Thesis for the Master of Biomedical Sciences

Investigating the molecular mechanisms of ADAR1 in colon cancer

The Graduate School of the University of Ulsan

Department of Biomedical Sciences

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Investigating the molecular mechanisms of ADAR1 in colon cancer

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Investigating the molecular mechanisms of ADAR1 in colon cancer

This certifies that the master's thesis of

Hyeon Ji Choi is approved.

Committee Member	**Prof. Sun Cheol Choi**	**(signature)**
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Committee Member	**Prof. Suhwan Chang**	**(signature)**

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Abstracts

ADAR1 (adenosine deaminase acting on RNA) binds to double-stranded RNA (dsRNA) and catalyzes the deamination of adenosine through RNA editing to generate inosine. ADAR1 is known to inhibit the IFN (type I interferon) response and restrict the interaction between ZBP1 and RIPK3, thus suppressing ZBP1-mediated necroptosis. However, its mechanism in colon cancer is not clearly understood. This study analyzed changes in IFN signaling and ZBP1-mediated necroptosis pathway in ADAR1-depleted colon cancer cells with inducible knockdown using the tet on shADAR1 system, to clarify how ADAR1 regulates IFN signaling and ZBP1-mediated necroptosis. However, in contrast to previous studies, these observations showed that ADAR1 knockdown in colon cancer cells did not lead to activation of IFN signaling and ZBP1-mediated necroptosis. Furthermore, it was noted that the activation of IFN signaling, and ZBP1-mediated necroptosis did not occur upon ADAR1 knockdown, even in the presence of IFN and CBL0137 treatment. Therefore, our results suggest that ADAR1 exists in colon cancer cells in a different pathway than the known type I IFN signaling pathway and ZBP1-mediated necroptosis through RNA editing.

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Introduction

Colorectal carcinoma (CRC) is a malignant cancer of the digestive tract. CRC is the third leading cause of cancer-related death worldwide. CRC death rate decreases during recent decade by about 2% per year. But the CRC death rate continues to increase by 1.2% per year in younger than 50 years and by 0.6% per year in those aged 50–54 years[1]. CRC is characterized by high inter-patient variability and high spatial heterogeneity. This can affect the molecular properties of tumor tissue, and molecular heterogeneity is known to influence response to treatment and prognosis. Therefore, explaining the molecular mechanism is important for CRC therapy and diagnosis[2,3].

Adenosine-inosine (A-to-I) RNA editing is deamination of adenosine to inosine, and adenosine deaminases acting on RNA (ADAR) catalyzes the A-to-I RNA editing on double-stranded RNA (dsRNA)[4]. A-to-I RNA editing, catalyzed by the ADAR family of enzymes, is the most common form of RNA editing in RNA[5]. ADAR have three ADAR protein families (ADAR1, ADAR2, ADAR3). Starting from the C-terminus, each protein has dsRNA binding domain. ADAR2 and ADAR3 contain two dsRNA binding domains and ADAR1 contain three dsRNA binding domains. The third dsRNA binding domain contain nuclear localization sequence in ADAR1. ADAR1 has two different isoforms, ADAR1p110 and ADAR1p150. ADAR1 p110 has a Z-DNA binding domain, Zβ in N terminus, While ADAR1 p150 has Zα and Zβ domain. This Zα domain includes a nuclear export sequence[4,6]. A-to-I editing of endogenous dsRNA by ADAR1 prevents the activation of the dsRNA response by endogenous transcripts, thus, endogenous dsRNA is edited and regarded as self dsRNA.

However, in the absence of ADAR1, endogenous dsRNA is recognized as non-self and activates A-form dsRNA (A-RNA) sensing proteins such as MDA-5 and PKR5, leading to interferon (IFN) signaling[7-10]. The suppression of ADAR1 activates the MDA5/MAVS related type 1 IFN signaling in cervical cancer[11].

Besides ADAR1 p150 isoform, the other mammalian protein that contains Zα domains is Z-DNA binding protein 1 (ZBP1)[12]. ZBP1 recognizes viral and endogenous Z-RNA and selectively enhances the expression of type I IFNs and

other innate immune-related genes, suggesting that it is an important role in their activation[9,13,14].

ADAR1 and ZBP1 are mammalian interferon-inducible proteins containing the Zα domain that regulates innate immune responses[9,13,14]. Also, recent studies have shown that ADAR1 prevents the generation of endogenous Z-RNA and inhibits ZBP1-mediated necroptosis by limiting interaction between ZBP1 and RIPK3[9,14]. These studies suggest that inhibition of ADAR1 makes tumors sensitive to immunotherapy and overcomes resistance to immune checkpoint blockades (ICBs) beneficial for cancer treatment[8] (Figure 1).

To confirm similarity with prior research, this study employed methods beyond ADAR1 knockdown (KD) to reduce expression levels, including the use of an ADAR1 inhibitor and other compounds. ZYS-1 is known as a direct ADAR1 inhibitor and inhibits deaminase activity by directly binding to ADAR1. ADAR1 mRNA level does not change and protein level decreases[15]. Another ZBP1 related small molecule is CBL0137. It is known as a compound that can activate ZBP1 by binding directly to DNA, converting it to Z-DNA and activating ZBP1. CBL0137 reported to induce ZBP1-mediated necroptosis in cancer-associated fibroblast and strongly reverse ICB in mouse models of melanoma. It is known that ZBP1 activation upon ADAR1 loss is similar to the ZBP1 activation upon CBL0137 treatment[9].

ADAR1 has been related to cancers, including cervical cancer[11], breast cancer[16], gastric cancer[17]. Although the function of ADAR1 in cancer has been actively studied, the study of RNA editing in CRC have not been clearly elucidated. ADAR1 is known to be increased in common solid tumors, and also CRC also showed high expression of ADAR transcripts[18]. These observations indicate the important role of RNA editing regulation in CRC progression and prognosis. However, the abnormal alterations and immune association of ADAR1 have not been clearly elucidated in CRC. Therefore, it was studied whether inhibition of ADAR1 in colon cancer cells affects the IFN signaling pathway and the ZBP1-mediated necroptosis pathway.

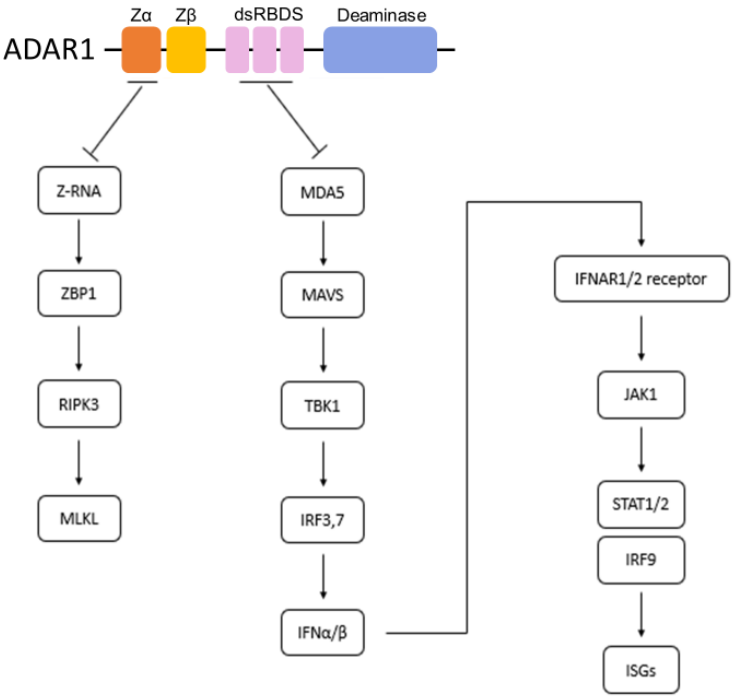


Figure 1. Schematic diagram of ADAR1 pathway

Materials and Methods

Cell line

Mouse colon cancer cell line, CT26 and MC38 cell were cultured in RPMI 1640 medium with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin.

Human embryonic kidney cell, HEK 293T was cultured in Dulbecco's Minimum Essential Medium (DMEM) with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin. All cells were cultured at 37 ℃ with 5% CO2.

Inducible knockdown of ADAR1 using shRNA

To ADAR1 knockdown, an inducible ADAR1 knockdown system using shADAR1 plasmid cloned into pLKO Tet-on vector was used. First, HEK-293T cells were plated at a density of 2 x 10^5 cells/well in a 100mm plate and incubated for 24h. Subsequently, HEK-293T cells were co-transfected with an shADAR1 plasmid (1500 ng) and the packaging mix of psPAX2 (700 ng) and the envelope plasmid pMD2.G (700 ng), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Viral supernatants were then harvested at 24–48h after transfection and were used to infect Mouse colon cancer cell line (CT26 and MC38). After infection, several stable clones of ADAR1 knockdown (shADAR1) were selected using puromycin (10 μg/ml). Experiments conditionally knocks down ADAR1 by doxycycline (Dox) treatment at 2 μg/mL for 72 h.

Drug

CBL0137(HY-18935A, MCE) is dissolved in DMSO in vitro and and stored at -80 ℃.

ZYS-1 is dissolved in D.W in vitro and and stored at 4 ℃. Doxycycline is dissolved

in D.W in vitro and and stored at -20 ℃

RNA isolation and Real-Time PCR

RNA extraction was performed using TRIzol (Invitrogen). RNA (1 μg) was used for cDNA synthesis (PrimeScript RT reagent kit, TaKaRa) according to the manufacturer's instructions. Real-time PCR was performed with SYBR Green (AMPIGENE® qPCR Green Mix Lo-ROX, Enzo life sciences) in a Bio-Rad real-time. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as an internal control.

Table 1. Real-Time PCR Primer Sequences

mADAR1 Forward 5'-3'	GGTGAGTTTCGAGCCATCAT
mADAR1 Reverse 5'-3'	CGTCCTGCTTGGCTACTTTC
mADAR1 p110 Forward 5'-3'	GCCCTAACCATTGATTCCTG
mADAR1 p110 Reverse 5'-3'	GAATCTTGGCCAGTGTCCTG
mADAR1 p150 Forward 5'-3'	CTTGCCGGCACTATGTCTC
mADAR1 p150 Reverse 5'-3'	GTCACACTCCTGGGGAACTC
mRPL13a Forward 5'-3'	GAGGTCGGGTGGAAGTACCA
mRPL13a Reverse 5'-3'	TGCATCTTGGCCTTTTCCTT
mGAPDH Forward 5'-3'	CATGGCCTTCCGTGTTCCTA
mGAPDH Reverse 5'-3'	GCGGCACGTCAGATCCA

Protein extract and Western blot

For detection of target proteins by Western blot, cells were lysed in RIPA (rapid immunopprecitation assay) lysis buffer containing protease inhibitor cocktail and phosphatase inhibitor tablets (Roche, Switzerland). All tissue extracts were stored on ice for 15 min and then centrifuged at 13,000 rpm at 4 °C for 15 min. Protein concentration was measured with a BCA protein assay kit (Bio-Rad Laboratories, USA). Western blot was performed by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and prepared samples were loaded 20μg of total protein per lane. Antibodies reactive with GAPDH (1:1,000; Santa Cruz Biotechnology), β-actin (1:1,000; Santa Cruz Biotechnology), ADAR1 (1:1,000; Santa Cruz Biotechnology), MDA5 (sc365333, 1:1,000; Santa Cruz Biotechnology), IRF3 (sc365333, 1:1,000; Santa Cruz Biotechnology), anti-pIRF3 (29047, 1:1,000; Cell Signaling Technology), STAT1 (29047, 1:1,000; Cell Signaling Technology), pSTAT1 (7649, 1:1,000; Cell Signaling Technology), TBK1 (3504, 1:1,000; Cell Signaling Technology), RIP3 (15828, 1:1,000; Cell Signaling Technology), pRIP3 (91702, 1:500; Cell Signaling Technology), MLKL (37705, 1:1,000; Cell Signaling Technology), pMLKL (37333, 1:500; Cell Signaling Technology). Western blot densitometry was performed using Image J software.

