이학석사 학위논문

대장암 세포의 유전자 변이와 항암제 반응성

Genetic alterations and sensitivity to anti-cancer drugs in colorectal cancer cells

2015년 2월

서울대학교 융합과학기술대학원

분자의학 및 바이오제약학과

권 연 주

ABSTRACT

Genetic alterations and sensitivity to anti-cancer drugs in colorectal cancer cells

Yeon-Ju Kwon

Department of Molecular Medicine and Biopharmaceutical Sciences

World Class University Graduate School of Convergence Science and Technology

Seoul National University

Colorectal cancer (CRC), the third most common cancer in the world, develops through complicated genetic mutations that occur in a stepwise progression. Therefore, examining cells for genetic alterations has been considered important in furthering research

into the treatment of CRC.

In support of such research, we first investigated the effects of mutation of *KRAS* oncogene, which is the most common genetic alteration in CRC. To determine the effects of *KRAS* substitutions in different CRC cell lines, we initially generated plasmids for 7 *KRAS* mutation subtypes that occur frequently. We then compared downstream signaling and *KRAS* activity in transfected HEK293 cells and CRC cell lines harboring *KRAS* mutations. In HEK293 cells, the G12D and Q61L mutant substitutions increased RAS downstream signaling, and *KRAS* activity was markedly increased in both G12S and G13D. In contrast, among 18 CRC cells, SW480 harboring *KRAS* G12V exhibited the greatest level of signaling activation and *KRAS* activity was noticeably high in HCT15 CRC cells harboring G13D. Therefore, it seems that mutation at codon 12 and 13 activates K-RAS signaling and activity in CRC.

To investigate further the relationship between genetic alterations and drug sensitivity, we initially confirmed the presence of genetic alterations of major 5 genes (*NRAS, KRAS, BRAF*, *PI3KCA* and *PTEN*) in 18 CRC cell lines by using Sanger sequencing. Then, we selected hot-spot mutation of 5 genes based

on Cancer Cell Line Encyclopedia data (CCLE). Drug sensitivities of the 18 cell lines to 6 anti-cancer drugs (5-FU, oxaliplatin, SN-38, AZD6244, regorafenib and PLX4720) were evaluated. Due to non-computable IC_{50} values, the drug sensitivities of the 18 cell lines were arranged by survival at clinically relevant concentrations. Mann Whitney *U* test results indicated a significant association between *PIK3CA* mutation and oxaliplatin sensitivity.

In addition, we confirmed that cell lines with a *BRAF* mutation showed sensitivity to AZD6244 and PLX4720.

In conclusion, the presence of different effects of *KRAS* substitutions on CRC and the presence of different relationships between genetic alterations and drug sensitivity are indicative of variability in CRC cell lines.

Keywords : colorectal cancer (CRC), KRAS, genetic alteration, anti-cancer drug

Student Number : 2012-22835

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Colorectal cancer (CRC) is a disease of the colon or rectum resulting from the abnormal growth of cells [1]. CRC is the third most common type of cancer in the world. It has a poor prognosis and can spread to other parts of the body [2]. The abnormal cell growth in CRC reflects genetic events that occur in stepwise progression. Thus, research into genetic alterations in CRC cells has been considered as being important in the development of appropriate treatment strategies.

The v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) is essential for normal signaling in cell proliferation, survival and maintenance. *KRAS* mutations are found in 30%-40% of CRC with the most common *KRAS* mutations being point mutations of codons 12 and 13 with, the most common amino acid substitution at codons 12 and 13 being an aspartate in place of a glycine residue [3]. Mutant KRAS has a critical role in cancer growth and resistance of cancer to therapy [4]. Due to its high affinity to GTP/GDP and the absence of a known allosteric regulatory site, studies directly targeting the *KRAS* oncogene have

reported little success [5]. Recently, several studies have targeted mutant *KRAS* by investigating differences between KRAS oncogene substitutions [6-8], screening for synthetic lethality [9], or identifying small molecules that inhibit KRAS activity [5].

Currently, the general strategies for CRC therapy involve chemotherapeutic treatments with drugs such as 5-fluorouracil, oxaliplatin and irinotecan.

The antimetabolite drug 5-fluorouracil (5-FU) prevents cell proliferation and is used in the treatment of many cancer types, particularly CRC. By inhibiting thymidylate synthase (TS) and incorporating of its metabolites, 5-FU produces anti-cancer effects [10]. Generally, 5-FU is combined with oxaliplatin or irinotecan to increase drug efficacy in the treatment of CRC. Oxaliplatin in combination with 5-FU and folinic acid is known as FOLFOX, which is used in the treatment of CRC [11]. The cytotoxicity of oxaliplatin results from the inhibition of DNA synthesis [12]. Irinotecan is mainly used in combination with other chemotherapy drugs such as 5-FU and leucovorin in the treatment of CRC [13]. When activated by hydrolysis, irinotecan can be turned into another form, SN-38, an inhibitor of topoisomerase I [14].

In addition to cytotoxic drugs, various molecular targeted drugs target specific genetic alterations in cancer cells. For example, selumetinib or AZD6244 is a *MEK1/2* selective inhibitor and blocks *ERK1/2* activation. Several studies have reported that, AZD6244 resistance is associated with weak *ERK1/2* activation or high activation of the PI3K pathway [15, 16], the combination of AZD6244 and a PI3K pathway inhibitor has been effective in the treatment of AZD6244 resistant cell lines [17]. Regorafenib is an oral multi-kinase inhibitor that shows anti-angiogenic activity as a result of VEGFR2-TIE2 tyrosine kinase inhibition [18] and is reported to have potential therapeutic value in many tumor types [19-21]. As an administered multi-kinase inhibitor, regorafenib has increased overall survival of metastatic CRC patients [22]. PLX4720 is an RAF inhibitor and has been studied in cancers harboring a BRAF V600E mutation and highly sensitive to PLX4720 [23]. For example, it has been shown to be beneficial in the treatment of human melanoma, which is often associated with BRAF V600E mutations.

Recently, due to the development of Next Generation Sequencing (NGS), it has become possible to obtain personalized genetic

information. With the availability of information on an individual's genetic alterations and the development of predictive drug markers, there is potential for the development of personalized treatment strategies. To date, there have been many reports on studies into predictive markers associated with anti-cancer drugs [24, 25]. In contrast to the presence of markers for CRC-targeted drugs, there is an absence of predictive markers associated with chemotherapy drugs.

In this study, we investigated the effects of 7 different KRAS mutants on CRC cell lines. Among the tested subtypes, downstream signaling, KRAS activity, and chemotherapy drug responses were compared. In addition to *KRAS*, we analyzed the relationships between gene mutation status and drug sensitivity for 5 confirmed genes mutations in CRC cell lines. At a variety of clinically relevant concentrations of 6 anti-cancer drugs, 18 CRC cell lines were arranged in order of their survival. By analyzing these results, we detected associations between gene mutations and CRC cell lines. The study results contribute to elucidation of relationships between gene alterations and drug sensitivity in CRC cell lines.

MATERIALS AND METHODS

Cell lines and cell culture

Human colorectal cancer cell lines (Colo-201, -205, LS174T, HCT-15, -116, HEK293, HT29, SNU-C1, -C2A, -C4, -C5, -61, -81, -175, -283, -407, -1033, LOVO, SW480) were obtained from the Korea Cell Line Bank (Seoul, Korea). All cell lines were cultured in DMEM or RPMI1640 (Hyclone Laboratories, Ind., Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS) and gentamicin (10㎍/mL) at 37℃ in a humidified 5% CO_2 atmosphere.

Plasmids and transfection

The pcDNA3 wt *KRAS* and pcDNA3 *KRAS* (G12D) were purchased from ADDGENE. Site-directed mutagenesis was conducted on the pcDNA3 wt *KRAS* plasmid using a QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol to generate the following *KRAS* mutations;

G12V, G12C, G12A, G12S, Q61L and G13D using the oligonucleotide primers described in Table 1. The mutant plasmid construct was confirmed by sequencing, and then transfected into each cell line. Transfection was conducted with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

Table 1. Primer sequences for site-directed mutagenesis

Mutation	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
G12V	TGGTAGTTGGAGCTGTTGGCGTAGGCAAGAG	CTCTTGCCTACGCCAACAGCTCCAACTACCA
G12C	GTGGTAGTTGGAGCTTGTGGCGTAGGCAAGA	TCTTGCCTACGCCACAAGCTCCAACTACCAC
G12A	GGTAGTTGGAGCTGCTGGCGTAGGCAAGA	TCTTGCCTACGCCAGCAGCTCCAACTACC
G12S	GTGGTAGTTGGAGCTAGTGGCGTAGGCAAGA	TCTTGCCTACGCCACTAGCTCCAACTACCAC
Q61L	TCGACACAGCAGGTCTAGAGGAGTACAGTGC	GCACTGTACTCCTCTAGACCTGCTGTGTCGA
G13D	TAGTTGGAGCTGGTGACGTAGGCAAGAGTGC	GCACTCTTGCCTACGTCACCAGCTCCAACTA

Western blot analysis

Cultured cells were washed with ice-cold PBS, and lysed with lysis buffer (50mM Tris-HCL, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 150mM NaCl, 50mM NaF, 1mM sodium pyrophosphate, 1mM EDTA, and protease/phosphatase inhibitors). Equal amounts of proteins were separated using SDS-PAGE. The resolved proteins were then transferred to nitrocellulose membranes. After blocking with 1% BSA and 1% skim milk/ TBST,

membranes were incubated with primary antibodies at 4℃ overnight. Antibodies against *p-CRAF, p-AKT* (pS-473), *p-ERK* (p44/42), *p-p90RSK*, *p-MEK1/2*, *p-JNK*, *p-p38* and *Cyclin D* were purchased from Cell signaling Technology (Beverley, MA, USA). Antibodies against *Actin* and GST came from Santa Cruz Biotechnology (Santa Cruz, CA ,USA). Anti-*c-KRAS* antibody was from Calbiochem. Quantitation of western band intensity was performed with Image J.

