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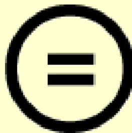

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Master of Science

# ADAR1 and interferon signaling suppression in gastric cancer

# 위암에서 ADAR1과 인터페론 신호 억제

The Graduate School

Of the University of Ulsan

Department of Medicine

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# ADAR1 and interferon signaling suppression in gastric cancer

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Master's thesis

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for the Degree of

Master of Science

by

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Department of Medicine

Ulsan, Korea

February 2019

ADAR1 and interferon signaling suppression in gastric cancer

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Ulsan, Korea

February 2019

## ABSTRACT

ADAR (Adenosine Deaminase Acting on RNA) is known to mediate deamination of adenosine to inosine through binding to double-stranded RNA (dsRNA), the phenomenon known as RNA editing. Moreover, another unique property of ADAR1 is its ability to suppress type I interferon (IFN) response.

To understand regulation between ADAR1 and type I interferon (IFN) signaling in gastric cancer, we sought to determine the specific target molecule(s) where ADAR1 exerts its activity on IFN signaling, and to determine whether the suppression of IFN response is related to its RNA editing function. We found that protein level of STAT1 and IRF9 was increased upon ADAR1 knockdown even in the absence of type I or type II IFN in AGS cell.

Mechanistically, we identified miRNA-302a-3p the level of which decreases upon ADAR1 knockdown, binds to the seed sequence of IRF9. We further found IRF9 UTR reporter level was decreased upon the addition of miRNA 302a-3p, which was ameliorated when the seed-binding sequence in UTR was mutated. In contrast, STAT1 UTR reporter level was not decreased upon the addition of miRNA-302a-5p, suggesting other mechanism of STAT1 over-expression exists. Interestingly, treatment of miRNA-302a-3p mimic to ADAR1 knocked-downed AGS cell reversed IRF9 as well as STAT1 protein level to that of control AGS cells.

Altogether, these results suggest that ADAR1 functions in gastric cancer through suppression of STAT1 and IRF9 via miRNA302a-3p, independently from the activation or editing of known IFN production pathway.

**Keywords**: RNA editing, gastric cancer, 3′-untranslated region, microRNA-302a-3p, type I interferon, IRF9, STAT1.

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# INTRODUCTION

Adenosine deaminase acting on RNA (ADAR) 1 is a double strand (ds) RNA-binding and editing protein. It has 2 major isoforms: a shorter ADAR1-p110 and a longer ADAR1-p150[1)]. The promoter of the longer ADAR1-p150 isoform is located in first exon and contains an interferon-stimulated response element and its translation start site (TSS) is also within exon 1A, whereas the ADAR1-p110 has a different promoter and its translation start from exon 2[2)]. The ADAR1-p110 isoform is present predominantly in the nucleus whereas the interferon-inducible isoform localizes to the cytoplasm, implying they have different functions[2, 3)]. The most prevalent type of RNA editing in mammalians is the conversion of adenosine to inosine by deamination (A-to-I editing), largely mediated by ADAR1 and to a lesser extent by ADAR2 or another type of ADAR. RNA editing is basically divided into 2 categories: infrequent site-specific and promiscuous non-site specific "hyper" editing of repetitive elements[2,4)].

Another unique property of the ADAR1 protein is its ability to suppress type I IFN signaling[5, 6)]. Viral infection or endogenously produced dsRNAs induces a series of cellular signaling events leading to production of type I IFNs, such as IFN-α and IFN-β, as well as other proinflammatory cytokines[7-9)]. These cytokines play a key role in the induction of innate immune responses to inhibit virus replication and possibly, activate autoimmunity[10, 11)]. Specifically, (pattern recognition receptors are activated after viral infection); RIG-I (retinoic acid-

inducible gene I), MDA5 (Melanoma Differentiation Associated Protein 5), or TLR3 (Toll-like receptor 3) detects and activates (Mitochondrial antiviral-signaling protein) MAVS, which in turn activate IRF3 (Interferon regulating factor 3) and/or IRF7 (Interferon regulating factor 7)[12-14]. Activation of IRF3 occurs via phosphorylation of its C-terminal, which in turn forms dimer and then translocate from the cytoplasm to the nucleus to activate transcription of type I IFNs[15]. Receptor for type I IFN signaling constitutes of heterodimers composed of IFNAR1 (Interferon Alpha and Beta Receptor Subunit 1) and IFNAR2 [16]. Downstream of the IFN receptors are receptor-associated tyrosine kinases – JAK1 (Janus kinase 1) and Tyk2 (Tyrosine Kinase 2), which are responsible for phosphorylation of STAT1 (Signal Transducer and Activator of Transcription 1) and STAT2[17]. Upon type I IFN stimulation, via JAK1 and Tyk2 activation, STAT1 and STAT2 form heterodimer which assemble a complex with IRF9 (Interferon regulating factor 9) to form IFN-stimulated gene factor 3 (ISGF3) complex[18-21]. ISGF3 then moves into the nucleus, binds to IFN-stimulated response element (ISRE) to promote transcription of numerous IFN stimulated genes (ISGs).