Flow cytometry

Apoptosis of colon cancer cells was analyzed using Annexin V-FITC apoptosis detection kits.
Briefly, 5 x 10^5/test of cells were resuspended in 500 μL binding buffer and labeled with Annexin V-FITC (1 μL) and propidium iodide (PI) (1 μL) for 15 minutes in the dark at room temperature.

Cell proliferation assay

Cells were seeded in a 96 well plate at a density of 500 cells/well in 100 μL culture media. Cells were treated with drug after 5 hours of seeding. To monitor cell proliferation, 1/10 volume of water-soluble tetrazolium salt (WST-8) was added directly to control and drug treated cells after 48, 72 hours of the drug treatment. The cells were detected by fluorescence measurements using a microplate fluorescence spectrophotometer.

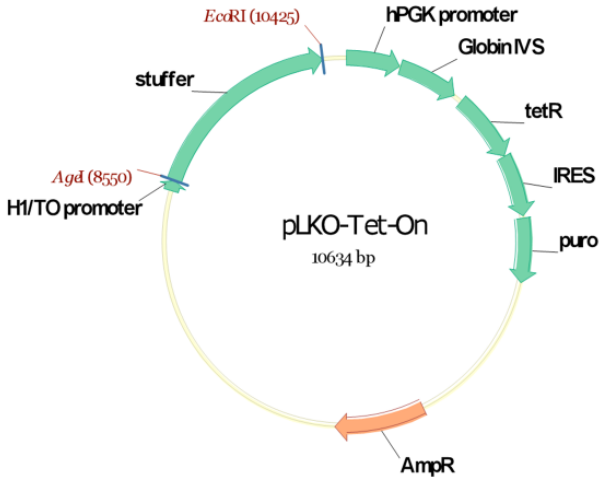
Result

1. Generation of an Inducible ADAR1 Knockdown System in colon cancer cell line

To investigate the molecular mechanism through ADAR1 knockdown, it used an inducible ADAR1 knockdown system using shADAR1 plasmid cloned into the pLKO Tet-on vector (Figure 2A). When CT26 tet on shADAR1 was treated with doxycycline, both mRNA levels (Figure 2B) and protein levels (Figure 2C) of ADAR1 decreased significantly upon knockdown. In this study, clonal cells were generated to select cells with stable ADAR1 knockdown. It was selected 6 clonal cells for CT26 and 10 clonal cells for MC38 to confirm whether knockdown was successful. It was confirmed that tet on shADAR1 cells were successfully knockdown through western blot and band densitometry results. In this study selected clonal cells 1, 4, and 5 that are prone to ADAR1 knockdown in CT26 (Figure 2D-E). In MC38, clonal cells 5 and 10 were selected, which showed successfully ADAR1 knockdown and relatively high

expression of RIP3 and MLKL which are part of the ZBP1-mediated necroptosis pathway (Figure 2F-G).

A



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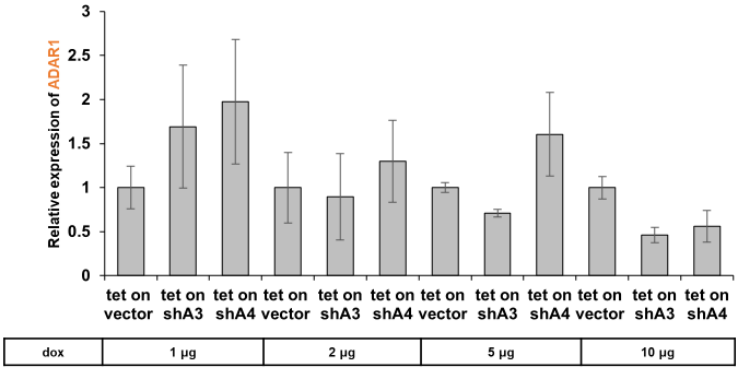
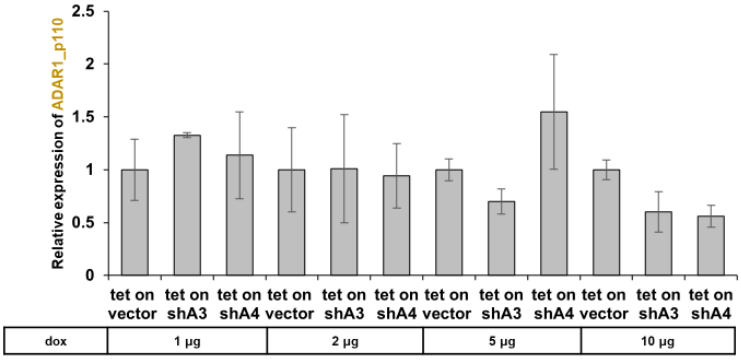
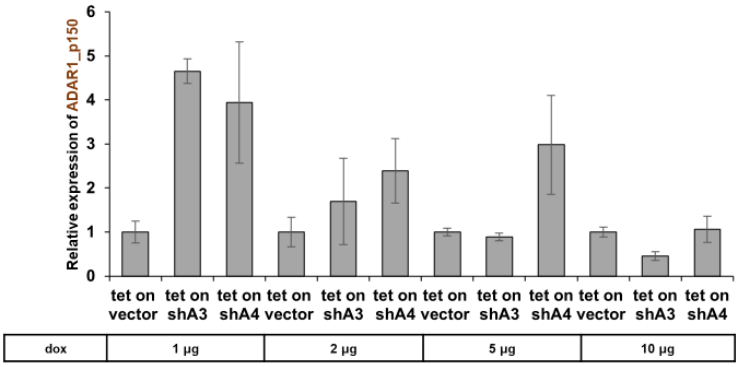
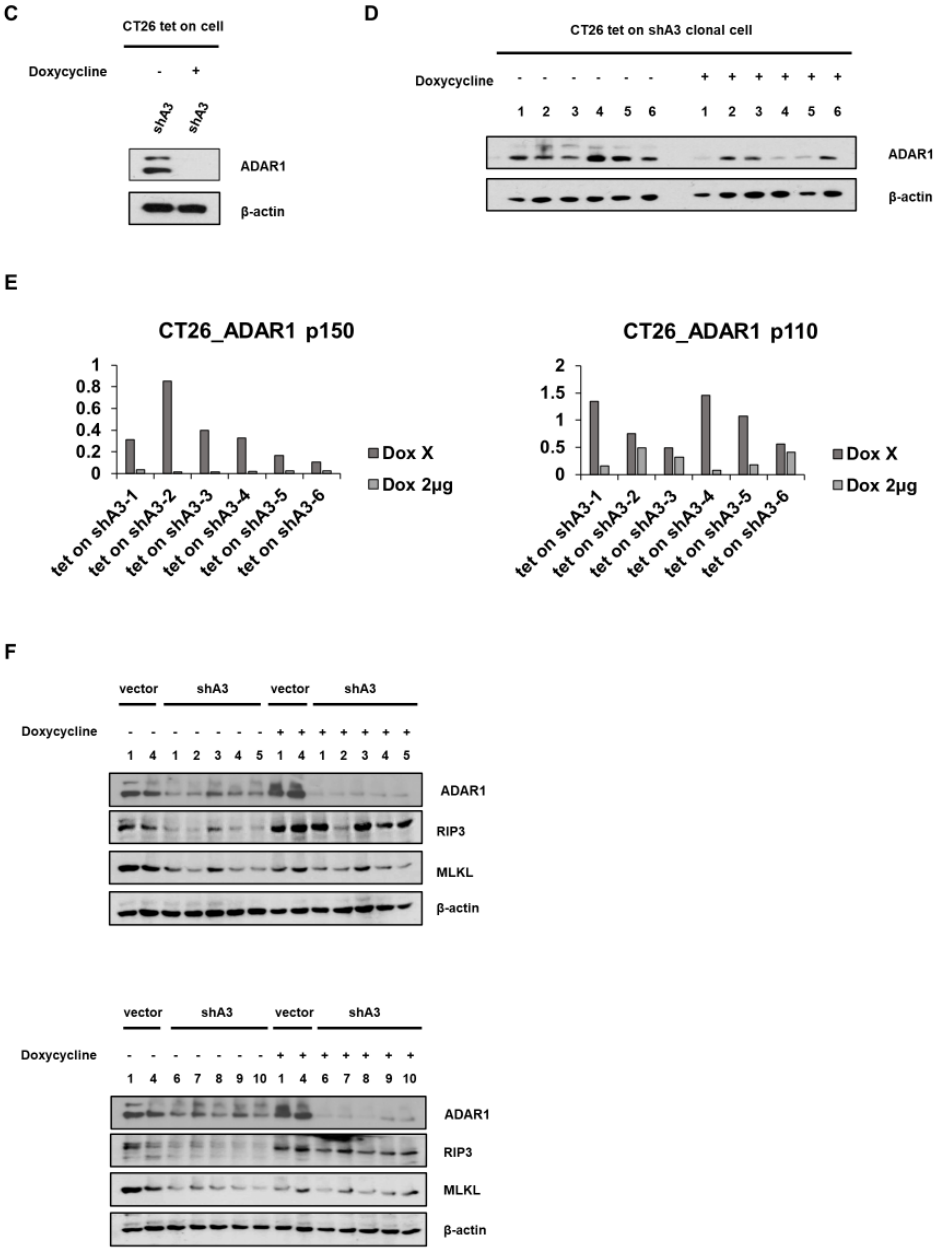

Relative expression of ADAR1
3
2.5
2
1.5
1
0.5
0
tet on vector
tet on shA3
tet on shA4
tet on vector
tet on shA3
tet on shA4
tet on vector
tet on shA3
tet on shA4
tet on vector
tet on shA3
tet on shA4
dox
1 µg
2 µg
5 µg
10 µg


Relative expression of ADAR1_p110
2.5
2
1.5
1
0.5
0
tet on vector
tet on shA3
tet on shA4
tet on vector
tet on shA3
tet on shA4
tet on vector
tet on shA3
tet on shA4
tet on vector
tet on shA3
tet on shA4
dox
1 µg
2 µg
5 µg
10 µg


Relative expression of ADAR1_p150
6
5
4
3
2
1
0
tet on vector
tet on shA3
tet on shA4
tet on vector
tet on shA3
tet on shA4
tet on vector
tet on shA3
tet on shA4
tet on vector
tet on shA3
tet on shA4
dox
1 µg
2 µg
5 µg
10 µg


C
CT26 tet on cell
Doxycycline
-
+
shA3
shA3
ADAR1
β-actin
D
CT26 tet on shA3 clonal cell
Doxycycline
- - - - - - + + + + + +
1 2 3 4 5 6 1 2 3 4 5 6
ADAR1
β-actin
E
CT26_ADAR1 p150
1
0.8
0.6
0.4
0.2
0
Dox X
Dox 2μg
tet on shA3-1
tet on shA3-2
tet on shA3-3
tet on shA3-4
tet on shA3-5
tet on shA3-6
CT26_ADAR1 p110
2
1.5
1
0.5
0
Dox X
Dox 2μg
tet on shA3-1
tet on shA3-2
tet on shA3-3
tet on shA3-4
tet on shA3-5
tet on shA3-6
F
vector
shA3
vector
shA3
Doxycycline
- - - - - - - + + + + + + +
1 4 1 2 3 4 5 1 4 1 2 3 4 5
ADAR1
RIP3
MLKL
β-actin
vector
shA3
vector
shA3
Doxycycline
- - - - - - - + + + + + + +
1 4 6 7 8 9 10 1 4 6 7 8 9 10
ADAR1
RIP3
MLKL
β-actin

