Combination strategy to enhance IGF signaling pathway inhibition in gastric cancer

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Combination strategy to enhance IGF signaling pathway inhibition in gastric cancer

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TABLE OF CONTENTS

Abstract·····1

I. INTRODUCTION·····4

II. MATERIALS AND METHODS·····8

1. Materials·····8
2. Cell lines and cultures·····8
3. Establishment of stable cell lines expressing wild type and mutant KRAS ·····8
4. Whole-exome sequencing (WES)·····9
5. RNA sequencing·····9
6. Immunoblotting·····9
7. GTP-Ras pull-down assay·····10
8. siRNA transfection of IGF-1R·····10
9. Cell viability·····11
10. Proliferation assay·····11
11. Migration assay·····11
12. Clonogenic assay·····12
13. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Real-time quantitative RT-PCR·····12
14. Enzyme-linked immunosorbent assay (ELISA) ·····13
15. Apoptosis assay·····13
16. Statistical analysis·····13

III. RESULTS·····14

1. Characterization of RAS alteration cell lines in the gastric cancer cell line panel·····16
2. Generation of WT and mutant KRAS stable cell lines in SNU-638·····22
3. Characterization of WT and mutant KRAS SNU-638 stable cell lines·····24
4. Inhibition of IGF1R signaling pathway using small interfering RNA or Xentuzumab·····29
5. Enhancement of IGF1R signaling pathway inhibition using a combination of a downstream signaling pathway in IGF1R ·····43

IV. DISCUSSION·····49

V. CONCLUSION·····53

REFERENCES·····54

ABSTRACT (IN KOREAN) ·····59

PUBLICATION LIST·····62

LIST OF FIGURES

Figure 1. Profiling of RTK and RAS-related copy number variant (CNV), single nucleotide variant (SNV) ·····17
Figure 2. mRNA sequencing analysis of 49 GC cell line panel·····18
Figure 3. RTK and downstream protein expression in RAS amplification and mutation group ·····21
Figure 4. Generation of WT and mutant KRAS SNU-638 stable cell line ·····23
Figure 5. Phenotype changes of WT and mutant KRAS cell lines ·····26
Figure 6. EGFR and IGF1R protein expressions in KRAS stable cell lines·····27
Figure 7. Protein expression of IGF1R downstream signaling factors·····28
Figure 8. Suppression of siRNA on the IGF1R level in WT and mutant KRAS SNU-638 stable cells ·····34
Figure 9. Phenotype changes in knockdown IGF1R·····36
Figure 10. Protein expression changes by IGF1R siRNA transfection·····37
Figure 11. Phenotype changes of IGF1R signaling inhibition by Xentuzumab·····39
Figure 12. Protein expression changes by IGF1R signaling pathway inhibition using Xentuzumab ·····40

Figure 13. Candidate Interference factors of efficacy when inhibition of IGF1R signaling pathway ·····42
Figure 14. Combination treatment of WT and Mutant KRAS cell lines·····46
Figure 15. Protein expression in combination treatment·····47
Figure 16. Measurement of apoptosis by flow cytometry through Annexin V-FITC and PI staining·····48

LIST OF TABLES

Table 1. Comparison of mRNA expression between no RTK amplification/ no RAS alteration group and RAS alteration group ……19
Table 2. Nucleotide sequences of siRNAs targeting IGF1R····33

ABSTRACT

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Variety of studies are underway to target receptor tyrosine kinase (RTK) amplification in gastric cancer which has high tumor heterogeneity. In gastric cancer, RAS alteration is known as important as RTK amplification. However, there is a lack of researches developing RAS inhibition because of a limitation in targeting RAS directly. Therefore, this study attempted to comprehend the characteristics of RAS altered gastric cancer cell lines and determine whether there is phenotypic changes and dependency on a specific RTK and downstream pathways when KRAS alteration is induced.

First of all, 49 gastric cancer cell lines were classified into 3 groups

based on RTK/RAS alteration status, 1) RTK amplification, 2) RAS alteration only, and 3) none group with no RTK amplification nor RAS alteration according to the genetic characteristics using whole exome sequencing data. Using RNA sequencing data, transcripts levels of PI3K/AKT and MAPK pathways were increased in the RAS alteration group compared to none group. In addition, increased phosphorylation of EGFR and IGF1R were observeed in the RAS alteration group by immunoblotting. This data suggested an association between RAS alteration and the activation of RTKs. To confirm this association, a cell line stably expressing KRAS wild type, G12D mutant, and Q61H mutant was established. As a result, immunoblotting confirmed that phosphorylation of IGF1R was increased but not EGFR in RAS altered cell line. In addition, analysis of cell proliferation, colony formation, and cell migration according to KRAS alteration was performed. Although there was a difference in degree, all phenotypes were increased in the KRAS altered cell lines compared to the vector. To verify that these increased phenotypes can be regulated through IGF1R pathway, siRNA of IGF1R and Xentuzumab (IGF-1 and -2 neutralizing antibody drug) were used. Upon inhibition of IGF1R, increased cell proliferation, colony formation and cell migration was significantly inhibited in KRAS Q61H mutant, KRAS wild type and KRAS G12D mutant cell lines, respectively. With IGF1R inhibition, we observed that the activation of IGF1R was decreased, but the downstream signaling molecules were less inhibited.

Therefore, a combination treatment of Xentuzumab with BYL-719 (PI3Kα inhibitor) and RMC-4550 (SHP-2 inhibitor) was performed to evaluate the effect of downstream pathway. Synergistic effect of the combination treatment was confirmed through cell viability analysis and inhibition of protein expression levels of target downstream signaling molecules were confirmed by immunoblotting. Compared to vector, KRAS WT, G12D mutant and Q61H mutant cell lines showed synergistic effect of both combination treatments of Xentuzumab with BYL-719 and RMC-4550. Expression of the downstream molecules of each drug target was inhibited in these 3 cell lines. According to FACS analysis results, the combination treatment compared to single treatment increased apoptosis more in KRAS WT, G12D mutant and Q61H mutant cell lines.

Based on these results, we found that RAS alteration in gastric cancer induces the increase of cell proliferation, colony formation and cell migration through the activation of IGF1R. In conclusion, IGF1R may be a potential target molecule with RAS alteration in gastric cancer, and suggests the possibility of enhancing its inhibitory effect through combination treatment with RTK downstream effectors such as PI3Kα and SHP-2.

Keywords: Gastric cancer, Receptor tyrosine kinase, KRAS, IGF1R

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I. INTRODUCTION

According to the GLOBOCAN 2018 database, gastric cancer is the fourth common cause of cancer-related deaths worldwide[1]. In addition, new cases and crude incidence rates per 100,000 are 29,685 cases and 57.9 cases in both sexes, respectively, which are the highest in Korea in 2017[2]. Despite its high incidence and high mortality, targeted treatment of gastric cancer is limited due to high tumor heterogeneity. Compared to other tumor types, infections of *Helicobacter pylori* or *Epstein-Barr* virus provoke high complexities in the tumor environment[3]. Among the many trials with targeted therapy, Trastuzumab is the first Food and Drug Administration (FDA) approved drug

that is targeted for HER2 positive metastatic adenocarcinomas of the stomach and gastroesophageal junction (GEJ)[4]. Likewise, the angiogenesis inhibitor Ramucirumab is the second FDA approved drug for treating patients with advanced or metastatic gastric cancer[5]. Therefore, discovery of essential new targets and understanding their mechanisms are crucial to overcome tumor heterogeneity in gastric cancer.

In recent years, many kinds of research have focused on inhibiting receptor tyrosine kinases (RTKs) in order to target P13K/AKT and MAPK pathways, which are the major downstream pathways of RTKs[6]. Since RTKs are mostly expressed on the cell membrane surface, it is easier to develop a targeted drug for RTKs. Moreover, RTK targeting antibody drugs have longer half-lives and fewer side effects than tyrosine kinase inhibitors (TKI) or chemotherapeutic agents[7]. To maximize these advantages, understanding RTKs and their pathways in various cell types is extremely important for developing new target drugs.

Niantao Deng *et al*. performed a comprehensive survey of genomic alterations in gastric cancer for suggesting distinct therapeutic targets. They found out that the most powerful targets are factors of the RTK/RAS signaling pathways; FGFR2, KRAS, ERBB2, EGFR, and MET amplification. Most of the RTKs and RAS amplifications were mutually exclusively expressed and these groups showed poor prognosis compared to the RTK/RAS absent group[8]. It is well known that not just RTKs, but RAS signaling is also a considerable target in the development of gastric cancer treatments. Furthermore, KRAS mutations were detected in about 6% of diffuse-type gastric cancer which has an aggressive character[9]. The Cancer Genome Atlas (TCGA) group studied the

molecular characterization of gastric adenocarcinoma with a large scale cohort. They emphasized that RTK-RAS alterations are important to activate the downstream signaling pathway[10]. As predicted, MKN-1 (KRAS amplified cell line) showed high total KRAS expression but relatively low expression of GTP-KRAS (active form of KRAS). When compared with MKN-1, AGS (KRAS G12D mutant cell line) expressed a lower total KRAS but a higher GTP-KRAS. According to the results, KRAS mutation is also important for KRAS activation[11]. Therefore, both KRAS amplification and mutation should be considered as an important target for gastric cancer research.

RTKs-targeting drugs were developed and are going through clinical trials for the treatment of gastric cancer[12]. However, many drugs directly targeting RAS have struggled in development because druggable pockets on the surface of RAS are limited. Moreover, it has a very active GTPase transmission process[13]. The development of RAS-targeting drugs still remains attractive and biologically important. That is the reason why many studies including colorectal, lung, and pancreas cancer with RAS alteration are targeting RTK such as EGFR or downstream signaling instead of directly targeting RAS[14-16]. Many studies revealed that inhibition with a single drug has intrinsic resistance. To overcome this limitation dual inhibition approaches combining EGFR inhibitors and downstream molecules were implemented. However, combination treatment showed limited efficacy in the KRAS alteration group compared to KRAS wild type group in pancreatic and gastric cancer cell lines[17,18]. Therefore, understanding the mechanisms of KRAS alteration and suggesting the effective candidate targets in gastric cancer are needed.

The aim of this study was to identify characteristics of RAS alteration and

find specific RTK pathway activations in the gastric cancer cell line panel. In addition, RTK activation and biological changes were explored in the KRAS alteration induced model. As a result, RTK pathway activation by RAS alteration may play a functional role in cell proliferation, colony formation, and cell migration, suggesting its possible role as a therapeutic target in RAS altered gastric cancer.

II. MATERIALS AND METHODS

1. Materials

Xentuzumab (BI836845) is a humanized monoclonal antibody that targets insulin-like growth factor (IGF) ligands IGF-1 and IGF-2. It was provided by Dr. Ulrike Weyer-Czemilofsky (Boehringer Ingelheim, Ingelheim am Rhein, Germany). BYL-719, a selective PI3Kα inhibitor, and RMC-4550, an allosteric inhibitor of SHP2, were commercially purchased (Selleckhem, TX, USA).

2. Cell lines and culture

Among the 49 human gastric cancer cell lines, 26 of cell lines were established by Songdang Institute for Cancer Center and 23 of cell lines were purchased from 3 different organizations worldwide (American Type Culture Collection, Korean Cell Line Bank, and Japanese Collection of Research Bioresources Cell Bank)[19]. Cell lines were maintained with their proper complete growth medium. Specifically, Eagle's Minimum Essential Medium (EMEM), Dulbecco's Modified Eagle Medium (DMEM), and Roswell Park Memorial Institute-1640 (RPMI-1640) (Gibco, MA, USA) containing 1 % of antibiotics (Lonza, Basel, Switzerland) and 5% of FBS (Lonza, Basel, Switzerland) were consumed for the maintenance of cell lines. Cell lines were cultured at 37℃ in a 5% CO_2 incubator.

3. Establishment of stable cell lines expressing wild type and mutant KRAS

SNU-638, which has no RTK/RAS amplification and no RAS, PIK3CA, and

PIK3R1 mutations, was seeded in 6-well plates and was incubated overnight. Next day, the cell line was transfected with 2 μg of plasmid DNA using Lipofectamine 3000 (Invitrogen, CA, USA) according to the manufacturer's protocol. After incubation for 48 hrs, cells were seeded as a single cell into each 96-wells and fed with growth medium containing G418 (500 μg/ml). Among the several colonies, one clone was selected by Sanger sequencing and KRAS expression was confirmed by immunoblotting.

4. Whole exome sequencing (WES)

DNA was extracted from the 49 gastric cancer cell lines. WES was performed with a HiSeq 2000 system (Illumina, Inc., CA, USA). Using WES data, copy number variant (CNV) and single nucleotide variant (SNV) were evaluated.

