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# MEK1/2 Inhibitors AS703026 and AZD6244 May Be Potential Therapies for *KRAS* Mutated Colorectal Cancer That Is Resistant to EGFR Monoclonal Antibody Therapy

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# Abstract

EGFR monoclonal antibodies (mAbs) are used widely to treat metastatic colorectal cancer (mCRC) patients, but it is now clear that patients harboring K-*ras* mutation are resistant to EGFR mAbs such as cetuximab (Erbitux) and panitumumab (Vectibix). For this reason, current recommendations for patient care involve diagnosing the K-*ras* mutational status of patients prior to EGFR mAb therapy. In this study, we investigated the ability of two MEK inhibitors currently in clinical trials, AS703026 and AZD6244, to address the challenge posed by the resistance of K-*ras* mutated colorectal cancers to EGFR mAb. AS703026 and AZD6244 were tested in various cell-based assays and tumor xenograft studies, focusing on isogenic human colorectal tumor cell lines that expressed only WT or mutant K-Ras (D-WT or D-MUT). The EGFR mAb cetuximab inhibited the Ras-ERK pathway and proliferation of D-WT cells *in vitro* and *in vivo*, but it did not inhibit proliferation of D-MUT cells in either setting. In contrast, AS703026 and AZD6244 effectively inhibited the growth of D-MUT cells *in vitro* and *in vivo* by specific inhibition of the key MEK downstream target kinase ERK. Inhibition of MEK by AS703026 or AZD6244 also suppressed cetuximab-resistant colorectal cancer cells attributed to K-*ras* mutation both *in vitro* and *in vivo*. Our findings offer proof-of-concept for the use of MEK inhibitors as an effective therapy in K-*ras* mutated CRC.

# Introduction

Colorectal cancer is a commonly diagnosed malignancy and a major cause of cancer-related-death (1, 2). During the past decade, the median overall survival time for patients with metastatic colorectal cancer (mCRC) has increased from 12 to 21 months, primarily due to chemotherapy with oxaliplatin, irinotecan, and 5-fluorouracil (3). Newly developed molecular targeted therapies using monoclonal antibodies (mAbs) such as cetuximab (Erbitux), panitumumab (Vectibix), trastuzumab (Herceptin) and Bevacizumab (Avastin) have provided clinically meaningful benefits in treating colorectal cancer (4-6). Most novel therapies have developed by targeting the vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) signaling involving angiogenesis and proliferation of cancer cells.

Since many human cancers, including colorectal cancer, involve abnormal expression of EGF receptor (EGFR), which is implicated in the development and prognosis of malignancy, EGFR is a rational molecular target for cancer treatment (7). However, the benefit from anti-EGFR mAb therapy using cetuximab and panitumumab is limited to only a small portion (8~23%) of patients. The patients with mCRC bearing K-*ras* activating mutation are resistant to EGFR mAb therapy (8-12). In July 2009, the FDA recommended that the K-*Ras* gene mutational status of mCRC patients be screened prior to treatment with EGFR mAb to avoid wasting time and disbursement (13).

Most K-*ras* mutations occur in the mid-adenoma stages in the colorectal tumorigenesis (14). Ras activation stimulates cell proliferation via the Raf-MEK-ERK and PI3 kinase-Akt pathways, which involves development of carcinoma (15-17). Cetuximab is a monoclonal antibody targeting EGFR, and its binding to the extracellular domain of EGFR prevents

ligand binding and inhibits signaling cascades such as the Ras-Raf-MEK-ERK and PIK3CA-Akt pathways (18). Considering the epistasis of the Ras-Raf-MEK-ERK cascade, we hypothesized that MEK inhibition may overcome cetuximab resistance attributed to K-*ras* mutations in some colorectal cancers.

To investigate the sensitivity of cetuximab resistance dependent on K-*ras* mutation in cells and tumors, we used isogenic DLD-1 colorectal cancer cell lines (D-WT and D-MUT) in which wild-type (WT) and mutant K-*Ras* alleles have been inactivated by homologous recombination (19). These cell lines share the same genetic background with the exception of one base pair of codon 13 in the K-*Ras* gene. Considering intact DLD-1 cells which are heterozygous for K-*Ras*, this paired colorectal cell line provides an ideal system to distinguish the responsiveness of a drug against K-*ras* mutation. In this study, we address the effect of K-*ras* mutation on the responsiveness of colorectal cancer cells to cetuximab using D-WT and D-MUT cells and tumors. We further evaluated the effect of MEK inhibition on the responsiveness of the D-WT and D-MUT cell lines using two MEK1/2 inhibitors, AS703026 and AZD6244, which are currently undergoing clinical trial-phase I and phase II, respectively (20, 21). We observed that the MEK inhibitors are effective in inhibiting tumor cell growth on the D-MUT cells harboring a K-*ras* mutation both *in vitro* and *in vivo*, in contrast to cetuximab. The MEK inhibitors reduced activities of the ATF2 transcription factors which were also not affected by cetuximab in K-*ras* mutated tumors. Our present study, based on the recent clinical observations related to cetuximab resistance (8-11, 22), is a pioneering study to investigate the responsiveness of an EGFR mAb to a K-*ras* mutation using isogenic colorectal cancer cell lines and their xenograft models.

