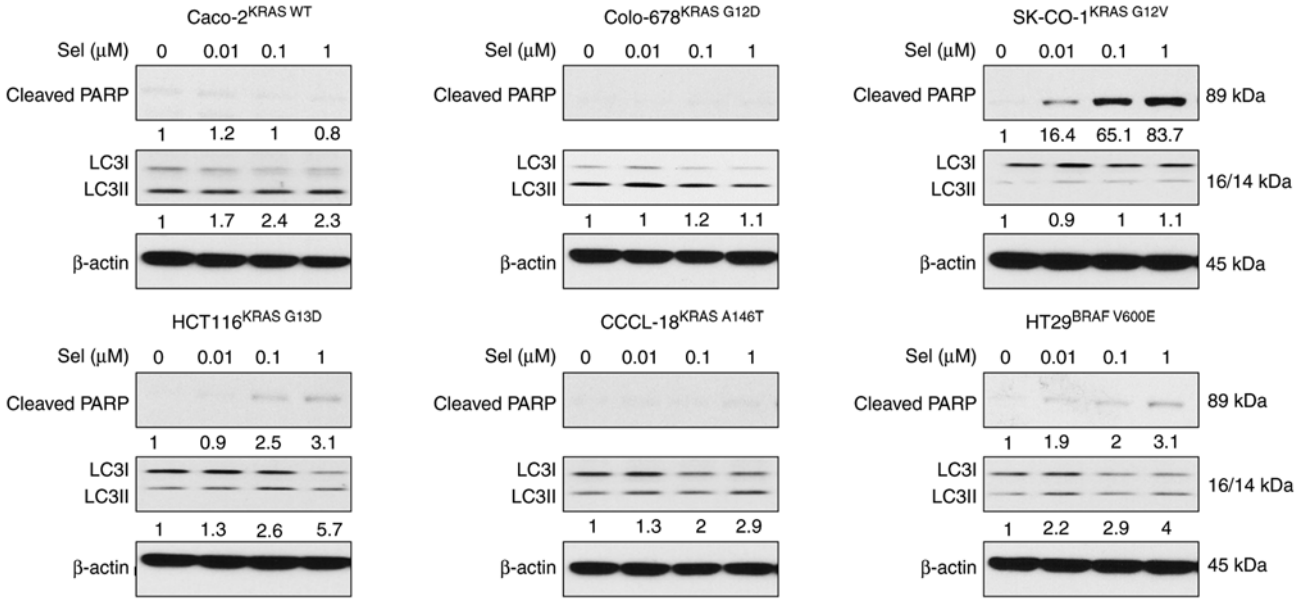

Figure 6. Inhibition of mitogen-activated protein kinase kinase modulates apoptosis and autophagy in a RAS allele-specific manner in colorectal cancer cell lines. Caco-2$^{KRAS\ WT}$, Colo-678$^{KRAS\ G12D}$, SK-CO-1$^{KRAS\ G12V}$, HCT116$^{KRAS\ G13D}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ cells were treated with Sel, and apoptosis and autophagy were examined as indicated by cleaved-PARP and LC3I and II by western blotting (n=3). Fold change was calculated in comparison to the control group treated with DMSO. Sel, selumetinib; PARP, poly ADP-ribose polymerase; WT, wild-type.

pathway in all tested cell lines, evident from the suppression of Erk phosphorylation; however, there were different responses to Sel treatment in the PI3K/Akt signaling pathway. The expression levels of p-Akt were decreased in Caco-2$^{KRAS\ WT}$, Colo-678$^{KRAS\ G12D}$ and SK-CO-1$^{KRAS\ G12V}$ cell lines, but were increased in the remaining cell lines. Furthermore, the effects of Sel on apoptosis and autophagy were tested across the CRC cell lines. Sel did not markedly affect apoptosis in Caco-2$^{KRAS\ WT}$, Colo-678$^{KRAS\ G12D}$ and CCCL-18$^{KRAS\ A146T}$ cell lines, as indicated by the unaffected PARP cleavage (Fig. 6). However, Sel induced cell apoptosis in SK-CO-1$^{KRAS\ G12V}$, HCT116$^{KRAS\ G13D}$ and HT29$^{BRAF\ V600E}$ cell lines, evident from the increase in the expression levels of cleaved PARP. Additionally, Sel promoted cell autophagy by increasing the conversion of LC3I to LC3II in Caco-2$^{KRAS\ WT}$, HCT116$^{KRAS\ G13D}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ cell lines. In combination, these results suggested that the MEK inhibitor exerted different regulatory effects on RAS signaling pathways, resulting in different responses to apoptosis and autophagy.