KRAS in vitro pull-down assay

Using GST-Ras binding domains (RBD) of Raf-1, measure the active (GTP-bound) *KRAS* protein that interaction with these effector in cell lysates. Plasmids containing pGEX-Raf-1 (ADDGENE) were transfected into Escherichia coli and induced their expression with IPTG (1M), incubating for 3h at 37℃ and pelleting at 3000 rpm for 15 min at 4℃. Cell pellets were lysed by sonication on ice for 5 x 15s and separated by 15s intervals. Cell debris was pelleted at 15,000 rpm for 30 min at 4℃. Supernatants containing the GST fusion proteins were incubated with glutathione agarose beads (GE Healthcare Life Science) overnight. After

protein absorption, the GSH sepharose was washed with 1ml of cold PBS. The purified GST-Raf-1 protein, immobilized on glutathione agarose beads was incubated with cell lysates supernatants at 4℃ for 2h with gentle mixing. The beads were washed twice with 1ml of RIPA buffer, twice with 1ml of PBS and centrifuged (6000 rpm, 2 min at 4℃). After boiling at 100℃ for 7 min, the beads were eliminated by centrifuging at 3000 rpm for 5 min.

Reagent

5-FU was purchased from JW pharmaceutical Co. (Seoul, Korea). Oxaliplatin was purchased from Sanofi-Aventis (U.S.). SN-38, AZD6244 and PLX4720 were purchased from Selleck. Regorafenib was purchased from Bayer. Stock solutions were prepared in saline solution or dimethyl sulfoxide (DMSO) and diluted in fresh media before each experiment.

Mutational analysis of *NRAS, KRAS, BRAF, PIK3CA* and *PTEN* gene

Total genomic DNA (gDNA) was extracted from the each cell line using QIAamp DNA mini kit (Qiagen) following the manufacturer's instructions and was dissolved in H_2O. Primers were synthesized by Macrogen Inc. (Seoul, Korea). The sequences of all primers used for PCR are listed in Table 2. All sequencing reactions were conducted in both the forward and reverse directions. Sequencing was carried out by Macrogen Inc. (Seoul, Korea). All mutations were confirmed at least twice from independent PCR isolates.

Table 2. Primer sequences

No.	Gene	Exon	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
1	NRAS	exon 3	AGCATTGCATTCCCTGTGGT	GTGTGGTAACCTCATTTCCCCA
2	KRAS	exon 2	TAGGCAAGAGTGCCTTGACG	CCCTCCCCAGTCCTCATGTA
3	BRAF	exon 15	GCATCTCACCTCATCCTAAC	GTAACTCAGCAGCATCTCAG
4	PIK3CA	exon 9	GCTATCGGCATGCCAGTGT	CAGTATAAGCAGTCCCTGCC
		exon 20	GGCCTGCTTTTGGAGTCCTAT	CTTCTAAACAACTCTGCCCCAC
5	PTEN	exon 5	TGGCTACGACCCAGTTACCA	GGAAGGATGAGAATTTCAAGCACT
		exon 7	CTCAGCCGTTACCTGTGTGT	TCACCAATGCCAGAGTAAGCA

Cell growth inhibition assay

Cells were seeded in 96-well plates and incubated overnight at 37℃ in 5% CO_2 atmosphere. 5-FU (Fluorouracil), SN-38, oxaliplatin, selumetinib (AZD6244), regorafenib and PLX4720 were treated and incubated for 3 days. After drug treatment, 3-(4,5-dimethylthiazol-2yl)-2,5- diphenyltetrazolium bromide (sigma-Aldrich, St. Louis, MO) solution was added to each well and incubated for 4h at 37℃ before media removal. 150 μl of DMSO was added and the absorbance of each well was measured at 540 nm with micro-plate reader (Molecular Devices; Sunnyvale, CA, USA). Graphs were generated by nonlinear regression of data points to a four-parameter logistic curve, using Sigma Plot software. Six replicate wells were utilized for each analysis; at least three independent experiments were conducted.

Statistical analysis

The statistical significance of the results was calculated using a Mann Whitney U test and p-value of <0.05 was considered to be statistically significant.

RESULT

Different effects of mutant forms of *KRAS* on downstream signaling and *KRAS* activity in HEK293 and *KRAS* mutant CRC cell lines

Initially, we investigated the different effects of a variety of *KRAS* mutant types on downstream signaling. 7 *KRAS* mutant substitutions (G12D, G12V, G12C, G12S, G12A, G13D, and Q61L) were separately transfected into human embryonic kidney (HEK293) cells. Following transfection, several downstream molecules related to cell proliferation and cell survival were analyzed in each mutant cell type by western blotting. Downstream molecules in the *KRAS* mutant-types were activated compared to *KRAS* wild-type (Fig. 1A). Transfection efficiencies of each of *KRAS* subtypes varied; therefore, to obtain a standardized comparison, each of the downstream signaling band intensities were quantitated and normalized by the expression in each *KRAS* subtype (Fig. 1B). The results showed that the *KRAS* G12D and Q61L mutants exhibited marked increases in the phosphorylation levels of *ERK* and *AKT*.

Typically, however, cancer cells are observed to have more than two genetic alterations. Therefore, we compared downstream signaling between HEK293 cells and *KRAS* mutant CRC cell lines.

To investigate the effects of downstream signaling in CRC cell lines, we selected 8 *KRAS* mutant CRC cell lines. Among the 8 CRC cell lines, 2 cell lines (LS174T and SNU-407) harbored the *KRAS* G12D mutation, 5 cell lines (HCT-15, -116, LOVO, SNU-283, and SNU-1033) had the *KRAS* G13D mutation, and one cell line (SW480) had the *KRAS* G12V mutation (Fig. 2A). Unlike the results obtained from the *KRAS* mutant transfected HEK293 cells, only SW480 expressing KRAS G12V showed high phosphorylation of *p-ERK*, *p-AKT* and *p-MEK1/2* (Fig. 2B).

In addition, we investigated the level of *KRAS* activity in the HEK293 and CRC cell lines by performing RAS pull-down assay (Fig. 3). *KRAS* activation, as indicated by the ratio of *KRAS* bound to Raf-RBD/total *KRAS*, was highest in HEK293 cells transfected with *KRAS* G12S and G13D (Fig. 3A). Among the 8 *KRAS* mutation bearing CRC cell lines, *KRAS* activity was highest in HCT15 cell harboring the *KRAS* G13D mutation (Fig. 3B). While the HEK293 cells contained no genetic alteration other than the transfected

KRAS mutant, the tested CRC cell lines harbored various genetic alterations. Through the results obtained from the transfected HEK293 cells, we detected the different effects associated with individual *KRAS* mutations only.

Figure 1. Activation and expression of molecules downstream of *KRAS* in HEK293 harboring 7 *KRAS* mutant substitutions. (A) Each of 7 *KRAS* mutant subtype was transfected in HEK293. And *KRAS* mutant subtypes showed very high levels of active downstream molecules compared to *KRAS* wild type. **(B)** The band intensities were quantitated and normalized by each *KRAS* expression. *KRAS* G12D and Q61L showed enhanced phosphorylation of *ERK* and *AKT* as compared with the others. Quantitation of band intensity was performed with Image J.

Fig. 1A

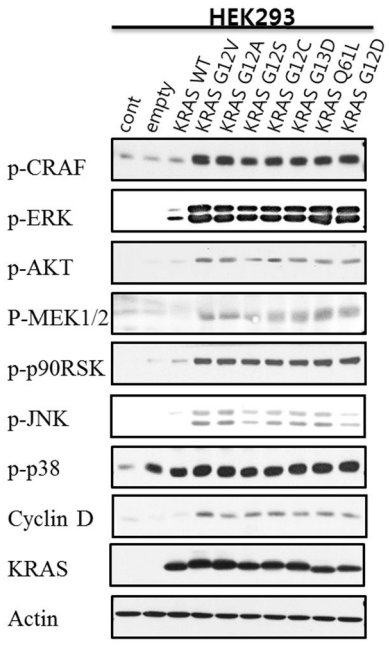

HEK293
cont
empty
KRAS WT
KRAS G12V
KRAS G12A
KRAS G12S
KRAS G12C
KRAS G13D
KRAS Q61L
KRAS G12D
p-CRAF
p-ERK
p-AKT
P-MEK1/2
p-p90RSK
p-JNK
p-p38
Cyclin D
KRAS
Actin

Fig. 1B

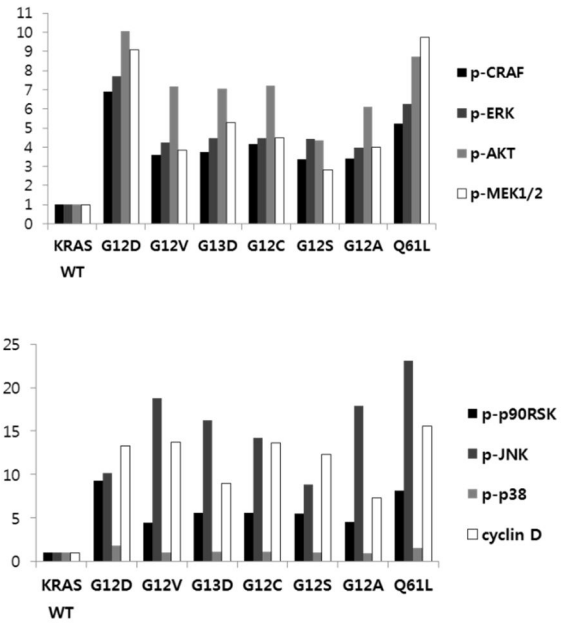

11
10
9
8
7
6
5
4
3
2
1
0
KRAS WT
G12D
G12V
G13D
G12C
G12S
G12A
Q61L
p-CRAF
p-ERK
p-AKT
p-MEK1/2
25
20
15
10
5
0
KRAS WT
G12D
G12V
G13D
G12C
G12S
G12A
Q61L
p-p90RSK
p-JNK
p-p38
cyclin D

Figure 2. Activation and expression of molecules downstream of *KRAS* in 8 colorectal cancer cell lines harboring KRAS mutation. (A) Among 8 colorectal cancer cell lines, SW480 harboring *KRAS* G12V mutation showed high levels of active *ERK*, *AKT* and *MEK1/2*. **(B)** The band intensities were quantitated and normalized by each Actin expression. SW480 showed enhanced phosphorylation of *ERK, AKT* and *MEK1/2* as compared with the others. Quantitation of band intensity was performed with Image J.

