이학석사 학위논문

Decreased Notch1 Activation Improves Response to Cetuximab in Colorectal Cancer Cells

대장암 세포에서 Notch1 활성 억제를 통한 세툭시맙 반응성 개선

울 산 대 학 교 대 학 원

의 과 학 과

최 수 은

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이 논문을 이학석사 학위 논문으로 제출함

2022년 02월

울 산 대 학 교 대 학 원

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최 수 은

최수은의 이학석사학위 논문을 인준함

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Abstract

Background and Purpose Epidermal growth factor receptor (EGFR)-targeted monoclonal antibody therapy is widely used in RAS wild-type metastatic colorectal cancer (mCRC). However, drug resistance represents a clinical challenge in the management of patients with colon cancer. Some studies suggested that Notch activation is related to tumorigenesis and drug resistance in colorectal cancer. However, whether Notch affects cetuximab response or not is still unknown. In this study, we hypothesized that inhibition of Notch1 activation may contribute to improve the effect of cetuximab by regulating AKT phosphorylation.

Methods We applied siRNA and Notch inhibitor DAPT to colorectal cancer cell lines (SNU503 and LS1034) to investigate the effect of Notch1 activation in colorectal cancer. Furthermore, the additive effects of combined cetuximab with Notch1 downregulation were confirmed by Western blotting, colony formation assay, thiazolyl blue tetrazolium bromide (MTT), growth curve, and immunofluorescence (IF) by comparing the results obtained by treatment with cetuximab and DAPT alone or in combination.

Results We first confirmed the importance of Notch1 in colorectal cancer. Notch1 is more activated in colon cancer cell lines (SNU503 and LS1034) than in normal colon epithelial cell line (CCD-18Co). When Notch1 was reduced by siRNA transfection in colorectal cancer cell lines, AKT phosphorylation was significantly decreased. Moreover, Western blotting and MTT assay suggested that pretreated siNotch1 enhanced cetuximab-mediated cell death. In addition, combination treatment of DAPT and cetuximab inhibited cell growth and induced the expression of apoptosis markers (cleaved caspase3 and cleaved PARP) and cell death compared with when cetuximab was treated alone.

Conclusions These results suggest that alternative targeting of Notch1 as a strategy for improvement of cetuximab response in colorectal cancer may be a potential approach.

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Introduction

Colorectal cancer (CRC) had the fourth-highest incidence rate (11.4% of the total cancer cases) and was the second most common cause of cancer-linked deaths in Korea as of 2018[1]. The epidermal growth factor receptor (EGFR) is one of the most important therapeutic targets in metastatic CRC (mCRC) and is commonly overexpressed in several cancers. Cetuximab, a chimeric mouse-human monoclonal antibody, is being widely used in mCRC patients with wild-type RAS. Cetuximab binds the extracellular domain of EGFR, which induces blocking of the downstream of EGFR related to cell proliferation, survival, apoptosis, migration, and tumorigenesis. As a first-line treatment for mCRC, cetuximab is used in combination with chemotherapy such as FOLFOX or FOLFIRI[2]. However, the therapeutic effect of cetuximab is ultimately limited by the occurrence of mutations such as RAS, BRAF, PIK3CA, and PTEN and other mechanisms associated with cetuximab resistance[3]. Hence, there is a need for additional strategies for improving the response to cetuximab treatment in addition to the existing treatment options.

Notch is a transmembrane receptor composed of functional extracellular, transmembrane, and intracellular domains. The Notch pathway is a highly conserved signaling pathway that plays a role in tissue homeostasis, stem cell maintenance, embryogenesis, cell proliferation, or cell fate determination[4]. The human Notch family consists of four receptors: Notch1, Notch2, Notch3, and Notch4. Among them, Notch1 is the most actively studied because aberrant Notch1 activation among human cancers is higher than other Notch members[5]. Notch1 is activated upon interaction with members of the DSL (Delta/Serrate/LAG-2) family or Jagged, which are the transmembrane ligands expressed on the surface of the neighboring cell. Once a Notch ligand binds to an extracellular domain of the Notch receptor, the Notch receptor is cleaved by γ-secretase and releases the Notch intracellular domain (NICD), which moves into the nucleus and

transcriptionally regulates Notch target genes[4]. Some studies have shown that aberrant Notch1 signaling is vital to tumorigenesis in many cancers, including CRC[6-8]. Furthermore, Notch1 is related to chemotherapy resistance[9]. For example, some studies have exhibited that Notch1 is involved in drug resistance towards regorafenib and 5-FU in CRC [10-12]. Additionally, in other cancers, activated Notch1 is known to induce resistance to EGFR tyrosine kinase inhibitors [13, 14]. However, the role of Notch1 in the drug resistance of EGFR inhibitors in CRC has not been well known.

In this study, we identified Notch1 as a potential target gene for improving cetuximab sensitivity in CRC. In two CRC cell lines, one is wild KRAS and the other is mutant KRAS, we studied the effectiveness of Notch1 on AKT phosphorylation related to the EGFR signaling pathway by inhibiting Notch1 activation using siRNA or γ-secretase inhibitor, DAPT. In addition, the effect of Notch1 downregulation combined with cetuximab was observed in terms of cell death and growth inhibition. Intriguingly, Notch1 downregulation was efficient at increasing cetuximab sensitivity by regulating AKT phosphorylation in two CRC cell lines regardless of KRAS mutation status.

Material and Methods

CRC Cell Lines

In this study, a human normal colon epithelial cell line (CCD-18co) was purchased from the American Type Culture Collection (ATCC) and human colon cancer cell lines (SNU503 and LS1034) were obtained from the Korean Cell Line Bank (KCLB). SNU503 is the wild-type KRAS/RAF genotype, and LS1034 is a homozygous A146T KRAS mutant cell line. CCD-18co was maintained in DMEM (Cat #LM 007-07, WELGENE, Gyeongsan, Korea), whereas SNU503 and LS1034 were cultured in RPMI-1640 (Cat #SH30027.01, HyClone, Utah, USA). Both media were supplemented with 10% fetal bovine serum (Cat #16000-044, Gibco, New York, USA) and 1% penicillin-streptomycin (Cat #SH40003.01, Cytiva, HyClone Laboratories, South Logan, Utah, USA). Cells were grown in a humidified incubator containing 5% CO_2 at 37°C.

Western blotting

Cells were harvested using scrapers from cultured dishes and were lysed using Mammalian Protein Extraction Reagent (M-PER) (Cat #78501, Thermo Scientific, Carlsbad, CA, USA), containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Sigma-Aldrich; Merck KGaA) to extract total protein. The protein concentration was determined using a Bradford assay. Cell lysates (15 μg protein/line) were separated by 8–12% SDS-PAGE and transferred onto nitrocellulose membranes using the Trans-Blot® RTA Transfer Kit (Cat #170-4270, Bio-Rad). The blotted membranes were blocked with 5% skim milk in TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature and were incubated overnight with primary antibodies at 4°C, using the following primary antibodies: Notch1 (1:1000, Cat #3268, Cell Signaling Technology), activated Notch1 (NICD) (1:1000, Cat #ab8925, Abcam), AKT (1:1000, Cat #9272, Cell Signaling

Technology), p-AKT (1:1000, Cat #4060, Cell Signaling Technology), EGFR (1:1000, Cat #2232, Cell Signaling Technology), p-EGFR (1:1000, Cat #2234, Cell Signaling Technology), and Actin (1:30000, Cat #A3854, Sigma-Aldrich, Merck KGaA). The primary antibodies were washed with TBST, and the protein was incubated with HRP-conjugated goat anti-Rabbit (1:10000, Cat #31460, Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Proteins were visualized with a detection system (LuminoGraph II, Cat #WSE-6200, ATTO Corporation) and using a controlling software (ImageSaver 6; Version 2.7.2; ATTO Corporation).