## Materials and Methods

### Reagents

The antibodies used in this study were anti-β–catenin (BD Bioscience, San Jose, CA); Pan-Ras (Upstate Biotechnology, Lake Placid, NY); α-tubulin (Calbiochem, San Diego, CA); p-ERK, p-ATF2 (T71) (Cell Signaling Technology, Beverly, MA); p-Akt, PCNA, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA); anti-rabbit AlexaFlour 488 and anti-mouse AlexaFlour 555 (Invitrogen). Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) was purchased from DAKO (Carpin-teria, CA). Cetuximab (Erbitux) and AS703026 were provided by Merck KGaA (Frankfurter, Darmstadt, Germany). AZD6244 was obtained from OTAVA (Toronto, Ontario, Canada).

### Cell culture

Human colorectal cancer cell lines with either wild-type (D-WT) or mutated K-*Ras* (D-MUT) were kindly provided by Dr. Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD) in 2009. These cell lines are derived from DLD-1 human colon cancer cell in which the endogenous wild type or mutant alleles had been inactivated through targeted homologous recombination (19). D-WT and D-MUT cells were grown in RPMI 1640 (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL of streptomycin at 37°C. Cells were transfected using Lipofectamine and Plus Reagent (Invitrogen, Carlsbad, CA). Cells were checked by morphology and were tested to be *Mycoplasma*-free using MycoFluor[TM] Mycoplasma Detection Kit (Invitrogen) within the last 6 months. D-WT and D-MUT cells were authenticated by mutational analysis of K-*Ras*, as described below.

**Mutational analysis of K-*ras***

For mutational analysis of K-*ras*, total genomic DNA was extracted from D-WT and D-MUT cells using the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany). Fifty ng of DNA were amplified in a 20 μL reaction mixture containing 2 μL 10x buffer (Roche, Mannheim, Germany), 1.7 to 2.5 mmol/L MgCl$_2$, 0.3 μM each primer pair (codon 12, 13, F: 5'-TTATGTGTGACATGTTCTAAT-3', R: 5'-AGAATGGTCCTGCACCAGTAA-3' codon 61, F: 5'-TCAAGTCCTTTGCCCATTTT-3', R: 5'-TGCATGGCATTAGCAAAGAC-3'), 250 μmol/L deoxynucleotide triphosphates, and 2.5 units of DNA polymerase (Roche Diagnostics, Mann- heim, Germany). Amplification was performed using a 5-minute initial denaturation at 94°C, followed by 30 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C, and a 10-minute final extension at 72°C. Polymerase chain reaction (PCR) products were separated on a 2% agarose gel and gel-purified with a QIAgen gel extraction kit (QIAGEN, Valencia, CA) before being subjected to DNA sequencing analysis.

**MTT assay and BrdU incorporation**

Cells were plated at a density of $4x10^4$ cells per 24 well and treated with MEK inhibitor, AS703026 or AZD6244 with/without cetuximab for 72 hours. MTT reagent (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; AMRESCO, Solon, OH) was diluted in RPMI 1640 at a concentration of 0.25 mg/mL. Cells were incubated for 3 hours at 37°C. Media was removed and insoluble purple formazan was solubilized with 500 μL DMSO for 30 minutes. The absorbance of formazan product was determined at 500 nm. For *In vitro* BrdU labeling, cells were treated with 10 μmol/L BrdU for 4 hours. Cell were fixed with 4%

paraformaldehyde for 30 minutes and treated with 0.1% Triton X-100 for 15 minutes. Then, cells were blocked by 5% bovine serum albumin (BSA) and 1% normal goat serum (NGS) in PBS for 30 minutes and incubated with anti-BrdU antibody (1:200), overnight at 4°C, followed by incubation with anti-mouse Alexa Flour 555 secondary antibody, and counterstained by DAPI (Boehringer Mannheim, Mannheim, Germany), and then mounted in Gel/Mount media (Biomeda Corporation, Foster city, CA). Visualization of fluorescence signal was performed using confocal microscopy (LSM510, Carl-Zeiss, Jena, Germany) at excitation wavelengths of 488 nm (Alexa Fluor 488), 543 nm (Alexa Fluor 555) and 405nm (DAPI). Each experiment was repeated three times. Data are given as mean ± SD.