Fig. 2A

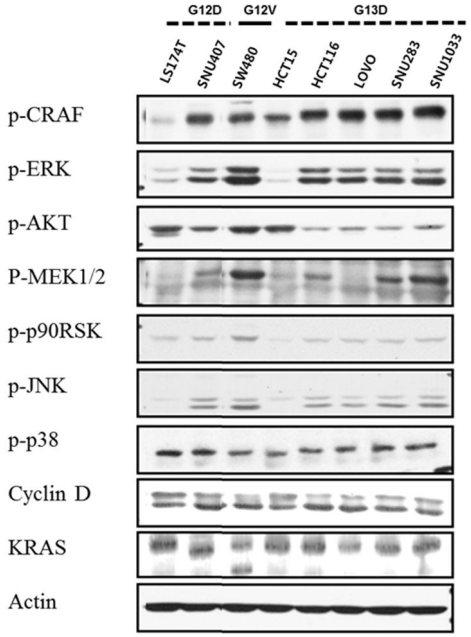

G12D
G12V
G13D
LS174T
SNU407
SW480
HCT15
HCT116
LOVO
SNU283
SNU1033
p-CRAF
p-ERK
p-AKT
P-MEK1/2
p-p90RSK
p-JNK
p-p38
Cyclin D
KRAS
Actin

Fig. 2B

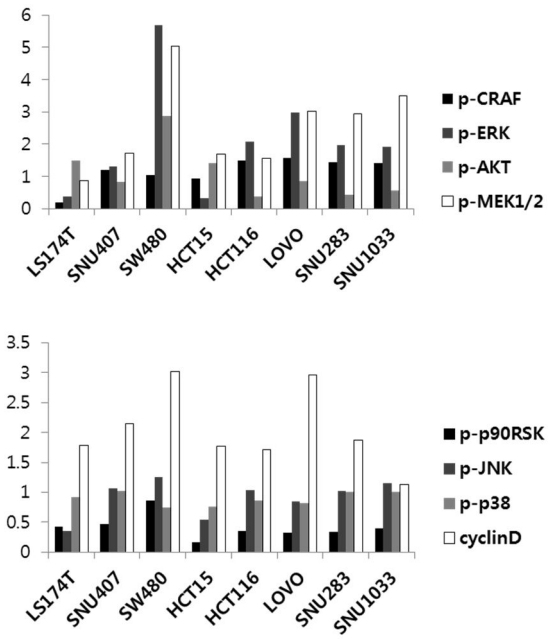

6
5
4
3
2
1
0
p-CRAF
p-ERK
p-AKT
p-MEK1/2
LS174T
SNU407
SW480
HCT15
HCT116
LOVO
SNU283
SNU1033
3.5
3
2.5
2
1.5
1
0.5
0
p-p90RSK
p-JNK
p-p38
cyclinD
LS174T
SNU407
SW480
HCT15
HCT116
LOVO
SNU283
SNU1033

Figure 3. Different activities in HEK293 transfected with mutant *KRAS* and 8 colorectal cancer cell lines harboring mutant *KRAS*. Pull-down experiments of GTP-bound *KRAS* proteins were performed to determine the level of active *KRAS*. **(A)** In HEK293 transiently harboring each *KRAS* mutation, *KRAS* G12S and G13D showed higher activation of *KRAS* than the others. **(B)** In 8 *KRAS* mutant colorectal cancer cell lines *KRAS* activity was high in HCT 15 harboring *KRAS* G13D. Below the western blot result, each graph indicated the band intensities quantitated and normalized by *KRAS* expression. Quantitation of band intensity was performed with Image J.

Fig. 3A

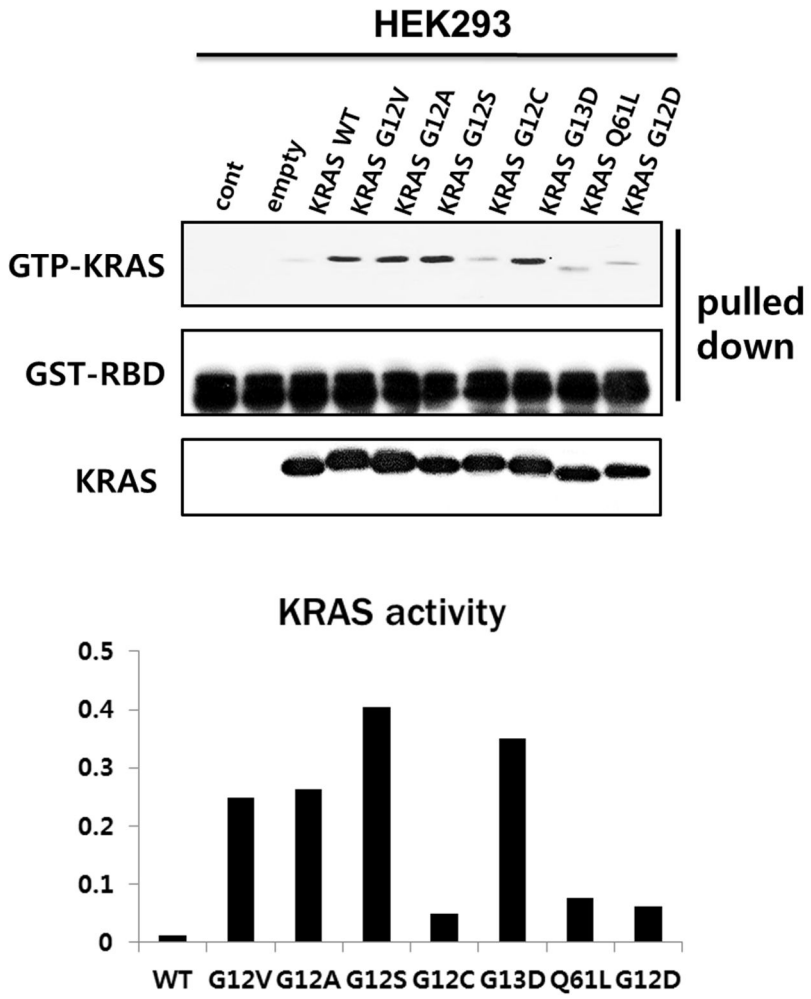
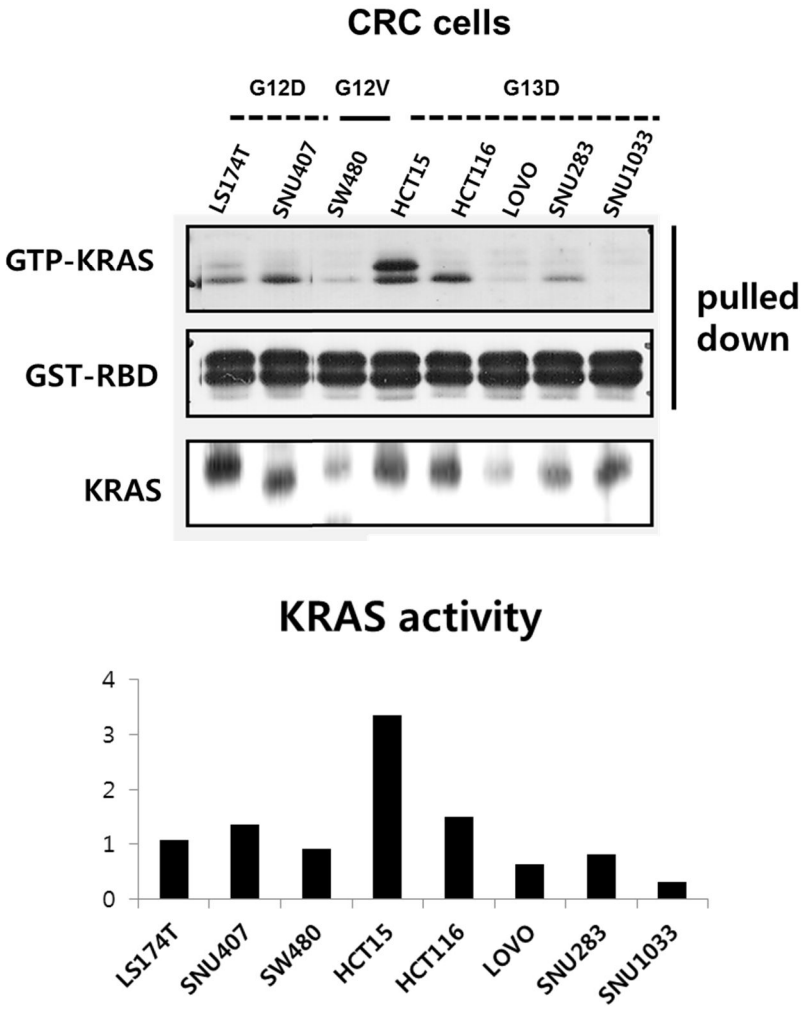

HEK293
cont
empty
KRAS WT
KRAS G12V
KRAS G12A
KRAS G12S
KRAS G12C
KRAS G13D
KRAS Q61L
KRAS G12D
GTP-KRAS
GST-RBD
KRAS
pulled down
KRAS activity
0.5
0.4
0.3
0.2
0.1
0
WT
G12V
G12A
G12S
G12C
G13D
Q61L
G12D

Fig. 3B


CRC cells
G12D
G12V
G13D
LS174T
SNU407
SW480
HCT15
HCT116
LOVO
SNU283
SNU1033
GTP-KRAS
GST-RBD
KRAS
pulled down
KRAS activity
4
3
2
1
0
LS174T
SNU407
SW480
HCT15
HCT116
LOVO
SNU283
SNU1033

Genetic alterations of 5 genes in 18 colorectal cancer cell lines

To confirm the presence of *NRAS, KRAS, BRAF, PIK3CA*, and *PTEN* genetic alterations in our 18 CRC cell lines, we conducted Sanger sequencing of the genomic DNA of each cell line (Table 3 and Fig. 4). The *NRAS* mutation was detected only in LOVO (Fig. 4A), whereas the *KRAS* exon 2 (G12D, G12V, and G13D) mutations were detected in 11 CRC cell lines (HCT-15, -116, LOVO, LS174T, SNU-C2A, -61, -175, -283, -407, -1033, and SW480) (Fig. 4B). Among the 18 CRC cell lines, 4 cell lines (Colo-201, -205, HT29, and SNU-C5) had the *BRAF* V600E mutation (Fig. 4C), 9 of the CRC cell lines (LOVO, LS174T, HCT116, SNU-C5, -61, -175, -283, -407, and SNU-1033) had the *PIK3CA* H1047R mutation, and only the HCT15 cell line had the *PIK3CA* E545K mutation (Fig. 4D). The *PTEN* mutation was detected in SNU-81 and SNU-C4 (Fig. 4E). These results showed that most CRC cell lines harbored *KRAS* and/or *PIK3CA* hot spot mutations. In addition, the results showed that 11 cell lines had RAS and PI3K pathway co-mutations, whereas 6 cell lines (Colo-201, -205, HT29, SNU-C1, -C2A, -C4, and SW480) had single mutations. The distribution

of genetic alterations in these CRC cell lines indicated a tendency toward multiple mutations in CRC cell lines.