5. RNA sequencing

Total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA sequencing was performed with an Illumina HiSeq 2500. Sequencing was done with 2 x 100-bp paired-end reads and the fragments per kilo base of exon per million fragments mapped (FPKM) values without normalization was generated. Hierarchical clustering was analyzed and Heatmap was performed with z-score.

6. Immunoblotting

First of all, 50 μg of whole-cell protein extracts in radio immunoprecipitation (RIPA) lysis buffer were size-fractionated by 8-15% SDS polyacrylamide gel

electrophoresis and transferred onto a polyvinylidene difluoride membrane by semi-transfer (Bio-rad, CA, USA). Membranes were blocked with 5% non-fat milk/ tris buffered saline buffer with tween 20 (TBST) for 1 hr at room temperature and then incubated overnight with the primary antibody at 4℃. After incubation with horseradish peroxidase-conjugated secondary antibody, protein signals were detected by enhanced ECL prime western blotting detection reagent (Amersham, Little Chalfont, United Kingdom).

7. GTP-RAS pull-down assay

Active RAS pull-down experiments were determined by RAS activation assay biochem kit (Cytoskeleton, CO, USA) following the manufacturer's instructions. Briefly, 5.0 x 10^5 cells were seeded on a 100 mm^2 dish and incubated for 3 days. About 500 μg of cell lysates were incubated at 4 ℃ for 1 hr with Raf-RBD beads. After washing twice with PBS, 20 μl of 2X sample buffer was added and boiled at 95 ℃ for 5 mins. Immunoblotting on 15% SDS polyacrylamide gel was performed.

8. siRNA transfection of IGF-1R

siRNA transfections were performed using Lipofectamine 3000 (Invitrogen, CA, USA) following the manufacturer's instruction. Briefly, cells were washed once with Opti-MEM (GIBCO-BRL/Invitrogen, CA, USA) and 750 μl of Opti-MEM was added to each well. For each transfection, 7.5 μl of Lipofectamine 3000 reagent was mixed with 125 μl of Opti-MEM and incubated for 5 mins at room temperature. In a separate tube, negative control (siNC) and siIGF1R were added to 125 μl of Opti-MEM containing 5 μl of

P3000 reagent and the siRNA solution was added to the Lipofectamine 3000 mixture. The siRNA mixture was incubated for an additional 5 mins at room temperature to allow complex formation. Subsequently, the solution was added to the cells in the 6-well plate. After 4 hrs incubation, the cells were replaced with 2 ml of standard growth media and cultured at 37°C. Cells were harvested at different time points of post-transfection for other experiments.

9. Cell viability

Cell viability was assayed by using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Appropriate numbers of cells (1.0×10^3) were seeded in 96 well plates and incubated for 24 hrs. Each well was treated with various concentration of drugs for 5 days. CCK-8 solution was then added and incubated for 2 hrs at 37℃. Using a Microplate reader (Tecan, Männedorf, Switzerland), the 96-well plate was measured of absorbance at 450 nm.

10. Proliferation assay

In a 48-well plate, 2.0×10^3 cells were seeded into each well and incubated at 37°C, 5% CO_2 incubator overnight. Every well was then replaced with a fresh culture medium and applied with 10 μl of MTT reagent. The optical density (O.D) value was measured at 540 nm by a Microplate reader (Tecan, Männedorf, Switzerland). Duplicate measurements were taken and they were evaluated every day for 5 days.

11. Migration assay

Cells were plated in the upper chamber of duplicate wells at a density of 2.5

x 10^5 in 500 μl of serum-free media. In the 24-well plates, each well was filled with 1 ml of chemoattractant containing drug in normal medium. After 48 hrs, the cells in the lower chamber including those attached to the undersurface of the membrane were stained with 0.5% crystal violet solution and counted.

12. Clonogenic assay

Five hundred cells were plated into 6-well plates and incubated for 24 hrs. Each drug treatment was performed on the next day. Until the formation of large clones (>1 mm), cells were incubated for 10 days. Colonies were stained with 0.5% crystal violet solution and counted.

13. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Real-time quantitative RT-PCR (qPCR)

With 2 μg of total RNA, cDNA was synthesized by using an OligodT primer and a Superscript II first-strand synthesis system supermix for RT-PCR (Invitrogen, CA, USA).

Relative quantification was performed by real-time qPCR with SYBR green using the MX3005P (Stratagene, CA, USA) following the manufacturer's instruction. The housekeeping gene GAPDH was used as the endogenous control for normalization. The lists of Real-time qPCR primers are listed as follows:

IGF-1 forward primer: 5'-AGGAAGTACATTTGAAGAACGCAAGT-3';

IGF-1 reverse primer: 5'-CCTGCGGTGGCATGTCA-3';

IGF-2 forward primer: 5'-TCCCCTGATTGCTCTACCCA-3';

IGF-2 reverse primer: 5'-TTCCGATTG CTGGCCATCTC-3';

14. Enzyme-linked immunosorbent assay (ELISA)

IGF-1 concentrations in conditioned media and lysate of cell lines were measured by the human IGF-1 Quantikine ELISA kit (R&D Systems, MN, USA). Briefly, 5.0 x 10^5 cells were seeded in 6 well plates and after 24 hrs, the plate was replaced with 1 ml of conditioned medium. The day after replacement, conditioned medium and cell lysate were collected separately. Prior to the assay, each sample was normalized with 50 μg of total protein from lysate and 200 μg of total protein from the conditioned medium. The assays were performed according to the manufacturer's protocol. A four-parameter logistic curve was used to analyze the results.

15. Apoptosis assay

Cells (5.0 × 10^5 cells) were seeded in 6-well plates and incubated for 24 hrs. Cells were then treated with drugs for 48 hrs and the supernatant (containing floating death cells) and adherent cells were collected together. The collected cells were washed twice with PBS, then centrifuged. The pellet was resuspended in 100 μl of AnnexinV binding buffer and stained using BD AnnexinV/ FITC apoptosis kit (BD Biosciences, NJ, USA). Apoptotic cells were measured using flow cytometry.

16. Statistical analysis

All data were analyzed using the Student *t-test* or ANOVA using SPSS statistics (IBM, NY, USA). All differences were considered statistically significant for p-values < 0.05.

III. RESULTS

1. Characterization of RAS alteration cell lines in the gastric cancer cell line panel

Among the 49 gastric cancer cell lines used in this study, 18 of cell lines (36.7%) had RTK amplifications in ERBB2, MET, EGFR, and FGFR2. These cell lines were defined as the RTK amplification group. The RAS alteration group (24.5%) was composed of 4 cell lines with KRAS amplification and 8 cell lines with KRAS and NRAS mutation. Nineteen of the cell lines (38.3%) were determined as the none group with no RTK amplification or no RAS alteration. In our gastric cancer cell line panel, KRAS G12D or G12S mutation, KRAS Q61H or Q61K mutation, and NRAS Q61L mutation were observed, but KRAS G13 mutation was not detected.

Most interestingly, RAS alteration and RTK amplification were mutually exclusively expressed in our gastric cancer cell line panel. In the downstream factors of RTK, PIK3CA and PIK3R1 mutations were observed in 12% and 27% of our panel, respectively. BRAF gene was mutated in 2 cell lines and RAF1 gene was mutated in 1 cell line (Fig. 1).

Analyzing the RNA sequencing data, 494 of differentially expressed genes (DEGs) were identified in the three groups. The relevant comparison among these three groups did not show any differences in RTK expression. However, KEGG pathway analysis revealed that the DEGs were enriched in ‘MAPK signaling pathway’ and ‘AMPK signaling pathway’ which are associated with RTK downstream signaling (Fig. 2A, B). Meanwhile, 16 of both PI3K/AKT and MAPK pathway-related genes, which are involved in the RTK

downstream signaling pathway, were shown with significant differences between two groups; none group with no RTK amplification or no RAS alteration and RAS alteration group. Most of the gene expressions in PI3K/AKT and MAPK pathway were significantly increased in the RAS alteration group except for MAP3K13, MAPK13, RELA, TAOK3, RSP6KA6 and FGF13 (Table 1). Although differences of RTK mRNA expression between two groups were not significant, RTK activation might have occurred on the analogy of higher mRNA expressions of RTK downstream factors in the RAS alteration group.

From the hypothesis, protein expressions of RTKs and its downstream factors were analyzed by immunoblotting. Compared to KRAS wild type cell lines, RAS altered cell lines showed up-regulation in phosphorylated EGFR and phosphorylated IGF1R. In the KRAS alteration group, total IGF1R expression was relatively lower in PIK3CA mutated cell lines (Fig. 3A). Most of RTK expression was down-regulated in PIKCA mutated cell lines in the KRAS altered group with the exception of HER3. Among the 6 of RTKs in the Figure 3B, IGF-1R was the only one showing statistical significance in protein expression between wild type and PIK3CA mutation cell lines in the KRAS alteration group. Protein expressions of RTK downstream signaling molecules were abundant across the cell lines. Only p-IRS-1 and IRS-1 were specifically expressed in the RAS alteration group. In the RAS alteration group, KRAS amplification cell lines showed higher total KRAS expression levels than RAS mutated cell lines. However, KRAS activation was observed in most of the RAS mutated cell lines and highly KRAS amplified cell lines, MKN-1 and YCC-1. In addition, YCC-2 and YCC-9, which has PIK3R1 mutation without

PIK3CA mutation in the RAS mutation group, showed a hyper-activation of phosphorylated ERK (Fig. 3C). In this regard, PIK3CA and PIK3R1 mutation affected the expressions of RTK and downstream signaling in RAS alteration cell lines.

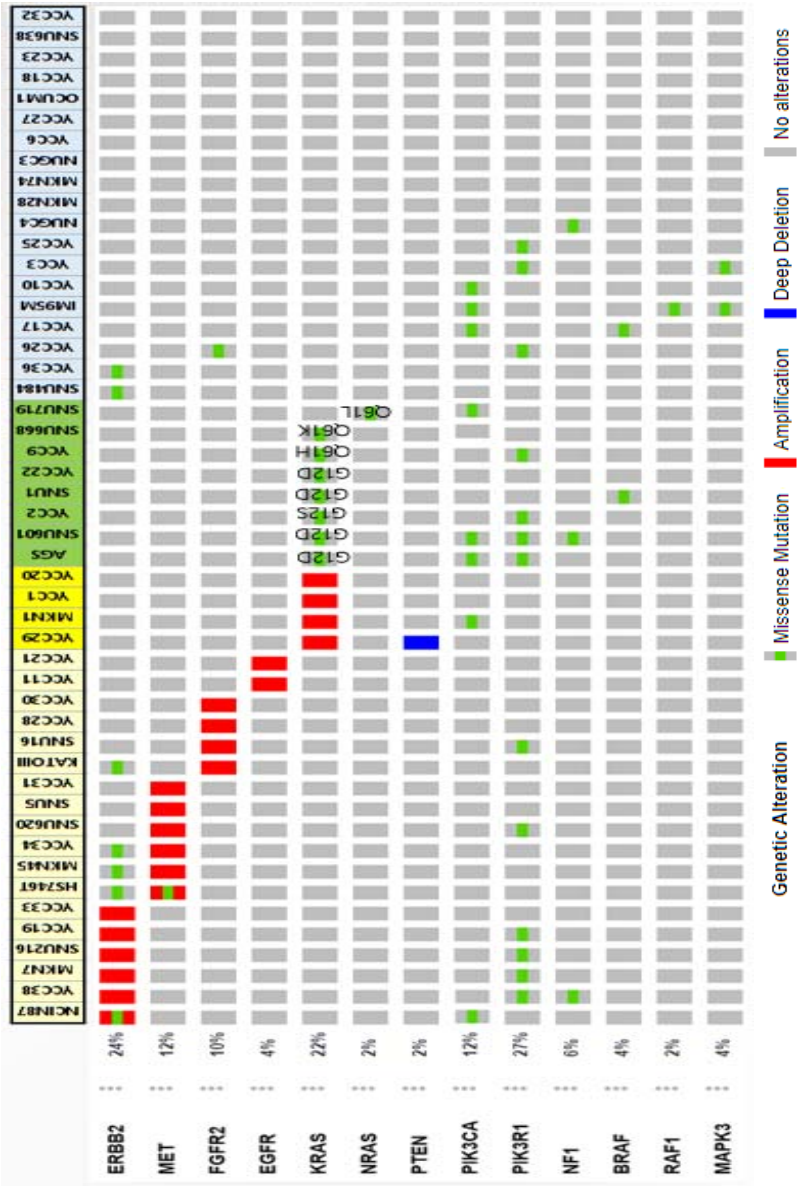


Figure 1. Profiling of RTK and RAS-related copy number variant (CNV), single nucleotide variant (SNV). Forty-nine cell lines were subgroup into three groups; (1) RTK amplification (Ivory), (2) RAS alteration including amplification (Yellow) and mutation (Green), and (3) no RTK amplification and no RAS alteration (Sky blue).