**Foci formation assay**

D-WT and D-MUT cells were seeded at a density of 500 cells in each well of 6 well plates. The cells were treated with MEK inhibitor, AS703026 or AZD6244 with/without cetuximab at every 3 days for 7 (D-MUT) or 14 (D-WT) days. Media was changed every 3 days. After 7 (D-MUT) or 14 (D-WT) days, cells were stained with 0.5% crystal violet in 20% ethanol.

**Ras activation assay and immunoblotting**

Cells were lysed with 2% Triton X-100, 100 mM HEPES, pH 7.5, 200 mmol/L NaCl, 10 mM $MgCl_2$, 2 mmol/L sodium orthovanadate, mammalian cell protease inhibitor mixture, and cleared by centrifugation at 14000xg, for 5 minutes at 4°C. Aliquots of lysates were to allow quantification of total Pan-Ras, α-tubulin, p-ERK, p-Akt, ERK by immunoblotting. The remainder of cell lysates was incubated with bead coated with fusion protein (GST-Raf1-RBD; Upstate Biotechnology, Inc.) at 4°C for 1 hour. Beads were washed three times with cold lysis buffer, and bounded protein was eluted with 4x laemmli sample buffer at 100°C for

5 minutes and analyzed by immunoblotting for Ras.


**Mouse xenograft assay**

This animal study was approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine. 4~6 week-old male *Balb C nu/nu* mice were purchased from Slc Inc. (Shizuoka, Japan). Mice were housed in filter-topped shoebox cages with a MSRS environmental control system from Orient Biotech. (Sungnam, Gyeong-gi do, Korea). Room temperature was maintained at 24°C with a relative humidity of 40~70%. The mice were given a standard maintenance diet from Dae Han Bio Link (Daejeon, Korea). After acclimatization for 1 week, $5 \times 10^6$ D-WT or MUT cells in 200 µL of PBS: Matrigel (1:1) were subcutaneously injected to dorsal flank of athymic nude mice. When the mean values of tumor were between 100 and 200 $mm^3$, mice were randomly divided into 4 groups (5 mice per group). Cetuximab was treated to mice intraperitonially at dose of 1 mg/mice, twice a week. AS703026 and AZD6244, in a suspension of 0.5% methyl cellulose and 0.4% Tween 80, were injected by oral gavages once and twice daily at dosage of 10 mg/kg and 15 mg/kg, respectively. Tumor volume and body weight of mice were measured every 4 days. Tumor volumes were measured by Verniar calipers, following the formula of $\pi/6$ x length x width x height. Animals were euthanatized when tumor volume exceeded 1500 $mm^3$. For *in vivo* BrdU labeling, mice were injected intraperitonially with 100 mg/kg BrdU 4 hours before sacrifice. Twenty-eight days after treatment mice were sacrificed and tumors were excised and weighed and then fixed in paraformaldehyde or snap frozen in liquid nitrogen for further analysis.

**Immunohistochemistry**

  For immunohistochemical analysis, 4 μm paraffin sections were treated with 0.1% triton X-100 in PBS for 15 minutes and then block by 5% BSA and 1% NGS in PBS for 30 minutes. Primary and secondary antibodies were diluted with 1% BSA and 1% NGS in PBS. The primary antibody was omitted for negative control slides, which resulted in very low background labeling. Sections were incubated with primary antibodies, overnight at 4°C, followed by incubation with anti-mouse Alexa Flour 488 (1:500) and anti-rabbit Alex Flour 555 secondary antibodies (1:500) for 1 hour at room temperature, and counter stain by DAPI (Boehringer Mannheim), and then mounted in Gel/Mount media (Biomeda Corporation). All incubations were carried out in dark and humid chambers. The fluorescence signal was visualized using confocal microscopy (LSM510) at excitation wavelengths of 488 nm (Alexa Fluor 488), 543 nm (Alexa Fluor 555) and 405 nm (DAPI). At least 3 fields per section were analyzed to establish statistical significance.