Combination of ALS and a MEK inhibitor regulates RAS signals, apoptosis and autophagy in a KRAS allele-specific manner. Following evaluation of the effects of ALS and Sel on RAS signals, the outcome of the dual inhibition of AURKA and MEK via ALS and Sel on RAS signaling pathways, and on apoptosis and autophagy, was examined. Caco-2$^{KRAS\ WT}$, Colo-678$^{KRAS\ G12D}$, SK-CO-1$^{KRAS\ G12V}$, HCT116$^{KRAS\ G13D}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ cells were first treated with Sel (0.1 μM) for 24 h followed by treatment with 0.1, 1 or 5 μM ALS for another 24 h. The RAS signaling output was evaluated by determining the levels of p-Akt and p-Erk, and apoptosis and autophagy were examined by determining the

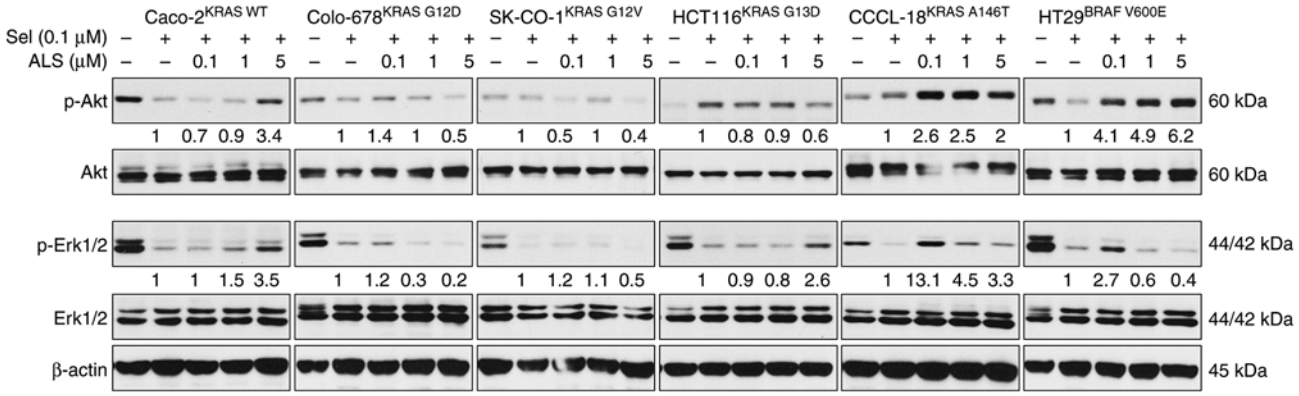

Figure 7. Combination of ALS and a mitogen-activated protein kinase kinase inhibitor modulates PI3K/Akt and MAPK signaling pathways in a RAS allele-specific manner in colorectal cancer cell lines. Caco-2$^{KRAS\ WT}$, Colo-678$^{KRAS\ G12D}$, SK-CO-1$^{KRAS\ G12V}$, HCT116$^{KRAS\ G13D}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ cells were treated with ALS and Sel, and proteins were subject to western blotting for the assessment of Erk and Akt phosphorylation (n=3). Fold change was calculated in comparison to the control group treated with Sel (0.1 μM). ALS, alisertib; Sel, selumetinib; p, phosphorylated; WT, wild-type.