Although the unique property of the ADAR1 protein is its ability to suppress type I IFN signaling, till now, it is unclear whether the ADAR1's editing activity is related to IFN signaling pathway. In addition, by which mechanism ADAR1 exerts its inhibitory action on IFN signaling pathway is not well-known. Therefore, we sought to determine the specific point(s) where ADAR1 exerts its anti-IFN activity, and to

determine whether the IFN suppressive function of ADAR1 is related to its RNA editing activity.

## MATERIALS AND METHODS

*Cell Cultures*

Human gastric cancer-derived AGS cells and HEK-293T human embryonic kidney cells were purchased from Korean Cell Line Bank (Seoul, Korea). AGS Cells were maintained in RPMI medium with 10% fetal bovine serum. HEK-293T cells were grown in DMEM containing 10% fetal bovine serum. All cells were cultured at 37 °C in a humidified incubator containing 5% $CO_2$.

*Transfection of AGS and MKN-45 cells with shRNA*

To stably knockdown ADAR1 in AGS and MKN-45 cells, HEK-293T cells were plated at a density of $1.5 \times 10^5$ cells/well in six-well plates, and were then incubated for 24 h. Subsequently, HEK-293T cells were co-transfected with the vector plasmid (shADAR1,TRCN0000050788, sequence:CCGGGCCCACTGTTATCTTCACTTTCTCGAGAAAGTGAAGATAAC AGTGGGCTTTTTG, Sigma, MO, USA, 1500 ng), the packaging plasmid psPAX2 (700 ng), and the envelope plasmid pMD2.G (700 ng) using Lipofectamine 2000 (Invitrogen, CA, USA). Viral supernatants were then harvested at 24–48 h after transfection and were used to transfect AGS and MKN-45 cells. The efficiency of 293T cell transfection was estimated by co-transfection with a green fluorescent protein. In addition, to knockdown ADAR1 p150 isoform, shRNA targeting p150 isoform specific sequences were used (shADAR1-p150, TRCN 0000050790, sequence:CCGGGCTGTTAGAATATGCCCAGTTCTCGAGAACTGGGCATATTC

TAACAGCTTTTTG, Sigma, MO, USA, 1500 ng). In IFN stimulation experiment, we used pLKO Tet-on vector control (Tet-shGFP), or Tet-on vector which ADAR1 could be conditionally knocked down by doxycycline treatment, at 1ug/mL (Tet-shADAR1).

*Overexpression of ADAR1 in AGS cell line*

To overexpress ADAR1 in AGS cells, cells were plated at $1.5 \times 10^5$ cells/well in 6-well plates, 24 h prior to transfection. 150ng of ADAR1 expression vectors encoding p110 or p150 isoform were transfected into AGS cell line using Lipofectamine 2000 (Invitrogen, CA,USA), and cells were harvested 48 h after the transfection.

*Preparation of human stomach samples*

Human gastric cancer samples and matched normal tissues from 16 patients were obtained from the Bio Resource Center (BRC) of Asan Medical Center, Seoul, Korea. The use of human samples was approved by the Internal Review Board of Asan Medical Center (IRB no: 2016-0931).

*RNA extraction, cDNA synthesis and quantitative real-time PCR*

Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA). cDNA was synthesized using the PrimeScript First Strand cDNA synthesis kit (Takara, Japan) and used for the quantitative real-time polymerase chain reaction (qRT-PCR) analysis of ADAR1, ADAR2, IRF9, STAT1, STAT2. The human GAPDH was used as an internal control. To confirm the amplification of specific transcripts, melting curve profiles

were produced at the end of each PCR, and relative quantification was calculated using the 2- ΔΔCT method. Quantitative assessments of miRNA-302a-3p, and miRNA-302a-5p were performed using TRIzol-isolated RNAs following reverse transcription with PrimeScript Kits (Heimbiotek, Korea), and cDNAs were analyzed using HB miR Multi Assay Kit™ System I (Heimbiotek, Korea) according to the manufacturer's instructions.

*RNA sequencing*

Transcriptome libraries were generated using Illumina TruSeq RNA Library Preparation Kits and quantified 10nM through validation of size-check (Agilent 2100) and qPCR (KAPA Library Quantification Kit), according to the standard manufacturer's protocol. The resultant libraries were sequenced using the Illumina HiSeq™ 2500 platform (Illumina Inc., San Diego, CA, USA), to generate paired-end 101 bp reads. Raw data processing was performed using Illumina RTA & Bcl2fastq software (versions 1.18.64 & 2.19, respectively) to convert image to fastq and demuliplex. Paired-end sequencing reads were demultiplexed and assigned to individual samples based on their index sequence.