# Results

**D-MUT colorectal cancer cells are resistant to cetuximab due to K-*ras* mutation**

  Cells retaining endogenous WT K-*Ras* (D-WT) and cells with a mutant allele of K-*ras* (D-MUT) in the genetic background of DLD-1 colorectal cancer cells (19) were adopted to study the sensitivity of anticancer drugs dependent on the mutational status of K-*ras*. DNA sequencing analysis confirmed the mutational status of K-*ras* in D-WT and D-MUT cells. (Supplementary Fig. S1). To determine responsiveness of EGFR mAb on K-*ras* mutation, D-WT and D-MUT cells expressing high level of EGFR were treated with cetuximab at every 24 hours for 72 hours. The proliferation of cells was measured by MTT analysis at 24, 48,

and 72 hours after cetuximab treatment. As expected, D-MUT cells harboring mutated K-*ras* clearly showed resistance to cetuximab, whereas proliferation of the isogenic D-WT cells harboring WT K-*Ras* was suppressed by cetuximab (Fig. 1A). D-WT cell growth was inhibited by 30% after cetuximab treatment as revealed by MTT analysis, while D-MUT cell growth was unchanged after 72 hours of treatment. To investigate the efficacy of cetuximab *in vivo*, mouse xenograft models were produced by injecting either D-WT or D-MUT cells subcutaneously into the dorsal flank of athymic nude mice. In the D-WT xenograft model, administration of cetuximab at dosage of 1 mg/mice twice a week for 8 weeks significantly reduced tumor size and tumor weight by 50% (Fig. 1B and 1C). In contrast, D-MUT tumors treated with cetuximab did not show a reduction in either volume (Fig. 1B) or weight (Fig. 1C) of tumors by the cetuximab therapy. Immunohistochemical analyses were performed to confirm the effect of cetuximab on D-WT or D-MUT xenograft tumors (Fig. 1D). Positive staining for p-ERK was observed specifically around tumor borders, where a relatively non-hypoxic condition is maintained. Therefore, all images were obtained from these regions. The p-ERK staining was decreased in D-WT tumors treated with cetuximab, but was unaffected in D-MUT xenograft tumors with the same treatment (Fig. 1D). The staining patterns for PCNA, a proliferation marker, were similar to that of p-ERK, revealing that proliferation of both D-WT and D-MUT tumors correlate with activation status of the ERK pathway (Fig. 1D). β-catenin, as a negative control of staining, did not show any significant change by treatment of cetuximab (Fig. 1D).

**AS703026 and AZD6244 inhibit ERK pathway, proliferation and transformation in cetuximab-resistant D-MUT cells**

MEK inhibitors currently in clinical development, AS703026 and AZD6244 (Fig. 2A),

which act downstream of K-Ras, were tested for their ability to overcome cetuximab resistance in D-MUT cells. In D-WT cells, cetuximab inhibited ERK activity by reducing GTP loading of Ras (Fig. 2B and 2C). In D-MUT cells, cetuximab did not reduce GTP loading of Ras or ERK activity attributed to the K-*ras* mutation (Fig. 2B and 2C). When D-MUT cells were also treated with AS703026 or AZD6244 (Fig. 2B and 2C), ERK activity was reduced, but no combination effect was observed. The inhibitory effect of 10 μmol/L AS703026 on ERK activity was higher than that of 50 μmol/L AZD6244 (Fig. 2B and 2C). The alternative MEK inhibitor U0126 showed identical effect on ERK activity (Supplementary Fig. S2). No significant change of Akt activity was observed when cetuximab, AS703026, or AZD6244 treated to both D-WT and D-MUT cells (Fig. 2B and 2C).

Effects of AS703026 and AZD6244 on cellular proliferation were measured using MTT and BrdU incorporation assays (Fig. 3). Treatment with cetuximab alone reduced proliferation of D-WT but not of D-MUT cells. Addition of AS703026 and AZD6244 reduced proliferation in both D-WT and D-MUT cells (Fig. 3A and 3B). When D-WT cells were treated with cetuximab, BrdU incorporation was decreased by 54-65%, but was unaffected in D-MUT cells. BrdU incorporation in D-WT cells was reduced by 62.3% with the addition of AS703026 and by 67.2 % with AZD6244, and in D-MUT cells by 63.2% with AS703026 and 61.3% with AZD6244 (Fig. 3C and 3D). The anchorage-independent growth of D-WT cells was inhibited by cetuximab, and that of D-MUT cells was not (Fig. 4A and 4B). Cellular transformation was reduced for both D-WT and D-MUT by treatment with AS703026 (Fig. 4A) or AZD6244 (Fig. 4B). However, no combination effects from treatment of cetuximab with either AS703026 or AZD6244 were observed in ERK activation, proliferation, or transformation of cells, although BrdU incorporation in D-WT cells did suggest combination

effect (Fig. 3 and 4)

**AS703026 and AZD6244 inhibit tumor growth of cetuximab resistant tumor attributed by K-*ras* mutation**

Anti-tumor effects of AS703026, AZD6244 and cetuximab were measured using mouse tumor xenograft models. The representative gross images for tumor on athymic mice bearing tumor are shown in Fig. 5A and B. Cetuximab treatment did not reduce the size of D-MUT xenograft tumors (Fig. 5C-D). However, both volume (Fig. 5C) and weight (Fig. 5E) of tumors caused by D-MUT cells were reduced by 60% by AS703026, and this correlates with the cell-based *in vitro* studies. Treatment with AZD6244 showed a similar growth inhibitory effect on tumor volume decreased by 71% (Fig. 5D) and weight by 68% (Fig. 5F). In agreement with the cell studies, no combination effect was observed when AS703026 or AZD6244 (Fig. 5). We did not observe any statistically significant changes in body weight of mice used through the both experiments (Supplementary Fig. S3).