Quantitative real-time PCR

For the mRNA analysis, total RNA was extracted using the QIAzol Lysis Reagent (Cat # 79306, Qiagen GmbH) and the RNeasy Mini Kit (Cat # 74106, Qiagen GmbH) according to the protocol provided by the manufacturer. The concentration and quality of the extracted RNA were measured using Nanodrop 2000 (Thermo Fisher Scientific, Inc.). cDNA was generated by reverse transcription of total RNA using the ReverTra Ace™ qPCR RT Master Mix (Toyobo Life Science) by incubating at 37°C for 15 min, heating at 50°C for 5 min, and cooling to 4°C. The transcripts were quantified by qPCR using the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) with SYBR Green qRT-PCR Master Mix (Solis BioDyne). The primer sequences were as follows: GAPDH forward, 5'-GAGTCAACGGATTTGGTCGT-3' and reverse, 5'- TTGATTTTGGAGGGATCTCG-3' and Notch1 forward, 5'-GGGTACAAGTGCGACTGTGA-3' and reverse, and 5'-CACGTAGCCACTGGTCATGT-3'.

siRNA transfection

Small interfering RNAs (siRNA) specific to either NOTCH1 (NOTCH1-siRNA) or a scrambled sequence (scrambled-siRNA) were prepared by Bioneer (Gyeonggi-do, Korea).

The siRNA target sequences were as follows: NOTCH1-siRNA sense, 5' CCAACCCAUGUCUGAACCA-3'; antisense, 5'-UGGUUCAGACAUGGGUUGG-3' and scrambled-siRNA sense, 5'-UCCCAGAUAGAGACUUCAATT-3'; and antisense, 5'-UUGAAGUCUCUAUCUGGGATT-3'. Cells were transfected with si-RNA (125 nM) using Lipofectamine 3000 (Thermo Fisher Scientific). The efficiency of siRNA-based interference with NOTCH1 synthesis was assessed by qRT-PCR and Western blot analyses.

Drug treatment

The gamma-secretase inhibitor compounds DAPT (D5942, Sigma-Aldrich, USA) and cetuximab (Merck KGaA) were used in this study. Cells were treated with drugs in 2% FBS media.

Cell Viability Assay

Thiazolyl blue tetrazolium bromide (MTT) assay was used to assess the effect of cetuximab combined with Notch1 silencing and cetuximab combined with a Notch inhibitor on cell viability. Cells were seeded in a 96-well plate at a density of 5000 cells per well. After 24 h of downregulation of Notch1 using siRNA and inhibitor, cetuximab was treated for 24 h. MTT (2.5 mg/ml) (Cat # M5655, Sigma Aldrich) was added to each well, and the plates were incubated for 4 h. Supernatants were eliminated from the wells, and the dye was solubilized in DMSO at 100 µl per well. Absorbance was measured at 550 nm using a Sunrise microplate reader (Tecan Group, Ltd.).

Colony-Formation Assay

A colony formation assay was used to assess the effect of cetuximab combined with a Notch inhibitor on cell proliferation. Cells were seeded in a 6-well plate at a density of 2000 cells per well. The cells treated with cetuximab and DAPT were incubated for 3 weeks. The cells

were fixed and dyed with 0.2% crystal violet containing methanol for 20 min at room temperature (22 ± 2 °C). The number of colonies (diameters > 120 μm) was counted using the Oxford Optronix GelCount™ system (v1.1.2.0; Oxford Optronix).

Growth curve

The growth curve was carried out in the SNU503 and LS1034 cell lines to determine the effect of cetuximab combined with the Notch inhibitor on cell growth inhibition. Cells were seeded in a 24-well plate at a density of 5000 cells per well. The cells treated with cetuximab and DAPT were incubated for 10 days and stained every 2 days. The cells were fixed in 10% neutral buffered formalin for 20 min at room temperature and then stained with 0.1% crystal violet for 20 min at room temperature. The dye extraction was carried out using 10% acetic acid, and the absorbance was measured at 595 nm using a Sunrise microplate reader (Tecan Group, Ltd.).

Immunofluorescence (IF)

Cells were fixed with freshly prepared ice-cold 10% neutral buffered formalin for 10 min at 4 and then exposed to 0.25% Triton X-100 in PBS for 10 min for permeabilization. Nonspecific antibody-binding sites were blocked by incubation in 1% BSA for 30 min and were then incubated for 1 h with Anti-activated Notch1 (Abcam; #ab8925) antibodies at a 1:200 dilution in PBS with 1% BSA. After incubation with Fluorescein Goat Anti-rabbit IgG (H+L), the cells were incubated with a secondary antibody (Invitrogen; #F-2765) for 30 min in a dark chamber. They were subsequently stained with Fluoroshield Mounting Medium with DAPI (Abcam; #ab104139) for 1 min at room temperature. Image acquisition at 20× magnification was performed using the EVOS FL Auto fluorescence microscope (Thermo Fisher Scientific, Inc.). The positive staining cells were counted using ImageJ software (v1.53a; National Institute of Health, USA).

Statistical Analysis

All data are presented as mean ± standard deviation (SD) from three independent experiments. The statistical significance of differences between two groups was analyzed with the unpaired Student's *t*-test, and one-way analysis of variance (ANOVA) was performed for experiments involving two or three factors. A *p*-value of <0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed by GraphPad Prism 6.0 software (GraphPad Software, Inc.).

Results

Notch1 activation is more commonly observed in colon cancer cells than in normal cell

To examine whether Notch1 is significantly activated in colon cancer cells compared with normal colon cells, we checked the Notch1 and NICD protein levels in the normal colon epithelial cell line CCD-18co and the colon cancer cell lines SNU503 and LS1034 by Western blotting. Notch1 has three domains, including an extracellular domain, a transmembrane domain, and an intracellular domain. Among them, when Notch1 is activated, the intracellular domain plays a key role in regulating the target genes of Notch1[15]. Our results showed that SNU503 and LS1034 have comparatively higher expressions of NICD (Notch1 intracellular domain), the product of Notch1 activation, compared with CCD-18co, independently of Notch1 (Notch1 full length and Notch1 transmembrane domain) protein expression levels (Fig 1A). We also assessed the expression levels and localization of NICD in normal colon cells and colon cancer cells using immunofluorescent staining. Once Notch1 is activated, the NICD moves to the nucleus and then regulates the transcription of Notch1 target genes. Immunofluorescent staining showed relatively high NICD expression in the nucleus of SNU503 and LS1034 compared to that of CCD-18co (Fig 1B). These results suggest that Notch1 activation occurs more frequently in colon cancer cells than in normal cells.

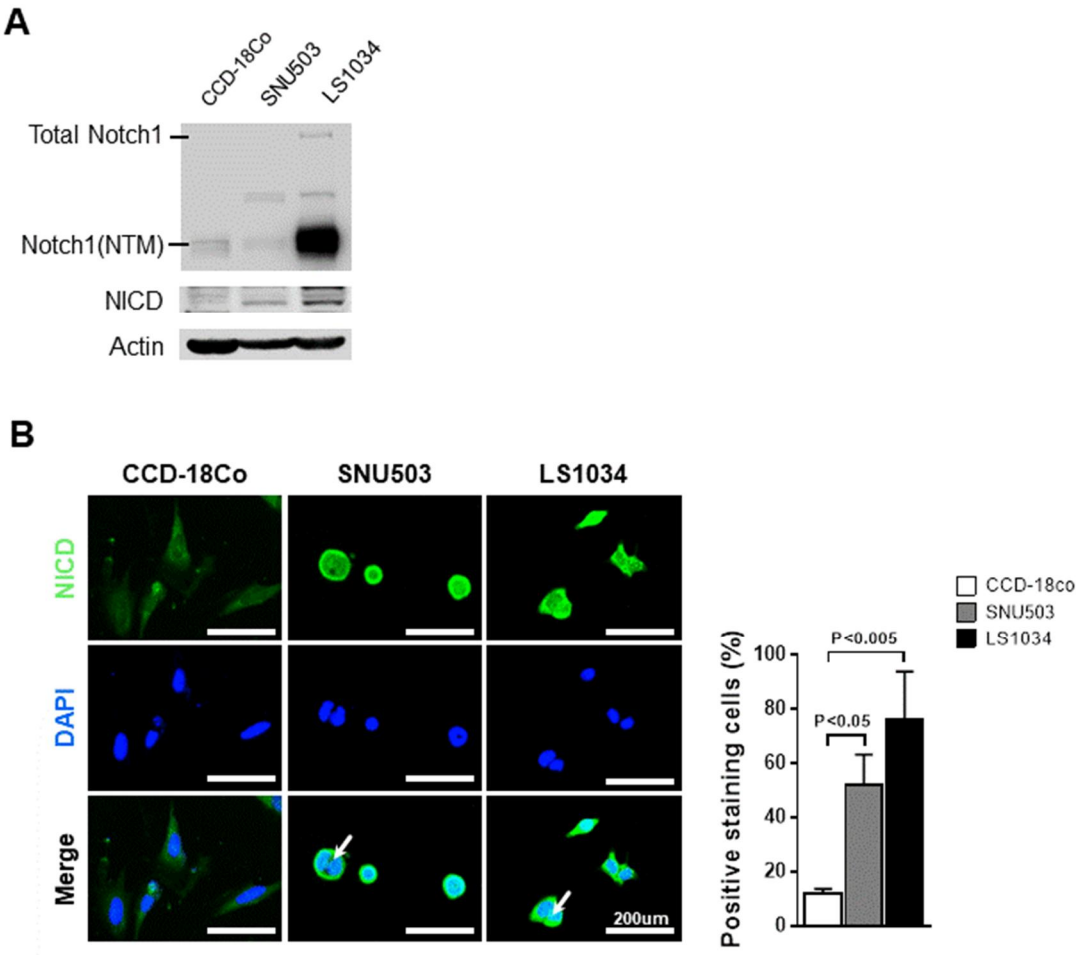


Figure 1. Notch1 activation in colon normal epithelial cells and colon cancer cells