G

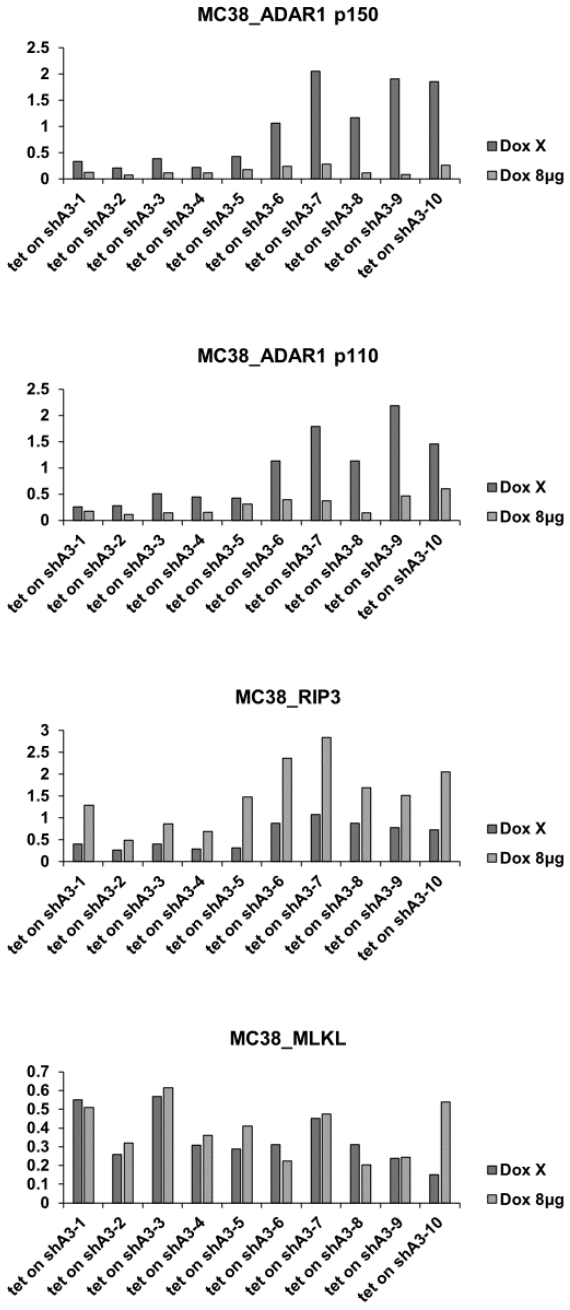


Figure 2. Inducible knockdown of ADAR1 in colon cancer cell

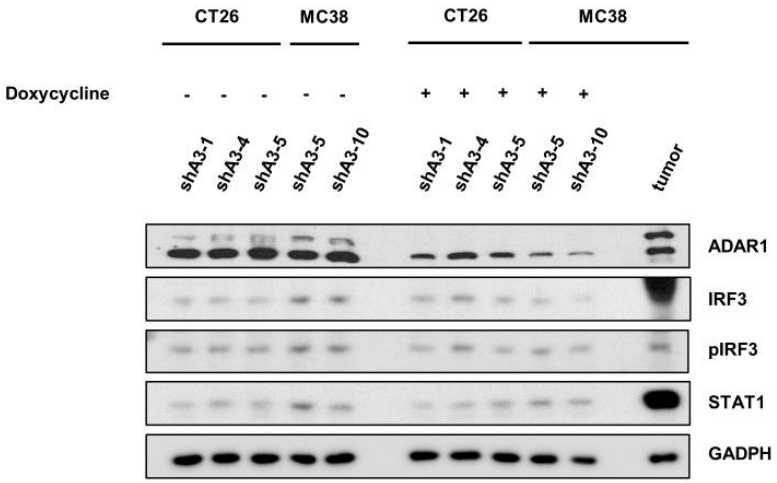
A schematic diagram of tet on vector. **B** Real-time PCR of ADAR1 expression by treatment different doxycycline dose in CT26 infected with tet on shADAR1 or control

(tet on vector) virus. **C** Western blot analysis of ADAR1 expression by treatment doxycycline 2 μg/ml in CT26 infected with tet on shADAR1 virus. **D** Western blot analysis of ADAR1 expression by treatment doxycycline 2 μg/ml in CT26 tet on shADAR1 clonal cells. **E** Densitometry of protein bands present on (D). **F** Western blot analysis for RIP3, MLKL, ADAR1 in MC38 tet on shADAR1 clonal cells. **G** Densitometry of protein bands present on (F).

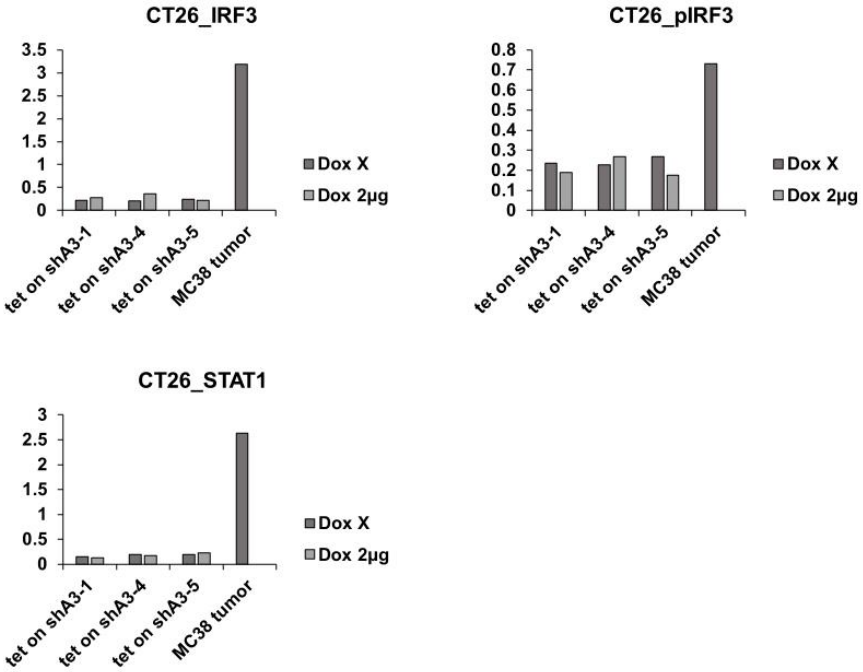
2. ADAR1 knockdown is challenging to exert an impact on the transcription and translation of interferon-stimulated genes in colon cancer cells

Since type 1 IFN signaling was activated when ADAR1 was inhibited in cervical cancer and breast cancer, the type 1 IFN signaling pathway of ADAR1 KD cells in CT26 and MC38 was checked to determine whether this was also possible in colon cancer (Figure 3A). As a result, it was confirmed that IRF3, pIRF3, and STAT1 all showed no differences in CT26 tet on shA3-1,4,5 (Figure 3B). The type 1 IFN signaling pathway was also confirmed in MC38. As a result, when both MC38 tet on shA3-5 and 10 were subjected to ADAR1 KD, IRF3, and pIRF3 decreased, while STAT1 showed no difference (Figure 3C). Based on these results, it can be observed that, for reasons not yet fully understood, activation of type 1 interferon signaling is not evident.

A


CT26
MC38
CT26
MC38
Doxycycline
- - - - -
+ + + + +
shA3-1
shA3-4
shA3-5
shA3-5
shA3-10
shA3-1
shA3-4
shA3-5
shA3-5
shA3-10
tumor
ADAR1
IRF3
pIRF3
STAT1
GADPH

B


CT26_IRF3
3.5
3
2.5
2
1.5
1
0.5
0
Dox X
Dox 2μg
tet on shA3-1
tet on shA3-4
tet on shA3-5
MC38 tumor
CT26_pIRF3
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Dox X
Dox 2μg
tet on shA3-1
tet on shA3-4
tet on shA3-5
MC38 tumor
CT26_STAT1
3
2.5
2
1.5
1
0.5
0
Dox X
Dox 2μg
tet on shA3-1
tet on shA3-4
tet on shA3-5
MC38 tumor

C

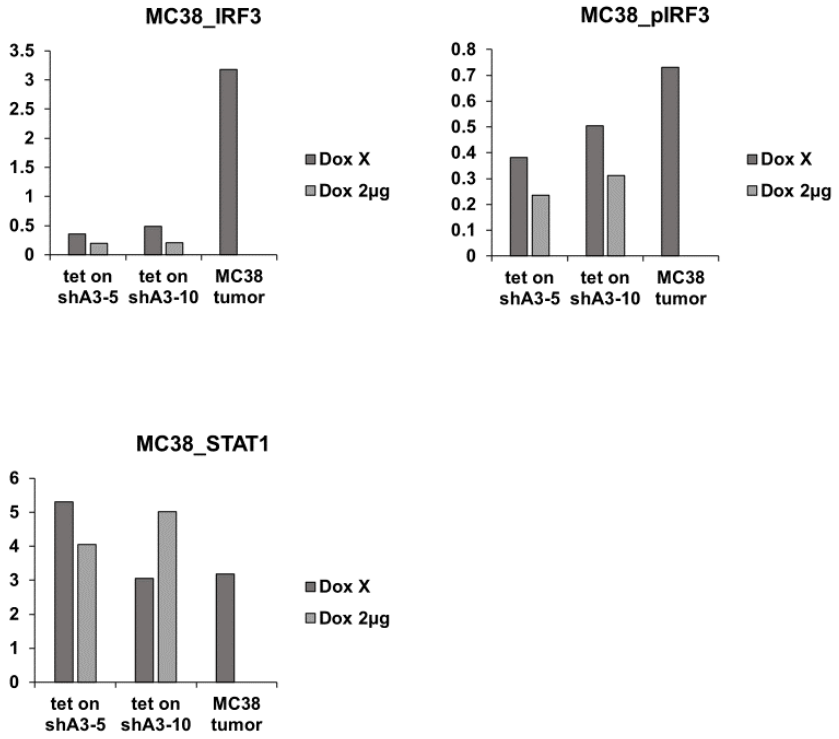


Figure 3. Identification of Type Ⅰ interferon signaling pathway upon ADAR1 Knockdown