Immunohistochemical analyses were performed to confirm the mechanisms of action of AS703026, AZD6244, and cetuximab on D-MUT xenograft tumors (Fig. 6). In AS703026 and AZD6244 administered D-MUT tumors, however, p-ERK staining decreased markedly (Fig. 6). The ATF2 activity monitored by anti-p-ATF2 (T71) antibody was reduced by treatment of AS703026 or AZD6244 in D-MUT tumors, indicating that ERK activity inhibits the regulation of target genes (Fig. 6). The Akt activity was not significantly reduced by cetuximab or MEK inhibitor treatment in D-MUT tumors (Fig. 6). β-catenin did not show any significant change by cetuximab therapy (Fig. 6).

## Discussion

Since the first MEK inhibitor, PD98059 have been developed, other numerous MEK inhibitors have also been developed. Undesirable pharmaceutical properties or side effects resulted in none of the early MEK inhibitors, including U0126 and RO 09-2210, progressing to clinical trials (23). By cell-based screening or modification from the original MEK inhibitor, eleven MEK inhibitors have entered clinical trials. CI-1040 (Pfizer) was the first MEK1/2 inhibitor to enter clinical trials (21). Despite higher selectivity of CI-1040 to MEK1/2 inhibition, this drug has been disclosed after Phase II because of insufficient anti-tumor activity, poor solubility and low efficacy (21). A following clinical trial of a second generation MEK1/2 inhibitor PD0325901 (Pfizer), an analogue of CI-1040, was also stopped in phase II by severe toxicity including blurred vision as well as neurotoxicity. Here, we investigated the use of AS703026 (Merck Serono) and AZD6244 (AstraZeneca), which are in clinical trials, to check the effects of MEK1/2 inhibition on K-*ras* mutated colorectal cancer (20, 21). The results of the phase I and II studies of AZD6244 were reported with evidence of only a single agent antitumor activity in patients with non-small cell lung cancer, advanced melanoma, or metastatic colorectal cancer as well as lack of radiographic response in patients with advanced or metastatic hepatocellular carcinoma (24-28). However, other phase II trials in a variety of tumor types are currently in process (21).

AS703026 was most recently developed as a highly selective, ATP non-competitive allosteric MEK1/2 inhibitor. Phase I clinical trials of AS703026 are underway to test its inhibitory effects on multiple solid tumors (29). Multiple myeloma (MM) cells are inhibited by AS703026 regardless of *Ras* and *BRAF* mutation when it is administered in combination with multiple conventional, novel, and emerging anti-MM therapies (30). Our present study

shows efficacy of both AZD6244 and AS703026 on colorectal cancer with K-*ras* mutation. When ligands bind to activate EGFR, both the ERK and PI3K-Akt pathways are activated (18). Ras protein directly interacts with the p110 subunit of PI3K to activate Akt (31). Both ERK and PI3K-Akt pathways are strongly implicated in cell proliferation and survival (32). Combined therapy with MEK and PI3K inhibitors had a synergistic inhibitory effect on non-small cell lung cancer cells affected by the K-*ras* mutation (33). Our study indicates that inhibitory effects of AS703026 and AZD6244 on tumor volume in the xenograft assay did not abolish tumor growth. We observed no inhibition of Akt activity by either cetuximab or MEK inhibitor in D-MUT tumors due to oncogenic mutation of K-*ras*. Our results indicate the simultaneous blockade of Ras-ERK and PI3K-Akt pathways may be more efficacious in the treatment of cancers attributed by K-*ras* mutation. The recent announcement of a collaboration between Merck and AstraZeneca to test combination cancer therapy using MEK inhibitor AZD6244 (AstraZeneca) and Akt inhibitor MK-2206 (Merck) further support importance of the combinatory therapy related with *ras* mutation (21).

We observed that both AS703026 and AZD6244 increased GTP-loading of Ras. This increment of GTP-loading of Ras may be caused by desensitization of Ras activation through the disassociation of the Grb2-Sos complex by phosphorylating Sos (34). Our observation also correlates with the previous report that MEK inhibition blocked phosphorylation and dissociation of Sos from Grb2, resulting in prolonged Ras activation (35). When AS703026 or AZD6244 were treated with a combination of cetuximab, this negative feedback regulation was thoroughly diminished by targeting EGFR, the upstream signaling regulator.