(A) Notch1 and activated Notch1 (NICD) expression in colon cancer cell lines (SNU503 and LS1034) and normal colon cell line (CCD-18Co). The protein levels of Notch1 and NICD were detected using Western blotting. (B) NICD is stained by immunofluorescence in CCD-18Co, SNU503, and LS1034. Scale bar: 200 μm (left). Activated Notch1-positive cells are indicated by a bar graph (n = 3), and values are presented as mean ± SD (right).

*Arrowheads: NICD nuclear translocation.

NICD, Notch1 intracellular domain; NTM, Notch1 transmembrane domain.

AKT phosphorylation is inhibited by Notch1 siRNA transfection

Aberrant activation of the MAPK pathway and the PI3K pathway is associated to generate cetuximab resistance[16, 17]. To identify whether Notch1 activation is directly involved in AKT phosphorylation, we performed Western blotting for phosphorylated AKT (p-AKT) in SNU503 and LS1034 knocked down Notch1. The p-AKT protein level was decreased in SNU503 transfected by Notch1 siRNA compared to the sample with scrambled siRNA (sc siRNA) (Fig 2A). To assess the efficiency of siRNA-based interference with NOTCH1 synthesis, we tested mRNA expression levels using qRT-PCR. When SNU503 cells were transfected with sc siRNA or Notch1 siRNA for 24 h, the Notch1 siRNA-transfected cells showed a 0.4-fold decreased Notch1 mRNA expression relative to the control ($p = 0.0011$) (Fig 2B). Similarly, after LS1034 cells were transfected with sc siRNA or Notch1 siRNA for 24 h, the cells were assessed for p-AKT protein level using Western blotting. When the Notch1 protein level was reduced by siRNA transfection, p-AKT was also decreased in LS1034 (Fig 2C). However, the effect of Notch1 knockdown on AKT phosphorylation was less in LS1034 than in SNU503. The Notch1 siRNA-transfected cells showed a 0.8-fold decreased Notch1 mRNA expression level relative to the control ($p < 0.0001$) (Fig 2D). Additionally, we also checked the alteration of the MAPK pathway according to the Notch1 knockdown in SNU503. Western blotting represented that Notch1 siRNA abolished not only p-AKT but also p-ERK1/2 and p-MEK1/2, which are key intermediaries in the MAPK pathway (Appendix figure 1). In this study, however, we focused on AKT phosphorylation because there were many references to the relationship between Notch1 and AKT phosphorylation in CRC, such as Notch1-related PTEN inactivation[18-20].

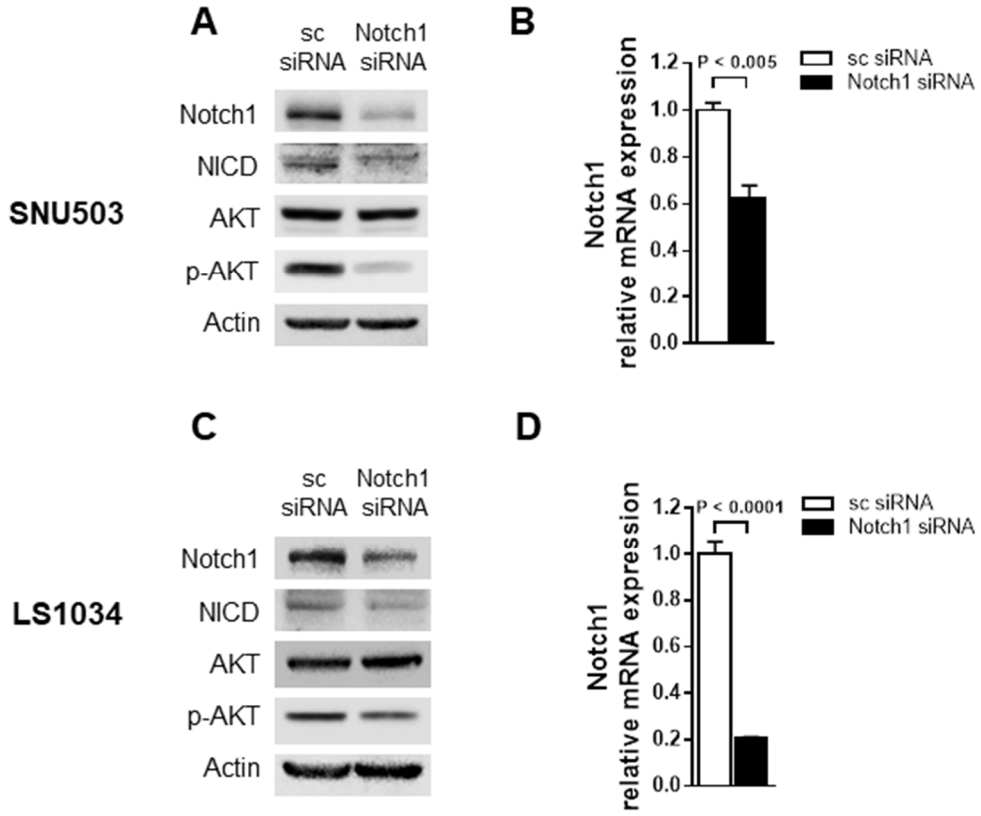


Figure 2. The effect of Notch1 knockdown on AKT phosphorylation in SNU503 and LS1034 cells

Notch1 activation was inhibited by siRNA transfection in colorectal cancer cell lines. (A) Western blot analysis of Notch1, NICD, AKT, and p-AKT expression in negative control- or siRNA-treated SNU503. (B) To identify the efficiency of siRNA-based interference with NOTCH1 synthesis, the Notch1 mRNA expression level was measured by qRT-PCR in SNU503. (C) Western blot analysis of Notch1, NICD, AKT, and p-AKT expression in negative control- or siRNA-treated LS1034. (D) To identify the efficiency of siRNA-based interference with NOTCH1 synthesis, the Notch1 mRNA expression level was measured by qRT-PCR in LS1034. The results are expressed as the mean ± SD, n = 3.

Knockdown of Notch1 increases cetuximab sensitivity in drug-induced cell death

To determine whether the knockdown of Notch1 affects the response of cetuximab, we performed an MTT assay and Western blotting in colon cancer cells. SNU503 and LS1034 cells were transfected with Notch1 siRNA or sc siRNA and then treated with cetuximab for 48 h. The MTT assay represented that combined Notch1 siRNA and cetuximab treatment led to significantly greater cell death than that with cetuximab treated alone in both SNU503 and LS1034 (Fig 3A and B). These results suggest that knockdown of Notch1 causes increased cetuximab-induced cell death. To confirm cetuximab-induced protein expression changes following Notch1 knockdown, Western blotting was performed in siRNA, cetuximab, or combined siRNA and cetuximab treated groups in SNU503 and LS1034 cells. EGFR, p-EGFR, Notch1, and NICD were confirmed to test drug efficiency. In SNU503 and LS1034 cells, Western blotting showed decreased p-AKT expression and increased active caspase3 in cells with combined Notch1 siRNA and cetuximab treatment. Cleaved PARP expression level increased in SNU503 treated with combined siRNA and cetuximab treatment compared to treatment with siRNA or cetuximab alone, but there was no difference in LS1034 between Notch1 siRNA treated alone and combined Notch1 siRNA and cetuximab treatment (Fig 3C and 3D). Taken together, these results suggested that knockdown of Notch1 increased cetuximab sensitivity by inducing apoptotic cell death in SNU503 and LS1034 cells.