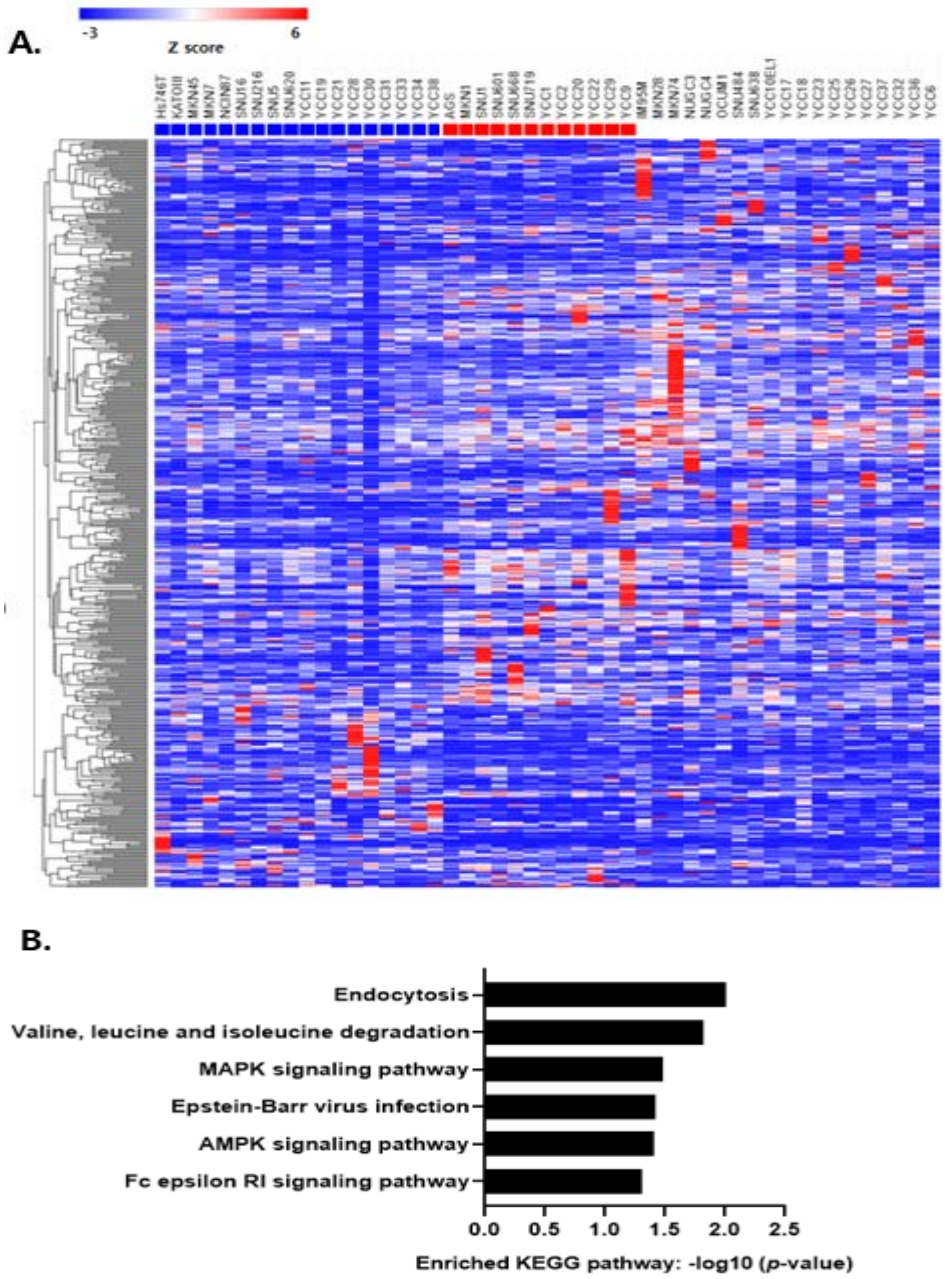
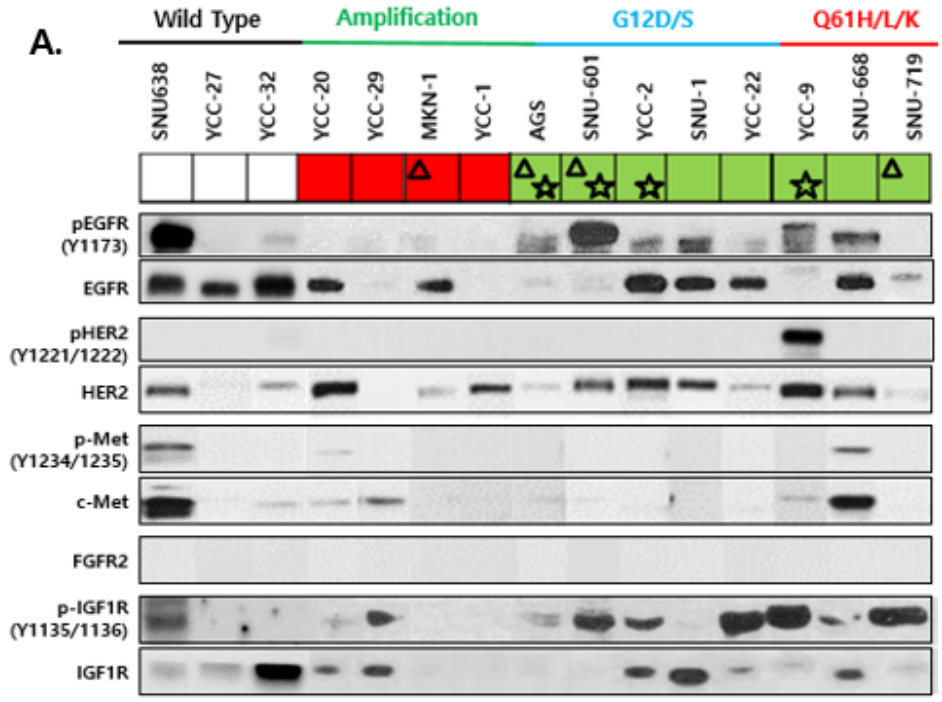
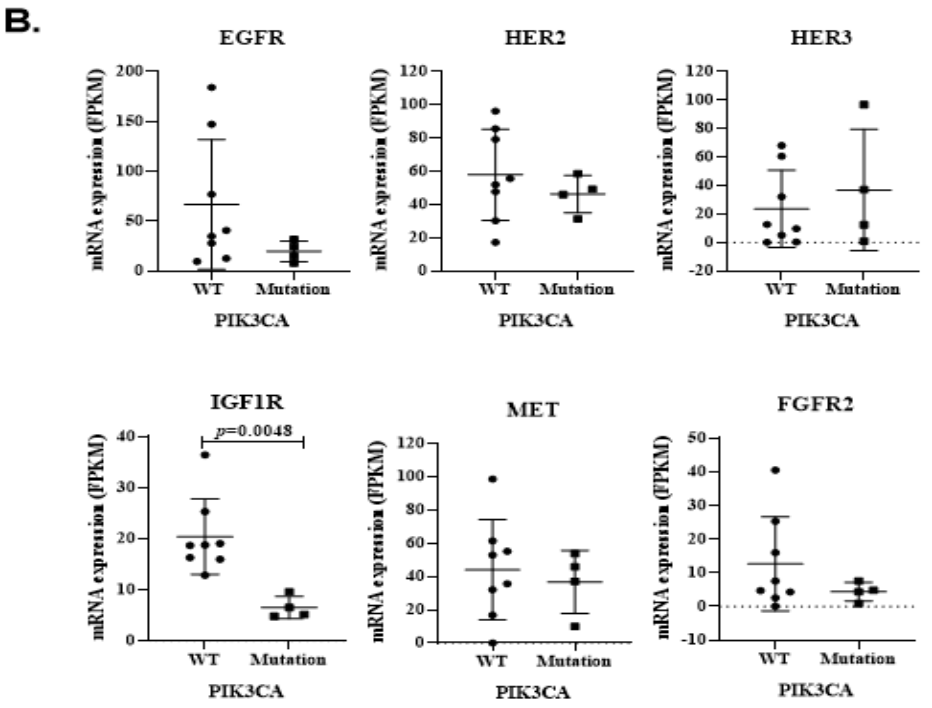


Figure 2. mRNA sequencing analysis of 49 GC cell line panel. (A) Hierarchical structure and heat map of 494 genes across the 3 groups. (B) KEGG pathway analysis of differentially expressed genes among the 3 groups.

Gene	*No RTK amplification/ No RAS alteration (19)*	*RAS alteration (12)*	*p-value*
PI3K/AKT pathway			
IRS1	12.10 ± 13.12	21.12 ± 9.57	0.035
AKT2	30.25 ± 9.78	38.75 ± 10.58	0.035
AKT1S1	23.88 ± 9.09	32.42 ± 8.62	0.014
MAPK pathway			
KRAS	15.03 ± 16.67	91.26 ± 158.14	0.044
MAP4K4	25.99 ± 12.45	38.26 ± 17.40	0.048
MAPK6	25.50 ± 11.62	34.74 ± 11.59	0.041
MAP3K13	4.00 ± 1.91	2.24 ± 1.38	0.006
MAPK13	48.23 ± 26.11	28.28 ± 23.85	0.038
GNA12	25.45 ± 10.10	32.44 ± 8.19	0.044
RELA	61.44 ± 35.49	41.59 ± 14.89	0.040
TAOK3	13.42 ± 7.83	8.41 ± 4.63	0.033
CACNA1I	0.02 ± 0.02	0.11 ± 0.14	0.043
CDC25B	40.23 ± 17.83	89.83 ± 55.65	0.011
PPP5C	46.07 ± 19.36	66.76 ± 29.35	0.045
RSP6KA6	0.68 ± 1.11	0.06 ± 0.14	0.026
FGF13	0.36 ± 0.56	0.06 ± 0.11	0.035

Table 1. Comparison of mRNA expression between no RTK amplification/ no RAS alteration group and RAS alteration group. 16 of PI3K/AKT and MAPK pathway-related genes showed significant differences between the 2 groups.





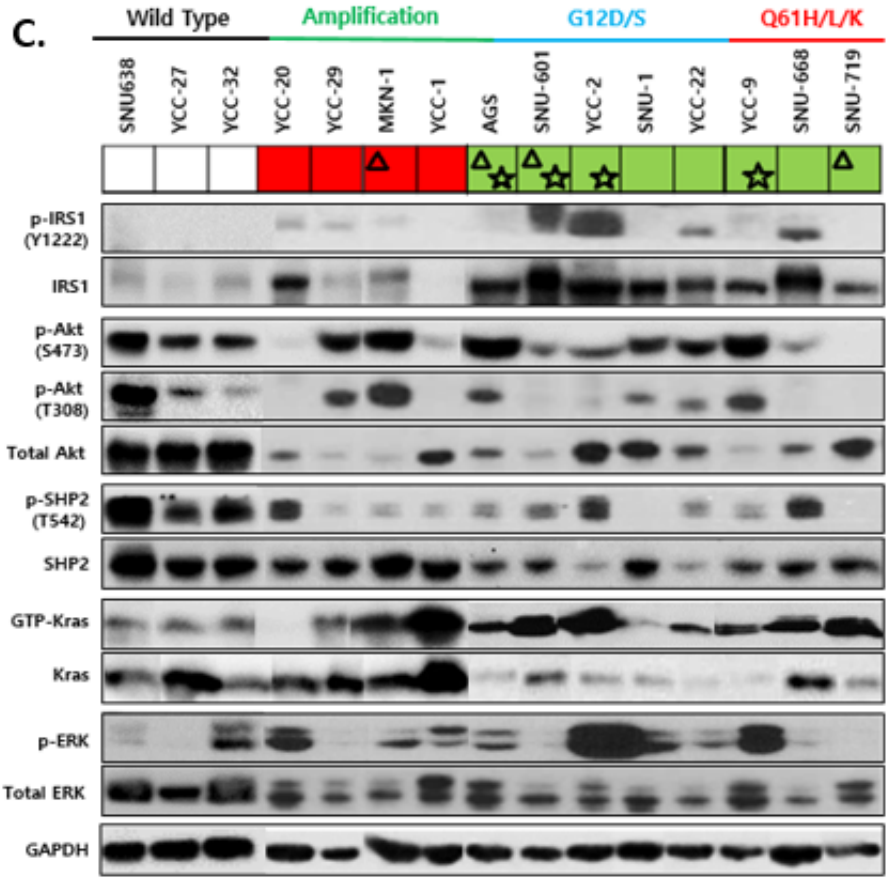


Figure 3. RTK and downstream protein expression in the RAS amplification and mutation group. (A) Phosphorylation and total protein expression of RTKs in RAS alteration cell lines with three of the no RTK amplification/ no RAS alteration cell lines. (B) Effects of PIK3CA mutation on RTK expression in KRAS alteration group. (C) Expression of downstream proteins of RTKs; IRS1, AKT, SHP2, KRAS, and ERK. GTP-KRAS was measured by Pull-down assay.