Table 3. NRAS, KRAS, BRAF, PIK3CA and PTEN genetic alterations of 18 colorectal cancer cell lines.

Cell line	RAS			PI3K	
	NRAS	KRAS	BRAF	PIK3CA	PTEN
Colo201			V600E		
Colo205			V600E		
HCT116		G13D		H1047R	
HCT15		G13D		E545K	
HT29			V600E		
LOVO	Q61R	G13D		H1047R	
LS174T		G12D		H1047R	
SNU 1033		G13D		H1047R	
SNU 175		G13D		H1047R	
SNU 283		G13D		H1047R	
SNU 407		G13D		H1047R	
SNU 61		G13D		H1047R	
SNU 81					R130Q/ R233Q
SNU C1					
SNU C2A		G12D			
SNU C4					F241S
SNU C5			V600E	H1047R	
SW480		G12V			

Figure 4. Validation of *NRAS, KRAS, BRAF, PIK3CA* and *PTEN* genes in genomic DNA of 18 colorectal cancer cell lines. **(A)** *NRAS* exon 3 was validated by PCR amplification and Sanger sequencing. **(B)** *KRAS* exon 2 was validated by PCR amplification and Sanger sequencing. **(C)** *BRAF* exon 15 was validated by PCR amplification and Sanger sequencing. **(D)** *PIK3CA* exon 9 and exon 20 were validated by PCR amplification and Sanger sequencing. **(E)** *PTEN* exon 5 and exon 7 were validated by PCR amplification and Sanger sequencing.

Fig. 4A

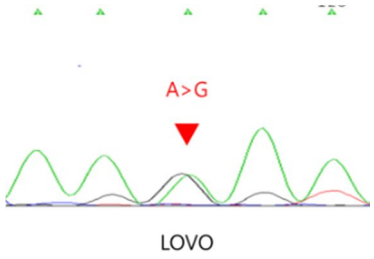
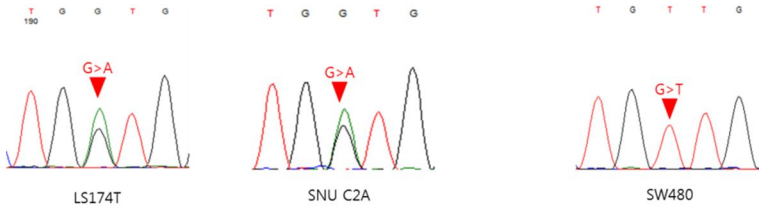
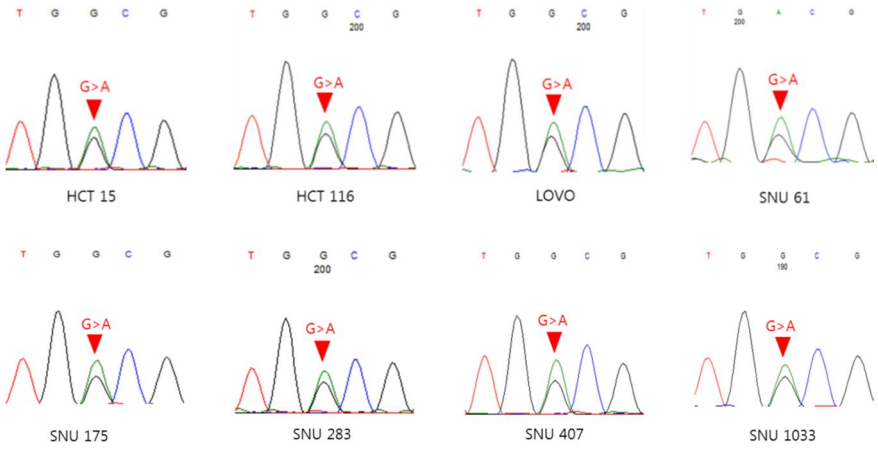


Fig. 4B



KRAS G12D KRAS G12V



KRAS G13D

Fig. 4C

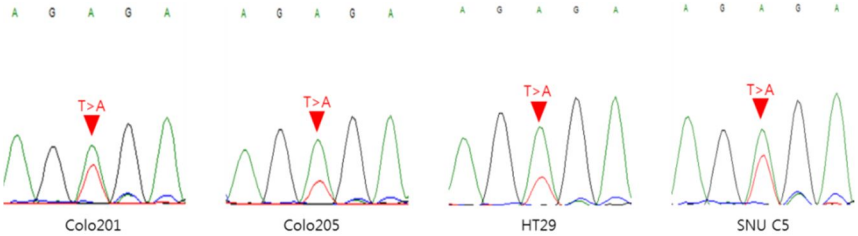
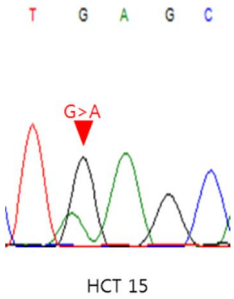
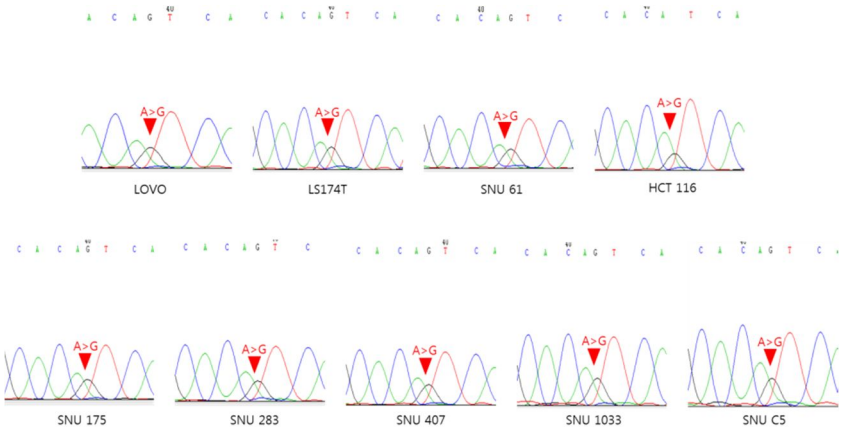


Fig. 4D

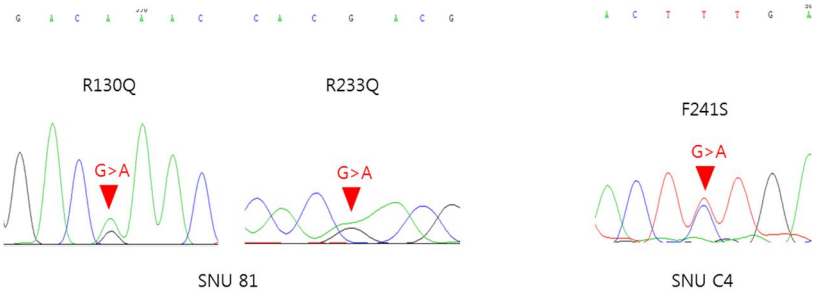


PIK3CA E545K



PIK3CA H1047R

Fig. 4E



Differential sensitivity to anti-cancer drugs in 18 colorectal cancer cell lines

To determine the effects of anti-cancer drugs on the proliferation of human CRC cell lines, we treated each CRC cell line with 3 chemotherapy drugs [5-FU (0.01-10 μM), oxaliplatin (0.001-10 μM), or SN-38 (0.001-1 μM)] and 3 targeted drugs [AZD6244 (0.01-10 μM), regorafenib (0.01-10 μM), or PLX4720 (0.01-10 μM)] for 72h. After treatment, cell viabilities were estimated by using the MTT assay (Fig. 5). To determine the effects on survival of the tested drug concentrations in each cell line, IC_{50} values for each drug were calculated (Table 4). However, some drugs' IC_{50} values could not be computed. Therefore, to assess the relative effectiveness of each drug, we examined results obtained from clinically relevant concentrations of each drug.

Figure 5. The growth inhibitory effects of 6 anti-cancer drugs in 18 colorectal cancer cell lines. Cells were incubated with 5-FU **(A)**, oxaliplatin **(B)**, SN-38 **(C)**, AZD6244 **(D)**, regorafenib **(E)**, PLX4720 **(F)** for 72 hours at the indicated concentrations. Growth inhibition was analyzed by the MTT assay. The results were presented as percentages of the vehicle control.

Fig. 5A

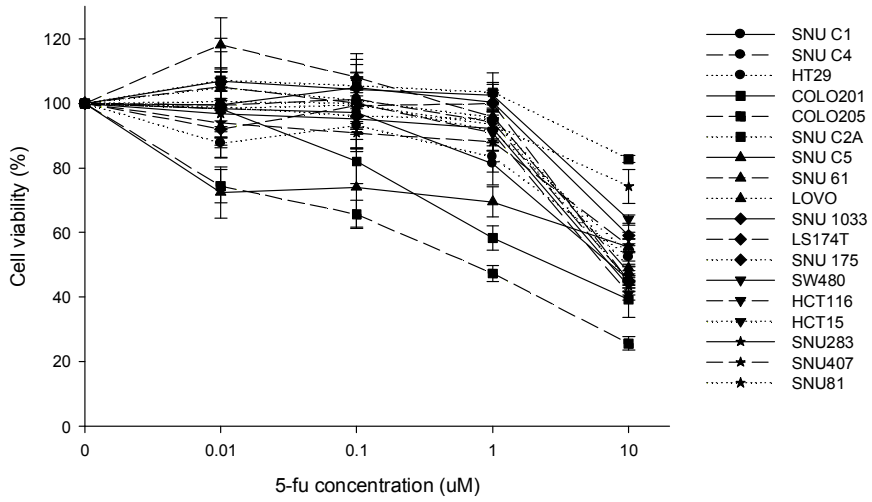

Cell viability (%)
120
100
80
60
40
20
0
0
0.01
0.1
1
10
5-fu concentration (uM)
SNU C1
SNU C4
HT29
COLO201
COLO205
SNU C2A
SNU C5
SNU 61
LOVO
SNU 1033
LS174T
SNU 175
SW480
HCT116
HCT15
SNU283
SNU407
SNU81