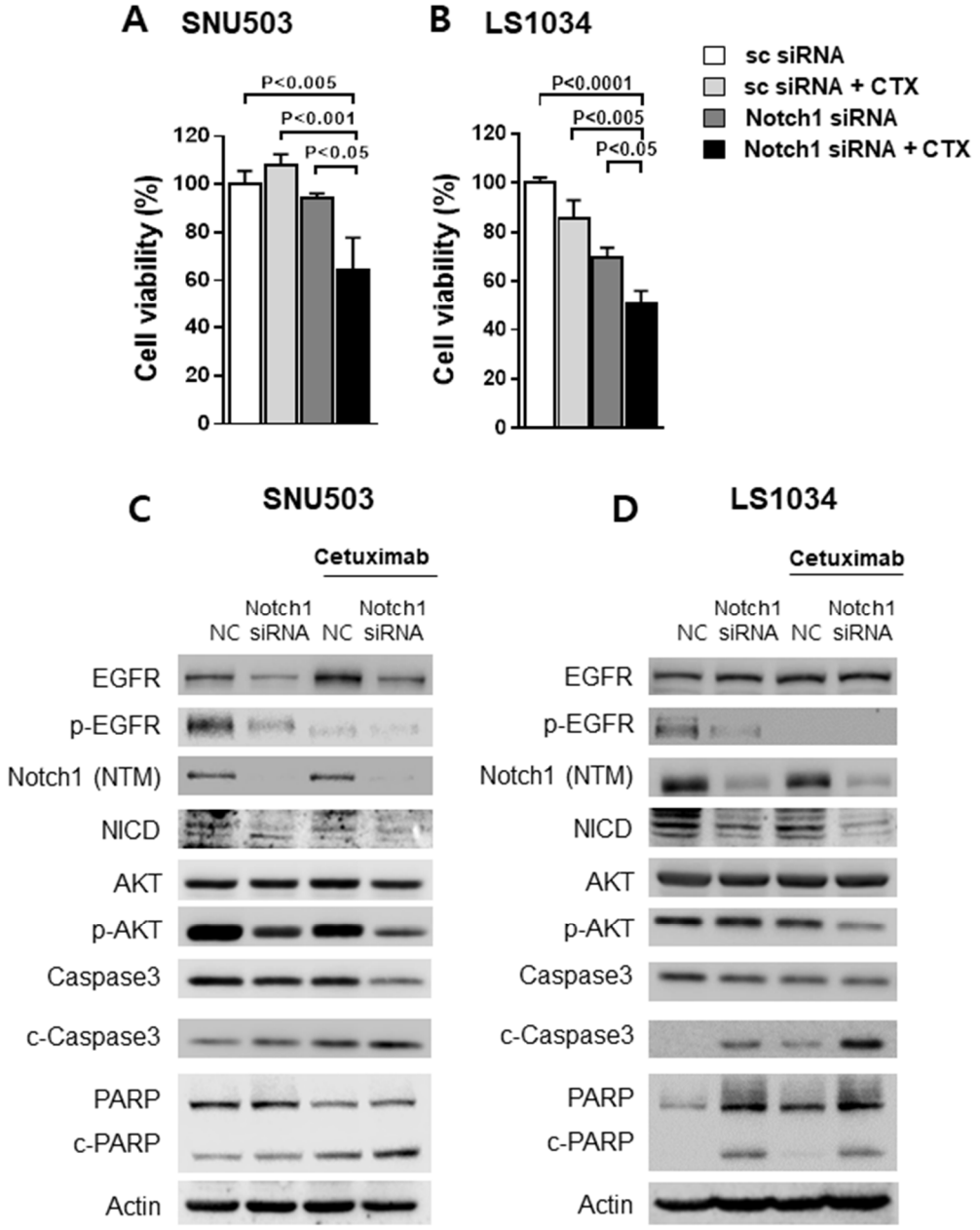


Figure 3. The effect of co-treatment with siNotch1 and cetuximab on cell viability and apoptosis-related signaling pathway in CRC cells

(A) MTT assay showed the cell viability of SNU503 treated with cetuximab (20 μg/ml) or vehicle after siRNA transfection. (B) MTT assay showed the cell viability of LS1034

treated with cetuximab (200 μg/ml) or vehicle after siRNA transfection. The results are expressed as the mean ± SD, n = 3. (C) p-AKT and apoptotic proteins (cleaved caspase3 and cleaved PARP) protein levels in SNU503 treated with 20 μg/ml cetuximab or vehicle for 24 h after sc siRNA or Notch1 siRNA transfection were determined by Western blotting. (D) p-AKT and apoptotic proteins (cleaved caspase3 and cleaved PARP) protein levels in LS1034 treated with 200 μg/ml cetuximab or vehicle for 24 h after sc siRNA or Notch1 siRNA transfection were determined by Western blotting.

AKT phosphorylation is inhibited in CRC cells treated with γ-secretase inhibitor

To confirm in more detail the effect of Notch1 downregulation in response to cetuximab, we conducted further research using γ-secretase inhibitor, DAPT. γ-secretase inhibitors abolish the production of NICD by blocking the S3 cleavage of Notch. So, the efficiency of DAPT can be evaluated by the expression of NICD. To identify whether DAPT affects AKT phosphorylation, we performed Western blotting for p-AKT in SNU503 treated with the indicated doses of DAPT (0, 2, 5, and 10 μM) for 48 h (Fig 4A). In addition, we performed Western blotting for p-AKT in SNU503 treated with 10 μM DAPT for the indicated time periods (0, 6, 12, 24, and 48 h) (Fig 4B). Western blotting results represented that p-AKT expressions were decreased when SNU503 was treated with DAPT. These data suggest that inhibition of Notch activation using γ-secretase inhibitor results in the suppression of AKT phosphorylation in colon cancer cells.