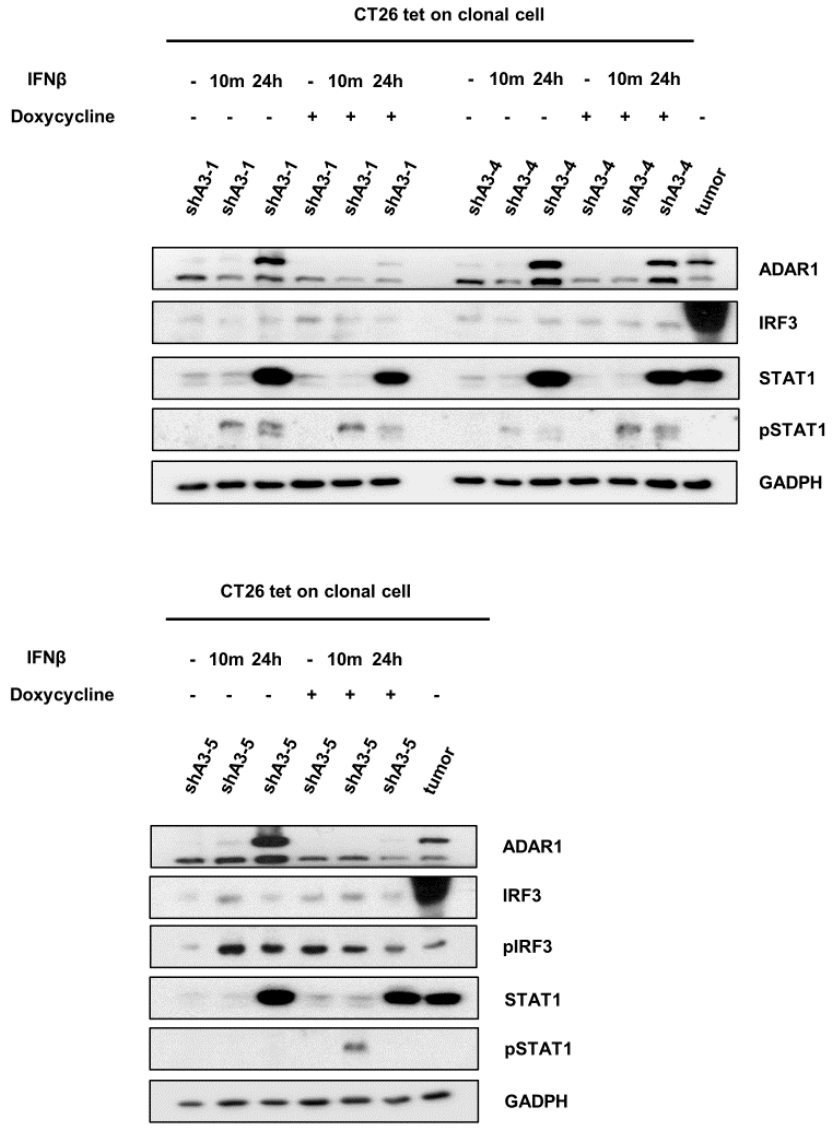
A Western blot analysis of CT26, MC38 tet on shADAR1 clonal cells for ADAR1, IRF3, pIRF3, STAT1 treated with 2 μg/ml doxycycline for 72 hours. MC38 tumor was a positive control. **B-C** Densitometry of protein bands present on (A).

3. ADAR1 knockdown is difficult to influence the transcription and translation of interferon-stimulated genes in colon cancer cells upon IFN treatment

In CT26 and MC38, when ADAR1 is inhibited, as in cervical cancer, not all type 1 IFN signaling appears to be activated, so additional experiments were conducted with treatment with IFN β. In CT26 and MC38, when ADAR1 is inhibited, as in cervical cancer, not all type 1 IFN signaling appears to be activated, so additional

experiments were conducted by treating with IFN β. It can be confirmed that ADAR1 has been successfully KD in clonal cells 1, 4, and 5. Comparing the ADAR1 knockdown, no differences were observed in IRF3, pIRF3, STAT1, and pSTAT1 levels in CT26 tet on shA3-1,4,5 (Figure 4A). This consistency with the earlier data suggests that, in CT26, the activation of type 1 interferon signaling remains unaltered. To confirm whether this result was correct, it conducted an experiment to confirm the type 1 IFN signaling pathway by treating it with IFN β. IFN β is secreted by type 1 IFN signaling and can bind to the receptor to activate STAT1 and pSTAT1, allowing downstream signaling to be confirmed even in clonal cells. Looking at the data treated with IFN β, there was a slight difference in IRF3 for each clonal cell, but STAT1 and pSTAT1 were increased both with and without ADAR1 KD, showing that the effect on IFN β was independent of the presence or absence of ADAR1 KD (Figure 4B). When examining the ADAR1 knockdown in MC38 tet on shADAR1 clonal cells 3 and 5, no differences were observed in the levels of STAT1, pSTAT1, IRF3, and pIRF3. When the experiment was conducted to check the type1 IFN signaling pathway by treating IFNβ, IRF3, pIRF3, STAT1, and pSTAT1 were both increased in both ADAR1 KD and not, so this study could see the effect on IFN β regardless of the presence or absence of ADAR1 KD (Figure 4C). Therefore, in CT26 and MC38, type 1 IFN signaling was still partially changed by ADAR1.

A


CT26 tet on clonal cell
IFNβ
- 10m 24h - 10m 24h - 10m 24h - 10m 24h
Doxycycline
- - - + + + - - - + + + -
shA3-1
shA3-1
shA3-1
shA3-1
shA3-1
shA3-1
shA3-4
shA3-4
shA3-4
shA3-4
shA3-4
shA3-4
tumor
ADAR1
IRF3
STAT1
pSTAT1
GADPH
CT26 tet on clonal cell
IFNβ
- 10m 24h - 10m 24h
Doxycycline
- - - + + + -
shA3-5
shA3-5
shA3-5
shA3-5
shA3-5
shA3-5
tumor
ADAR1
IRF3
pIRF3
STAT1
pSTAT1
GADPH

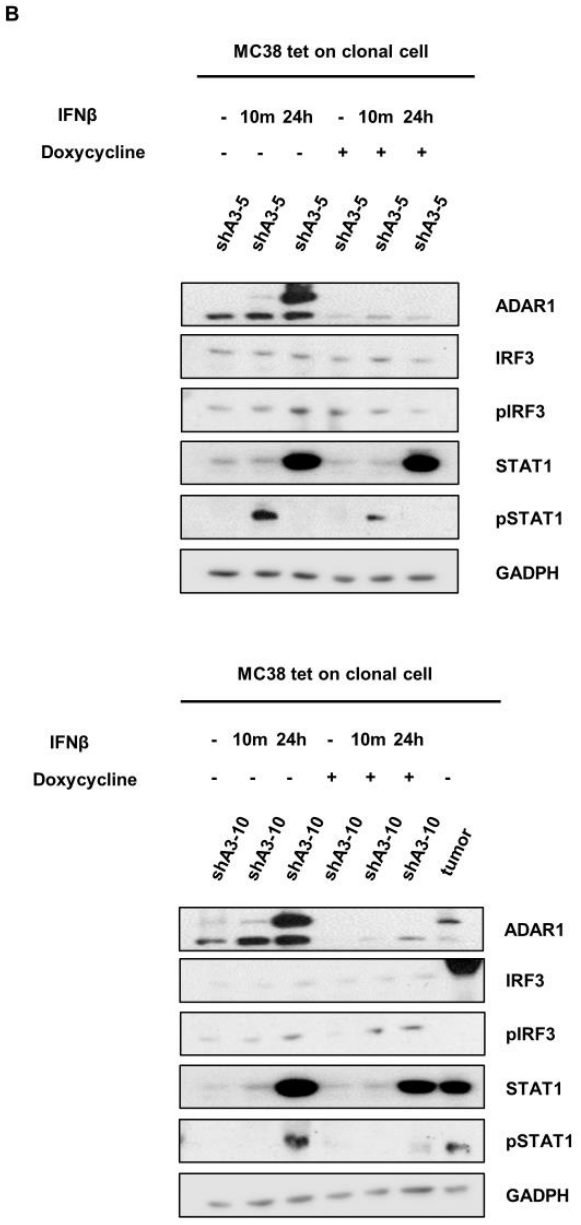


Figure 4. Identification of Type Ⅰ interferon signaling pathway upon ADAR1 Knockdown in IFN β treatment

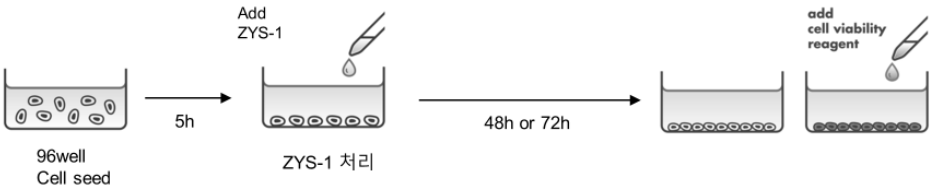
A Western blot analysis of CT26 tet on shADAR1 clonal cells for ADAR1, IRF3, pIRF3, STAT1, pSTAT1 treated with 2 μg/ml doxycycline for 72 hours and IFN β for 10min, 24 hours. MC38 tumor was a positive control. **B** Western blot analysis of

MC38 tet on shADAR1 clonal cells for ADAR1, IRF3, pIRF3, STAT1, pSTAT1 treated with 2 μg/ml doxycycline for 72 hours and IFN β for 10min, 24 hours. MC38 tumor was a positive control.

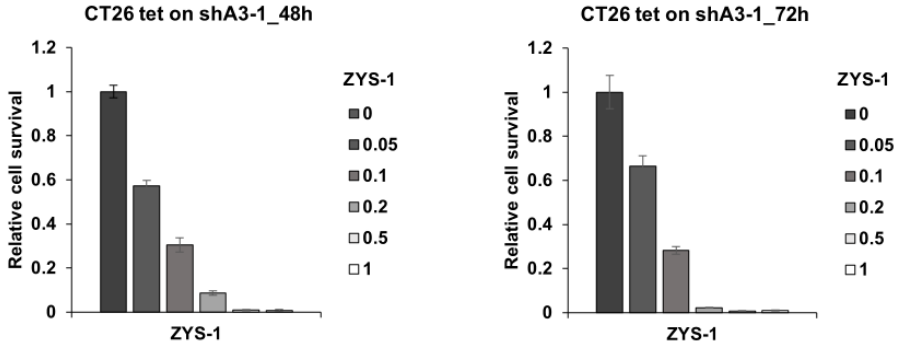
4. Identification of ZYS-1 chemical effect toxicity

To select the ZYS-1 dose, a proliferation assay was performed. The proliferation assay was performed 48 and 72 hours after ZYS-1 treatment. (Figure 5A). ZYS-1 reduced cell proliferation. When CT26 tet on shADAR1 clonal cells were treated with ZYS-1 at 0.5μg/ml, cell viability decreased to less than 20%, so these study first selected a high concentration of 0.5μg/ml and conducted the experiment (Figure 5B-D). When MC38 tet on shADAR1 clonal cells were treated with ZYS-1 at 0.2μg/ml, the cell viability decreased to less than 20%, so the experiment was first selected at a high concentration of 0.2μg/ml (Figure 5E-F).