2. Generation of WT and mutant KRAS stable cell lines in SNU-638

In order to identify specific RTK pathway dependency with KRAS alteration, cell line SNU-638 was selected. SNU-638 is known for having no other RTK amplification, PIK3CA mutation, and PIK3R1 mutation. KRAS wild type, G12D mutation, and Q61H mutation genes were transfected into SNU-638 and single cells were selected to create the stable cell lines. All of the stable cell lines were confirmed by Sanger sequencing (Fig. 4A). Flag-tagged exogenous KRAS was detected by immunoblotting. And KRAS activation was observed in G12D and Q61H mutation stable cells by GTP-KRAS pull-down assay (Fig. 4B).

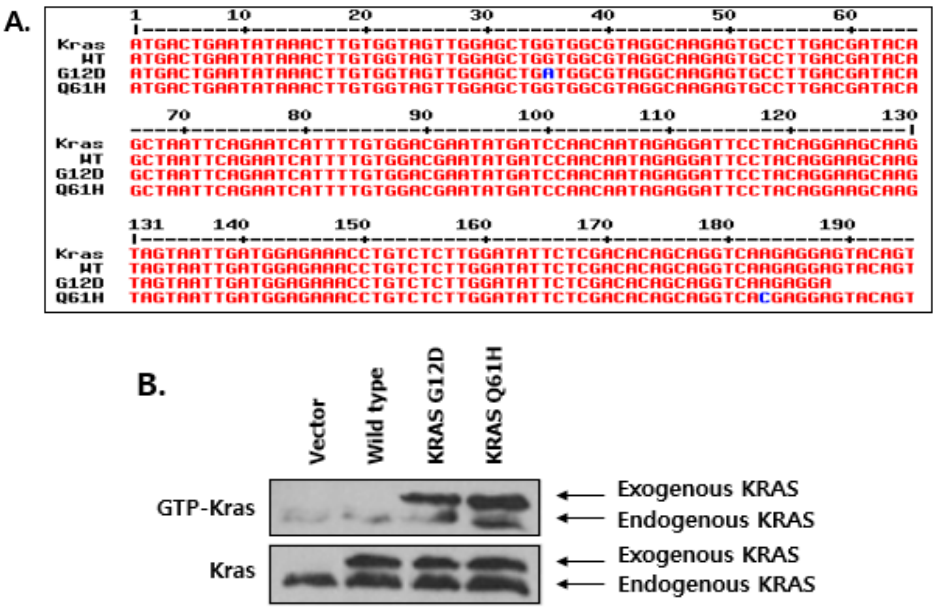


Figure 4. Generation of WT and mutant KRAS SNU-638 stable cell line. (A) Sanger sequencing was peformed to confirm WT and mutant KRAS expression. (B) Detection of KRAS and active KRAS by immunoblotting and GTP-Kras pull-down assay, respectively.

3. Characterization of WT and mutant KRAS SNU-638 stable cell lines

Compared to the empty vector cell line, proliferation activity was increased in WT and mutant KRAS cell lines. Among the three cell lines, Q61H mutant showed the highest proliferation activity (Fig. 5A). To evaluate the clinical significance, clonogenic assay was performed. Colony formation of WT and mutant KRAS cell lines was significantly increased compared to that of the empty vector cell line. G12D mutant cell line showed the highest colony numbers (Fig. 5B). To explore the role of KRAS in GC cell migration, trans-well migration assay was performed. Compared to empty vector, WT and mutant KRAS significantly promoted cell migration. Particularly, G12D mutant showed the highest migration activity (Fig. 5C). In these results, both WT and mutant KRAS induced a stimulation of cell proliferation, colony formation, and cell migration in gastric cancer cell lines.

For finding a selective RTK target of WT and mutant KRAS, we measured protein expression levels of EGFR and IGF1R which showed selectivity on the RAS alteration group in the prior result (Fig. 3A). Interestingly, EGFR was not activated in WT and mutant KRAS cell lines. Nevertheless, protein expression levels of phospho-IGF1R and IGF1R were increased in WT and mutant KRAS compared to empty vector. Moreover, mutant KRAS cell lines were highly phosphorylated on IGF1R (Fig. 6).

Protein expression levels of downstream factors of the IGF1R signaling pathway were measured by immunoblotting. In PI3K/AKT pathway factors, phospho-AKT (T308) was increased in WT and mutant KRAS (Fig. 7A). In the MAPK pathway, KRAS and ERK activation were specifically stimulated in G12D and Q61H mutation cell lines (Fig. 7B). In the result, WT and mutant

KRAS transfection led to activation of IGF1R and its downstream signaling pathway.

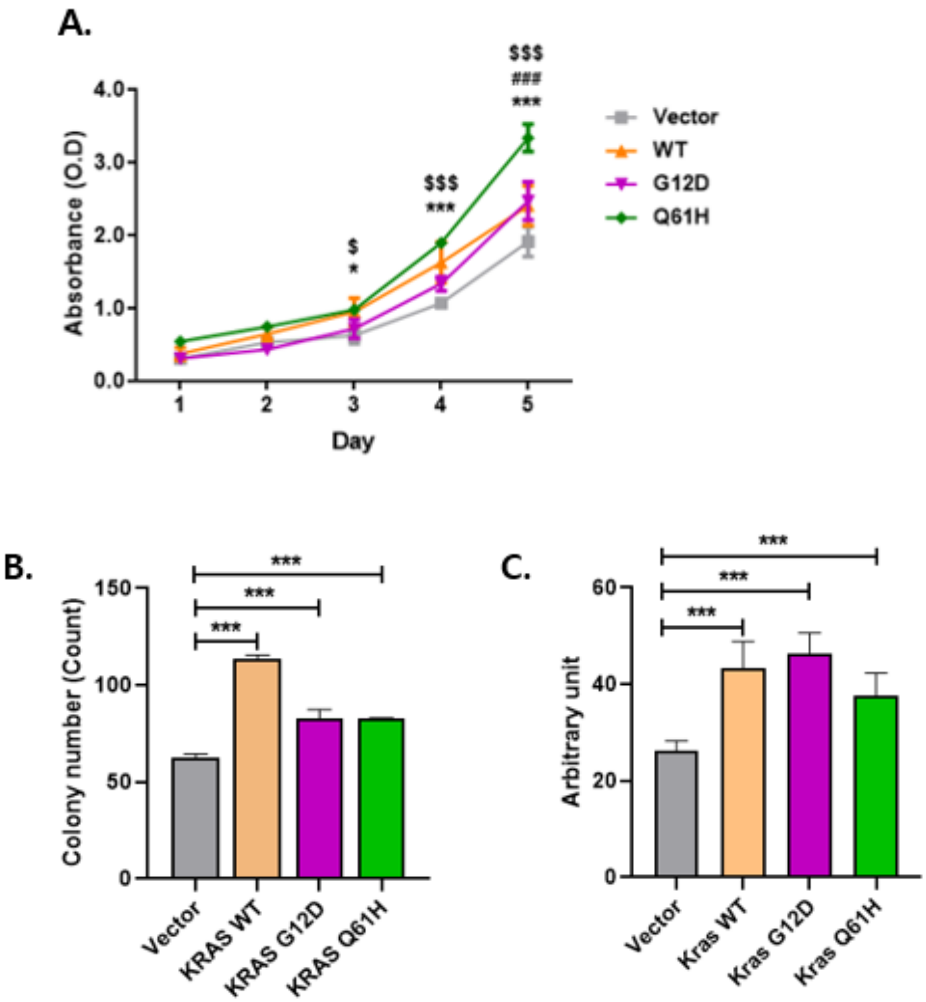


Figure 5. Phenotype changes of WT KRAS and mutant KRAS cell lines. (A) Proliferation was measured for 5 days. *p-value < 0.05 ***p-value <0.001 vector compared to WT. ###p-value <0.001 vector compared to KRAS G12D. $$p$-value < 0.05 $$$$p$-value <0.001 vector compared to Q61H. (B) Colony formation ability was measured by clonogenic assay. ***p-value <0.001 (C) Migration activities were analyzed by trans-well migration assay. ***p-value <0.001

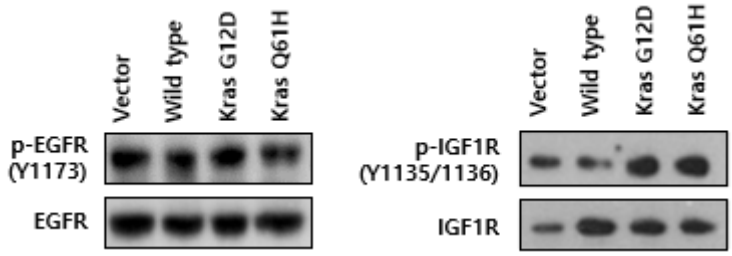


Figure 6. EGFR and IGF1R protein expressions in KRAS stable cell lines. EGFR expression was not increased both total and phosphorylation form. In contrasrt, an increase of phosphorylated IGF1R and IGF1R was observed in WT and mutant KRAS cell lines when compared with vector.

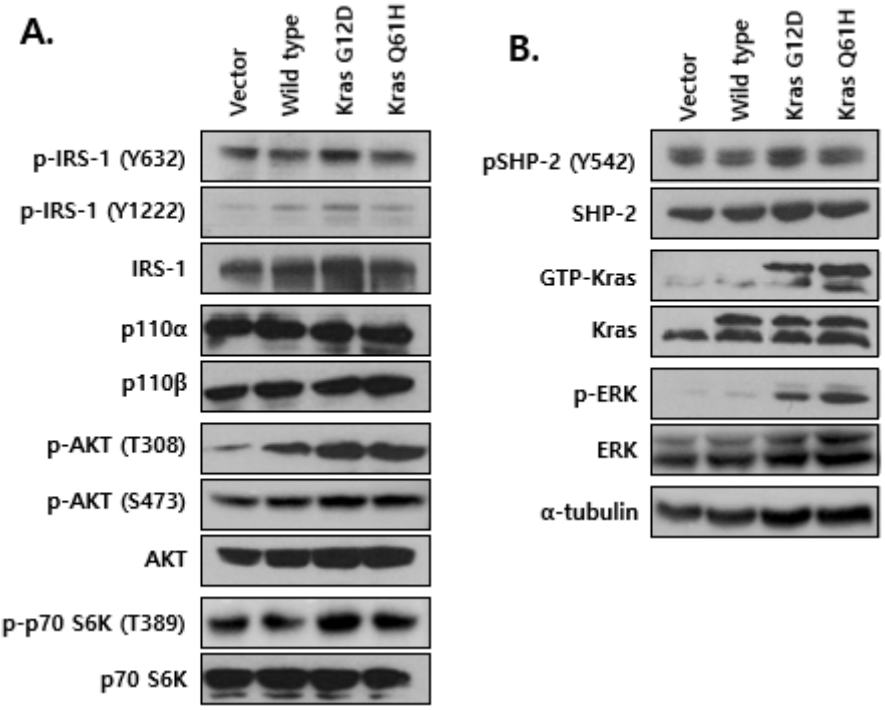


Figure 7. Protein expression of IGF1R downstream signaling factors. (A) PI3K/AKT pathway downstream factors. (B) MAPK pathway downstream factors.

4. Inhibition of IGF1R signaling pathway using small interfering RNA or Xentuzumab

In this part, knockdown of IGF1R by RNA interference and treatment with Xentuzumab were observed to inhibit IGF1R signaling pathway. To examine whether IGF1R is associated with phenotypical changes in WT and mutant KRAS, 3 different siRNAs targeting IGF1R were used for experiments after 72 hrs post-transfection (Table 2). To evaluate the protein expression levels of IGF1R, total IGF1R and phospho-IGF1R was measured by immunoblotting. Compared with the siRNA negative control (siNC), the IGF1R siRNAs not only significantly inhibited the expression of IGF1R, but also inhibited the activation of IGF1R. Only KRAS G12D mutant cell line transfected with siRNA#3 was observed with no inhibition of IGF1R and phospho-IGF1R (Fig. 8).