*Luciferase reporter assay and miRNA mimic treatment*

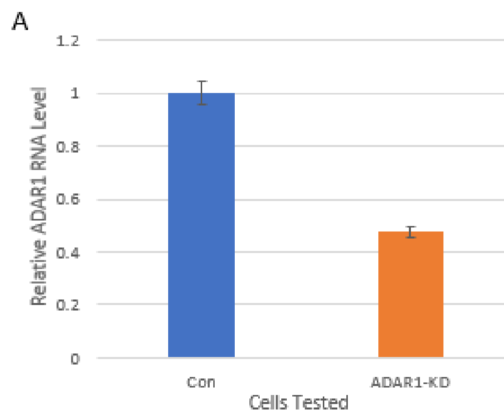
AGS cells were plated in 24-well plates, 24 h prior to transfection. On the following day, 100ng of pMIR-REPORT™ Luciferase vector (Life techonologies, MD, USA), wild type UTR containing pMIR vector or

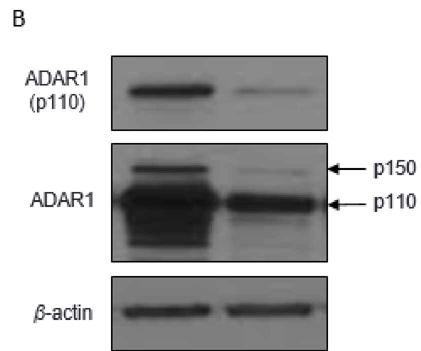
pMIR vector containing mutated seed sequence were cotransfected with 10ng of TK-luc plasmid using lipofectamine 2000 (Invitrogen, CA, USA). miRNA-302a mimic or scramble RNA duplexes were also co-transfected when necessary (Genolution, Korea). The cells were harvested 24 to 48 h later, suspended in passive lysis buffer, and the activity of each luciferase reporter was measured by the Dual Luciferase Reporter Assay system (Promega, WI, USA), following the manufacturer's instruction. Relative luciferase expression was determined as the ratio of firefly to Renilla luciferase activity. When miRNA 302a-3p was transfected, AGS cells were plated in 6-well plates, 24 h prior to transfection. On the following day, miRNA mimic or scrambled control were transfected using lipofectamine 2000 (Invitrogen, CA, USA) for 48hrs.

# RESULTS

## *1. Stable ADAR1 knockdown in AGS gastric cancer cell line.*

To investigate ADAR1-dependent editing targets and in the interferon signaling pathway, both of p110 and p150 isoforms of ADAR1 were stably knocked-down in AGS cells by lentiviral shRNA, which targets 3′-UTR of ADAR1 gene. Both mRNA levels (Figure 1A) and protein levels (Figure 1B) of ADAR1 decreased significantly upon knockdown. In contrast, ADAR2 mRNA level was unaffected and protein was not detectable (data not shown). Immunofluorescence analysis revealed most of the ADAR1 were abundant in nucleus, especially in the nucleolar portion, which was clearly decreased upon knockdown (Figure 1C, bottom left panel).