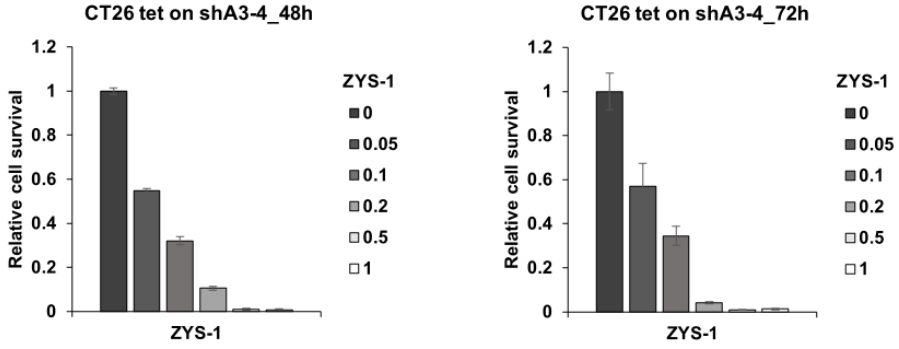
A


Add
ZYS-1
add
cell viability
reagent
5h
48h or 72h
96well
Cell seed
ZYS-1 처리

B


CT26 tet on shA3-1_48h
CT26 tet on shA3-1_72h
Relative cell survival
ZYS-1
0
0.05
0.1
0.2
0.5
1
0
0.2
0.4
0.6
0.8
1
1.2

C


CT26 tet on shA3-4_48h
CT26 tet on shA3-4_72h
Relative cell survival
ZYS-1
0
0.05
0.1
0.2
0.5
1
0
0.2
0.4
0.6
0.8
1
1.2

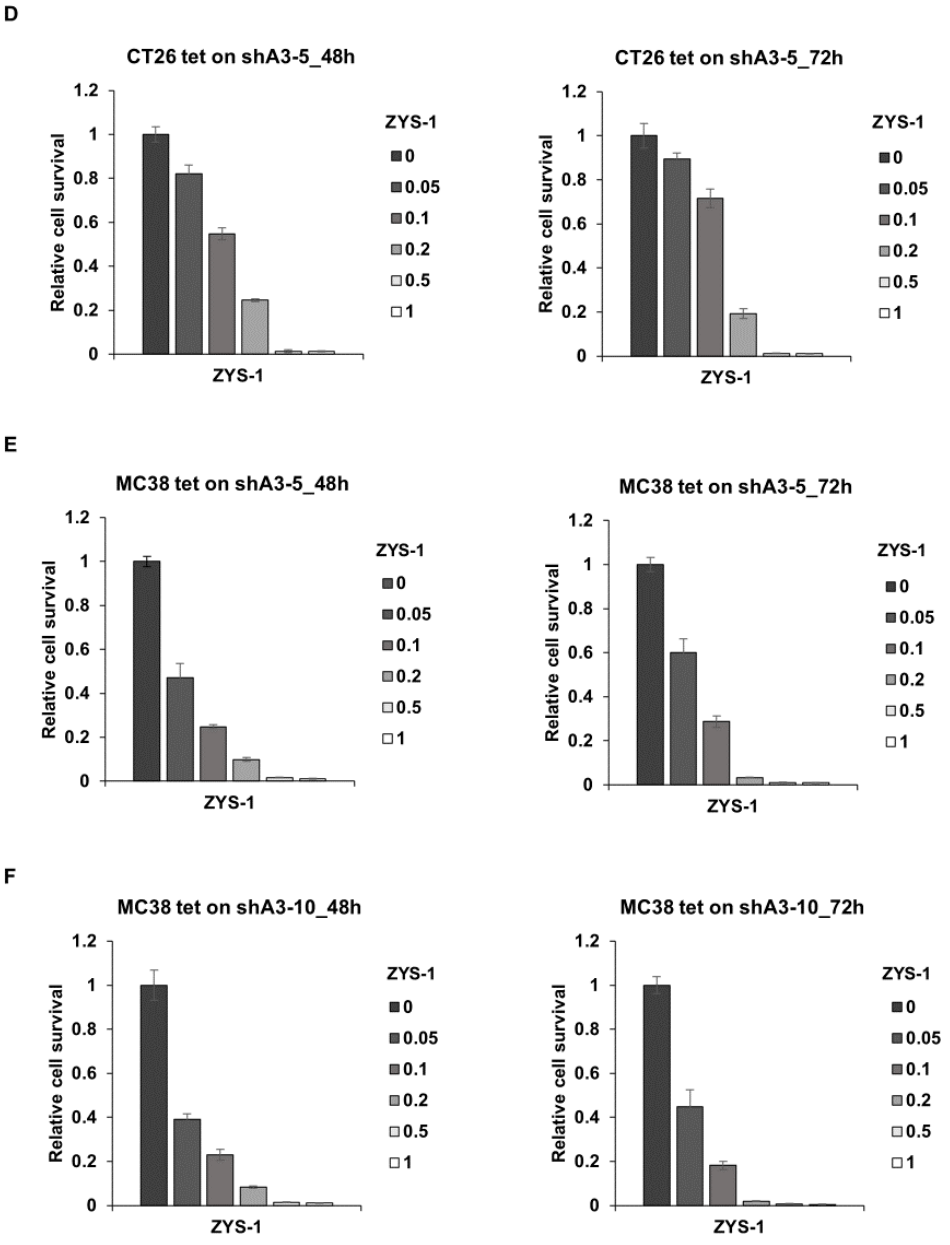
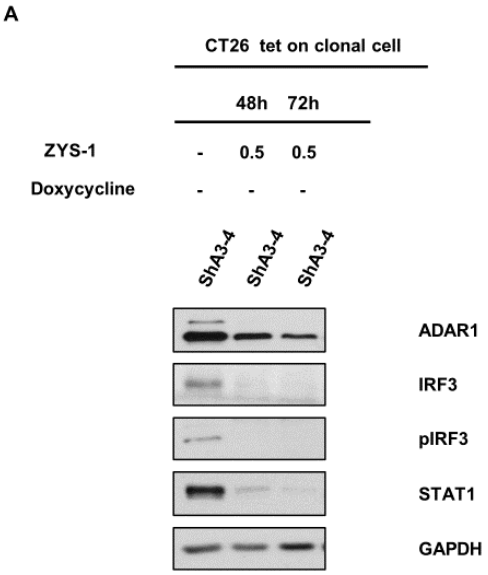


Figure 5. CT26 and MC38 Tet-on proliferation assay in ZYS-1 treatment

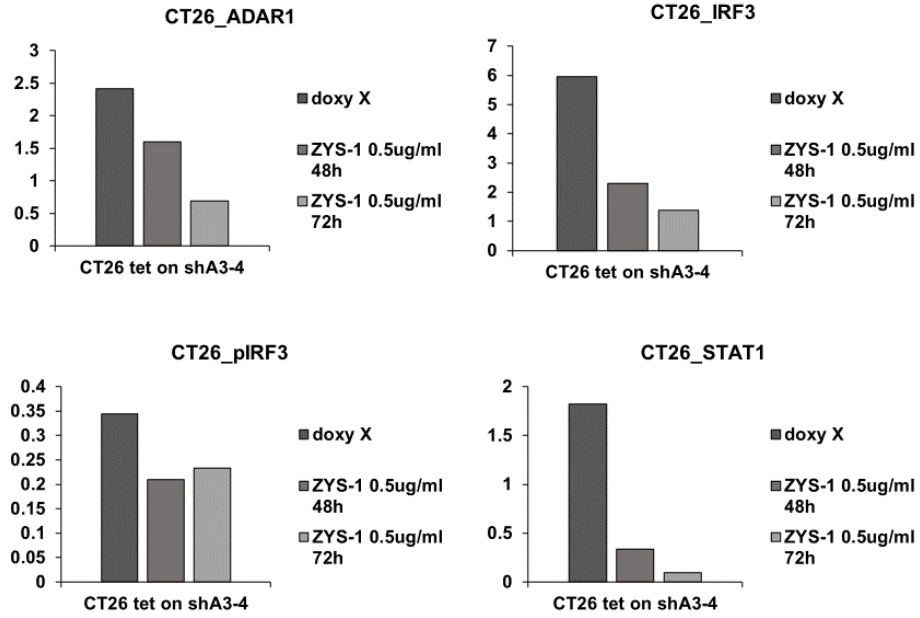
A Schematic summary of proliferation with ZYS-1. **B-D** Proliferation assay result with ZYS-1 (0, 0.05, 0.1, 0.2, 0.5, 1 μM) for 48, 72 hours in CT26 tet on shADAR1 clonal cell. **E-F** Proliferation assay result with ZYS-1 (0, 0.05, 0.1, 0.2, 0.5, 1 μM) for 48, 72 hours in MC38 tet on shADAR1 clonal cell.

5. ADAR1 inhibition with ZYS-1 does not induce type 1 IFN signaling

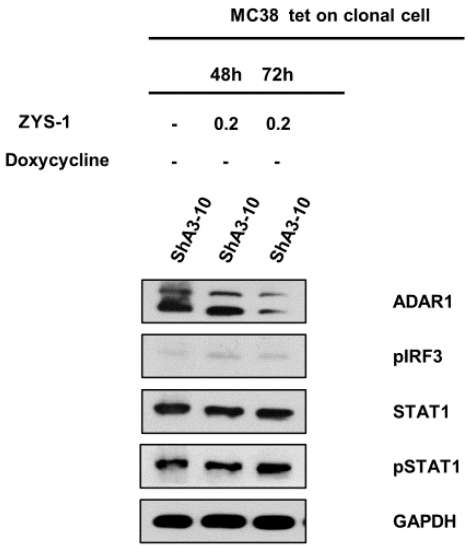
By treating ZYS-1, which directly inhibits ADAR1, the Type I interferon signaling pathway was confirmed by western blot (Figure 5A, C). By observing ADAR1 knockdown, it can be seen that ZYS-1 is functioning effectively. Additionally, it can confirm the reduction of IRF3, pIRF3, and STAT1 in CT26 tet on shADAR1 3-4 upon treatment with ZYS-1 (Figure 5B). In MC38 tet on shADAR1 3-10, it can be seen that pIRF3, STAT1, and pSTAT1 are decreased upon ZYS-1 treatment (Figure 5D). Considering the similar results in the experiments (Figure 4A, C), it can be indirectly inferred that ADAR1 is not being regulated. This suggests the possibility of the involvement of a pathway other than type 1 IFN signaling.



B


CT26_ADAR1
3
2.5
2
1.5
1
0.5
0
CT26 tet on shA3-4
doxy X
ZYS-1 0.5ug/ml 48h
ZYS-1 0.5ug/ml 72h
CT26_IRF3
7
6
5
4
3
2
1
0
CT26 tet on shA3-4
doxy X
ZYS-1 0.5ug/ml 48h
ZYS-1 0.5ug/ml 72h
CT26_pIRF3
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
CT26 tet on shA3-4
doxy X
ZYS-1 0.5ug/ml 48h
ZYS-1 0.5ug/ml 72h
CT26_STAT1
2
1.5
1
0.5
0
CT26 tet on shA3-4
doxy X
ZYS-1 0.5ug/ml 48h
ZYS-1 0.5ug/ml 72h

C


MC38 tet on clonal cell
48h 72h
ZYS-1 - 0.2 0.2
Doxycycline - - -
ShA3-10
ShA3-10
ShA3-10
ADAR1
pIRF3
STAT1
pSTAT1
GAPDH