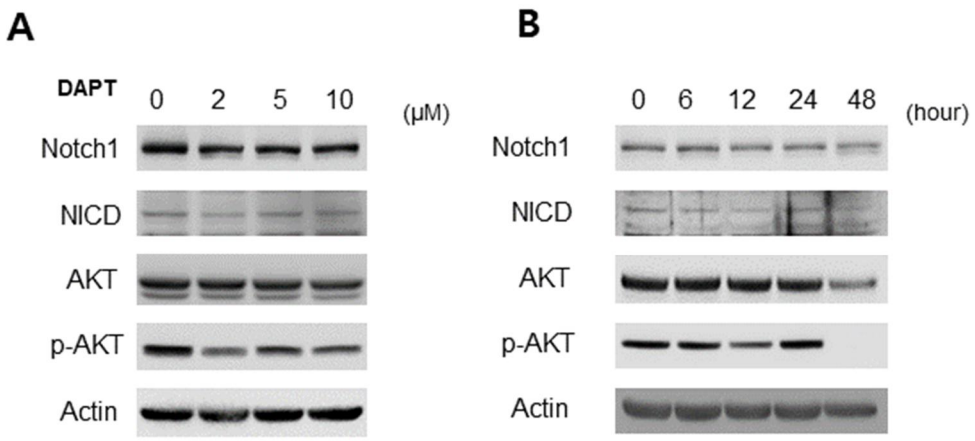
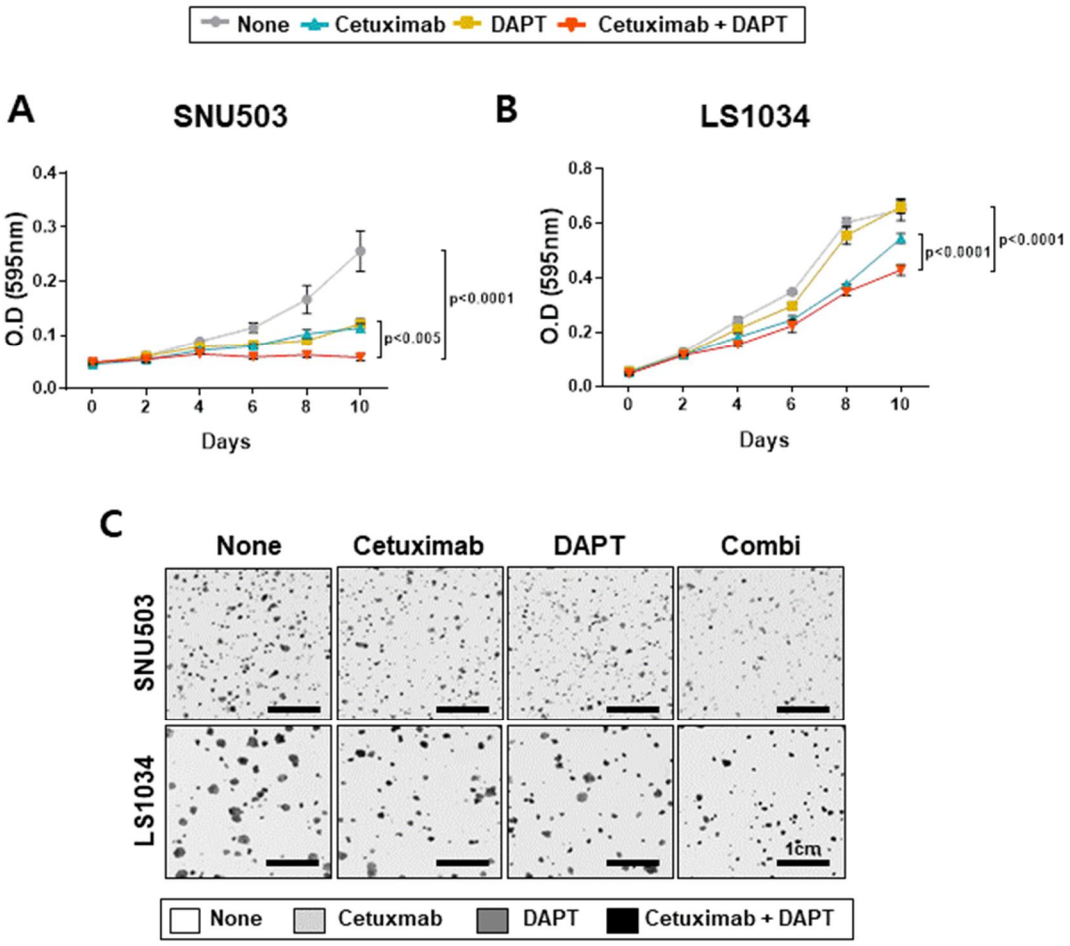


Figure 4. The effect of DAPT on AKT phosphorylation

(A) SNU503 was treated with indicated doses of DAPT (0, 2, 5, and 10 μM) for 48 h. Western blotting analysis showed the alteration of p-AKT protein expression. (B) SNU503 was treated with 10 μM DAPT for indicated times (0, 6, 12, 24, and 48 h). Western blotting analysis showed the alteration of p-AKT protein expression.

The combination of cetuximab with DAPT improves the cell growth inhibition and cell death in colon cancer cells

We hypothesized that combining cetuximab and DAPT would result in favorable responses in cell growth inhibition and cell death. Accordingly, we assessed the additive effects of cetuximab and DAPT on cell growth inhibition and cell proliferation using growth curve and colony formation assay in SNU503 and LS1034 cells. As expected, cetuximab (200 μg/ml) and DAPT (10 μM) significantly induced cell growth inhibition compared to vehicle or single treatment groups for 10 days in both cell lines (Fig 5A and B). Furthermore, the colony formation assay showed a significant decrease in colony numbers and colony size in cells treated with both cetuximab and DAPT compared to cells treated with vehicle, DAPT, or cetuximab alone (Fig 5C, D, and E). In addition, the MTT assay was performed to evaluate drug-induced cell death. Cell viability in SNU503 and LS1034 cells was significantly declined by using the combination of cetuximab with DAPT compared to cells treated alone (Fig 6A and B). Additionally, we performed immunofluorescent staining to confirm the efficiency of DAPT and DNA fragments by drug-induced apoptosis. In terms of drug efficiency, immunofluorescent staining showed that DAPT treatment decreased NICD expression in nucleus of SNU503. Furthermore, DNA fragments induced by drug treatment are more emerged in cells treated with both cetuximab and DAPT than in cells treated with drug alone (Fig 6C).


None
Cetuximab
DAPT
Cetuximab + DAPT
A
SNU503
O.D (595nm)
Days
p<0.0001
p<0.005
B
LS1034
O.D (595nm)
Days
p<0.0001
p<0.0001
C
None
Cetuximab
DAPT
Combi
SNU503
LS1034
1cm
None
Cetuxmab
DAPT
Cetuximab + DAPT

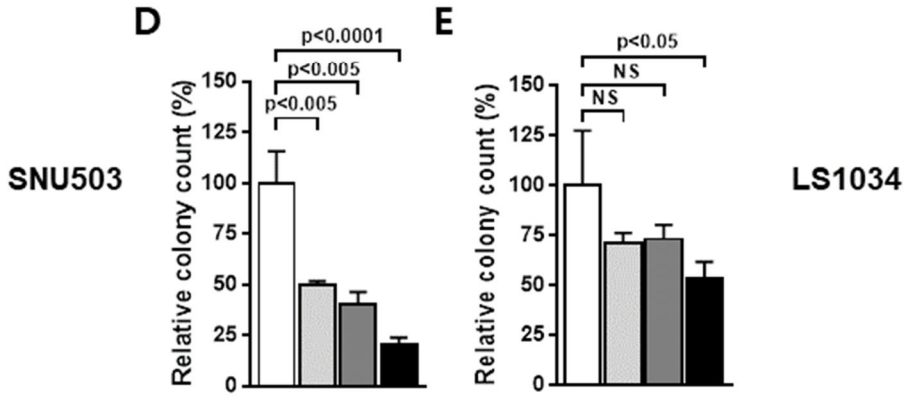

D
SNU503
Relative colony count (%)
p<0.0001
p<0.005
p<0.005
E
LS1034
Relative colony count (%)
p<0.05
NS
NS

Figure 5. The combination effects of cetuximab and DAPT in colon cancer cell growth and proliferation

(A) Cell growth of SNU503 cells treated with cetuximab, DAPT, or both was evaluated by staining using crystal violet solution every 2 days for 10 days. (B) Cell growth of LS1034 cells treated with cetuximab, DAPT, or both was evaluated by staining using crystal violet solution every 2 days for 10 days. (C) In SNU503 and LS1034 cells, 2000 cells seeded in a 6-well plate were treated with cetuximab, DAPT, or both until colonies were formed. After 2 weeks, the cells were stained with a 0.2% crystal violet solution. (D and E) Colony counts were measured using cell counter software. Data are presented as the mean ± SD, n = 3.