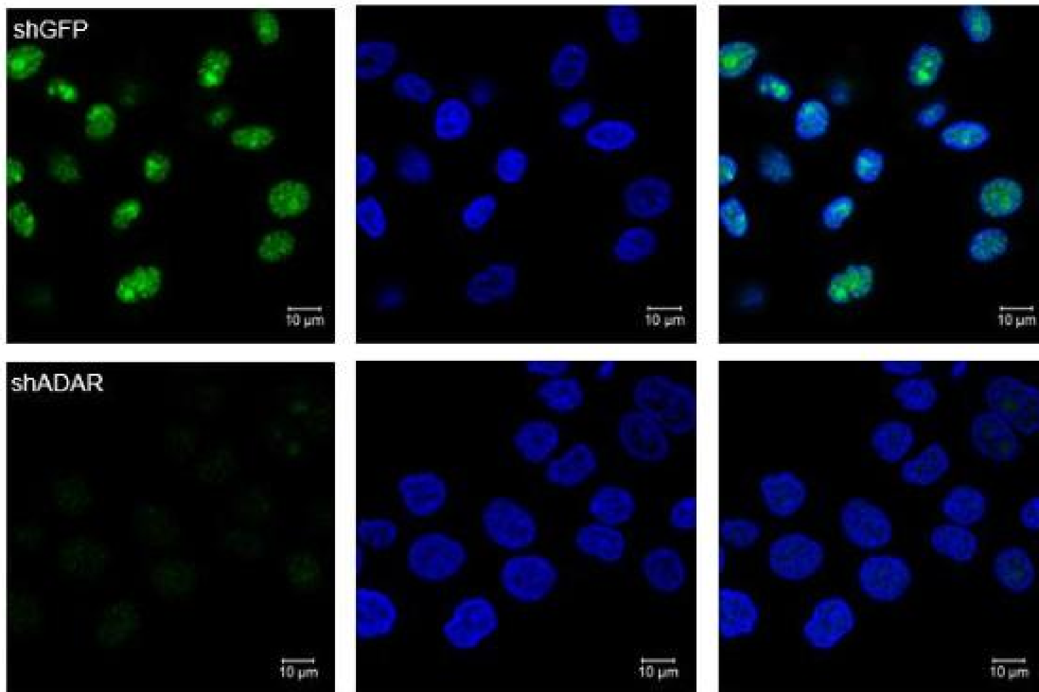



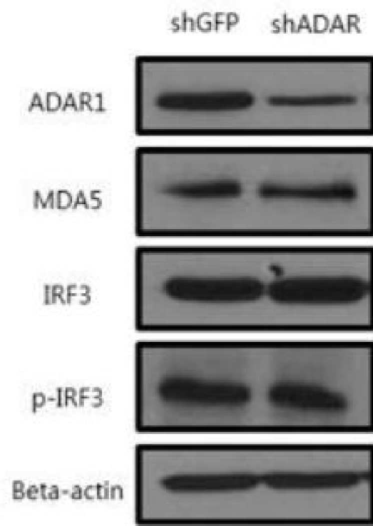

**Figure 1. Knockdown of ADAR1 in AGS cells by shRNA.** qRT-PCR(A) immunoblot(B) and immunofluorescence(C) analysis of ADAR1

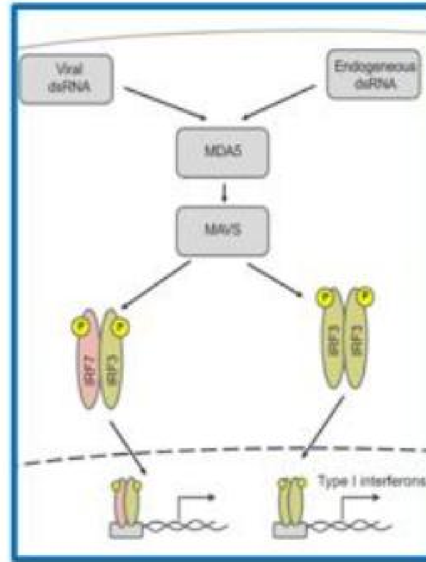
expression in AGS infected with shADAR1 or control (shGFP) virus. In (C), green signal on left indicate ADAR1 protein and the middle one is for Hoescht staining. Merged image is shown on right. Scale bar; 10uM (Data from 조정제 선생님)

***2. ADAR1 knockdown leads to elevated STAT1 and IRF9 protein level without increase in IFN level.***

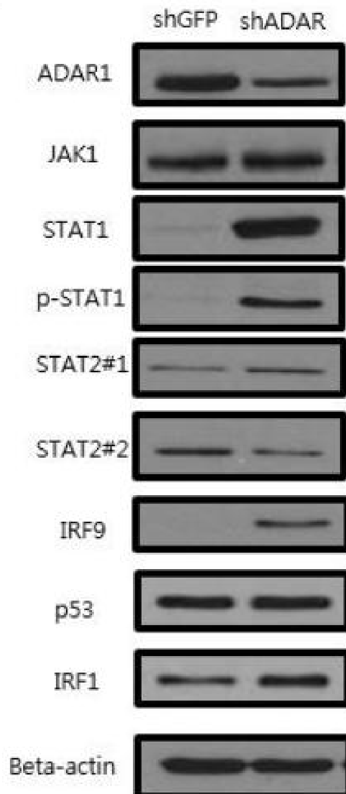
In our previous experiments, RNA-seq data (for example, PHACTR4 mRNA-sequencing in ADAR1 knockdown AGS cells) revealed alteration in mRNA level of many IFN signaling pathway components. Hence, we went on to determine if the pattern recognition receptors and their downstream IFN signaling pathways were activated upon ADAR1 knockdown. To this end, immunobloting for MDA5 as well as unphosphorylated and phosphorylated form of IRF3 was performed. In the absence of IFN stimuli, levels of examined proteins were not altered. (Figure 2A) We further went on to determine if the signaling pathway of type I IFN production was activated. Interestingly, STAT1, phosphorylated STAT1 as well as IRF9 levels were elevated upon ADAR1 knockdown. (Figure 2B). However, JAK1 level was unaffected, which imply that ADAR1's suppressive role on STAT1 and IRF9 is JAK1 independent. STAT2 level was also evaluated but the result was inconsistent, suggesting that certain factor other than ADAR1 may be related to this change. Protein level of IRF1, a known downstream target of STAT1, was also increased (Figure 2B). However, another downstream target of STAT1, p53, was unchanged (Figure 2B). To confirm that these alterations upon ADAR1 knockdown were not due to IFN production, we measured serum IFN level by ELISA, revealing undetectable level of IFN alpha or beta(Figure 2C). The level of IFN gamma (type II IFN) was also undetectable (data not shown).