Interestingly, new DNA synthesis of D-WT cells, monitored by BrdU incorporation, was synergistically reduced by MEK inhibitor and cetuximab although we did not observe the synergy effects in the measurement of cellular transformation by foci formation assay. The

synergy effect observed in these studies indicates that an alternative route for transmission of proliferation signal is present only in the wild-type DLD-1 cells. Somatic mutation of *PI3K* in the parent DLD-1 cells may not allow transferring downstream signaling to Akt (36). Therefore, PI3K-Akt pathway may not be involved in this synergistic effect. One potential route attributing the synergy effect may be the pathway involving RalGDS. The RalGDS can directly interact with GTP-bound active form of Ras protein through their Ras binding domain (RBD) (37), and interaction between GTP-Ras and RalGDS promote the translocation of RalGDS to the plasma membrane where it activates Ral (38). The RalGDS-Ral pathway has been shown to be involved in regulation of various transcription factors, such as Rlf, c-Jun, and ATF2, to promote cell proliferation (39-41). In addition, RalGDS promote Akt activation by PDK1 as a scaffold protein (42).

Here, we used D-WT and D-MUT cell, the isogenic colorectal cancer cell lines, as pair. The usage of these cell lines eliminates the possibility of partial effect by wild type K-*Ras* in K-*ras* mutated cancer cells, when compared to the several previous trials related with therapeutic potential of MEK inhibitors on various cancer cells (43-46). The data indicates clear resistance to EGFR mAb in the K-*ras* mutated cell line D-MUT, an advantage over most colorectal cancer cells that have heterozygous K-*ras* mutations. The BRAF inhibitor Sorafenib was previously suggested to overcome the cetuximab resistance of K-*ras* mutated colorectal cancer, although the study involved only an *in vitro* system (47). Our present study provides *in vivo* evidence for the cetuximab resistance of K-*ras* mutations and therapeutic strategy to overcome it using xenograft tumor model of CRC harboring an isogenic K-*ras* mutation. In the era of personalized medicine, our results suggest that the paired isogenic colorectal cancer cell can be used as a drug screening system to identify the overcoming effect related with cetuximab resistance in K-*ras* mutated colorectal cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgements

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**Figure Legends**

**Figure 1.** The K-*ras* mutation affects the responsiveness of D-WT and D-MUT cells to cetuximab. A, sensitivity of D-WT and D-MUT cells to cetuximab. D-WT and D-MUT cells were grown in RPMI, and treated with DMSO (control) or Cetuximab (5μg/mL) at every 24 h for 72 h. MTT assays were performed at 24, 48, and 72 h after treatment of drugs. B and C, *in vivo* effect of cetuximab on volume (B) and weight (C) of D-WT or D-MUT tumor in mouse xenograft model. Xenograft tumor model was established as described in *Materials and Methods*. Cetuximab were administrated to the nude mice bearing tumors intraperitonially at dosage of 1 mg/mice, twice a week for 8 weeks (D-WT) or 4 weeks (D-MUT). Tumor volume was measured using callipers every week through experiment. D, paraformaldehyde fixed paraffin sections were incubated with anti-p-ERK, PCNA, or β-catenin antibody, followed by incubation with goat anti-mouse-Alexa 555 secondary antibodies. Nuclei were counter stained by DAPI. Images were captured from non-hypoxic area and the tumor borders using laser scanning confocal microscopy (Carl Zeiss LSM510). Original magnification: x400.

**Figure 2.** Effects of AS703026 and AZD6244 on Ras-ERK pathway in D-WT and D-MUT cells. A, chemical structures of AS703026 (upper) and AZD6244 (lower). B and C, effect of AS703026 (B) and AZD6244 (C) on Ras-ERK pathway in D-WT and D-MUT cells. D-WT and D-MUT cells were grown in RPMI, and treated for 24 h with DMSO as a control, 5 μg/mL cetuximab with/without 10 μmol/L AS703026 or 50 μmol/L AZD6244. The Effects of AS703026 or AZD6244on Ras-ERK pathway in D-WT or D-MUT cells were measured by immunoblot analysis. GTP-loaded active Ras

with a GST fusion-protein corresponding to the human Ras Binding Domain of Raf-1 (GST-Raf 1-RBD) evaluated Ras activation. Immuno blot analyses were performed using whole-cell lysates with anti-Pan-Ras, -p-ERK, -ERK or -α-tubulin antibody. The GTP-loaded Pan-Ras protein (RAS-GTP) was detected using anti Pan-Ras antibody.