Fig. 5B

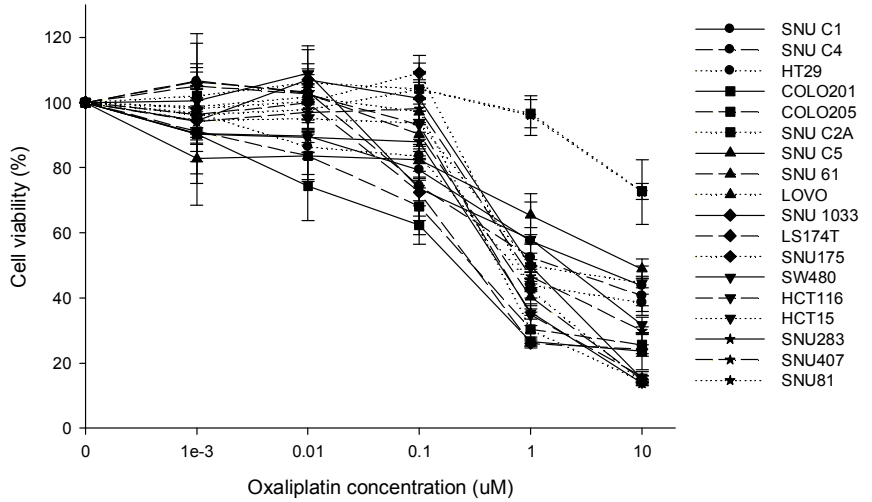

Cell viability (%)
120
100
80
60
40
20
0
0
1e-3
0.01
0.1
1
10
Oxaliplatin concentration (uM)
SNU C1
SNU C4
HT29
COLO201
COLO205
SNU C2A
SNU C5
SNU 61
LOVO
SNU 1033
LS174T
SNU175
SW480
HCT116
HCT15
SNU283
SNU407
SNU81

Fig. 5C

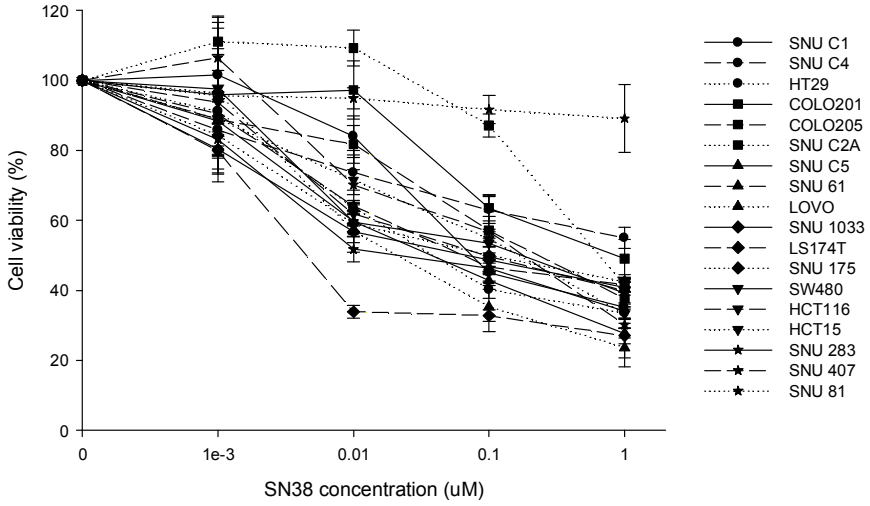

Cell viability (%)
120
100
80
60
40
20
0
0
1e-3
0.01
0.1
1
SN38 concentration (uM)
SNU C1
SNU C4
HT29
COLO201
COLO205
SNU C2A
SNU C5
SNU 61
LOVO
SNU 1033
LS174T
SNU 175
SW480
HCT116
HCT15
SNU 283
SNU 407
SNU 81

Fig. 5D

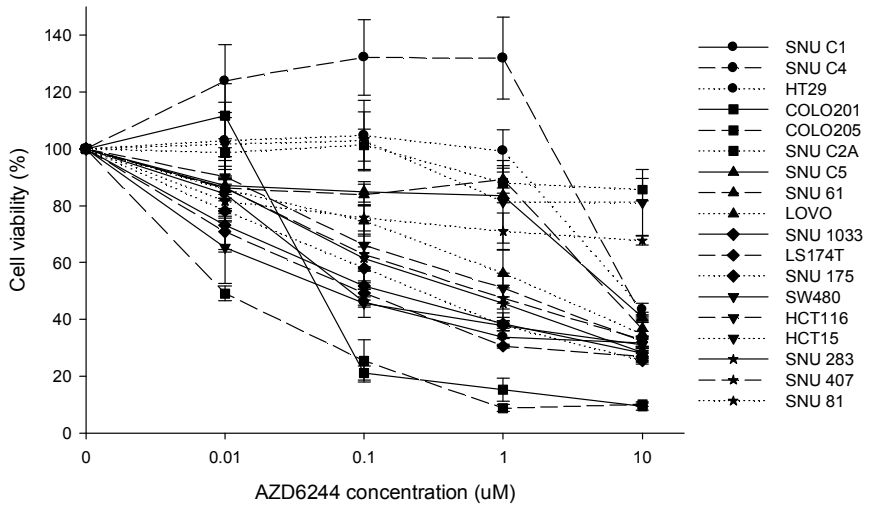

Cell viability (%)
140
120
100
80
60
40
20
0
0
0.01
0.1
1
10
AZD6244 concentration (uM)
SNU C1
SNU C4
HT29
COLO201
COLO205
SNU C2A
SNU C5
SNU 61
LOVO
SNU 1033
LS174T
SNU 175
SW480
HCT116
HCT15
SNU 283
SNU 407
SNU 81

Fig. 5E

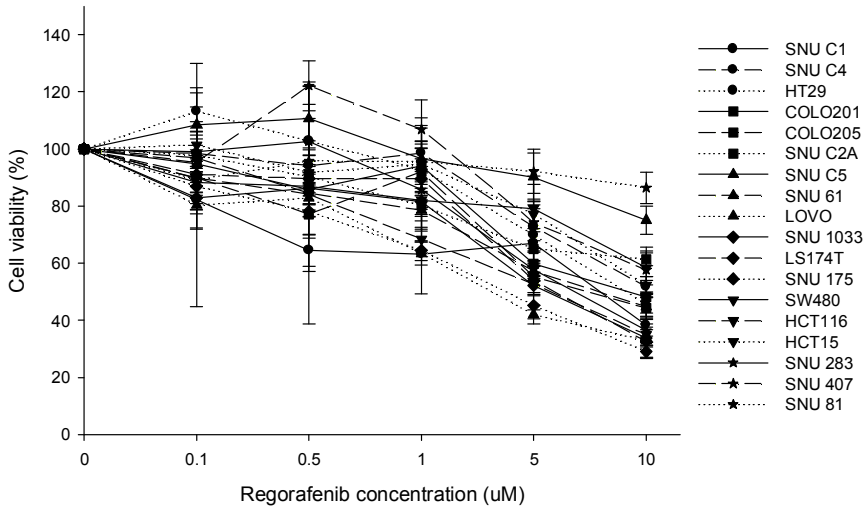

Cell viability (%)
140
120
100
80
60
40
20
0
0
0.1
0.5
1
5
10
Regorafenib concentration (uM)
SNU C1
SNU C4
HT29
COLO201
COLO205
SNU C2A
SNU C5
SNU 61
LOVO
SNU 1033
LS174T
SNU 175
SW480
HCT116
HCT15
SNU 283
SNU 407
SNU 81

Fig. 5F

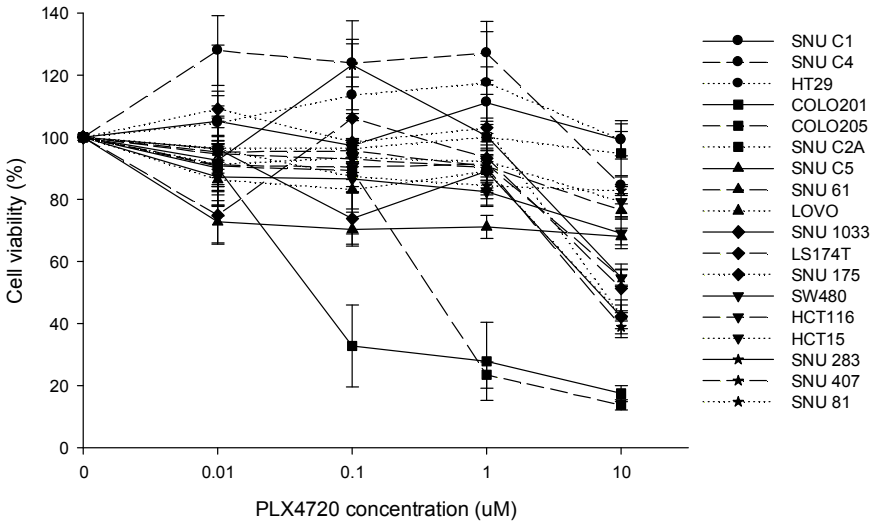

Cell viability (%)
140
120
100
80
60
40
20
0
0
0.01
0.1
1
10
PLX4720 concentration (uM)
SNU C1
SNU C4
HT29
COLO201
COLO205
SNU C2A
SNU C5
SNU 61
LOVO
SNU 1033
LS174T
SNU 175
SW480
HCT116
HCT15
SNU 283
SNU 407
SNU 81

Table 4. IC_{50} values of anti-cancer drugs in 18 colorectal cancer cell lines.