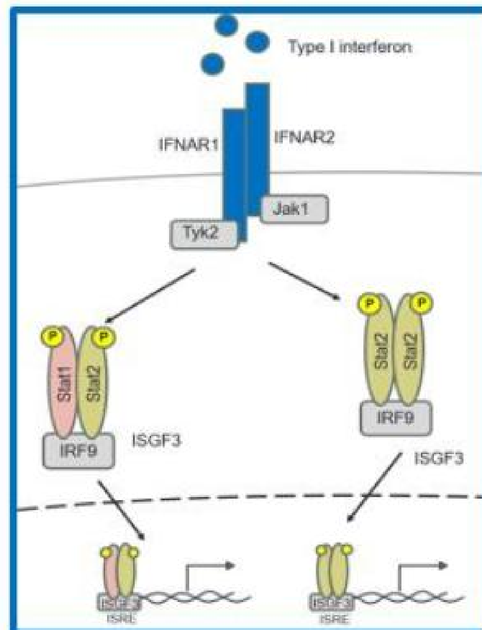
A




B



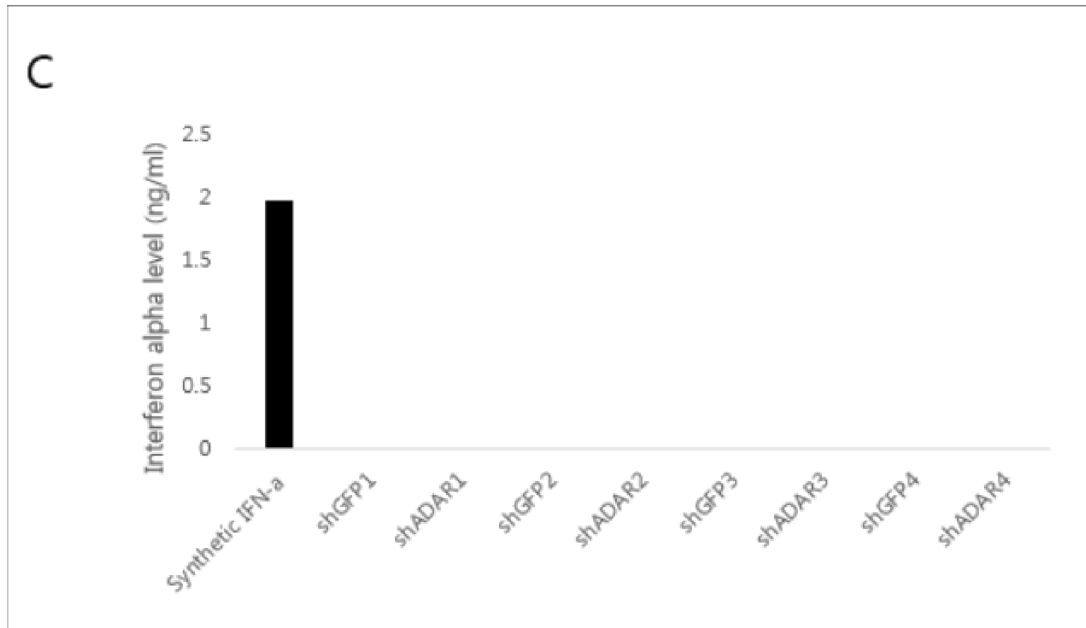
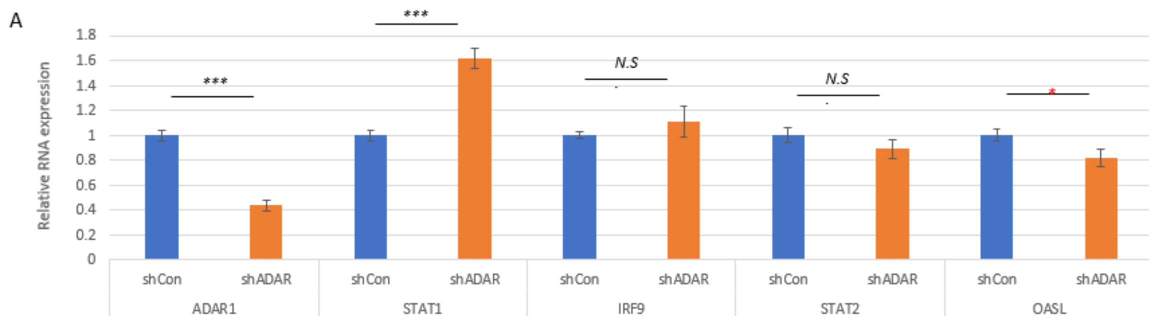