To obseved the phenotypical changes by siIGF1R transfection, cell viability assay, clonogenic assay, and migration assay were performed. Cell viabilities of empty vector of all siIGF1R groups were promoted up to 150% of siNC. Adversely, cell viabilities were significantly reduced by siIGF1R transfection in KRAS Q61H mutant cell line (Fig. 9A). From results of the clonogenic assay, the empty vector did not show any change after siIGF1R transfection. However, KRAS WT and KRAS mutant cell lines showed less colony formation after siIGF1R transfection, especially KRAS WT cell line was significantly reduced (Fig. 9B). Compared with siNC, all groups of IGF1R siRNA transfection exhibited impairment on cell migration ability but the effect is particularly noticeable in the KRAS G12D mutant group (Fig. 9C). These data are consistent with the different phenotype changes by KRAS WT and mutant induction that were reduced due to IGF1R depeletion.

To investigate the regulation of protein expression by IGF1R inhibition using siRNA, immunoblotting was performed at 72 hrs posttransfection. All four cell lines with siRNA-mediated IGF1R knockdown had attenuated phospho-IGF1R and IGF1R expression. Downstream p-AKT was not inhibited by knockdown of IGF1R, however, pERK was down-regulated in the KRAS WT and Q61H mutant cell line (Fig. 10).

To determine the effect of Xentuzumab treatment on cell viability, cell lines were treated with a concentration of 0.01, 0.1, 1, 10, and 100 μg/ml for 5 days. In contrast to vector, KRAS WT and KRAS mutants had noted declines in cell viability. Even though the proportion of inhibition was about 10%, it might be a remarkable inhibition rate for Xentuzumab which is a cytostatic drug (Fig 11A). Compared to vector, colony numbers were significantly decreased in KRAS WT and mutants cell lines (Fig. 11B). Migration assay was performed with 100 μg/ml of Xentuzumab. In KRAS G12D mutant cell lines, migration abilitiy was inhibited by Xentuzumab treatment (Fig. 11C). Xentuzumab treatment also showed inhibition on phenotype changes of both WT and mutant KRAS cell lines as well.

To analyze the protein expression of phospho-IGF1R and IGF1R, Xentuzumab was added to all cell lines at 1 hr, 4 hrs, and 24 hrs. No siginificant changes were observed in the protein expression of IGF1R. Vector and KRAS WT cell lines showed inhibition of phospho-IGF1R at 1hr and 4 hrs timepoints. At 24 hrs, phospho-IGF1R has recovered their expression. In KRAS G12D mutant cell line, similar phospho-IGF1R expression was observed at every timepoints. On the other hand, phospho-IGF1R levels of KRAS Q61H were inhibited at all timepoints (Fig. 12A). As shown in the occurrence of phospho-IGF1R recovery

at 24 hrs timepoint, downstream signaling factors were not down-regulated. Interestingly, KRAS Q61H cell line showed no significant suppression of downstream factors even when p-IGF1R was continually inhibited (Fig. 12B).

Inhibition of the IGF1R signaling pathway showed suppression of the phenotype; however, the efficacy was not dramatically high. Many factors could be the reason for the disruption of efficacy. Ligand reproduction might be one possible reason. For the evaluation of IGF1R ligand expression, siIGF1R#1 was used for experiments which showed the highest ihhibition of IGF1R and p-IGF1R expression in previous result. When IGF1R was inhibited by siRNA#1, mRNA expressions of KRAS G12D cell line and KRAS Q61H cell line were promoted in IGF-1 and IGF-2, respectively (Fig. 13A). In Xentuzumab treatment, IGF-1 mRNA expression increased in all type of cell lines except KRAS Q61H, and IGF-2 mRNA expression increased in all type of cell lines except KRAS WT. Furthermore, mRNA expression levels are 2-fold higher with Xentuzumab treatment than when IGF1R inhibited by siRNA#1. (Fig. 13B).

With the IGF1R inhibition by siRNA, IGF-1 secretion was increased in KRAS WT and KRAS mutants (Fig. 13C). In the Xentuzumab treatment group, there were no significant changes in KRAS WT and KRAS mutants. However, the vector showed a high secretion of IGF-1 after Xentuzumab treatment. This might be one of the reasons that Xentuzumab mono-therapy was not effective for the empty vector (Fig. 13D).

Moreover, Kras activation occurred in KRAS Q61H when knockdown by siRNA transfection of IGF1R. This might be the explanation of dephosphoryaltion of IGF1R at 24 hrs but downstream signaling still being

activated at the same time (Fig. 13E).

SiRNA	***Sequencing (5'-3')***
IGF1R-siRNA#1	Sense: GAAGAAUCGCAUCAUACUA
	Antisense: UAUGAUGAUGCGAUUCUU
IGF1R-siRNA#2	Sense: CUGUGAACCCGGAGUACUU
	Antisense: AAGUAGUCCGGGUUCACAG
IGF1R-siRNA#3	Sense: CUGGAUUUCUACAGAUCAU
	Antisense: AUGAUCUGUAGAAAUCCAG

Table 2. Nucleotide sequences of siRNAs targeting IGF1R

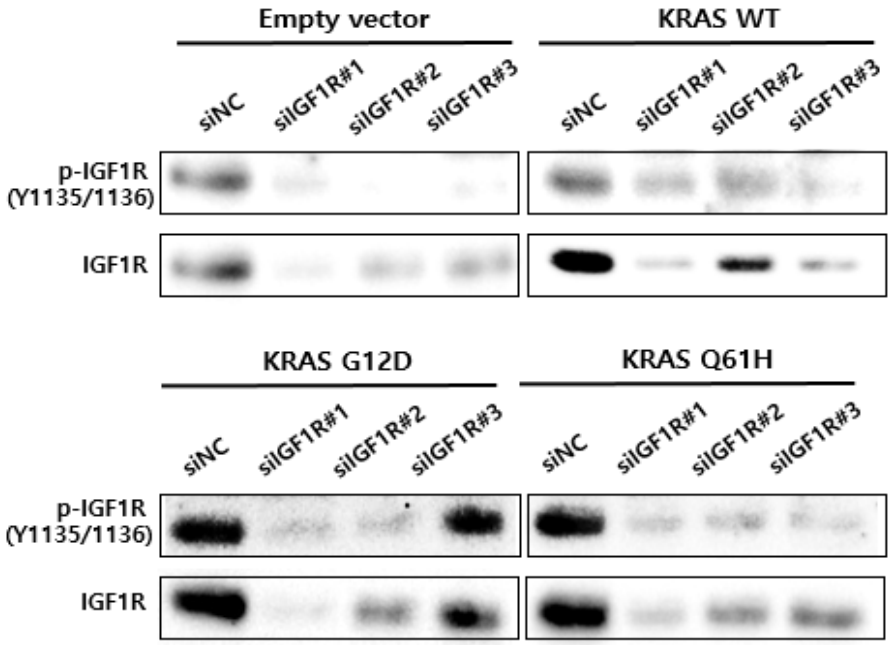
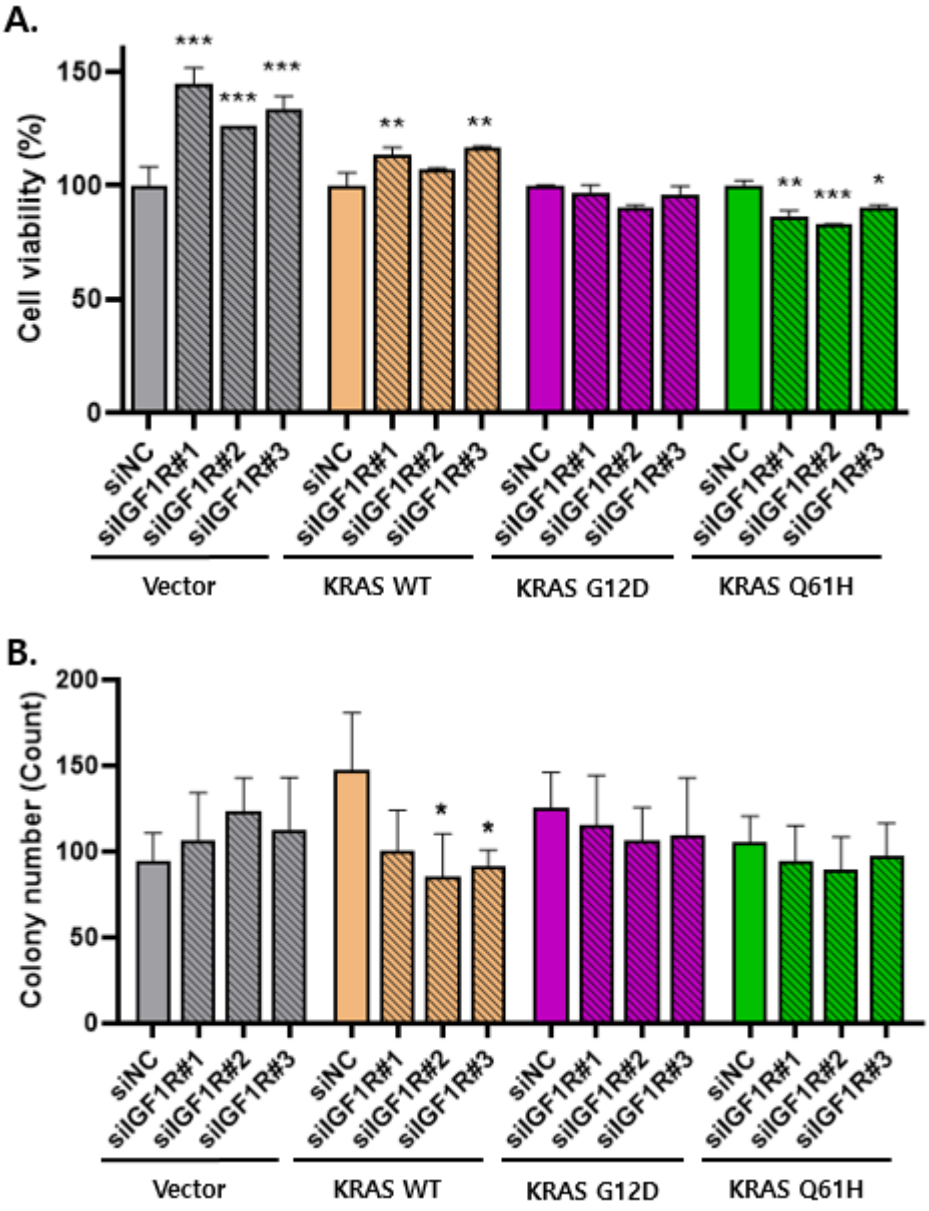


Figure 8. Suppression of siRNA on the IGF1R level in WT and mutant KRAS SNU-638 stable cells. IGF1R protein expression, as indexed by immunoblotting, was reduced at cell harvest in 3 siRNA samples collected 72 hrs post-transfection.



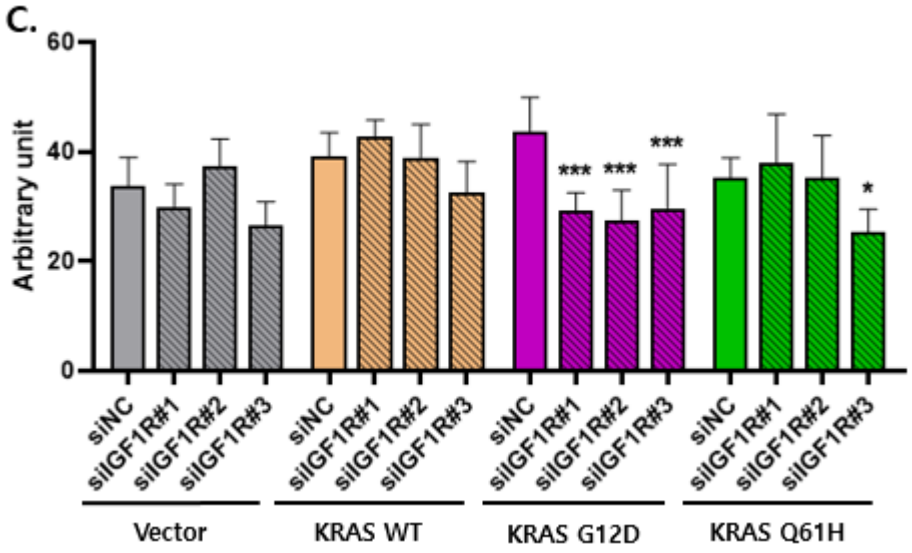


Figure 9. Phenotype changes of knockdown IGF1R. (A) Colony formation ability was evaluated by Clonogenic assay. The number of colonies was counted after 10 days posttransfection. (B) Migration assay of 4 cell lines using a transwell system. *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$ compared with siNC.