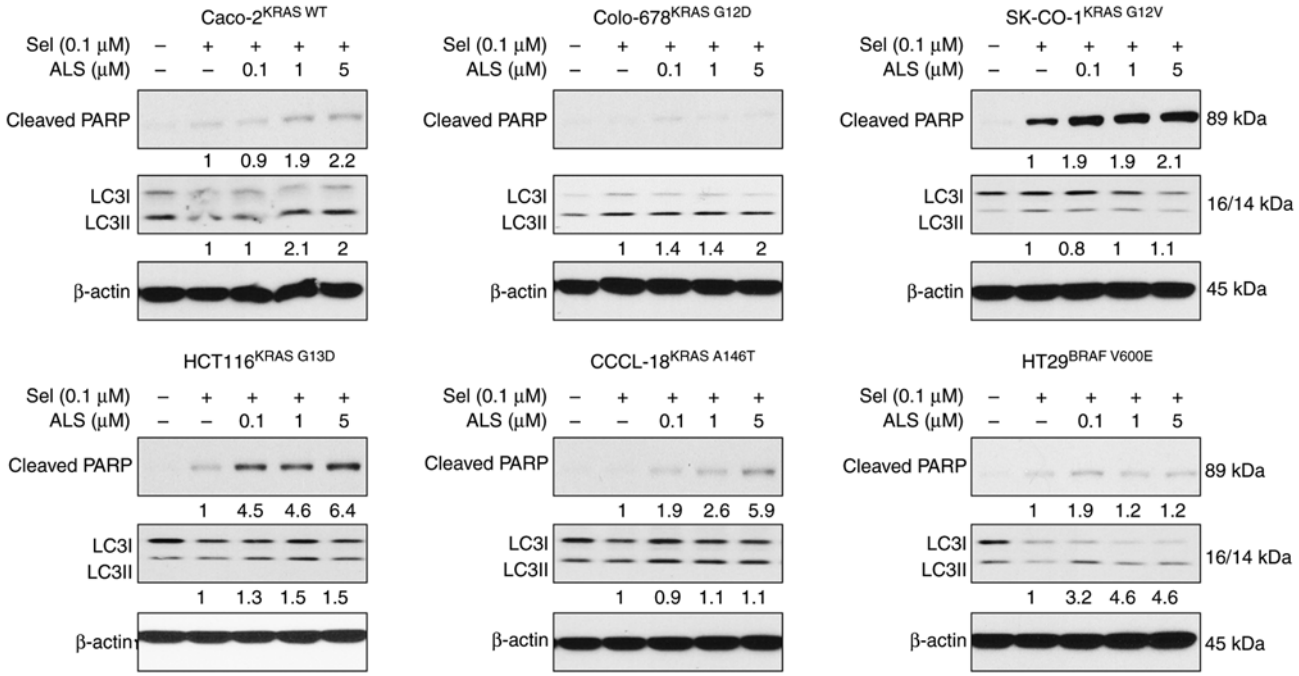

Figure 8. Combination of ALS and a mitogen-activated protein kinase kinase inhibitor regulates apoptosis and autophagy in a RAS allele-specific manner. Caco-2$^{KRAS\ WT}$, Colo-678$^{KRAS\ G12D}$, SK-CO-1$^{KRAS\ G12V}$, HCT116$^{KRAS\ G13D}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ cells were treated with ALS and Sel, and apoptosis and autophagy were examined as indicated by cleaved-PARP and LC3I and II by western blotting (n=3). Fold change was calculated in comparison to control group treated with Sel (0.1 μM). ALS, alisertib; Sel, selumetinib; PARP, poly ADP-ribose polymerase; WT, wild-type.

levels of cleaved PARP and LC3I/II. ALS counteracted the inhibitory effect of Sel on the PI3K/Akt signaling pathway in Caco-2$^{KRAS\ WT}$, CCCL-18$^{KRAS\ A146T}$ and HT29$^{BRAF\ V600E}$ cells, with an increase in the expression levels of p-Akt, whereas ALS enhanced the suppressive effect of Sel on the PI3K/Akt signaling pathway in Colo-678$^{KRAS\ G12D}$ and SK-CO-1$^{KRAS\ G12V}$ cells, with a further reduction in the expression levels of p-Akt (Figs. 7 and S3). In addition, ALS strengthened the inhibitory effect of Sel on the MAPK signaling pathway in Colo-678$^{KRAS\ G12D}$, SK-CO-1$^{KRAS\ G12V}$ and HT29$^{BRAF\ V600E}$ cells, with further suppression of Erk phosphorylation, although in the effects on Erk phosphorylation in SK-CO-1$^{KRAS\ G12V}$ cells were not significant, whereas ALS was counteractant to Sel in the remaining cell lines. Furthermore, ALS enhanced the apoptotic effects in the presence of Sel in all cell lines except for Colo-678 cells (Fig. 8). In particular, ALS enhanced the cleavage of PARP in KRAS WT-expressing Caco-2 cells, which did not occur in the presence of Sel alone. ALS enhanced autophagy in the presence of Sel in Caco-2$^{KRAS\ WT}$, Colo-678$^{KRAS\ G12D}$, HCT116$^{KRAS\ G13D}$ and HT29$^{BRAF\ V600E}$ cells, but there was no marked alteration in SK-CO-1$^{KRAS\ G12V}$ and CCCL-18$^{KRAS\ A146T}$ cells. The combination of ALS and Sel regulated RAS signal output, apoptosis and autophagy in a RAS allele-specific manner.