**Figure 2. ADAR1 knockdown increases the expression of STAT1, IRF9 and IRF1.** (A) Immunoblot images of targets upstream of type I IFN production upon ADAR1 knockdown, showing no significant change. (B) Immunoblot images for the downstream targets of type I IFN showing increase of STAT1, phosphorylated STAT1, IRF9 and IRF1 protein level. (C) ELISA analysis for type I IFN (IFN alpha and beta) showing undetectable type I IFN level, regardless of ADAR1 knockdown status. (Data from 조정제 선생님)

***3. Increased IRF9 and STAT1, triggered by ADAR1 knockdown, was mediated by miRNA-302a-3p suppression.***

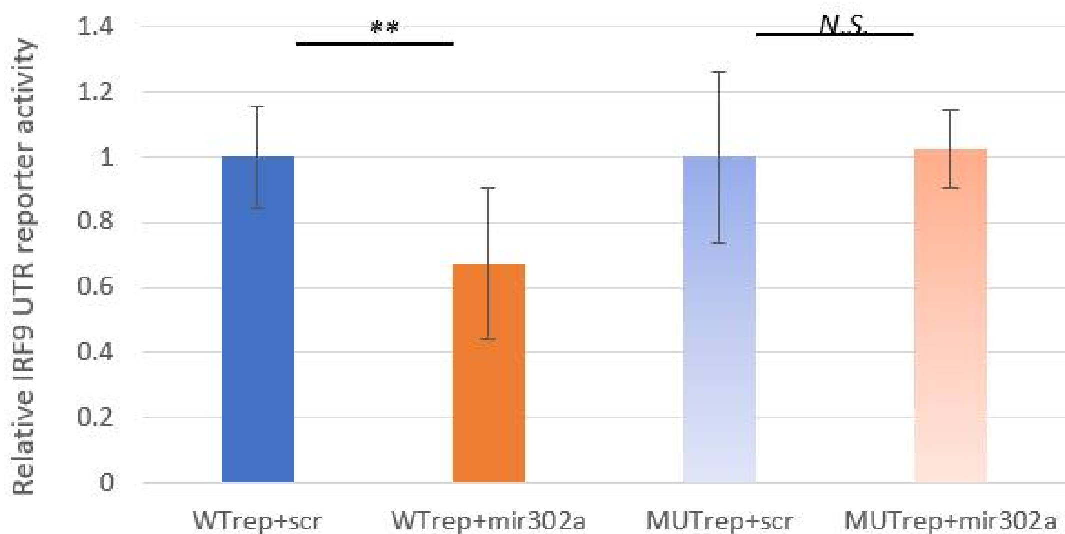
We next examined whether the dramatic change of STAT1 and IRF9 protein level was due to increased transcription. The mRNA level of STAT1 was elevated by less than 2-fold (Figure 3A). Additionally, the mRNA level of IRF9 or STAT2 was unaffected. Interestingly, OASL mRNA (oligoadenylate synthetase-like protein) level was decreased upon ADAR1 knockdown, which was different from previous RNA-seq data. Although two-fold increase in STAT1 mRNA was noted, we reasoned the elevation was insufficient to draw increased STAT1 protein level. We therefore sought for a factor that could affect post-transcriptional regulation of the targets. Since miRNA 302a-3p and miRNA 302-5p were decreased miRNA upon ADAR1 knockdown (Figure 3 the left panel of C2 and C3), we sought to determine whether miRNA-302a-3p and miRNA 302-5p could post-transcriptionally regulate IRF9 and STAT1, respectively. Indeed, 8-mer seed sequence for miRNA-302a-3p and miRNA 302-5p were found in the 3′-UTR of IRF9 and STAT1 (Figure 3B). Therefore, WT and Mutants of IRF9 and STAT1 UTR reporter vector for 8-mer seed binding sequence of miR302a-3p were generated (Figure 3B). In the luciferase assay, a significant decrease in the luciferase activity of WT reporter was restored by the mutagenesis of the seed binding sequence in IRF9 gene, but there was no decrease in the luciferase activity of WT reporter treated by addition of miRNA 302-5P (Figure 3 C and C1). Finally, we added miRNA-302a-3p mimic or