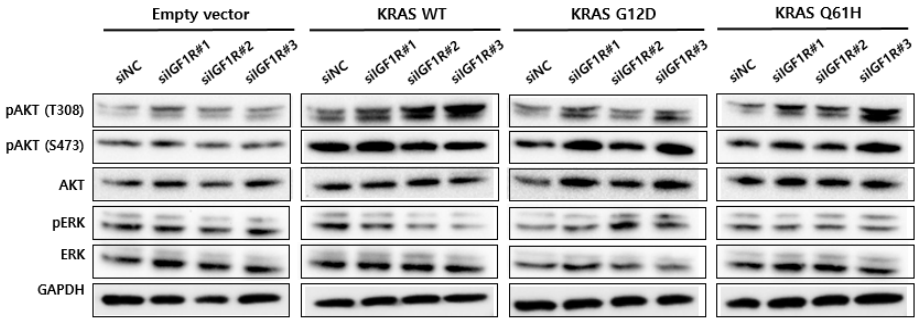
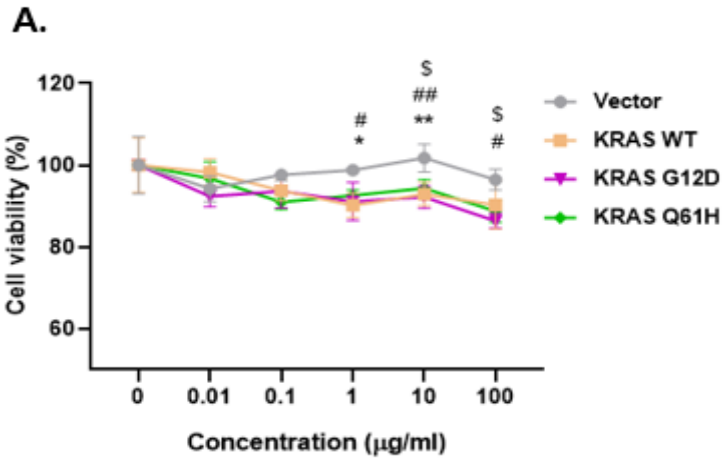
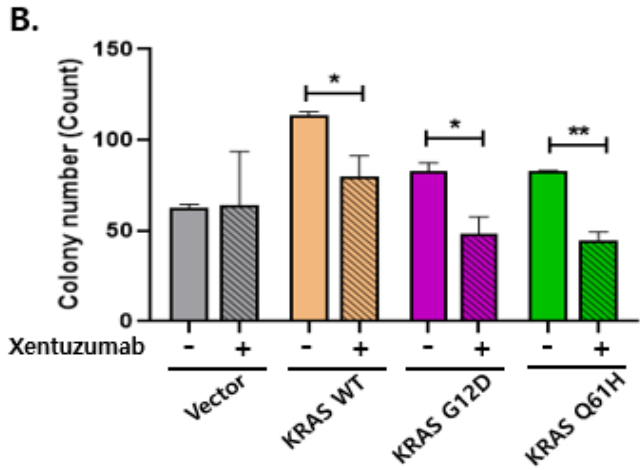


Figure 10. Protein expression changes by IGF1R siRNA transfection. At 72 hrs posttransfection, IGF1R, and p-IGF1R protein expression were suppressed in all groups.





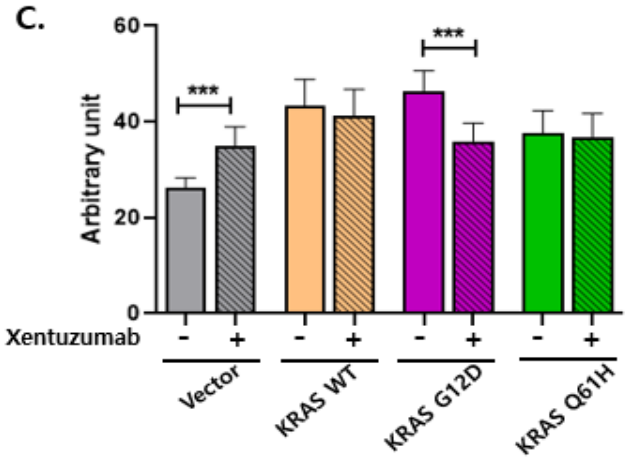


Figure 11. Phenotype changes of IGF1R signaling inhibition by Xentuzumab (A) Cell viability of Xentuzumab was evaluated by CCK-8 assay. **p*-value < 0.05, ***p*-value <0.01 vector compared to WT. #*p*-value < 0.05, ##*p*-value <0.01 vector compared to KRAS G12D. $*p*-value < 0.05 vector compared to Q61H. (B) Colony formation ability was evaluated by Clonogenic assay. The number of colonies was counted after 10 days of Xentuzumab treatment. (C) Cell migration was measured after 24 hrs of Xentuzumab treatment. **p* < 0.05, ***p* < 0.01 ****p* < 0.001 compared with control.

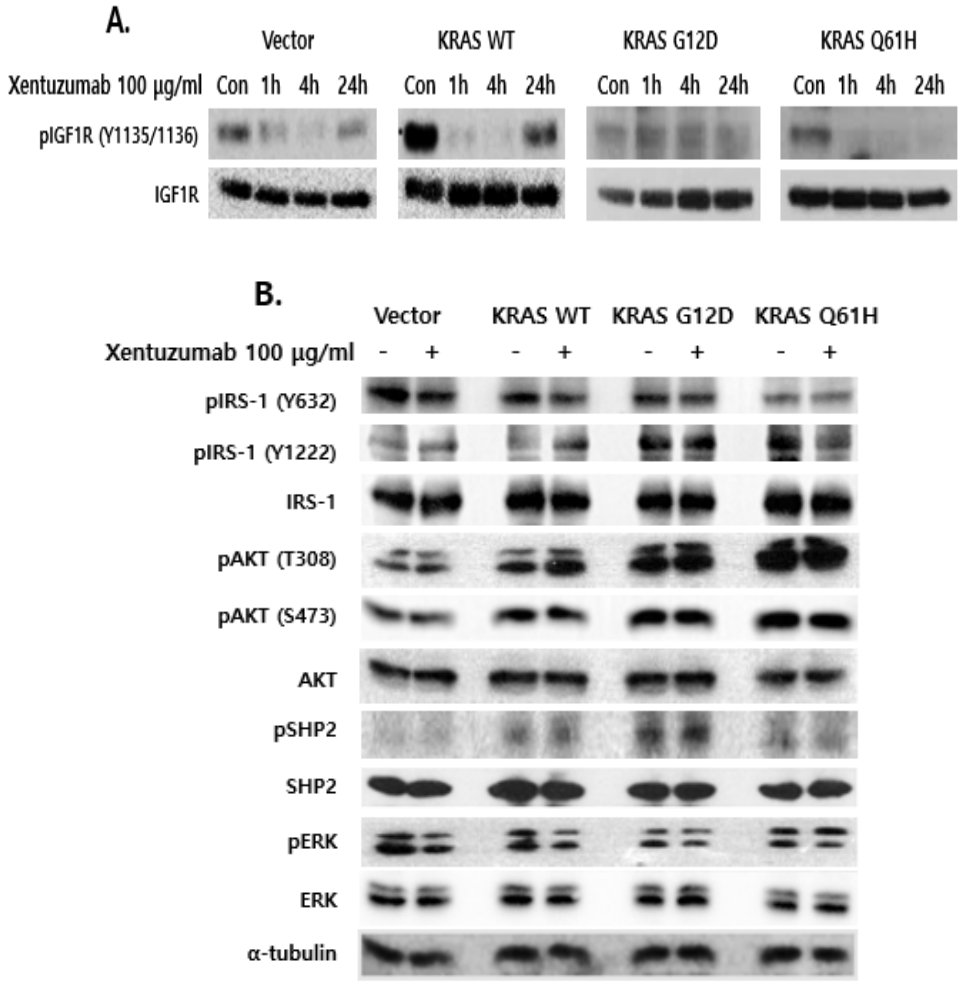
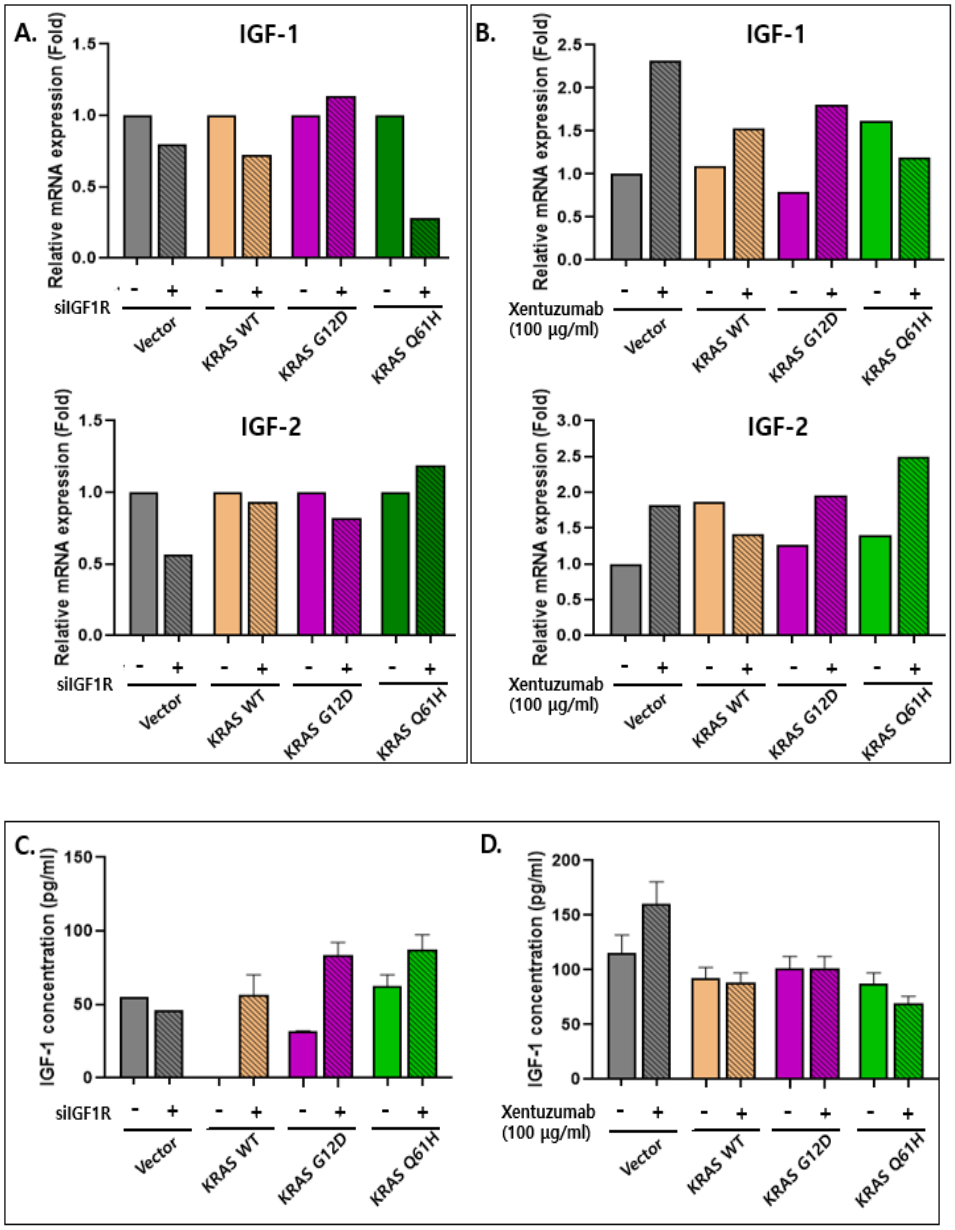


Figure 12. Protein expression changes by IGF1R signaling pathway inhibition using Xentuzumab. (A) Inhibition of phosphorylated IGF1R expression was observed with time-dependent Xentuzumab treatment. (B) Protein expression changes in downstream factors of IGF1R observed at 24 hrs after the treatment of Xentuzumab.



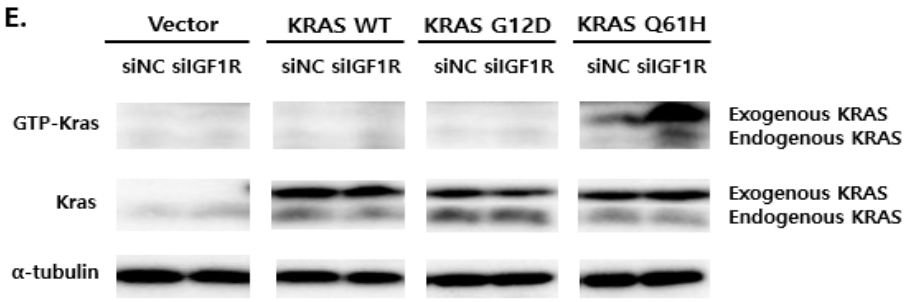


Figure 13. Candidate Interference factors of efficacy when inhibition of IGF1R signaling pathway. (A) mRNA expression levels of IGF-1 and IGF-2 were measured by real-time qPCR with knockdown by siRNA of IGF1R. (B) inhibition by Xentuzumab. (C) secreted IGF-1 concentration was analyzed by ELISA with knockdown by siRNA of IGF1R. (D) inhibition by Xentuzumab. (E) GTP-Kras was evaluated by GTP-Kras pull-down assay with knockdown by siRNA of IGF1R.