Combination of ALS and a MEK inhibitor exerts a synergistic cell proliferation inhibitory effect. Since the commonly occurring KRAS mutants (G12D and A146T) exhibit distinct biological features in CRC, such as intermediate

hyperproliferative phenotype of KRAS A146T in colons and high hyperproliferative phenotype of KRAS G12D in colons (36), Flp-In T-REx cells were engineered to express KRAS WT, G12D and A146T along with mCherry to assess the effects of ALS and Sel, alone or in combination, on cell proliferation. In a dose escalation assay, ALS and Sel monotherapy exerted a stronger inhibitory effect on cell proliferation in the context of KRAS G12D and A146T compared with KRAS WT (Fig. 9A and B). Notably, combination treatment with ALS and Sel displayed a synergistic effect on cell proliferation inhibition in KRAS WT, G12D and A146T-expressing cells at concentrations from 0.1-10 μM (Fig. 9C), indicated by CI<1. Collectively, these results suggested that the dual inhibition of AURKA and MEK could generate an enhanced effect.

Discussion

The notion that each RAS protein is unique has been acknowledged, and increasing evidence has shown that the RAS allele-specific approach for cancer monotherapy or combination therapy may result in encouraging outcomes in preclinical and clinical settings (37-39). In particular, a $KRAS^{G12C}$-targeted therapy in lung cancer treatment has suggested that the RAS allele can be directly and specifically targeted (40). However, this $KRAS^{G12C}$-targeted therapy is only applicable to a cysteine mutation and causes drug resistance to sotorasib and adagrasib (41-43), which limits the application of this therapy in other RAS mutant-driven cancers. The rationale of the present study stems from the concept of a 'RAS allele-specific therapeutic approach for cancer therapy'. Given the increasing evidence showing that RAS proteins are biochemically and structurally unique (37), it has been suggested that each RAS protein represents a unique biological function. Therefore, the present study aimed to assess the effects of ALS against a panel of CRC cell lines bearing different KRAS mutations. Whilst the present pilot study has shown a KRAS allele-specific response, further *in vivo* studies are needed.

KRAS mutations are present in ~45% of CRC cases, with G12D, G12V, G13D and A146T being the most common. KRAS-driven CRC is associated with substantial morbidity and mortality (44), and therefore requires urgent, novel therapeutic strategies with reduced side effects. Increasing evidence has shown that Aurora kinases are promising cancer therapeutic targets (45-52), and the selective inhibition of these targets in a RAS allele-specific manner may have profound therapeutic advantages for cancer treatment. It has previously been shown that AURKA knockdown decreases MAPK signal output, whereas AURKA overexpression promotes MAPK signaling in nasopharyngeal carcinoma (33). In the present study, the RAS allele-specific regulatory effects of ALS on RAS signaling pathways were observed against a panel of CRC cell lines bearing different KRAS mutations. Furthermore, Davis *et al* (31) showed a varied response in the combined inhibition of MEK and AURKA in KRAS/phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α double-mutant CRC. The present study also found that ALS alone or in combination with a MEK inhibitor exerted different regulatory effects on cell proliferation, and PI3K/Akt and MAPK signaling pathways, and differentially induced apoptosis and autophagy, suggesting that the KRAS mutational profile is important for ALS treatment. Notably, the inhibitory effect on the PI3K/Akt signaling pathway differed in the Caco-2 cell line when the cells were treated with ALS alone or in combination with Sel. This differential inhibitory effect on Caco-2 cells could suggest that the PI3K/Akt signaling pathway is the main route for RAS signaling, compared to the MAPK signaling pathway, and that AURKA interplays with the PI3K/Akt signaling pathway more than the MAPK signaling pathway in a WT KRAS setting. Together, the data suggested that WT KRAS-mediated PI3K/Akt signaling pathway is more susceptible to ALS inhibition. However, simultaneous inhibition with ALS and Sel showed the opposite effect, suggesting that there may be a feedback activation loop between MAPK and the PI3K/Akt signaling pathway. Indeed, the feedback activation loop has been reported in regulation of the RAS signaling pathway, which has been proposed as a potential therapeutic targeting strategy in CRC (36). However, this observation in the context of WT KRAS needs further study.