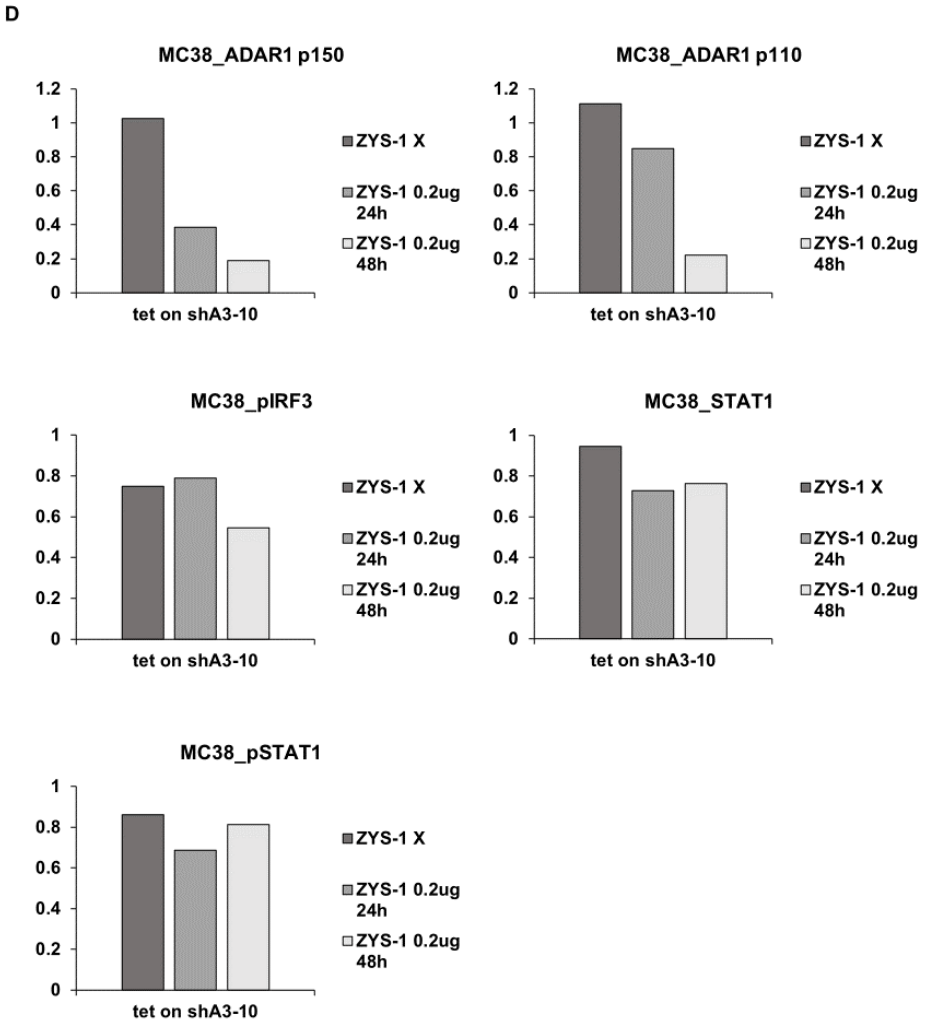
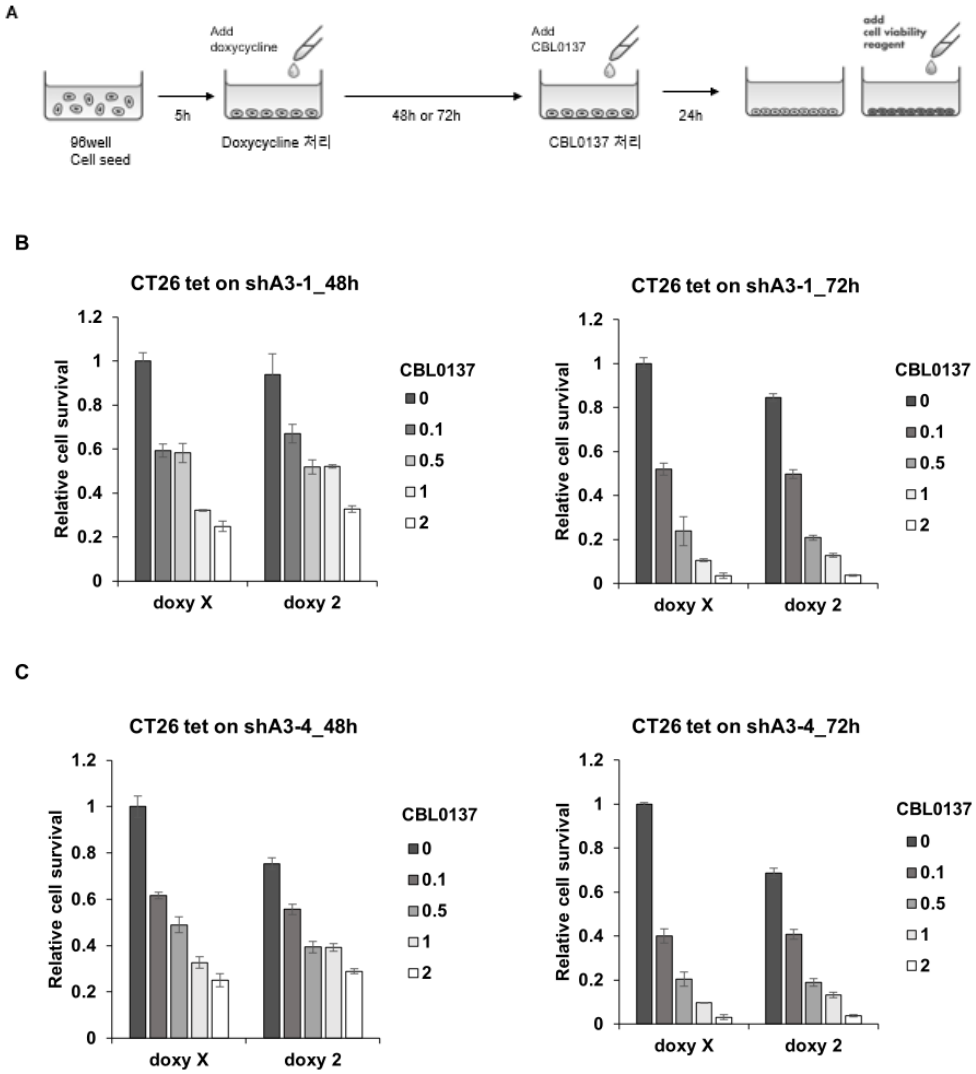


Figure 6. Identification of Type Ⅰ interferon signaling pathway upon ADAR1 Knockdown in ZYS-1 treatment

A Western blot analysis of CT26 tet on shADAR1 clonal cells for ADAR1, IRF3, pIRF3, STAT1, treated with 0.5 μg/ml ZYS-1 for 48, 72 hours. **B** Densitometry of protein bands present on (A). **C** Western blot analysis of MC38 tet on shADAR1 clonal cells for ADAR1, pIRF3, STAT1, pSTAT1 treated with 0.2 μg/ml ZYS-1 for 48, 72 hours. **D** Densitometry of protein bands present on (C).

6. Identification of CBL0137 chemical effect toxicity

To select the CBL0137 dose, a proliferation assay was performed. The proliferation assay was performed 48 and 72 hours after CBL01371 treatment (Figure 7A). CBL0137 slightly reduced cell proliferation. When CT26 tet on shADAR1 clonal cells and MC38 tet on shADAR1 clonal cells were treated with CBL0137 at different concentrations, the cell viability of 1 and 2uM was similar, so 0.1 and 0.5μg/ml were selected and western blot was performed (Figure 7B-E).



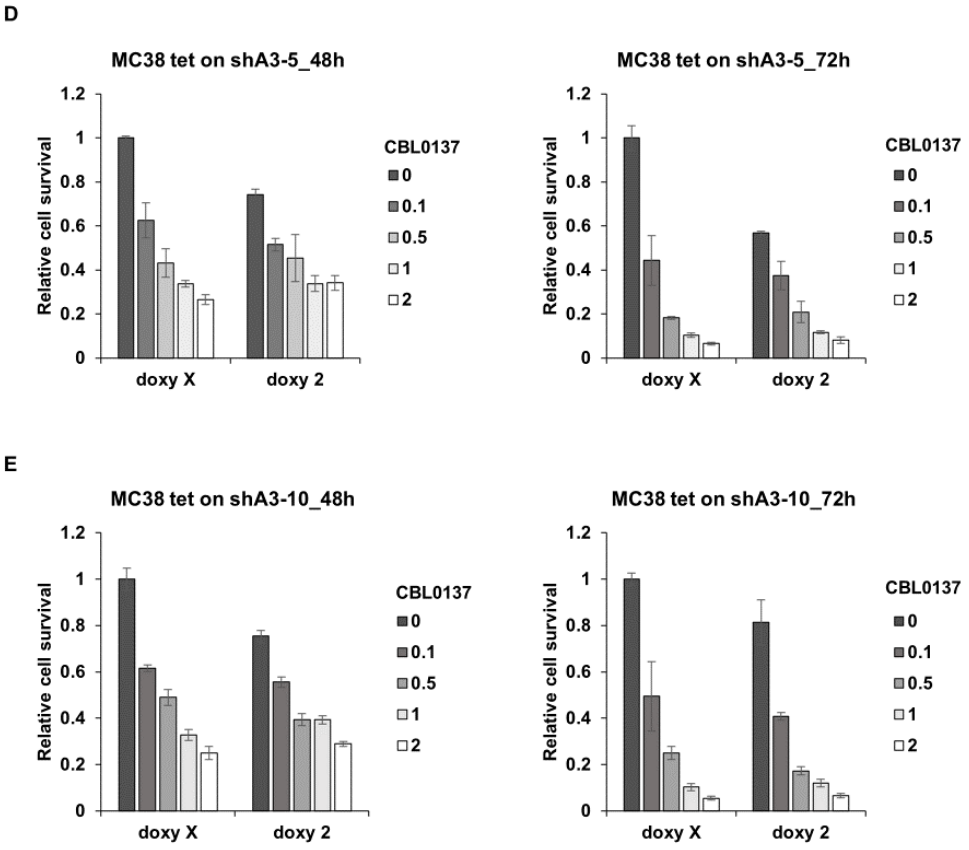


Figure 7. CT26 and MC38 Tet-on proliferation assay in CBL0137 treatment

A Schematic summary of proliferation with CBL0137. **B-C** Proliferation assay result with CBL0137 (0, 0.1, 0.5, 1, 2 μM) for 48, 72 hours in CT26 tet on shADAR1 clonal cell. **D-E** Proliferation assay result with ZYS-1 (0, 0.1, 0.5, 1, 2 μM) for 48, 72 hours in MC38 tet on shADAR1 clonal cell.

7. ADAR1 knockdown has a partial impact on the ZBP1-mediated necroptosis pathway in colon cancer cell lines

The ZBP1-mediated necroptosis pathway was confirmed in ADAR1 KD CT26 and MC38 tet on shADAR1 clonal cells. As a result, RIP3 showed no difference in CT26 tet on shADAR1 3-1, 4, and 5, and MLKL showed a slight increase or decrease depending on the clonal cell. In MC38 tet on shADAR1 clonal cells 3-5 and 10, an

increase in RIP3 was observed, while MLKL showed no significant difference (Figure 8A). Based on these results, it can be inferred that the inhibition of ADAR1 may partially regulate or not regulate the ZBP1-mediated necroptosis pathway.