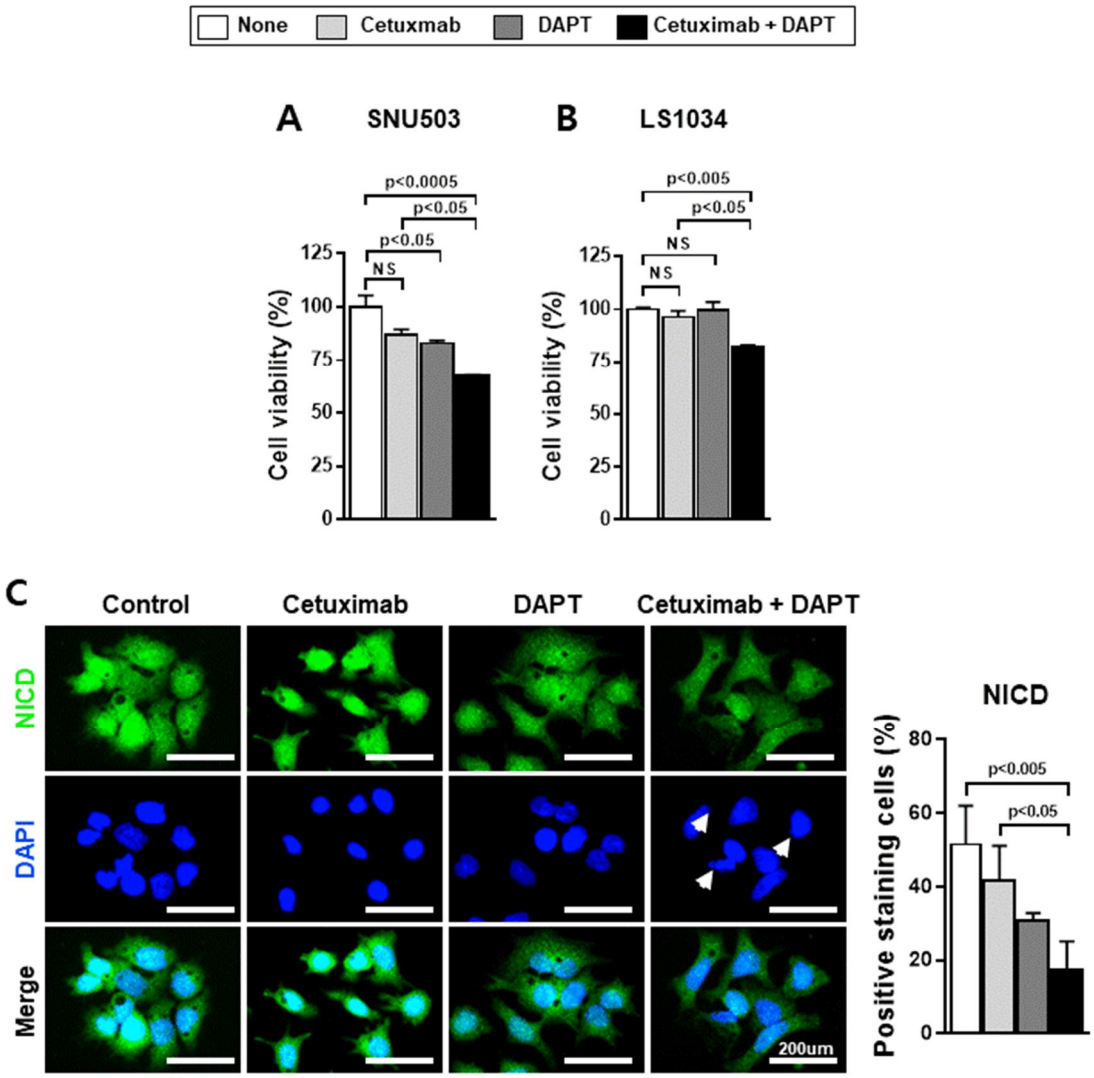


Figure 6. The combination effects of cetuximab with DAPT in drug-induced cell death

(A) MTT assay showed that the cell viability of SNU503 treated with combined cetuximab and DAPT or treated alone. (B) MTT assay showed that the cell viability of LS1034 treated with combined cetuximab and DAPT or treated alone. (C) Activated Notch1 is stained with immunofluorescence staining in SNU503 with cetuximab, DAPT, and a combination. Scale bar: 200 μm (left). Activated Notch1-positive cells are indicated by a bar graph (n = 3), and the values are presented as mean ± SD (right).

* Arrowheads: DNA fragmentation.

Combination of DAPT and cetuximab exhibits additive apoptotic effect by abolishing AKT phosphorylation

As shown in Figure 6C, immunofluorescent staining suggested the possibility of a combination effect on cell death after treatment with cetuximab and DAPT. To further examine whether DAPT treatment contributes to cause colon cancer cells to become more sensitive to cetuximab by inducing apoptotic cell death, SNU503 and LS1034 were treated with either cetuximab, DAPT, or both. Then, Western blotting was further performed to determine the expression of several apoptosis-related proteins (Fig 7A (SNU503) and B (LS1034)). The results showed that cetuximab and DAPT cotreatment resulted in a noticeable inhibition of AKT phosphorylation compared with cetuximab or DAPT alone. Moreover, cetuximab and DAPT cotreatment induced an increase in cleaved caspase3 and cleaved PARP expression levels, indicating apoptosis occurrence by drug treatment. EGFR, p-EGFR, Notch1, and NICD were confirmed to test drug efficiency. These results suggest that the Notch inhibitor DAPT and cetuximab enhanced apoptotic cell death in colorectal cancer cells.

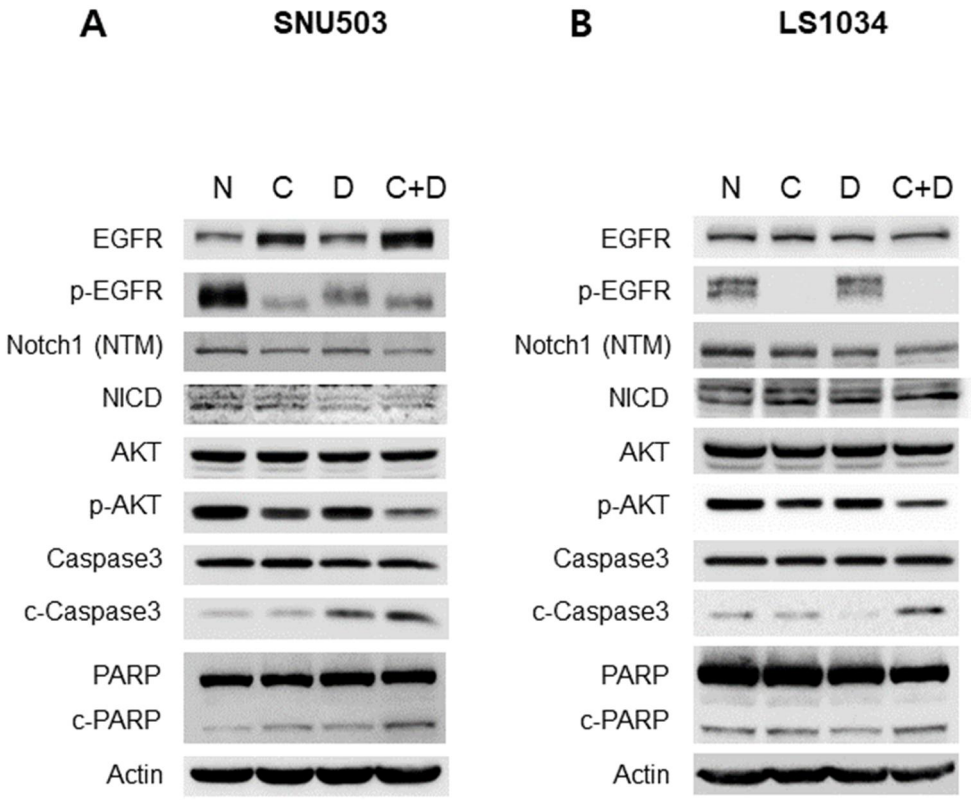


Figure 7. Effects of cetuximab and DAPT on AKT phosphorylation and apoptosis

SNU503 and LS1034 were treated with cetuximab or DAPT as indicated. (A) p-AKT and apoptotic proteins (cleaved caspase3 and cleaved PARP) protein levels in SNU503 treated with 20 μg/ml cetuximab or 10 μM DAPT for 48 h were determined using Western blotting. (B) p-AKT and apoptotic proteins (cleaved caspase3 and cleaved PARP) protein levels in LS1034 treated with 200 μg/ml cetuximab or 10 μM DAPT for 48 h were determined by Western blotting.

C, cetuximab; D, DAPT; N, none.

Discussion

In this study, we assessed the potential roles of Notch1 in the sensitivity of colon cancer cells to cetuximab treatment. The current study revealed that Notch1 is activated in colon cancer cells compared to noncancerous colon cells. This study also found that knockdown of Notch1 can inhibit the phosphorylation of AKT and increase cetuximab sensitivity in SNU503 and LS1034 cells. As γ-secretase produces NICD by cleaving the total Notch receptor, we treated DAPT to inhibit γ-secretase. Along with the results in Notch1 siRNA, the treatment of DAPT also inhibited AKT phosphorylation. Finally, this study found that the combined treatment of cetuximab and DAPT can inhibit the growth of colon cancer cells and induce apoptotic cell death. Altogether, the current study suggested Notch1 can be promising in EGFR therapies targeting CRC.