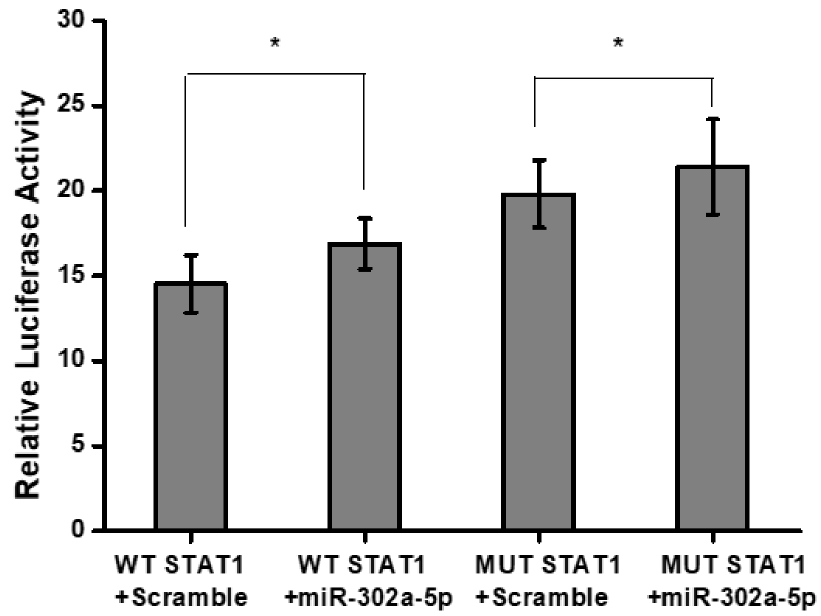
scrambled control to the control (shGFP) and ADAR1 knocked down (shADAR1) cells. As expected, the increased IRF9 protein level by ADAR1 knockdown was abolished when miRNA-302a-3p mimic was treated. Similarly, the increased STAT1 protein level was also restored by the treatment of miRNA-302a-3p mimic. (Figure 3D)

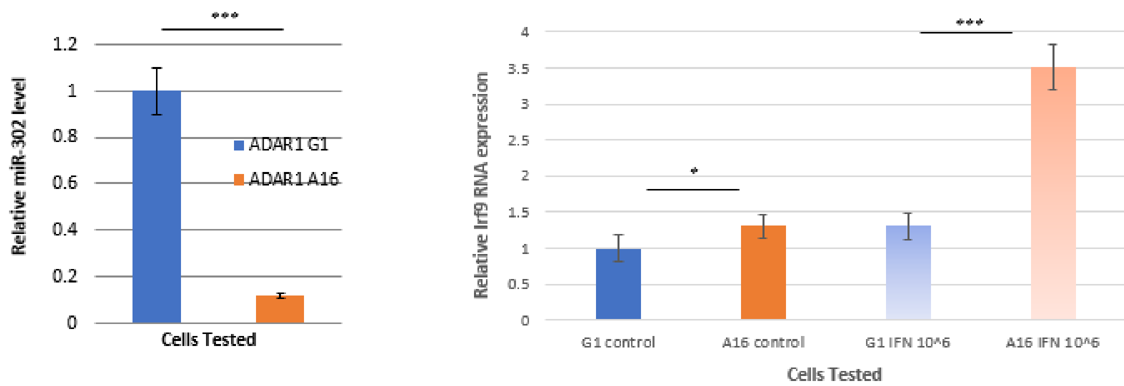


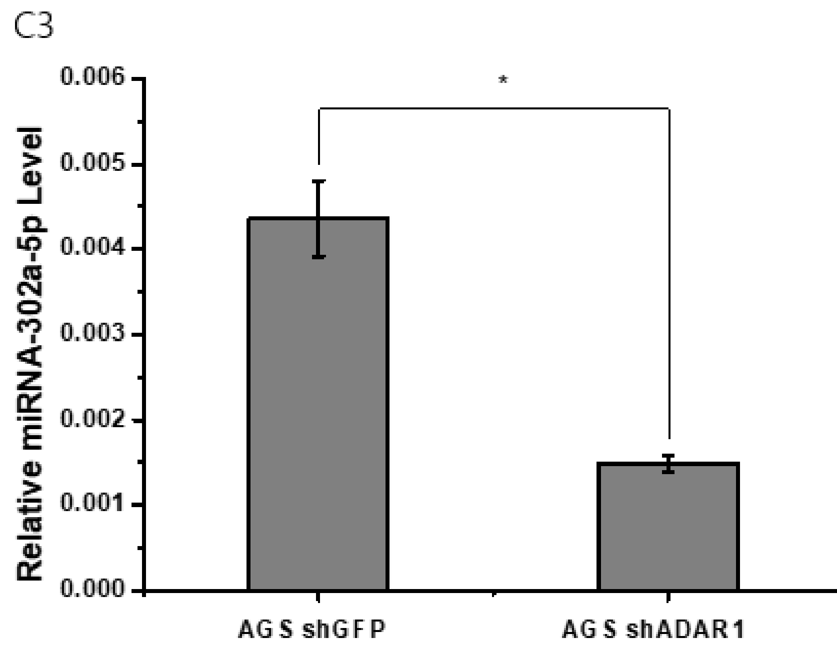

B

| | Predicted consequential pairing of target region (top) and miRNA (bottom) | |
|---|---|---|
| Position 3219-3225 of STAT1 3' UTR<br>hsa-miR-302a-5p | 5' ...CCAAGAGACUUUACUUUUAAGAA...<br>\|\|\|\|\|\|<br>3' UCGUUCAUGUAGGUGCAAAUUCA | |
| Position 304-310 of IRF9 3' UTR<br>hsa-miR-302a-3p | 5' ...CCAACUCUAAAGCCAAGCACUUU...<br>\|\|\|\|\|\|\|<br>3' AGUGGUUUUGUACCUUCGUGAAU | 7mer-m8 |