The RAS subfamily consists of KRAS, NRAS and HRAS, all of which are mutated in human cancer (53). These isoforms demonstrate a high degree of sequence identity, except at the C-terminus. Nevertheless, they are mutated in cancer in a non-random distribution, suggesting context-dependent differences in biological function. NRAS mutations are most common in malignant melanoma, hematopoietic malignancies and thyroid cancer; while HRAS mutations are most common in head and neck, and bladder cancers (53). KRAS mutations are the most common mutations in cancer, occurring in up to 22% of all human cancer cases, predominately in lung cancer, CRC and pancreatic cancer (53). KRAS mutations typically occur in exons 2 and 3, at codons 12, 13 and 61 (37,53). KRAS G12D, G13D, G12V and A146T mutations often occur in CRC, and these mutants have unique biological features, such as tissue-specific effects on homeostasis (36), which further supports the notion of RAS allele specificity. For example, KRAS A146T-driven CRC may be more susceptible to a negative feedback regulation by ribosomal S6 kinases (36). Various responses were also observed in different KRAS-mutated CRC cell lines.

Epidemiological and prospective clinical studies have shown that cancer expressing different mutant forms of KRAS exhibits distinct clinical behaviors (37,54). These differences are believed to arise from the rewiring of signal transduction networks in a RAS mutation-dependent manner, which is encouraging studies on the RAS context dependency in signal output. For example, a comparison of KRAS G12C, G12V and G12D mutations in patient-derived non-small cell lung cancer cell lines demonstrated the activation of MAPK and PI3K/Akt in G12D lines, whereas G12C and G12V exhibited little PI3K/Akt signaling and prominent RAL activation (55). Another study reported similar RAS allele-specific rewiring (56). While these studies are critical for establishing that isoform or allele-specific effects occur, the mechanisms and translational implications have not been explored. Given this lack of understanding, RAS allele-specific biology is currently a major research focus, and RAS context dependency in monotherapy or combination therapy of other key node inhibitors with RAS signaling pathway inhibitors has been a dominant topic in the RAS research community.