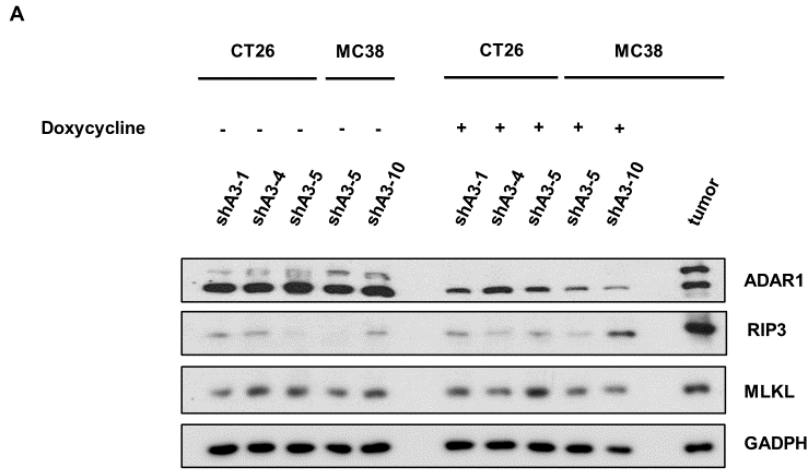


Figure 8. Identification of ZBP1-mediated necroptosis pathway upon ADAR1 Knockdown

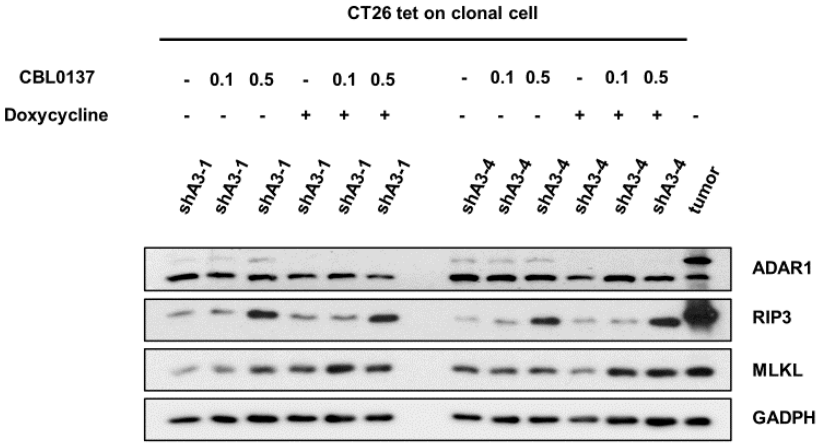
A Western blot analysis of CT26, MC38 tet on shADAR1 clonal cells for RIP3, MLKL treated with 2 μg/ml doxycycline for 72 hours. MC38 tumor was a positive control.

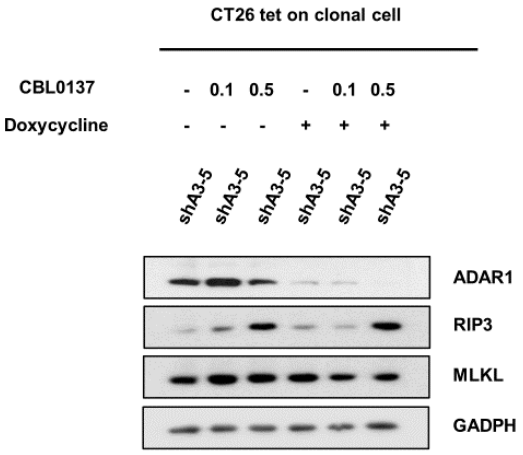
8. ADAR1 knockdown has a partial impact or is challenging to influence the ZBP1-mediated necroptosis pathway in colon cancer cell lines upon treatment with CBL0137

When ADAR1 was inhibited, additional experiments were conducted by treating with CBL0137. Comparing ADAR1 KD in CT26 tet on clonal cells, it was confirmed that there was no difference in RIP3 when ADAR1 was KD in tet on clonal cells 1, 4, and 5. MLKL can be seen slightly increasing in tet on clonal cell 1, but no difference can be seen in tet on clonal cells 4 and 5. Looking at these results, it can be seen that in CT26, when ADAR1 is KD, the ZBP1-mediated necroptosis pathway is not activated at all. To confirm whether this result was correct, these study conducted an experiment to check the ZBP1-mediated necroptosis pathway by treating CBL0137.

CBL0137 is known to activate ZBP1, so downstream signaling can be confirmed even in clonal cells. Looking at the data processed with CBL0137, RIP3 was increased both with and without ADAR1 KD and no difference was seen, so the effect on CBL0137 could be seen regardless of the presence or absence of ADAR1 KD. MLKL also shows a slight increase or no difference when treated with CBL0137, and a decrease or no difference can be seen compared to when ADAR1 is inhibited (Figure 9A). When ADAR1 is KD in MC38 tet on shADAR1 clonal cells, RIP3 increases, but MLKL shows no difference. When an experiment was conducted to confirm the ZBP1-mediated necroptosis pathway by treating CBL0137, RIP3 was found to be increased both with and without ADAR1 KD upon CBL0137 treatment, showing the effect on CBL0137 independent of the presence or absence of ADAR1 KD. MLKL shows a slight increase when treated with 0.1uM in tet on clonal cell 5, but there is no difference in other bands, and tet on clonal cell 10 shows an increase when treated with CBL0137 but decreases when compared to ADAR1 KD. You can see this (Figure 9B). Considering these results, one could infer that the inhibition of ADAR1 is not associated with necroptosis.

A


CT26 tet on clonal cell
CBL0137
Doxycycline
- 0.1 0.5 - 0.1 0.5 - 0.1 0.5 - 0.1 0.5
- - - + + + - - - + + + -
shA3-1 shA3-1 shA3-1 shA3-1 shA3-1 shA3-1 shA3-4 shA3-4 shA3-4 shA3-4 shA3-4 shA3-4 tumor
ADAR1
RIP3
MLKL
GADPH


CT26 tet on clonal cell
CBL0137
Doxycycline
- 0.1 0.5 - 0.1 0.5
- - - + + +
shA3-5 shA3-5 shA3-5 shA3-5 shA3-5 shA3-5
ADAR1
RIP3
MLKL
GADPH

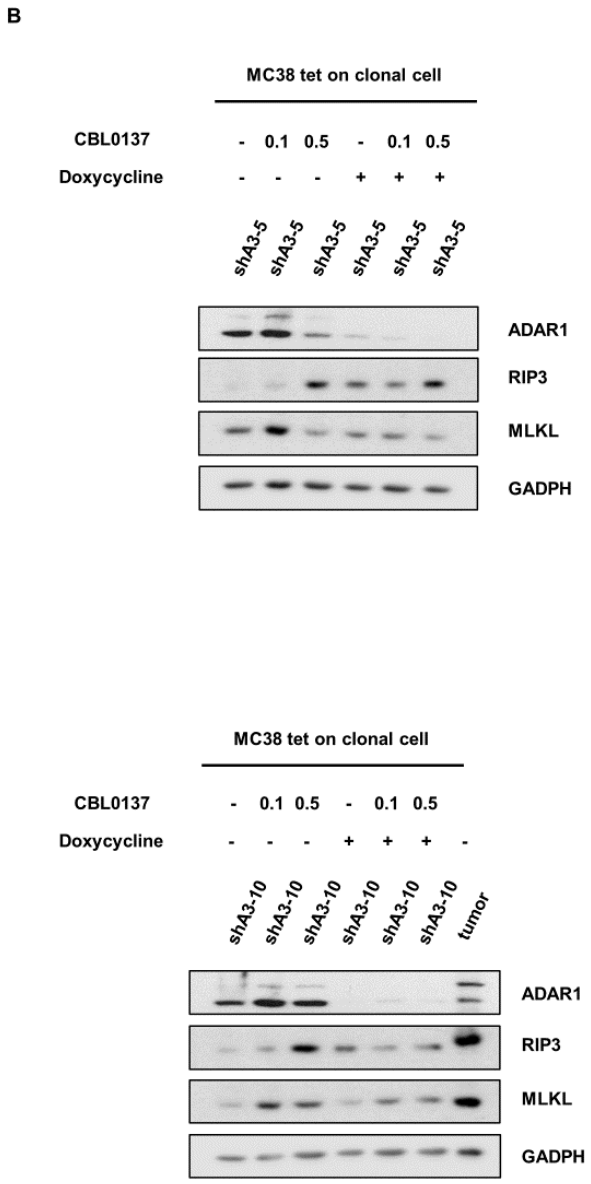


Figure 9. Identification of ZBP1-mediated necroptosis pathway upon ADAR1 Knockdown in CBL0137 treatment

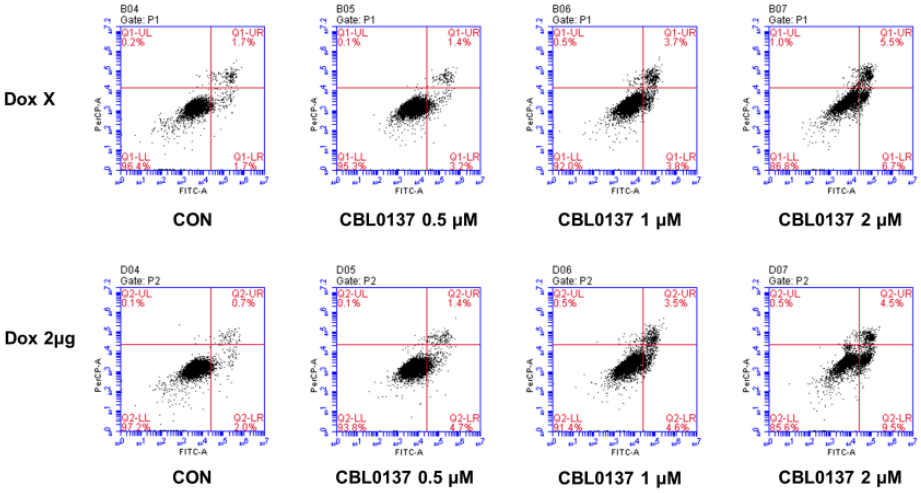
A Western blot analysis of CT26 tet on shADAR1 clonal cells for RIP3, MLKL treated with 2 μg/ml doxycycline for 72 hours and 0.1, 0.5uM CBL0137 for 24 hours. MC38 tumor was a positive control. **B** Western blot analysis of MC38 tet on shADAR1

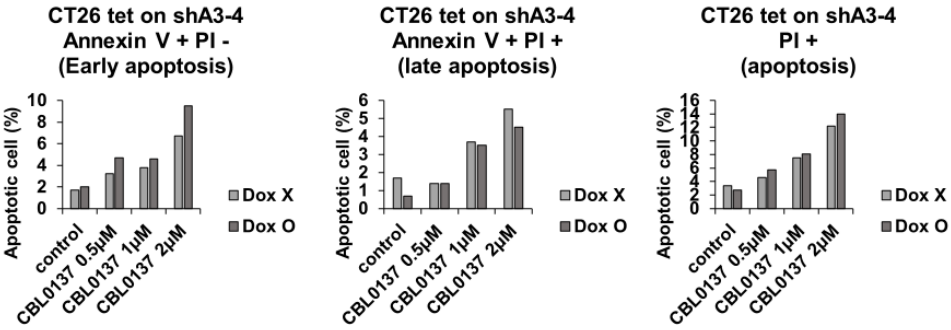
clonal cells for RIP3, MLKL treated with 2 μg/ml doxycycline for 72 hours and 0.1, 0.5uM CBL0137 for 24 hours. MC38 tumor was a positive control.

9. ADAR1 knockdown induces apoptosis in colon cancer cell lines upon treatment with CBL0137

Due to the possibility that the cell death induced by the inhibition of ADAR1 or CBL0137 may be influenced by a pathway other than necroptosis, annexin V staining FACS was conducted to verify the occurrence of cell death. As the results of the proliferation assay showed that cell viability was lowered when treated with CBL0137 at a high rate, FACS was performed by treating with CBL0137 at 0.5, 1, and 2uM for 48 hours. Looking at CT26 tet on shADAR1 clonal cells, there appears to be no difference when ADAR1 is KD in the control, but you can see that more apoptosis occurs as the concentration of CBL0137 increases (Figure 8A). Looking at MC38 tet on shADAR1 clonal cells, there appears to be no difference when ADAR1 is KD in the control, but you can see that more apoptosis occurs as the concentration of CBL0137 increases (Figure 8B). Since there was an increase in apoptosis in the FACS assay results, this observation suggests that there is an possibility of apoptosis rather than necroptosis.