**Figure 3.** Effects of AS703026 and AZD6244 on cell proliferation of D-WT and D-MUT cells. A and B, cetuximab at 5 µg/mL was applied to D-WT and D-MUT cells every 24 h for 72 h in combination with either 10 µmol/L AS703026 (A) or 50 µmol/L AZD6244 (B). MTT assays were performed at 24, 48, and 72 h after drug treatments. C and D, D-WT and D-MUT cells were treated for 24 h with DMSO (control) or 5 µg/mL cetuximab in combination with either 10 µmol/L AS703026 (C) or and 50 µmol/L AZD6244 (D). Then BrdU was added at a final concentration of 20 µmol/L for 4 h, and cells were fixed, permeabilized, incubated with anti-BrdU mAb, and finally incubated with goat anti-mouse-Alexa 555 secondary antibodies. Cell nuclei were counterstained with DAPI. Cells were visualized by a laser scanning confocal microscopy (Carl Zeiss LSM510). The percentage of BrdU-positive cells was calculated from the total number of DAPI-stained cells (lower panel), and error bars indicate the standard deviations of three independent analyses. Original magnification: C, x400; D, x200.

**Figure 4.** Effects of AS703026 and AZD6244 on cellular transformation of D-WT and D-MUT cells. A and B, D-WT and D-MUT cells were treated every 3 days for 7 days (D-MUT) or 14 days (D-WT) with DMSO (control) or 5 µg/mL cetuximab combined with either 10 µmol/L AS703026 (A) or 50 µmol/L AZD6244 (B). Cells were stained

with 0.5% crystal violet in 20% ethanol, and then foci were photographed. Quantitative data for the relative percentages of foci numbers are represented in the lower panel, with error bars indicating the standard deviation of three independent analyses.

**Figure 5.** Effects of AS703026 and AZD6244 on the tumor size of cetuximab-resistant tumor caused by K-*ras* mutation. Five athymic nude mice were treated for 28 days by oral gavage with vehicle or with cetuximab combined with either 10 mg/kg AS703026 (Left) once daily or 15 mg/kg AZD6244 (Right) twice daily. A, gross images showing representative cases treated with AS703026 (A) and AZD6244 (B) with enlarged tumor images for each case presented in the lower panel. C and D, the tumors volume of mice treated with vehicle or cetuximab with/without either AS703026 (C) or AZD6244 (D) were measured with vernier callipers every 4 days. E and F, after sacrifice, tumor weights were measured by an electronic balance from the tumors of mice treated with vehicle or cetuximab with/without either AS703026 (E) or AZD6244 (F). Gross images from representative excised tumors are shown in the lower panel.

**Figure 6.** Effect of AS703026 or AZD6244 on the activations of downstream pathways of Ras in D-MUT xenograft tumors**.** Immunohistochemical analyses of activation of the Ras downstream components in the Ras-ERK and PI3K-Akt pathways. Paraformaldehyde fixed paraffin sections were incubated with anti-p-ERK, p-Akt, p-ATF2, PCNA, or β-catenin antibody, followed by incubation with goat anti-mouse-Alexa 555 secondary antibodies. Nuclei were counter stained by DAPI.