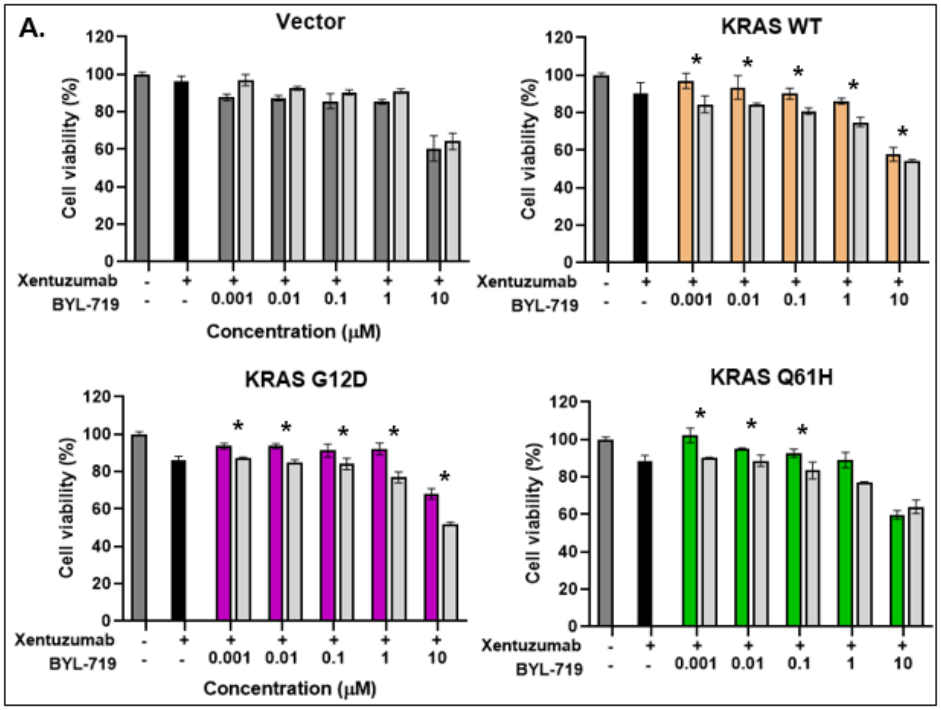
5. Enhancement of IGF1R signaling pathway inhibition using a combination of the downstream signaling pathway in IGF1R

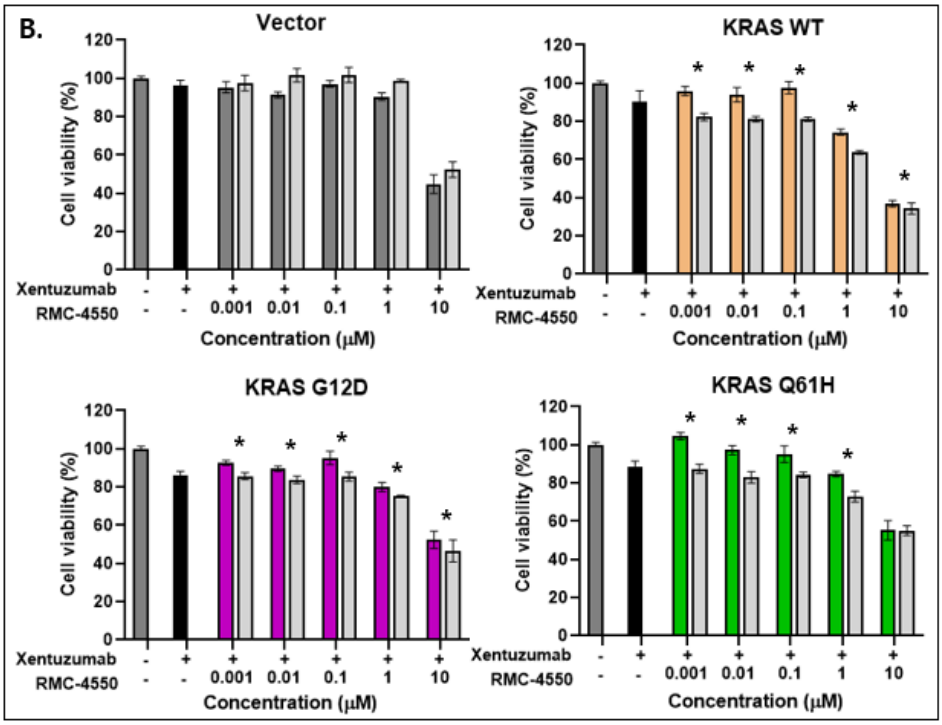
From the previous results, inhibition of IGF1R alone was not completely inhibited downstream of IGF1R signaling pathway. To enhance IGF1R signaling pathway inhibition, both p110α inhibitor (BYL-719) and SHP2 inhibitor (RMC-4550) was combined with Xentuzumab. Except for empty vector (antagonistic effect), the other three cell lines showed a synergistic effect with the combination of BYL-719 (Fig. 14A) and RMC-4550 (Fig. 14B). Combination index (CI) values of KRAS WT and KRAS G12D also indicated synergism with both combination treatments with BYL-719 and RMC-4550. In KRAS Q61H, most of the lower concentrations showed synergism except at the highest concentration (combination with BYL-719 at 1 μM and 10 μM and RMC-4550 at 10 μM) (Fig. 14C).

In addition, the vector showed that pAKT (S473) was increased when Xentuzumab was treated in combination with BYL-719, and pERK was increased when combined with RMC-4550. Combination treatment of other three cell lines showed that downstream signaling was suppressed similar to their single treatment (Fig. 15).

In an attempt to assess whether the inhibited cell viability was associated with cell apoptosis, cells were stained with Annexin V and PI. Annexin V positive and PI negative cells were indicative of early apoptosis and Annexin V positive and PI-positive cells were indicative of late apoptosis. As shown in figure 16, cell apoptosis rates in combination treatment of KRAS WT and KRAS mutants were increased compared to empty vector. Particularly, the KRAS WT cell showed 2.12% apoptosis rate in BYL-719 and increased up to 32.53% when

used in combination. Additionally, RMC-4550 mono treatment exhibited 11.42% of apoptosis rate and it was increased to 19.65% when used in combination (Fig. 16).





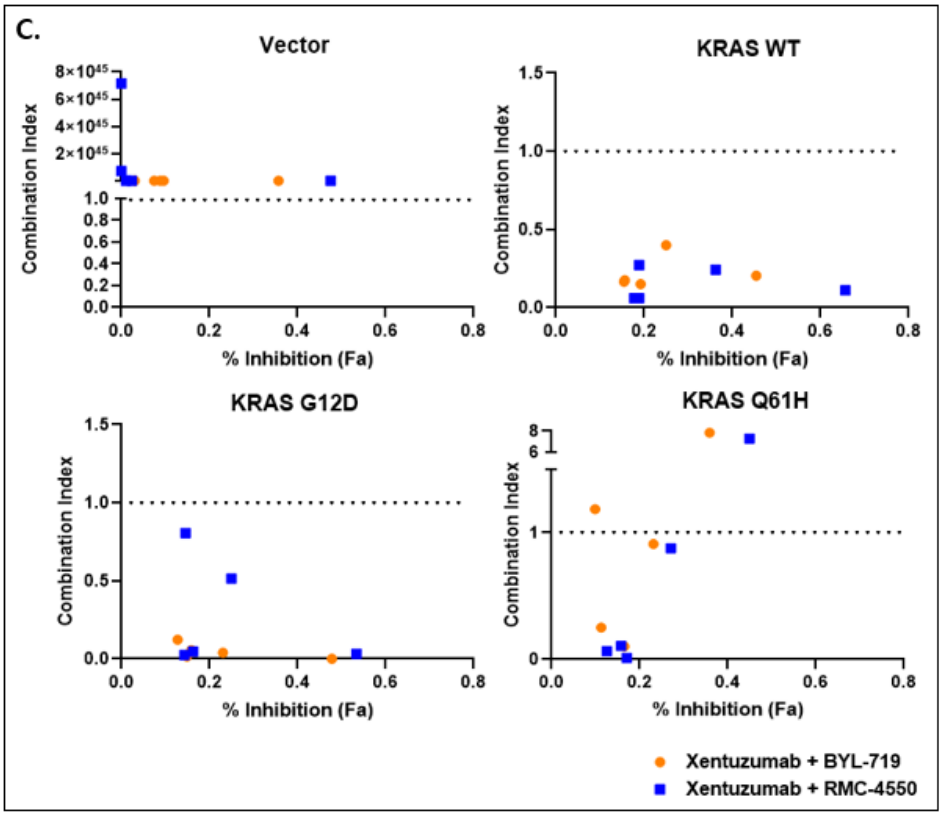


Figure 14. Combination treatment of WT and Mutant KRAS cell lines. (A) Xentuzumab plus BYL-719 (p110α selective inhibitor) combination treatment. * indicates synergism between single and combination treatment. (B) Xentuzumab plus RMC04550 (SHP2 allosteric inhibitor) combination treatment. * indicates synergism between single and combination treatment. (C) Combination Index values were generated by CalcuSyn. CI=1, additivity; CI>1, antagonism; CI<1, synergy.

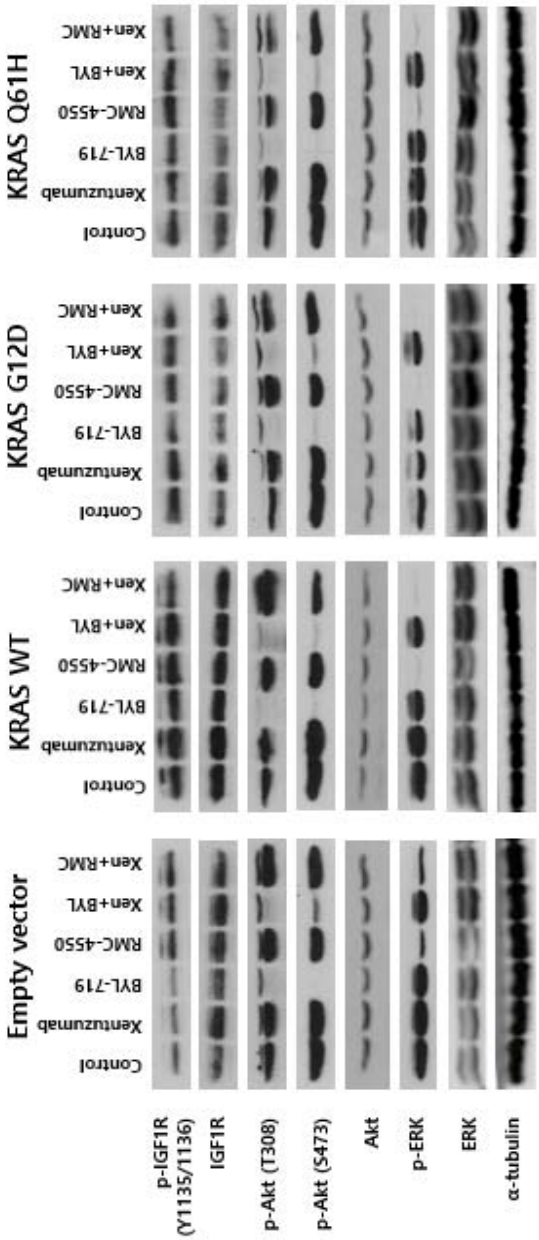


Figure 15. Protein expression in combination treatment. Immunoblotting of four cell lines treated with 100 μg/ml Xentuzumab, 1 μM BYL-719, and 1 μM RMC-4550, alone and in combination versus untreated control for 24 hrs duration.