A


Dox X
B04
Gate: P1
Q1-UL 0.2%
Q1-UR 1.7%
Q1-LL 98.4%
Q1-LR 1.7%
B05
Gate: P1
Q1-UL 0.1%
Q1-UR 1.4%
Q1-LL 92.3%
Q1-LR 7.2%
B06
Gate: P1
Q1-UL 0.5%
Q1-UR 3.7%
Q1-LL 92.9%
Q1-LR 2.8%
B07
Gate: P1
Q1-UL 1.0%
Q1-UR 5.5%
Q1-LL 88.8%
Q1-LR 4.7%
PerCP-A
FITC-A
CON
CBL0137 0.5 μM
CBL0137 1 μM
CBL0137 2 μM
Dox 2μg
D04
Gate: P2
Q2-UL 0.1%
Q2-UR 0.7%
Q2-LL 97.2%
Q2-LR 2.0%
D05
Gate: P2
Q2-UL 0.1%
Q2-UR 1.4%
Q2-LL 93.8%
Q2-LR 4.7%
D06
Gate: P2
Q2-UL 0.5%
Q2-UR 3.5%
Q2-LL 91.4%
Q2-LR 4.6%
D07
Gate: P2
Q2-UL 0.5%
Q2-UR 4.5%
Q2-LL 85.6%
Q2-LR 9.5%
PerCP-A
FITC-A
CON
CBL0137 0.5 μM
CBL0137 1 μM
CBL0137 2 μM


CT26 tet on shA3-4
Annexin V + PI -
(Early apoptosis)
CT26 tet on shA3-4
Annexin V + PI +
(late apoptosis)
CT26 tet on shA3-4
PI +
(apoptosis)
Apoptotic cell (%)
control
CBL0137 0.5μM
CBL0137 1μM
CBL0137 2μM
Dox X
Dox O

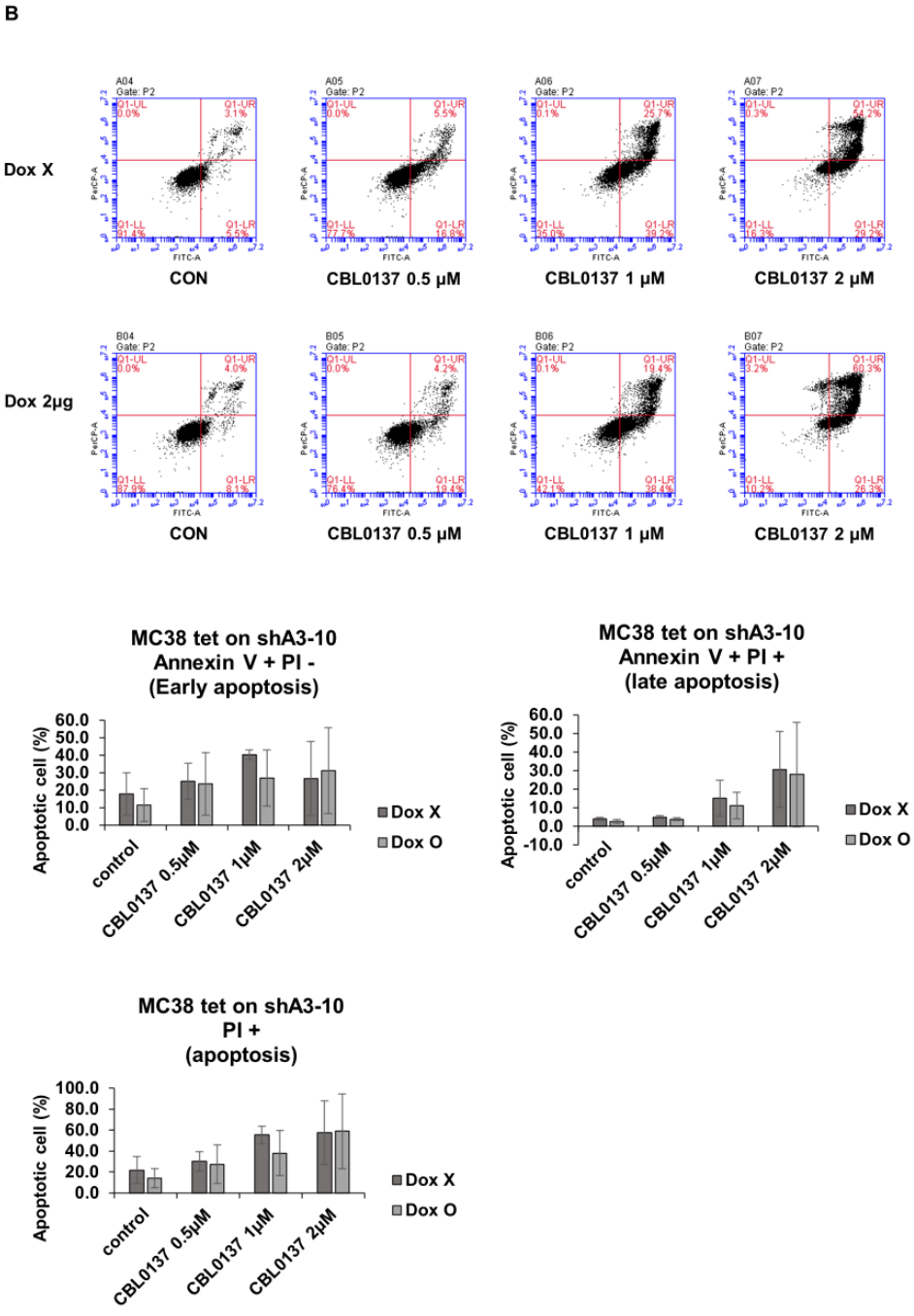


Figure 10. CT26 and MC38 Tet-on Annexin V staining Flow cytometry in CBL0137 treatment

A Annexin V staining FACS assay with CBL0137 (0, 0.5, 1, 2 μM) for 48 hours in CT26 tet on shADAR1 clonal cell. **B** Annexin V staining FACS assay with CBL0137 (0, 0.5, 1, 2 μM) for 48 hours in MC38 tet on shADAR1 clonal cell.

Discussion

Previous reports have shown that in the absence of ADAR1, endogenous dsRNA is recognized as non-self and triggers interferon (IFN) signaling by activating dsRNA sensing proteins such as MDA-5 and PKR5[7-10]. In addition, it is known that ADAR1 inhibition inhibits ZBP1-mediated necroptosis by limiting the interaction between ZBP1 and RIPK3[9]. ADAR1 is upregulated in many solid cancers and is also upregulated in colon cancer[18]. In colon cancer, it can be seen that the mRNA and protein levels of ADAR1 are increased compared to normal cells, but it is currently unknown by what mechanism ADAR1 is regulated in colon cancer. Based on the published research results, these study conducted research to determine what pathway mechanism occurs when ADAR1 is regulated in colon cancer (Figure 1).

In this study, an inducible ADAR1 knockdown system using Tet-on shADAR1 plasmid was used to confirm the type 1 IFN signaling pathway and ZBP1-mediated necroptosis pathway through ADAR1 knockdown (Figure 2). When ADAR1 was inhibited in colon cancer cell lines, type 1 IFN signaling was confirmed to be not activated (Figure 3). An experiment was conducted to identify the type 1 IFN signaling pathway by treating with IFN β. Although the signal responded after treatment with IFN β, there was no effect through ADAR1 inhibition (Figure 4). These results showed that CT26 and MC38 have different outcomes in cervical cancer[11] and breast cancer[16] when ADAR1 is inhibited.

An experiment was conducted to confirm whether type 1 IFN signaling occurred similarly by treating ZYS-1, which directly inhibits ADAR1. First, through proliferation assay, the ZYS-1 dose was selected as 0.5μg/ml for CT26 and 0.2μg/ml for MC38 (Figure 5). Although the Type I interferon signaling pathway was not investigated in previous studies[15] using ZYS-1, you can see that the signaling of the data I KD using tet on shADAR1 and the data KD by processing ZYS-1 are similar (Figure 6). So, This study indirectly proved that ADAR1 is not regulated, but additional experiments

are needed to confirm other type 1 IFN signaling pathways, and it is conceivable that pathways other than type 1 IFN signaling may be involved.

In addition, since it has been reported that ZBP1 activation occurs when ADAR1 expression is inhibited, this study attempted to confirm the ZBP1-mediated necroptosis pathway using ADAR1 KD cells in CT26 and MC38 and CBL0137 to determine whether this is a possibility in colon cancer. CBL0137 doses of 0.1 and 0.5uM were selected through proliferation assay (Figure 7). Initially, when ADAR1 was inhibited and compared in CT26 and MC38 tet on shADAR1 clonal cells, there was no significant difference observed in RIP3 and MLKL in CT26, indicating an unchanged status in the ZBP1-mediated necroptosis pathway. However, in MC38, the observed increase in RIP3 suggests that the inhibition of ADAR1 might partially regulate or not regulate the ZBP1-mediated necroptosis (Figure 8). CBL0137 is known to activate ZBP1, so the ZBP1-mediated necroptosis pathway was confirmed after CBL0137 treatment. When treated with CBL0137, RIP3 and MLKL are increased, but when compared with ADAR1 inhibition, they are decreased or show no difference (Figure 9). Considering these results, it can be contemplated that the inhibition of ADAR1 is not associated with necroptosis. Given the possibility that cell death induced by ADAR1 inhibition or CBL0137 might be influenced by a pathway other than necroptosis, annexin V staining FACS was conducted to confirm cell death. The results revealed an increasing occurrence of apoptosis with the escalating concentration of CBL0137 (Figure 10).

It is conceivable that a pathway other than ZBP1-mediated necroptosis might be involved, suggesting a contrasting result with previous studies. This research underscores the need for further investigations into the immunomodulatory role of ADAR1 in CRC, considering the conflicting outcomes with prior research findings.

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Korean Abstractss

ADAR1(RNA 에 작용하는 아데노신 탈아미노화효소)는 이중 가닥 RNA(dsRNA)와의 결합해서 RNA editing 을 통해 아데노신의 탈아미노화를 촉매하여 이노신을 생성합니다. ADAR1 은 IFN (type I interferon) 반응을 억제하는 기능과 ZBP1 과 RIPK3 상호작용을 제한해서 ZBP1-mediated necroptosis 을 억제하는 기능이 알려져 있지만, 대장암에서의 그 메커니즘은 명확하게 이해되지 않습니다. 우리는 ADAR1 이 IFN 신호전달과 ZBP1-mediated necroptosis 을 어떻게 조절하는지 밝히기 위해 tet on shADAR1 system 사용하여 inducible 하게 ADAR1 이 knockdown 될 수 있는 대장암 세포로 연구를 진행하였습니다. 그러나 이전 연구들과 다르게 대장암 세포에서 ADAR1 knockdown 시 IFN 신호전달과 ZBP1-mediated necroptosis 이 activation 되지 않는 것을 관찰했습니다. 또한, IFN β와 CBL0137 처리 시에도 ADAR1 knockdown 했을 때 IFN 신호전달과 ZBP1-mediated necroptosis 이 activation 되지 않는 것을 관찰했습니다. 따라서, 우리의 결과는 대장암 세포에서 ADAR1 이 알려진 type I IFN signaling pathway 와 ZBP1-mediated necroptosis 의 RNA editing 과는 다른 pathway 가 존재할 수도 있음을 시사합니다.