Images were captured from non-hypoxic area and the tumor borders using laser scanning confocal microscopy (Carl Zeiss LSM510). Original magnification: x400.
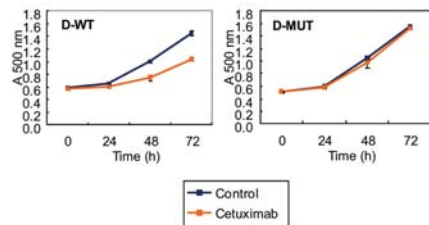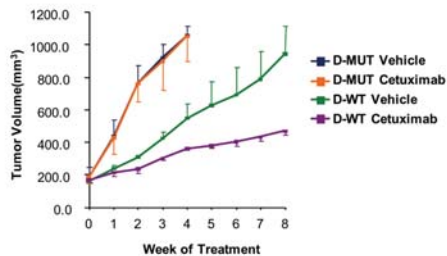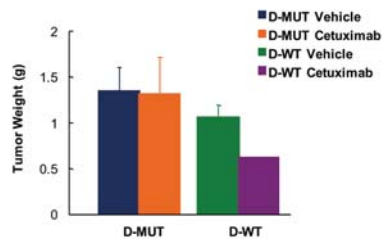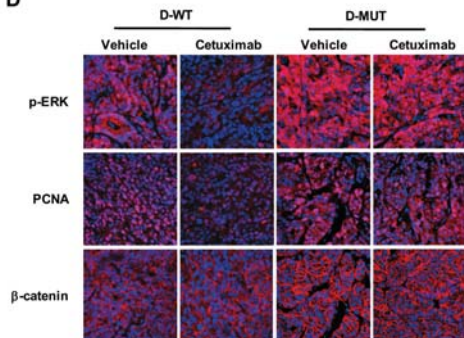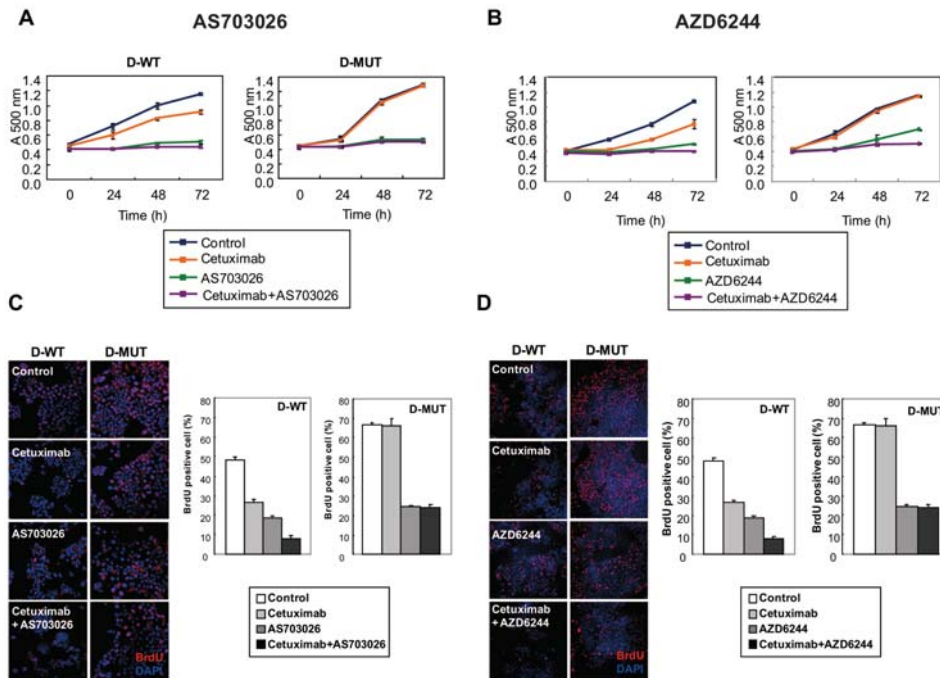
**Figure 1**

**Figure 2**



A

AS703026

AZD6244

B

|  | control | Cetuximab | AS703026 | Cetuximab+AS703026 | control | Cetuximab | AS703026 | Cetuximab+AS703026 |

GTP-Ras — Pull-down GST-Raf1-RBD
GST-RBD

p-ERK — WCL
ERK
p-Akt
α-tubulin

D-WT    D-MUT

C

|  | control | Cetuximab | AZD6244 | Cetuximab+AZD6244 | control | Cetuximab | AZD6244 | Cetuximab+AZD6244 |

GTP-Ras — Pull-down GST-Raf1-RBD
GST-RBD

p-ERK — WCL
ERK
p-Akt
β-actin

D-WT    D-MUT
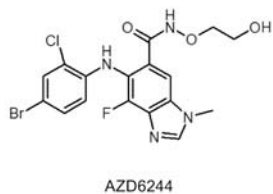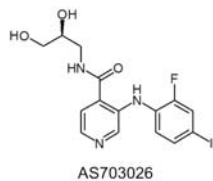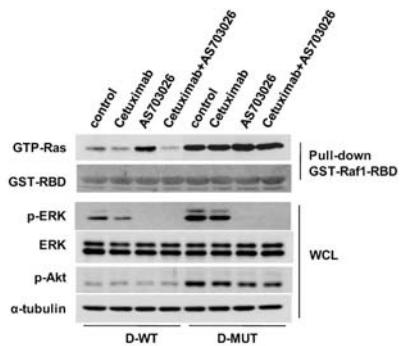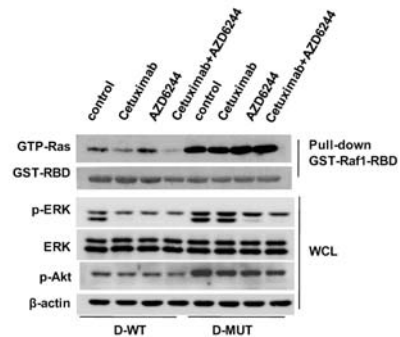
Figure 3

**Figure 4**

**A**

**AS703026**



**B**

**AZD6244**

**Figure 5**



A  AS703026

B  AZD6244

C  D-MUT

D  D-MUT

E  D-MUT

F  D-MUT

Figure 6