Cell lines	IC50 (μmol/L)					
	5-fu	Oxaliplatin	SN38	AZD6244	Regorafenib	PLX4720
Colo201	1.38	0.16	0.3165	0.091	>10	0.06
Colo205	0.2	0.02	0.1345	0.087	6.25	0.45
HCT116	>10	0.25	0.0039	0.02	5.39	>10
HCT15	7.21	0.89	0.007	>10	>10	>10
HT29	>10	0.66	0.0183	0.083	7.2	>10
LOVO	8.1	0.56	0.0054	1.27	4.52	>10
LS174T	>10	0.18	0.0044	0.03	9.33	>10
SNU 61	8.89	0.73	0.0217	0.911	>10	>10
SNU 81	>10	>10	>1	>10	8.41	>10
SNU C1	5.76	1.44	0.0688	>10	>10	>10
SNU C2A	>10	>10	0.5445	>10	>10	>10
SNU C4	9.02	0.4	>1	>10	>10	>10
SNU C5	>10	2.52	0.0149	>10	3.55	>10
SNU1033	4.39	0.34	0.0044	0.1	2.87	>10
SNU175	3.99	0.5	0.0044	0.13	4.38	>10
SNU283	5.39	0.28	0.0044	0.17	7.09	>10
SNU407	8.54	0.42	0.007	0.18	8.49	>10
SW480	>10	0.24	0.0039	0.02	>10	>10

Oxaliplatin had a valid correlation between gene mutation and drug sensitivity

The clinically relevant concentration of each drug used in our study was based on previously reported clinical concentration studies. The clinically effective concentration of 5-FU was 5 μM [26], whereas the clinically effective doses of oxaliplatin and SN-38 were 3 μM [27] and 20nM, [28] respectively. For each respective dose for each drug, the results from the 18 cell lines were arranged in ascending survival rate (*vs.* vehicle) order from drug sensitive to drug resistant (Fig. 6). Mann Whitney *U* test was used to assess viability differences between drug treated wild-type and mutant-type cell lines. Treatment with 5 μM of 5-FU produced no significant difference ($p > 0.05$) in drug sensitivity between wild-type and mutant-type for the 5 gene mutations (Fig. 6A). Sensitivity to 3 μM of oxaliplatin sensitivity did show significant differences ($p < 0.05$) between wild-type and mutant-type for the *KRAS, PIK3CA* and RAS pathway mutants (Fig. 6B). In comparison to the *PIK3CA* mutation results, the average survival rates in *KRAS* wild-type and RAS pathway mutation cells showed more modest oxaliplatin effects indications that the *PIK3CA* mutation could

increase sensitivity to oxaliplatin. Although there were significant differences ($p < 0.05$) between wild-type and *KRAS, PIK3CA* and PI3K pathway mutations, the mean survival rate changes only indicated a modest effect of treatment with 20nM of SN-38 (Fig. 6C), suggesting that there was no useful relationship between cell line genetic alterations and SN-38 treatment.

Figure 6. Correlation diagrams between 5 gene alterations and 3 anti-cancer drugs sensitivity. Cell lines were incubated with each drug for 72 hours at clinically revelant concentration. **(A)** The effective dose of 5-fu was 5μM. **(B)** The effective dose of oxaliplatin was 3μM. **(C)** The effective dose of SN-38 was 20nM. The results were presented as survival points of vehicle control.

Fig. 6A

Cell line	SNU 283	SNU 61	Colo205	LOVO	HCT116	SNU 1033	Colo201	LS174T	HCT15	SNU C4	SNU C1	SNU 407	SNU C5	SNU 175	HT29	SNU C2A	SW480	SNU 81
5-FU(5uM)	0.280238	0.370519	0.381696	0.389859	0.392608	0.42646	0.4291242	0.442176	0.465669	0.478014	0.48166	0.5039	0.525854	0.532829	0.627591	0.638831	0.799367	1
NRAS				Q61R														
KRAS	G13D	G13D		G13D	G13D	G13D		G12D	G13D			G13D		G13D		G12D	G12V	
BRAF			V600E				V600E						V600E		V600E			
PIK3CA	H1047R	H1047R		H1047R	H1047R	H1047R		H1047R	E545K			H1047R	H1047R	H1047R				
PTEN										F241S								R130Q/R233Q
RTK-RAS																		
PI3K																		

sensitive → resistant

	Mann-Whitney	Mean	
	p-value	MT	WT
KRAS	0.342	0.47659	0.57162
BRAF	1	0.49107	0.51997
PIK3CA	0.062	0.45070	0.61230
PTEN	0.206	0.77772	0.48052
RTK-RAS	0.214	0.480448	0.679033
PI3K	0.261	0.501011	0.546136

Fig. 6B

Cell line	SNU 283	HCT116	SNU 1033	LOVO	SNU 61	LS174T	SNU 175	SNU 407	Colo201	HT29	Colo205	HCT15	SNU C4	SNU C5	SW480	SNU C1	SNU C2A	SNU 81
Oxali(3uM)	0.116601	0.175767	0.17744	0.186258	0.186293	0.201109	0.2219559	0.254623	0.305465	0.316343	0.359283	0.397188	0.404406	0.448572	0.598534	0.685018	0.73682	0.935988
NRAS				Q61R														
KRAS	G13D	G13D	G13D	G13D	G13D	G12D	G13D	G13D				G13D			G12V		G12D	
BRAF									V600E	V600E	V600E			V600E				
PIK3CA	H1047R	H1047R	H1047R	H1047R	H1047R	H1047R	H1047R	H1047R				E545K		H1047R				
PTEN													F241S					R130Q/R233Q
RTK-RAS																		
PI3K																		

sensitive → resistant

	Mann-Whitney	Mean	
	p-value	MT	WT
KRAS	0.026	0.29569	0.49358
BRAF	0.524	0.35742	0.37700
PIK3CA	0.003	0.24383	0.57507
PTEN	0.092	0.67020	0.33545
RTK-RAS	0.028	0.31215	0.675138
PI3K	0.053	0.309426	0.537024

Fig. 6C

Cell line	LS174T	SNU 1033	LOVO	SNU 283	HCT116	SNU 61	HCT15	SNU 175	SNU C5	SNU 407	Colo205	SNU C1	SNU C4	Colo201	HT29	SNU C2A	SW480	SNU 81
SN38(20nM)	0.221661	0.341453	0.344449	0.344715	0.377306	0.395139	0.46778	0.470034	0.504372	0.544125	0.544869	0.593525	0.601423	0.614511	0.681004	0.796584	0.802485	0.816653
NRAS			Q61R															
KRAS	G12D	G13D	G13D	G13D	G13D	G13D	G13D	G13D		G13D						G12D	G12V	
BRAF									V600E		V600E			V600E	V600E			
PIK3CA	H1047R	H1047R	H1047R	H1047R	H1047R	H1047R	E545K	H1047R	H1047R	H1047R								
PTEN													F241S					R130Q/R233Q
RTK-RAS																		
PI3K																		

sensitive → resistant

	Mann-Whitney	Mean	
	p-value	MT	WT
KRAS	0.021	0.464157	0.622337
BRAF	0.243	0.586189	0.508381
PIK3CA	0.001	0.426549	0.681436
PTEN	0.092	0.709038	0.502751
RTK-RAS	0.086	0.496699	0.670534
PI3K	0.009	0.470009	0.670395

A correlation between targeted drug sensitivity and genetic alterations could be observed but not valid

We conducted a similar assessment to determine the drug sensitivity of CRC cell lines associated with 3 targeted drugs (Fig. 7). The cell lines were treated with clinically relevant concentrations of AZD6244, regorafenib and PLX4720 (1 μM [29], 5 μM [20], and 1 μM [30], respectively). None of the three drugs produced a significant change in cell viability between wild-type and mutant-type CRC cell lines; however, each drug was associated with tendency toward drug sensitivity. It has been reported that if a cancer has *BRAF* mutation instead of a *PIK3CA* mutation then, drug sensitivity to AZD6244 is increased [17]. In our results, the Colo-201 and Colo-205 cell lines with the *BRAF* V600E mutation but no *PIK3CA* or *KRAS* mutation were the cell lines most sensitive to AZD6244 (Fig. 7A). Similarly, Colo-201 and Colo-205 cells showed higher sensitivity to PLX4720 than that in the other cell lines (Fig. 7C), indicating that Colo-201 and Colo-205 were sensitive to both AZD6244 and PLX4720. In contrast, there was no marked regorafenib sensitivity detected among the cell lines (Fig. 7B).

Figure 7. Correlation diagrams between 5 gene alterations and 3 targeted drugs sensitivity. Cell lines were incubated with each drug for 72 hours at clinically revalent concentration. **(A)** The effective dose of AZD6244 was 1 μM. **(B)** The effective dose of regorafenib was 5 μM. **(C)** The effective dose of PLX4720 was 1 μM. The results were presented as survival points of vehicle control.

Fig. 7A

Cell line	Colo205	Colo201	LS174T	SNU C1	SW480	SNU 175	SNU 1033	SNU 283	SNU 407	HCT116	LOVO	SNU C2A	SNU C5	SNU 81	HCT15	SNU 61	HT29	SNU C4
AZD (1uM)	0.09	0.15	0.31	0.33	0.38	0.38	0.38	0.46	0.47	0.51	0.56	0.71	0.81	0.84	0.88	0.89	0.99	1
NRAS											Q61R							
KRAS			G12D		G12V	G13D	G12D	G13D	G13D	G13D	G13D	G12D			G13D	G13D		
BRAF	V600E	V600E											V600E				V600E	
PIK3CA			H1047R			H1047R	H1047R	H1047R	H1047R	H1047R	H1047R		H1047R		E545K	H1047R		
PTEN														R130Q/R233Q				F241S
RTK-RAS																		
PI3K																		

sensitive → resistant

	Mann-Whitney	Mean	
	p-value	MT	WT
KRAS	0.821	0.4793	0.443
BRAF	0.595	0.5358	0.445
PIK3CA	0.824	0.4601	0.4715
PTEN	0.068	0.146	0.5051
RTK-RAS	0.374	0.49438	0.319374
PI3K	0.146	0.377013	0.694533

Fig. 7B

Cell line	LS174T	HT29	HCT116	SNU 175	LOVO	Colo205	SNU 1033	SNU 61	Colo201	SNU C1	SNU 283	SNU C4	SNU 407	SNU C2A	HCT15	SNU C5	SW480	SNU 81
Regora (SuM)	0.32	0.47	0.49	0.49	0.55	0.55	0.56	0.57	0.6	0.67	0.71	0.73	0.74	0.77	0.77	0.9	0.91	0.92
NRAS					Q61R													
KRAS	G12D		G13D	G13D	G13D		G12D	G13D			G13D		G13D	G12D	G13D		G12V	
BRAF		V600E				V600E			V600E							V600E		
PIK3CA	H1047R		H1047R	H1047R	H1047R		H1047R	H1047R			H1047R		H1047R		E545K	H1047R		
PTEN												F241S						R130Q/R233Q
RTK-RAS																		
PI3K																		

sensitive → resistant

	Mann-Whitney	Mean	
	p-value	MT	WT
KRAS	0.556	0.3736	0.3078
BRAF	0.595	0.3684	0.3422
PIK3CA	0.286	0.3886	0.2973
PTEN	0.122	0.1756	0.3696
RTK-RAS	0.173	0.37223	0.226915
PI3K	0.925	0.366485	0.299978