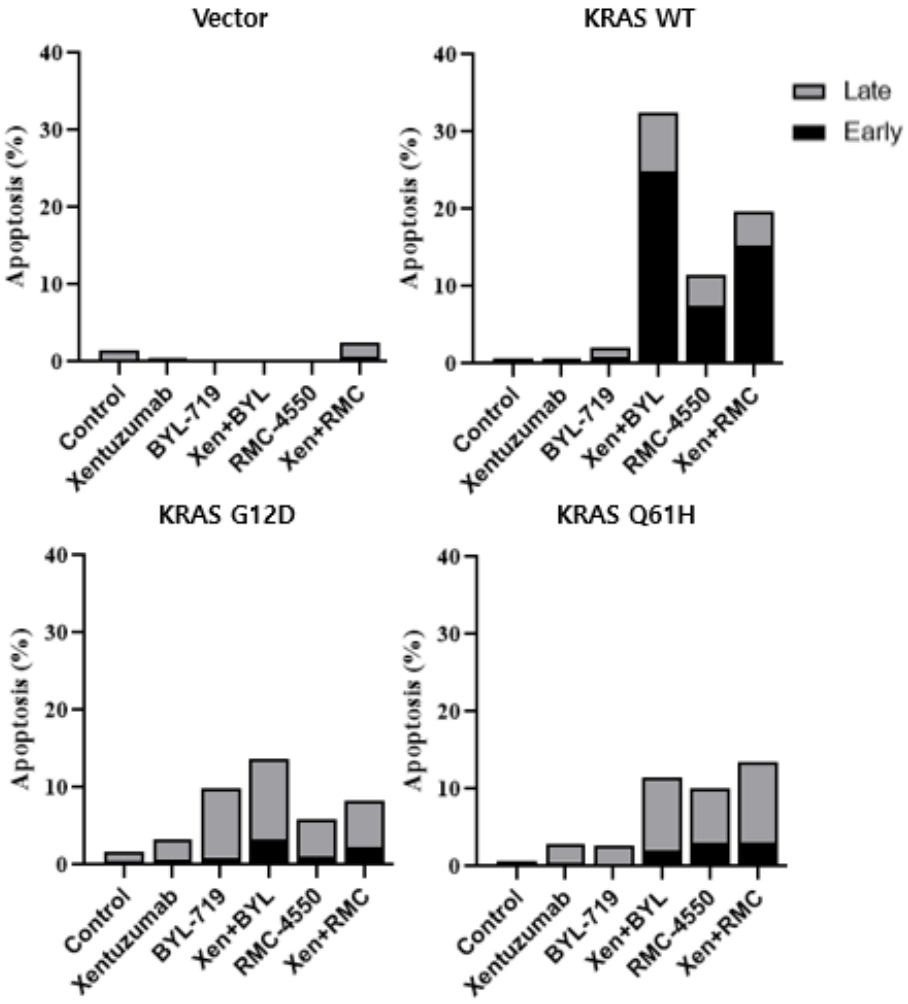


Figure 16. Measurement of apoptosis by flow cytometry through Annexin V-FITC and PI staining. After the treatment of 48 hrs, cells were harvested and stained. In this analysis, early and late apoptotic cells (Annexin V positive) were calculated.

IV. DISCUSSION

This thesis outlines the classification of gastric cancer cell line panel into 3 groups and the study of its distinctive features such as RTK amplification and RAS alteration. In humans, there are 58 known RTKs but only a few are functionally important for cancer[20]. RTKs are activated by specific mutation and amplification, which allows the detection of therapeutic targets and the development of targeted therapeutics[21]. Some representative therapies include cetuximab in EGFR positive colorectal cancer[22], capmatinib in MET exon 14 skipping metastatic non-small cell lung cancer[23] and trastuzumab in HER2 positive breast cancer[24] and gastric cancer[25].

Besides RTK amplification, RAS signaling pathway also plays an important role in transformation and tumorigenesis. Within the RAS family, HRAS, KRAS and NRAS, are among the most altered genes in human cancer[26]. Specifically, in gastric cancer, the importance and incidence of RAS mutations as well as RAS amplification have been reported[27]. RAS alteration and RTK amplification is expressed mutually exclusive so RAS alteration should be dealt with a different approach than RTK amplification[28]. One reasons for the lack of research in gastric cancer is difficulty in developing drugs that target RAS directly. In recent years, drugs have been developed to traverse RAS-specific mutations but they are still in the preclinical phase[29]. Therefore, methods that indirectly target RAS through suppression of various RAS related RTKs or downstream signals are being studied[30]. In gastric cancer, KRAS alteration is a representative response marker that has resistance to cexuximab[31]. Because of the high resistance of RTK targeting inhibitors in

KRAS altered gastric cancer, this study sought to find RTKs that are specifically upregulated. In particular, in the mRNA sequencing analysis, the factors of PI3K/AKT and MAPK pathway were highly expressed, showing that there was no RTK amplification, but RTK activation is potentially expected to occur (Table 1).

Stolze *et al.* made various KRAS mutations in MCF10A human mammary epithelial cell line. According to the study, KRAS was activated with several mutations and there was an activation of EGFR and increase of EGF-dependent growth in KRAS G13D mutation. In addition, various phenotype changes occurred according to the mutation type[32]. In our results, it affects phenotype changes according to the alteration type. In addition, KRAS G12D and Q61H mutations have an IGF1R dependency in our gastric cell line model (Fig.5, 6).

In gastric cancer, studies focused on RAS gene alteration correlate with the MAPK/ERK pathway[33] and KRAS alteration is a biomarker of intrinsic resistance to targeted drugs[34,35]. According to the study, when RAS gene alteration was present, the factors of the MAPK/ERK pathway were activated, and when a cell line with KRAS alterations was treated with PI3K inhibitor, activation of ERK and STAT3 occurred. This is why monotherapy with the PI3K inhibitor was resistant to KRAS altered cell lines and this resistance can be overcome with a combination treatment of STAT3 inhibitor. Thus, it was found that RAS alteration has drug resistance through activation of the RTK downstream pathway. In addition, as a result of analyzing a group of HER2 positive gastric cancer patients who had resistant to trastuzumab treatments, it was confirmed that KRAS alteration was present.

To date, studies on KRAS alteration in gastric cancer revealed that KRAS altered cell lines have resistance to various inhibitors[36,37], and many studies have been conducted to examine the effect that combinations of EGFR inhibitors and other inhibitors have in many different carcinomas[38]. When MEK inhibition was performed in RAS altered cell lines, the PI3K pathway was activated, which may be due to RAS by itself or dependent RTK signaling[39]. In particular, the combination treatment of EGFR inhibitor and MEK inhibitor in gastric cancer cell lines showed an antagonistic effect in cell lines with KRAS mutation[40]. For this reason, it is important to find specific RTK activation by RAS alteration and understand its biological role in gastric cancer.

In our gastric cancer cell line panel and KRAS altered stable cell lines, figure3A and figure 6 showed activation of IGF1R and it had different phenotype changes according to KRAS overexpression and mutation type in KRAS altered cell lines (Fig. 5). In addition, KRAS WT and mutant transfection induced phenotype changes but depletion of IGF1R signaling inhibited those increased phenotype changes (Fig. 9, 11). Similarly, cell proliferation and survival was enhanced in KRAS G12D mutated mouse pancreatic ductal epithelial cells. They also suggested that IGF2 which induces autocrine activation of IGF1R induced the PI3K/AKT signaling pathway. Individual inhibition of MEK or IGF1R was not sensitive, but their combination treatment reduced survival[41]. Our result also indicated that IGF-1 and IGF-2 expression or KRAS activation might be a reason for limited reduction of phenotype changes by IGF1R inhibition (Fig. 13). And our results were also able to enhance IGF1R pathway inhibition in combination treatment

with downstream molecules (Fig. 14)

In fact, many studies investigated the combination treatment of IGF1R inhibitor and MEK inhibitor in KRAS alteration. For instance, the synergistic effect of IGF1R and MEK inhibitor was confirmed in leukemia cell lines with NRAS G12D and KRAS G12D mutations[42]. Basal level of IGF1R activity determined PI3K activity in KRAS mutant lung cancer. Moreover, combinations of IGF1R and MEK inhibitors had a synergistic effect on KRAS mutant lung cancer cell lines, but not in wild type[43]. In our study, IGF ligand inhibitor had a synergistic effect with both PI3K inhibitor and SHP2 inhibitor in KRAS overexpression and mutant cell lines (Fig. 14).

V. CONCLUSION

In this study, the KRAS amplification and mutation have shown to have an important role in gastric cancer biology such as proliferation, colony formation and migration. Moreover, KRAS alteration in gastric cancer activated their downstream signaling pathway through IGF1R activation. This study modulated IGF1R signaling with siRNA transfection and targeted drug, and examined the cell biological changes mediated by KRAS alteration. By inhibiting the IGF1R signaling pathway, reduction of phenotype changes by KRAS alteration was induced. And the possibility of a combination strategy for KRAS alteration in gastric cancer was suggested to enhance of IGF signaling inhibition. These findings add to the practical fundamental insights for the understanding of the IGF signaling pathway in KRAS altered gastric cancer.

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ABSTRACT (IN KOREAN)

위암에서의 IGF signaling pathway의 표적 치료의 효과 증진을 위한 combination strategy

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종양 이질성 (Tumor heterogeneity) 이 높은 위암에서 수용체 티로신 키나아제 (Receptor tyrosine kinase) 증폭을 표적 치료하려는 연구들이 활발하게 진행되고 있다. 위암에서 RAS alteration은 수용체 티로신 키나아제 증폭 못지않게 중요성이 강조되는데 비해, 직접 표적하기 어렵다는 한계가 있어 연구가 부족한 상황이다. 따라서 본 연구에서는 RAS alteration 위암세포주의 특성을 확인하고, 실제 KRAS alteration을 유발하였을 때 표현형 (Phenotype)의 변화와 특정 수용체 티로신 키나아제에 대한 의존성이 있는지 확인 해 보고자 하였다.

먼저 49개 위암 세포주를 Whole exome sequencing 결과를 이용하여 수용체 티로신 키나아제와 RAS alteration의 유전적 상태에 따라 3군으로 분류하였는데, 1) 수용체 티로신 키나아제 증폭군과 2) RAS

alteration군과 3) 두 특성이 모두 없는 군으로 분류하였다. RNA sequencing 결과를 이용하여 RAS alteration군에서 수용체 티로신 키나아제 증폭과 RAS alteration이 모두 없는 군에 비해 PI3K/AKT와 MAPK pathway의 다수의 transcripts 발현이 증가되어 있는 것을 확인하였다. 또한, RAS alteration군에서 EGFR과 IGF1R의 단백질 활성이 증가되어 있는 것을 immunoblotting을 통해 확인 할 수 있었다. 이는 RAS alteration과 수용체 티로신 키나아제의 활성 사이에 연관이 있음을 보여주었다. 이러한 연관성을 확인해보기 위해 KRAS wild type, G12D mutant, Q61H mutant를 안정적으로 발현하는 세포주를 제작하였다. RAS altered 세포주에서 EGFR의 활성에는 차이를 보이지 않았지만 IGF1R의 활성을 증가시키는 것을 immunoblotting을 통해 확인 할 수 있었다. 또한 KRAS alteration에 따른 세포 증식, 종양 생성, 세포 이동 능력 분석을 진행하였다. 정도의 차이는 있었지만 KRAS vector에 비해 KRAS alteration 세포주에서 표현형이 증가되었다. 이렇게 증가 된 표현형을 IGF1R 억제를 통해 조절 가능한지 확인하기 위해 siRNA와 Xentuzumab (IGF-1과 -2 중화 항체 약물)을 이용해 확인하였다. IGF1R 억제 시, 증가 된 세포 증식은 KRAS Q61H mutant에서, 종양 생성은 KRAS wild type에서, 세포 이동 능력은 KRAS G12D mutant에서 각각 유의미하게 감소하였다. IGF1R을 억제 시키면, IGF1R의 활성은 감소하였지만 그 하위 신호의 활성 억제는 미미하였다. 그래서 Xentuzumab과 BYL-719 (PI3Kα 억제제) 또한 Xentuzumab과 RMC-4550 (SHP-2 억제제)과의 병합 처치를 수행하여

하위 pathway의 억제 효과를 확인하였다. 세포 생존력 분석을 통해 병합 처치의 시너지 효과를 확인하였으며, 표적 하위 신호 분자들의 단백질 발현 억제 또한 immunoblotting을 통해 확인하였다. 세포 생존력 분석에서 vector를 제외한 KRAS WT, G12D mutant, Q61H mutant 세포주의 병합 처치에서 시너지 효과를 나타내었다. 세개의 세포주에서 각 약제가 표적하는 pathway의 단백질의 억제가 일어나는 것을 확인 할 수 있었다. 병합 처치의 경우 세개의 세포주에서 세포 사멸을 단일 처치보다 더 증가 시키는 것을 FACS 분석을 통해 확인하였다.

이러한 결과들로 미루어볼 때, 위암에서 RAS alteration이 IGF1R의 활성을 통해 세포의 증식, 종양 생성 및 세포 이동에 있어 중요한 역할을 하는 것을 알 수 있었다. 결론적으로 RAS alteration에 의한 위암 세포의 증식, 종양 생성 및 세포 이동에 대해 IGF1R이 잠재적 표적 분자가 될 수 있고, PI3Kα 및 SHP-2와 같은 하위 신호 분자와의 병행 처치를 통해 그 억제 효과 증진의 가능성을 제시하였다.

핵심되는 말 : 위암, 수용체 티로신 키나아제, KRAS, IGF1R

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