Notch1 has been known to be associated with resistance of drugs such as oxaliplatin and regorafenib, in CRC[10, 12]. However, in the case of EGFR-targeted therapies, the effect of Notch1 activation on drug resistance has not been previously studied in CRC. A previous study in non-small cell lung cancer has shown that Notch1 contributes to EGFR-TKIs-acquired resistance by mediating epithelial-mesenchymal transition (EMT) and the combined effect of EGFR-TKIs and Notch1 inhibition[14]. However, as evidence has shown that Notch1 activation is related to the PI3K pathway by PTEN inactivation in several cancer cells, including CRC[18-20], we focused on the combined effect of Notch inhibition and cetuximab in terms of AKT phosphorylation. We assumed that Notch1 would be related to AKT phosphorylation in CRC. Notch1 downregulation using siRNA and inhibitor inhibited AKT phosphorylation in colon cancer cells, as expected. Furthermore, we checked the linkage of the Notch1/HES1 and PTEN/AKT signaling pathways. As for PTEN, we expected that the inhibition of the Notch1 signaling pathway could induce PTEN expression. However, the results for PTEN were not derived as expected (Appendix figure 2). So,

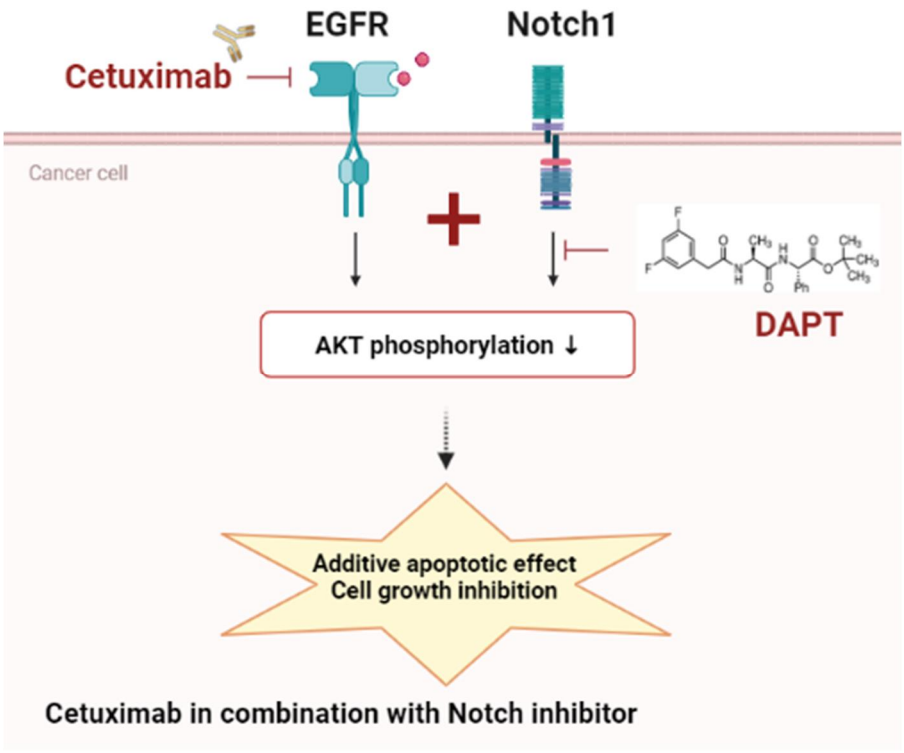
further studies on the mechanisms between the Notch1 signaling pathway and AKT phosphorylation are needed.

The protein levels of AKT phosphorylation and apoptosis markers were measured to evaluate the additive effect of cetuximab and γ-secretase inhibitor (DAPT) treatment. Cetuximab and DAPT cotreatment resulted in an increase in cleaved caspase3 and cleaved PARP expression levels, indicating that cetuximab in combination with DAPT treatment-induced apoptosis. The growth curve and colony formation assay showed that cetuximab in combination with DAPT significantly inhibited cell growth and proliferation compared with that of cetuximab or DAPT alone. Taken together, these results suggest that the possibility of combined treatment with cetuximab and γ-secretase inhibitor could be a strategy to improve the response of anti-EGFR therapies in CRC.

This study showed the results that Notch inhibition can enhance the effect of cetuximab treatment by inhibiting AKT phosphorylation. However, since no dramatic effect was seen when the drugs were treated together, it is considered that there is an additive effect rather than a synergistic effect. And this study has a limitation that though the inhibition of AKT phosphorylation and increment in apoptosis-related proteins in the combined treatment of Notch inhibitor and cetuximab, the study could not reveal further underlying mechanisms. Therefore, further research on the effect of Notch signaling on the combination of cetuximab is needed.

Intriguingly, not only the KRAS wild-type cell line but also the KRAS mutant cell line showed the additive effect of Notch1 inhibition and cetuximab in this study. Furthermore, some studies have shown that the inhibition of Notch1 and DLL4, a main ligand for the Notch receptors, is a therapeutic strategy in lung cancer and colorectal tumors with oncogenic KRAS mutations [21, 22]. Therefore, targeting Notch1 may hopefully be a prospective strategy for patients with CRC with RAS mutations.

Conclusion



In this study, we identified Notch1 as a potential target gene associated with response of cetuximab in CRC. Downregulation of Notch1 using siRNA and inhibitor inhibited AKT Phosphorylation in colon cancer cells. AKT phosphorylation is an important factor of cetuximab resistance. Furthermore, cetuximab and downregulation of Notch1 exhibited additive effects on growth inhibition and death via inhibition of AKT phosphorylation in colon cancer cell lines. Taken together, this study suggests that Notch1 is a target for the new therapeutic strategies to treat in combination with cetuximab.

References

1. S. S. Kweon, **Updates on Cancer Epidemiology in Korea, 2018**. *Chonnam Med J*, 2018. 54(2): p. 90-100.
2. Y. H. Xie, Y. X. Chen, and J. Y. Fang, **Comprehensive Review of Targeted Therapy for Colorectal Cancer**. *Signal Transduct Target Ther*, 2020. 5(1): p. 22.
3. S. M. Bray, J. Lee, S. T. Kim, J. Y. Hur, P. J. Ebert, *et al.*, **Genomic Characterization of Intrinsic and Acquired Resistance to Cetuximab in Colorectal Cancer Patients**. *Sci Rep*, 2019. 9(1): p. 15365.
4. R. A. Kovall, B. Gebelein, D. Sprinzak, and R. Kopan, **The Canonical Notch Signaling Pathway: Structural and Biochemical Insights into Shape, Sugar, and Force**. *Dev Cell*, 2017. 41(3): p. 228-241.
5. P. A. Shah, C. Huang, Q. Li, S. A. Kazi, L. A. Byers, *et al.*, **Notch1 Signaling in Head and Neck Squamous Cell Carcinoma**. *Cells*, 2020. 9(12).
6. Y. Zhang, B. Li, Z. Z. Ji, and P. S. Zheng, **Notch1 Regulates the Growth of Human Colon Cancers**. *Cancer*, 2010. 116(22): p. 5207-18.
7. A. Katarkar, G. Bottoni, A. Clocchiatti, S. Goruppi, P. Bordignon, *et al.*, **Notch1 Gene Amplification Promotes Expansion of Cancer Associated Fibroblast Populations in Human Skin**. *Nat Commun*, 2020. 11(1): p. 5126.
8. L. Gharaibeh, N. Elmadany, K. Alwosaibai, and W. Alshaer, **Notch1 in Cancer Therapy: Possible Clinical Implications and Challenges**. *Mol Pharmacol*, 2020. 98(5): p. 559-576.
9. S. Cho, M. Lu, X. He, P. L. Ee, U. Bhat, *et al.*, **Notch1 Regulates the Expression of the Multidrug Resistance Gene Abcc1/Mrp1 in Cultured Cancer Cells**. *Proc Natl Acad Sci U S A*, 2011. 108(51): p. 20778-83.
10. G. Mirone, S. Perna, A. Shukla, and G. Marfe, **Involvement of Notch-1 in Resistance to Regorafenib in Colon Cancer Cells**. *J Cell Physiol*, 2016. 231(5): p. 1097-105.

11. C. Sethy and C. N. Kundu, **5-Fluorouracil (5-Fu) Resistance and the New Strategy to Enhance the Sensitivity against Cancer: Implication of DNA Repair Inhibition**. *Biomed Pharmacother*, 2021. 137: p. 111285.

12. R. D. Meng, C. C. Shelton, Y. M. Li, L. X. Qin, D. Notterman*, et al.*, **Gamma-Secretase Inhibitors Abrogate Oxaliplatin-Induced Activation of the Notch-1 Signaling Pathway in Colon Cancer Cells Resulting in Enhanced Chemosensitivity**. *Cancer Res*, 2009. 69(2): p. 573-82.