Fig. 7C

Cell line	Colo205	Colo201	SNU C5	SW480	SNU 81	SNU 1033	LOVO	SNU 407	SNU 61	HCT116	HCT15	LS174T	SNU C2A	SNU 283	SNU 175	SNU C1	HT29	SNU C4
PLX(1uM)	0.23	0.28	0.71	0.82	0.84	0.89	0.89	0.9	0.91	0.91	0.92	0.93	1	1	1	1	1	1
NRAS							Q61R											
KRAS				G12V		G12D	G13D	G13D	G13D	G13D	G13D	G12D	G12D	G13D	G13D			
BRAF	V600E	V600E	V600E														V600E	
PIK3CA			H1047R			H1047R	H1047R	H1047R	H1047R	H1047R	E545K	H1047R		H1047R	H1047R			
PTEN					R130Q/R233Q													F241S
RTK-RAS																		
PI3K																		

sensitive → resistant

	Mann-Whitney	Mean	
	p-value	MT	WT
KRAS	0.684	0.9288	0.8039
BRAF	0.111	0.6001	0.9602
PIK3CA	0.999	0.9103	0.8426
PTEN	0.574	0.922	0.8581
RTK-RAS	0.214	0.841152	1.075562
PI3K	0.708	0.953299	0.690218

DISCUSSION

Colorectal cancer is the third most common cancer in the world. There are many factors that may contribute to the development of CRC including genetic and acquired factors, but CRC progression has been particularly associated with genetic factors. The relative importance of the relationship between genetic alteration and CRC has resulted in many studies into relationships between genetic status of CRC cells and sensitivity of those cells to treatment drugs [24]. In support of those investigations, we investigated the effects of *KRAS* oncogene substitutions, mutations that occur frequently in CRC. We also investigated relationships between CRC gene mutation status and cell responses to chemotherapy and targeted drugs. Our results contributed to elucidating the association between genetic alterations and drug sensitivity in CRC.

To investigate the effects of the different *KRAS* substitutions, we generated plasmids for 7 *KRAS* mutation subtypes that occur frequently. Of the *KRAS* mutation subtypes transfected into HEK293 cells, *KRAS* G12D and Q61L increased RAS pathway signaling to the greatest extent. In contrast to the HEK293 results,

among the 8 CRC cell lines harboring *KRAS* mutations, only the SW480 cells expressing *KRAS* G12V showed a marked increase in downstream signaling. We also compared *KRAS* activity among cell lines by performing RAS pull-down assay. Among the 7 *KRAS* mutant subtypes tested, *KRAS* activity was significantly high in HEK293 cells expressing *KRAS* G12S and G13D. In the 8 CRC cell lines with different *KRAS* mutant subtypes, *KRAS* activity was notably high in HCT15 cells harboring *KRAS* G13D. In contrast to HEK293 cells, all 8 CRC cell lines harbored multiple-mutations. The presence of more than one mutation suggested that the effects of each *KRAS* subtypes could differ among cell lines. The results from the transfected HEK293 cells allowed observation of *KRAS* dependent effects in the absence of other mutations. As there were only 3 *KRAS* mutation subtypes in the CRC cell lines used in our study, we added other *KRAS* mutation subtypes to those cell lines in order to obtain more varied results.

To investigate further the relationship between gene mutation status and drug response, we selected 5 frequently mutated genes common in CRC cell lines: *NRAS, KRAS, BRAF, PIK3CA*, and *PTEN*. Although *APC* and *TP53* are also frequently mutated in CRC cell

lines, the *APC* mutation frequency was deemed too high for inclusion in our study, and as validation of *TP53* was difficult it was also excluded. In the 18 human CRC cell lines assessed in our study, *NRAS* and *PTEN* mutations were rare and *KRAS* and *PIK3CA* mutations were common.

Subsequently, cell viability assay was conducted with 6 anti-cancer drugs (5-FU, oxaliplatin, SN-38, AZD6244, regorafenib and PLX4720), of which three (5-FU, oxaliplatin and SN-38) are widely used in CRC therapy [13]. We attempted to obtain IC_{50} values for the 6 anti-cancer drugs in order to rank their effects on CRC cell lines; however, some IC_{50} values could not be calculated. Therefore, to determine the relative drug sensitivity/drug resistance of the tested cell lines we selected fixed, clinically relevant concentrations, of each drug. Dosage was based on previous clinical reports that included C_{max} information .

In this study, relationships and tendencies between mutation status and drug sensitivity were investigated by testing CRC cells with 3 chemotherapy drugs (5-FU, oxaliplatin, SN-38) and 3 targeted drugs (AZD6244, regorafenib, PLX4720). The results indicated that 5-FU sensitivity was not affected by genetic alterations. In

contrast, oxaliplatin did show a significant association between presence of the *PIK3CA* mutation and drug sensitivity. However, the frequency of *PIK3CA* mutations was much higher in our study than in previous studies [31]. Therefore, although we detected a relationship between oxaliplatin sensitivity and *PIK3CA* mutation, the strength of that relationship may change if more cell lines are tested. Previously, excision repair cross-complementation group 1 (*ERCC1*) expression was reported to be a predictive marker of oxaliplatin [32] and another study demonstrated that ERCC1 expression was related to *KRAS* mutation status in CRC [33]. However, in our study, there was no significant association between *KRAS* mutation status and oxaliplatin sensitivity. Although *KRAS, PIK3CA* and PI3K pathway mutations were significantly associated with SN-38 sensitivity, the mean survival rate exhibited a modest change (Fig. 7C). Therefore, we suggested there was no meaningful relationship between genetic alterations and SN-38 sensitivity. Although AZD6244, regorafenib, and PLX4720 data indicated no valid associations between sensitivity to those drugs and genetic alterations, we inferred that there were tendencies toward such relationships. In the tested CRC cell lines with *BRAF* V600E

mutations, AZD6244 and PLX4720 sensitivities were increased. One of the mechanisms of drug resistance in AZD6244 is activation of the PI3K pathway [15] and, CRC cell lines with *BRAF* and *PIK3CA* co-mutation showed resistance to AZD6244. Even though BRAF V600E is reported to be a sensitive marker of PLX4720, there is also a report indicating that CRC cell lines with *PIK3CA* or *PTEN* mutations are less sensitive to PLX4720 [34]. In support of previous studies, our results showed that CRC cell lines harboring *BRAF* and *PIK3CA* co-mutation were more resistant than other CRC cell lines to PLX4720. As a multi-tyrosine kinase inhibitor, regorafenib has been studied in many cancer types. However, no clearly defined predictive marker of regorafenib has been reported. In this study, we did not detect an association between regorafenib sensitivity and genetic alterations of 5 genes. Additional screening of other CRC treatment drugs may identify predictive markers for each drug and possibly for multi-drug combinations. And determining drug sensitivity on the basis of only 5 genes may seem to be an oversimplification; however, those 5 genes are commonly mutated in CRC. Moreover, the relationships between the genetic alterations of those genes and CRC cell drug sensitivity have not

been described. Therefore, even though this study was conducted with a limited number of gene mutations, the results could be used as basis for further research.

Our research was limited by the number of genes and drugs investigated. We suggested that investigation of more genes, cell lines, and drugs will elucidate drug effectiveness in CRC therapy.

In conclusion, further analyses of genetic status in CRC cell lines could provide insights into effective use of drug and drug combinations in CRC treatment.

REFERENCE

1. Watson AJ, Collins PD: **Colon cancer: a civilization disorder.** *Digestive diseases* 2011, **29**(2):222-228.
2. Stewart BW, Wild C, International Agency for Research on Cancer, World Health Organization: **World cancer report 2014.**
3. Tentler JJ, Nallapareddy S, Tan AC, Spreafico A, Pitts TM, Morelli MP, Selby HM, Kachaeva MI, Flanigan SA, Kulikowski GN *et al*: **Identification of predictive markers of response to the MEK1/2 inhibitor selumetinib (AZD6244) in K-ras-mutated colorectal cancer.** *Mol Cancer Ther*, **9**(12):3351-3362.
4. Ihle NT, Byers LA, Kim ES, Saintigny P, Lee JJ, Blumenschein GR, Tsao A, Liu S, Larsen JE, Wang J *et al*: **Effect of KRAS oncogene substitutions on protein behavior: implications for signaling and clinical outcome.** *J Natl Cancer Inst* 2012, **104**(3):228-239.
5. Ostrem JM, Peters U, Sos ML, Wells JA, Shokat KM: **K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions.** *Nature*, **503**(7477):548-551.
6. Chen CC, Er TK, Liu YY, Hwang JK, Barrio MJ, Rodrigo M, Garcia-Toro E, Herreros-Villanueva M: **Computational analysis of KRAS mutations: implications for different effects on the KRAS p.G12D and p.G13D mutations.** *PLoS One*, **8**(2):e55793.
7. Ihle NT, Byers LA, Kim ES, Saintigny P, Lee JJ, Blumenschein GR, Tsao A, Liu S, Larsen JE, Wang J *et al*: **Effect of KRAS oncogene substitutions on protein behavior: implications for signaling and clinical outcome.** *J Natl Cancer Inst*, **104**(3):228-239.
8. Cespedes MV, Sancho FJ, Guerrero S, Parreno M, Casanova I, Pavon MA, Marcuello E, Trias M, Cascante M, Capella G *et al*: **K-ras Asp12 mutant neither interacts with Raf, nor signals through Erk and is less**

tumorigenic than K-ras Val12. *Carcinogenesis* 2006, **27**(11):2190-2200.