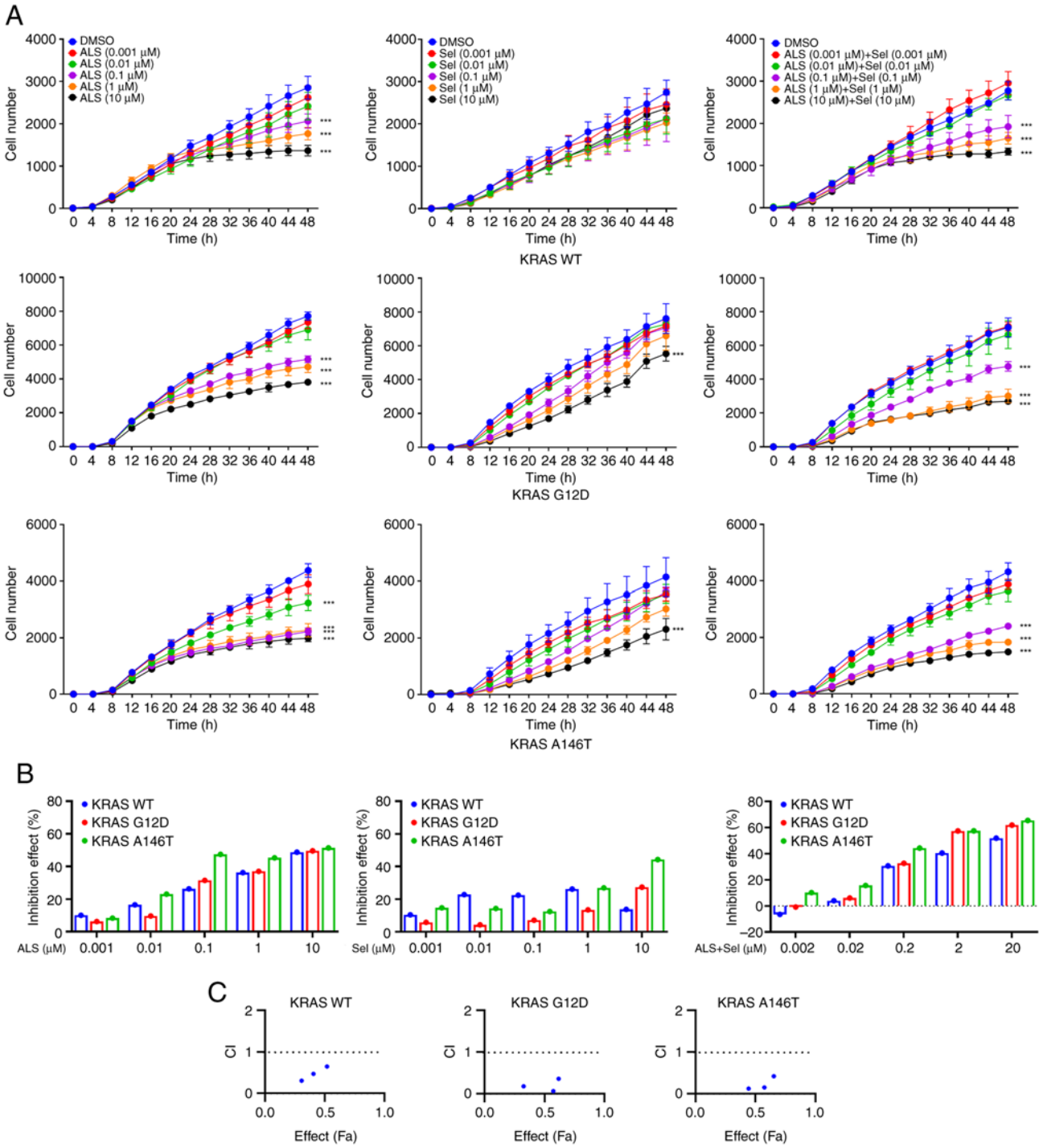

Figure 9. Dual inhibition of Aurora kinase A and mitogen-activated protein kinase kinase exerts synergistic effect on cell proliferation inhibition. (A) Treatment of ALS and Sel alone or in combination inhibits cell proliferation. Cell proliferation was examined in engineered Flp-In T-REx cells expressing KRAS WT, G12D and A146T in a dose escalation study. Cells were treated with ALS or Sel alone, or in combination and the cell proliferation was monitored in real-time using IncuCyte. (B) Inhibitory effect of monotherapy or combination therapy. (C) Combination therapy of ALS and Sel shows a synergistic effect. CI was calculated via Chou-Talalay method using CompuSyn. CI<1 defines synergism. ***P<0.001 compared with DMSO (n=3). ALS, alisertib; Sel, selumetinib; CI, combination index; WT, wild-type; Fa, fractional.

The present study is inspired by the aforementioned notion. In particular, previous studies on the preferential signaling output of KRAS G12D vs. KRAS Q61H in lung cancer (57) and distinct biological features of KRAS G12D vs. KRAS A146T in CRC (36) clearly demonstrate the role of RAS allele specificity in cancer treatment. For example, the present study demonstrated that the KRAS G13D-expressing CRC cell line was the most susceptible to treatment with ALS, which could be ascribed to the dual suppression of the PI3K/Akt and MAPK signaling pathways. In the KRAS A146T-expressing CRC cell line, ALS enhanced both the PI3K/Akt and MAPK signaling pathways, which could disturb the cell viability, resulting in over-activated signal outputs (58,59). However, ALS suppressed both PI3K/Akt and MAPK signal outputs in the HT29 cell line, which only

showed moderate sensitivity in cell proliferation. This moderate sensitivity could be ascribed to the BRAF V600E mutant. In addition, the crosstalk between other key nodes and RAS signaling pathways may be involved in the response to ALS treatment.

Furthermore, there are limitations in the present study. First, the lack of cell line authentication by STR profiling could affect the accuracy of the results. Second, the HT29 cell line may not be the most appropriate as a CRC cell model, because it has been indicated that the HT29 cell line originated from an adenocarcinoma of the rectosigmoid part of the intestine. Third, the lack of an AURKA knockdown experiment is also a limitation, although it has been reported in other studies (26-30). Furthermore, *in vivo* studies are needed to support the effects observed *in vitro* in future.

In conclusion, the present findings revealed a potential RAS allele-specific therapeutic role for AURKA inhibition and RAS signaling modulation in CRC treatment. Given that KRAS is the most common oncogene in human cancer, including CRC, novel therapeutic strategies are urgently needed. Such a strategy could not only target a single kinase but also be therapeutically effective by harnessing oncogenic KRAS with an allele-specific approach.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BR, YG and SC developed the study concept and design. BR, YG, SC, ZG, KZ, YY, QL, JF and ZL acquired the data. Analysis and interpretation of data was performed by BR, YG, SC, ZG, KZ, YY, QL, JF and ZL. BR, YG and SC drafted the manuscript, and statistical analysis was performed by BR, YG and SC. Technical and material support was provided by QL, JF and ZL. YJ and ZH designed and supervised the study, and finalized the manuscript. BR, YJ and ZH confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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