13. E. Bousquet Mur, S. Bernardo, L. Papon, M. Mancini, E. Fabbrizio*, et al.*, **Notch Inhibition Overcomes Resistance to Tyrosine Kinase Inhibitors in Egfr-Driven Lung Adenocarcinoma**. *J Clin Invest*, 2020. 130(2): p. 612-624.

14. M. Xie, C. S. He, S. H. Wei, and L. Zhang, **Notch-1 Contributes to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor Acquired Resistance in Non-Small Cell Lung Cancer in Vitro and in Vivo**. *Eur J Cancer*, 2013. 49(16): p. 3559-72.

15. L. Liu, H. Wada, N. Matsubara, K. Hozumi, and M. Itoh, **Identification of Domains for Efficient Notch Signaling Activity in Immobilized Notch Ligand Proteins**. *J Cell Biochem*, 2017. 118(4): p. 785-796.

16. Q. Liu, S. Yu, W. Zhao, S. Qin, Q. Chu*, et al.*, **Egfr-Tkis Resistance Via Egfr-Independent Signaling Pathways**. *Mol Cancer*, 2018. 17(1): p. 53.

17. I. Pinilla-Macua, A. Grassart, U. Duvvuri, S. C. Watkins, and A. Sorkin, **Egf Receptor Signaling, Phosphorylation, Ubiquitylation and Endocytosis in Tumors in Vivo**. *Elife*, 2017. 6.

18. A. Tyagi, A. K. Sharma, and C. Damodaran, **A Review on Notch Signaling and Colorectal Cancer**. *Cells*, 2020. 9(6).

19. T. Palomero, M. Dominguez, and A. A. Ferrando, **The Role of the Pten/Akt Pathway in Notch1-Induced Leukemia**. *Cell Cycle*, 2008. 7(8): p. 965-70.

20. Z. Koveitypour, F. Panahi, M. Vakilian, M. Peymani, F. Seyed Forootan*, et al.*, **Signaling Pathways Involved in Colorectal Cancer Progression**. *Cell Biosci*,

2019. 9: p. 97.

21. C. Ambrogio, G. Gomez-Lopez, M. Falcone, A. Vidal, E. Nadal*, et al.*, **Combined Inhibition of Ddr1 and Notch Signaling Is a Therapeutic Strategy for Kras-Driven Lung Adenocarcinoma**. *Nat Med*, 2016. 22(3): p. 270-7.

22. M. Fischer, W. C. Yen, A. M. Kapoun, M. Wang, G. O'Young*, et al.*, **Anti-Dll4 Inhibits Growth and Reduces Tumor-Initiating Cell Frequency in Colorectal Tumors with Oncogenic Kras Mutations**. *Cancer Res*, 2011. 71(5): p. 1520-5.

국문요약

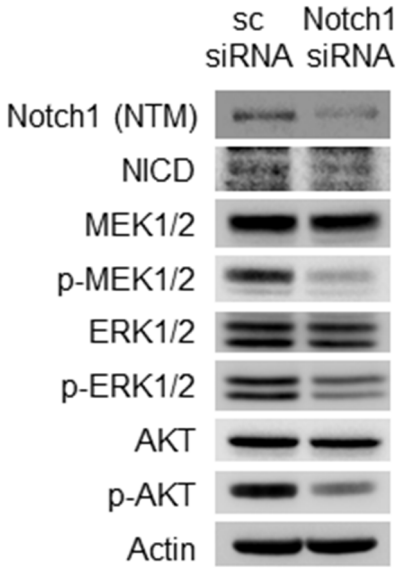
대장암 (Colorectal cancer, CRC)은 2018년 기준으로 국내에서 4번째로 높은 발병률을 보이고 있으며, 최근 식생활이 서구화됨에 따라 발병률은 증가하고 있는 추세이다. 세툭시맙 (Cetuximab, Erbitux®)은 EGFR의 세포 외 영역에 결합하여 EGFR 신호 경로의 활성화를 억제하는 단클론항체 치료제로서 FDA의 승인을 받은 대표적인 전이성 대장암 치료제이지만 세툭시맙에 대한 반응률은 약 60% 정도에 불과하고 기존 세포독성항암제와 비교하면 평균 4개월 수명을 연장시키는 한계를 보이고 있다. 이에 대장암 환자에게서 세툭시맙의 치료 효과를 향상시키기 위해 세툭시맙의 저항성 기전 연구 및 약물의 반응성을 높일 수 있는 병용 치료의 타겟을 발굴하는 것이 중요한 과제로 남아있다. Notch1은 막관통 수용체로 세포의 항상성, 발달, 사멸과 같은 세포사와 관련해서 중요한 역할을 하며 최근에는 암세포에서 다양한 약물의 저항 기전과 관련되어 있다고 보고되어 있다. 본 연구에서는 Notch1이 세툭시맙과 관련된 신호 경로에 영향을 끼치는 것을 보이고, 세툭시맙의 반응성을 개선할 수 있는 새로운 치료 전략으로 Notch1을 이용할 수 있음을 제시하고자 한다.

본 연구에서는 대장암 세포주 중 SNU503과 LS1034 세포주를 이용하여 대장암에서 Notch1과 EGFR 신호 경로 사이의 연관성 확인 및 Notch1의 저하와 세툭시맙의 상가 효과를 확인하였다. 우선 대장암에서 Notch1의 중요도를 확인하기 위해 정상 대장 세포주와 두 가지의 대장암 세포주에서 Notch1의 활성을 비교하였고 대장암 세포주에서 정상 대장 세포보다 Notch1의 신호 경로가 활성화되어 있는 것을 관찰했다. 또한, 대장암에서 세툭시맙이 주된 기능을 하는 경로에 Notch1이 끼치는 영향을 확인하기 위해 siRNA와 Notch 억제제를 이용하여 Notch1의 활성을 저하시켰을 때 AKT의 인산화에 대한 변화를 확인하였다. 그 결과, 대장암 세포주에서 Notch1의 활성이 저하되었을 때 AKT의 인산화가 억제되는 것을 관찰했다.

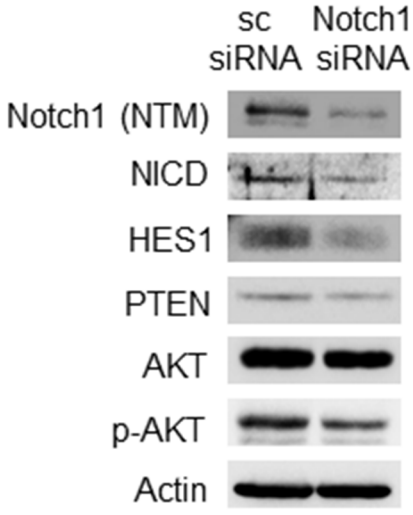
나아가 Notch1의 저하가 세포 죽음, 성장, 증식에 세툭시맙과 병합 효과를 보이는지 확인하기 위해 1) 대조군 2) 세툭시맙 단독 처리 실험군 3) Notch1 siRNA 또는 Notch 억제제 단독 처리 실험군 4) 세툭시맙과 Notch1 siRNA 또는 Notch 억제제 병합 처리 실험군으로 나누어 연구를 수행하였다. Notch1 저해제와 세툭시맙의 병합 처리는 약물의 단독 처리 실험군 보다 세포의 생존률을 저하시켰으며, 세포의 성장 및 증식이 억제되는 결과를 보였다. 또한, 세포 사멸을 보여주는 단백질인 caspase3와 PARP의 활성화가 약물의 단독 처리 실험군 보다 병합 처리 실험군에서 증가하였다. 이와 같은 연구를 통해 대장암에서 Notch1 저해제와 세툭시맙을 병합 투여했을 때 세툭시맙을 단독으로 투여하는 것 보다 치료 효과를 더욱 증진 시킬 수 있음을 보인다.

중심단어: 대장암, 세툭시맙, Notch1, 저항성, 병합요법

Appendix



Appendix figure1) Alteration of Expression of MAPK pathway and AKT phosphorylation after siNotch1 transfection in SNU503 cell line.



Appendix figure2) Expression of Notch1-HES1-PTEN signaling pathway-related proteins after Notch1 siRNA transfection in SNU503 cell line.