9. Corcoran RB, Cheng KA, Hata AN, Faber AC, Ebi H, Coffee EM, Greninger P, Brown RD, Godfrey JT, Cohoon TJ *et al*: **Synthetic lethal interaction of combined BCL-XL and MEK inhibition promotes tumor regressions in KRAS mutant cancer models**. *Cancer Cell*, **23**(1):121-128.
10. Longley DB, Harkin DP, Johnston PG: **5-fluorouracil: mechanisms of action and clinical strategies**. *Nature reviews Cancer* 2003, **3**(5):330-338.
11. Maindrault-Goebel F, Tournigand C, Andre T, Carola E, Mabro M, Artru P, Louvet C, de Gramont A: **Oxaliplatin reintroduction in patients previously treated with leucovorin, fluorouracil and oxaliplatin for metastatic colorectal cancer**. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 2004, **15**(8):1210-1214.
12. Graham J, Mushin M, Kirkpatrick P: **Oxaliplatin**. *Nature reviews Drug discovery* 2004, **3**(1):11-12.
13. Zhang N, Yin Y, Xu SJ, Chen WS: **5-Fluorouracil: mechanisms of resistance and reversal strategies**. *Molecules* 2008, **13**(8):1551-1569.
14. Pommier Y, Leo E, Zhang H, Marchand C: **DNA topoisomerases and their poisoning by anticancer and antibacterial drugs**. *Chemistry & biology* 2010, **17**(5):421-433.
15. E J, Xing J, Gong H, He J, Zhang W: **Combine MEK inhibition with PI3K/mTOR inhibition exert inhibitory tumor growth effect on KRAS and PIK3CA mutation CRC xenografts due to reduced expression of VEGF and matrix metallopeptidase-9**. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* 2014.
16. Grasso S, Tristante E, Saceda M, Carbonell P, Mayor-Lopez L, Carballo-Santana M, Carrasco-Garcia E, Rocamora-Reverte L,

Garcia-Morales P, Carballo F *et al*: **Resistance to Selumetinib (AZD6244) in Colorectal Cancer Cell Lines is Mediated by p70S6K and RPS6 Activation**. *Neoplasia* 2014, **16**(10):845-860.

17. Balmanno K, Chell SD, Gillings AS, Hayat S, Cook SJ: **Intrinsic resistance to the MEK1/2 inhibitor AZD6244 (ARRY-142886) is associated with weak ERK1/2 signalling and/or strong PI3K signalling in colorectal cancer cell lines**. *International journal of cancer Journal international du cancer* 2009, **125**(10):2332-2341.
18. Grothey A, Van Cutsem E, Sobrero A, Siena S, Falcone A, Ychou M, Humblet Y, Bouche O, Mineur L, Barone C *et al*: **Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial**. *Lancet* 2013, **381**(9863):303-312.
19. Schmieder R, Hoffmann J, Becker M, Bhargava A, Muller T, Kahmann N, Ellinghaus P, Adams R, Rosenthal A, Thierauch KH *et al*: **Regorafenib (BAY 73-4506): antitumor and antimetastatic activities in preclinical models of colorectal cancer**. *International journal of cancer Journal international du cancer* 2014, **135**(6):1487-1496.
20. Rey JB, Launay-Vacher V, Tournigand C: **Regorafenib as a single-agent in the treatment of patients with gastrointestinal tumors: an overview for pharmacists**. *Targeted oncology* 2014.
21. Tai WT, Chu PY, Shiau CW, Chen YL, Li YS, Hung MH, Chen LJ, Chen PL, Su JC, Lin PY *et al*: **STAT3 Mediates Regorafenib-Induced Apoptosis in Hepatocellular Carcinoma**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2014, **20**(22):5768-5776.
22. Sastre J, Argiles G, Benavides M, Feliu J, Garcia-Alfonso P, Garcia-Carbonero R, Gravalos C, Guillen-Ponce C, Martinez-Villacampa M, Pericay C: **Clinical management of regorafenib in the treatment of patients with advanced colorectal cancer**. *Clinical & translational oncology : official publication of the Federation of Spanish Oncology*

Societies and of the National Cancer Institute of Mexico 2014, **16**(11):942-953.

23. Tsai J, Lee JT, Wang W, Zhang J, Cho H, Mamo S, Bremer R, Gillette S, Kong J, Haass NK *et al*: **Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity**. *Proceedings of the National Academy of Sciences of the United States of America* 2008, **105**(8):3041-3046.
24. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, Wilson CJ, Lehar J, Kryukov GV, Sonkin D *et al*: **The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity**. *Nature* 2012, **483**(7391):603-607.
25. Guagnano V, Kauffmann A, Wohrle S, Stamm C, Ito M, Barys L, Pornon A, Yao Y, Li F, Zhang Y *et al*: **FGFR genetic alterations predict for sensitivity to NVP-BGJ398, a selective pan-FGFR inhibitor**. *Cancer discovery* 2012, **2**(12):1118-1133.
26. Jodrell DI, Stewart M, Aird R, Knowles G, Bowman A, Wall L, McLean C: **5-fluorouracil steady state pharmacokinetics and outcome in patients receiving protracted venous infusion for advanced colorectal cancer**. *British journal of cancer* 2001, **84**(5):600-603.
27. Ehrsson H, Wallin I, Yachnin J: **Pharmacokinetics of oxaliplatin in humans**. *Medical oncology* 2002, **19**(4):261-265.
28. Dumez H, Awada A, Piccart M, Assadourian S, Semiond D, Guetens G, de Boeck G, Maes RA, de Bruijn EA, van Oosterom A: **A phase I dose-finding clinical pharmacokinetic study of an oral formulation of irinotecan (CPT-11) administered for 5 days every 3 weeks in patients with advanced solid tumours**. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 2006, **17**(7):1158-1165.
29. Adjei AA, Cohen RB, Franklin W, Morris C, Wilson D, Molina JR, Hanson LJ, Gore L, Chow L, Leong S *et al*: **Phase I pharmacokinetic and pharmacodynamic study of the oral, small-molecule mitogen-**

activated protein kinase kinase 1/2 inhibitor AZD6244 (ARRY-142886) in patients with advanced cancers. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2008, **26**(13):2139-2146.

30. Johannessen CM, Boehm JS, Kim SY, Thomas SR, Wardwell L, Johnson LA, Emery CM, Stransky N, Cogdill AP, Barretina J *et al*: **COT drives resistance to RAF inhibition through MAP kinase pathway reactivation.** *Nature* 2010, **468**(7326):968-972.

31. Karakas B, Bachman KE, Park BH: **Mutation of the PIK3CA oncogene in human cancers.** *British journal of cancer* 2006, **94**(4):455-459.

32. Dabholkar M, Vionnet J, Bostick-Bruton F, Yu JJ, Reed E: **Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy.** *The Journal of clinical investigation* 1994, **94**(2):703-708.

33. Lin YL, Liau JY, Yu SC, Ou DL, Lin LI, Tseng LH, Chang YL, Yeh KH, Cheng AL: **KRAS mutation is a predictor of oxaliplatin sensitivity in colon cancer cells.** *PLoS One* 2012, **7**(11):e50701.

34. Mao M, Tian F, Mariadason JM, Tsao CC, Lemos R, Jr., Dayyani F, Gopal YN, Jiang ZQ, Wistuba, II, Tang XM *et al*: **Resistance to BRAF inhibition in BRAF-mutant colon cancer can be overcome with PI3K inhibition or demethylating agents.** *Clinical cancer research : an official journal of the American Association for Cancer Research* 2013, **19**(3):657-667.

국문 초록

세계에서 세 번째로 발생빈도가 높은 대장암은 여러 순차적 단계에 거친 유전적 변이로 인해 발생하게 된다. 그러므로 대장암 치료에 있어 유전적 변이를 분석하는 것은 앞으로의 연구에 중요한 실마리로 여겨지고 있다. 이러한 연구들을 뒷받침하고자 본 연구에서는 여러 *KRAS* 유전자 변이들의 영향력을 관찰하고 대장암 세포주에서 유전자 돌연변이와 항암제 반응성과의 관계를 보여주고자 하였다.

KRAS 돌연변이들의 영향력을 알아보기 위해 발생 빈도수가 높은 *KRAS* 유전자 변이 7가지를 선정하여 플라스미드를 제작하였다. 형질주입한 HEK293세포와 *KRAS* 돌연변이 대장암 세포주 간의 하위 신호전달 경향성과 *KRAS* activity를 각각 비교하여 각 *KRAS* 돌연변이들의 특성을 살펴볼 수 있었다. 형질 주입한 HEK293 세포에선 G12D, Q61L 돌연변이가 RAS 하위 신호가 가장 증가된 반면, 대장암 세포주에선 G12V 돌연변이를 지닌 SW480 세포의 RAS 하위 신호가 가장 활성화되어 있음을 확인할 수 있었다. *KRAS* activity의 경우 HEK293 세포에선 G12S와 G13D 돌연변이가, 대장암 세포주에선 G13D 돌연변이가 있는 HCT15 세포가 가장 큰 *KRAS* 활성을 나타내었다.

다음으로, 항암제 반응성과 유전적 변이간의 연관성을 규명하기 위해 먼저 18개의 대장암 세포주에 대해 Sanger 시퀀싱을 통한 유전적 변이

를 조사하였다. 대장암에서 변이빈도수가 높은 다섯 가지 유전자들(*NRAS, KRAS, BRAF, PIK3CA, PTEN*)을 가지고 유전적 변이를 검증하였으며, CCLE 자료를 바탕으로 각 유전자들의 잘 알려진 hot-spot 변이 위치를 확인하였다. 다음으로 6가지 항암제 (5-FU, oxaliplatin, SN-38, AZD6244, regorafenib, PLX4720)에 대한 대장암 세포주의 감수성을 알아보기 위해 MTT 실험을 진행하였다. 각각의 MTT 결과를 가지고 IC_{50}를 계산하여 각 항암제들에 대한 세포주의 감수성을 비교하려 하였으나 계산 불가능한 IC_{50}값들로 인해 감수성 검증에 어려움이 있었다. 그리하여 실제 각 항암제들을 가지고 임상에서 실험한 데이터를 바탕으로 정해진 농도를 가지고 다시 MTT 실험을 진행하였다. 각 농도에서 살아남은 정도를 숫자로 환산하여 6가지 항암제들에 대한 감수성을 확인할 수 있었다. 유전적 변이 여부에 따라 항암제 반응성에 대해 Mann Whitney *U* test 검증을 실시하였고 그 결과, *PIK3CA* 유전자 변이가 있을 경우 oxaliplatin에 대한 감수성과 유의한 관계가 있음을 검증할 수 있었으며, 비록 표적치료 항암제의 경우 통계적으로 유효한 의미가 있는 관계를 검증하진 못하였지만 BRAF 유전자 변이가 있을 경우 표적치료 항암제에 대한 감수성이 증가하는 경향성이 있음을 확인할 수 있었다.

요약하면, 본 연구에서 확인한 각 *KRAS* 돌연변이들에 대한 영향력과 대장암 세포주에서 유전자 분석과 각 항암제에 대한 세포 독성과의 관계

분석을 통해 앞으로 대장암 연구에 대한 근거로 사용될 수 있음을 보여 주고 있다.

주요어 : **대장암, 유전적 변이, *KRAS*, 화학 항암제, 표적치료 항암제,**

학번 : 2012-22835