C1
Relative Luciferase Activity
30
25
20
15
10
5
0
*
*
WT STAT1 +Scramble
WT STAT1 +miR-302a-5p
MUT STAT1 +Scramble
MUT STAT1 +miR-302a-5p


C2
Relative miR-302 level
1.2
1
0.8
0.6
0.4
0.2
0
***
ADAR1 G1
ADAR1 A16
Cells Tested
Relative Irf9 RNA expression
4
3.5
3
2.5
2
1.5
1
0.5
0
*
***
G1 control
A16 control
G1 IFN 10^6
A16 IFN 10^6
Cells Tested



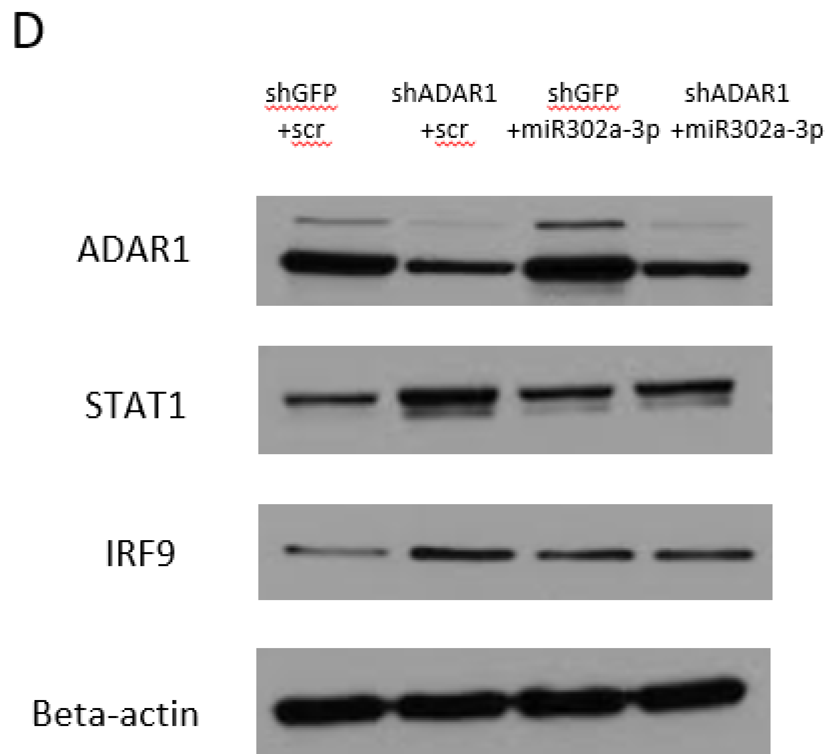
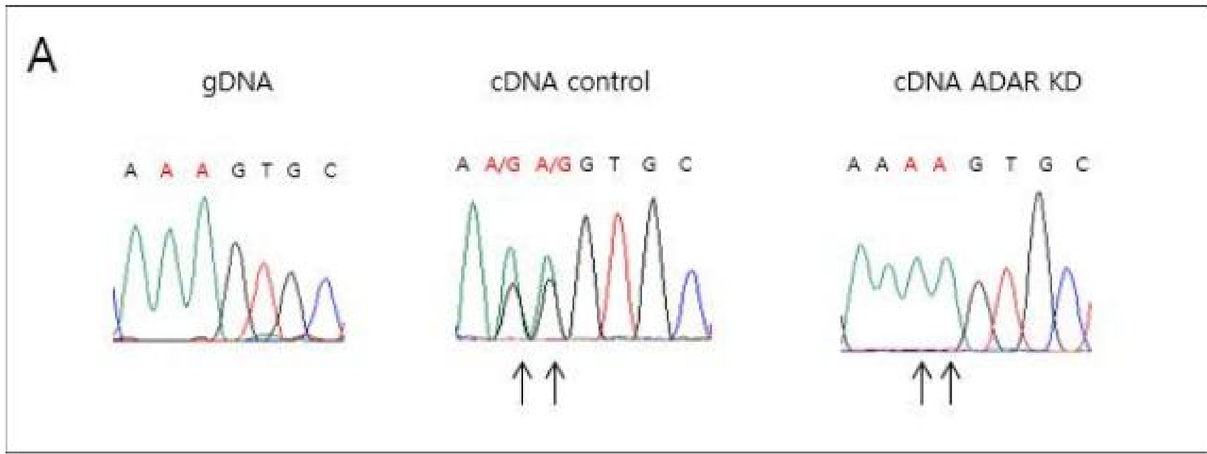



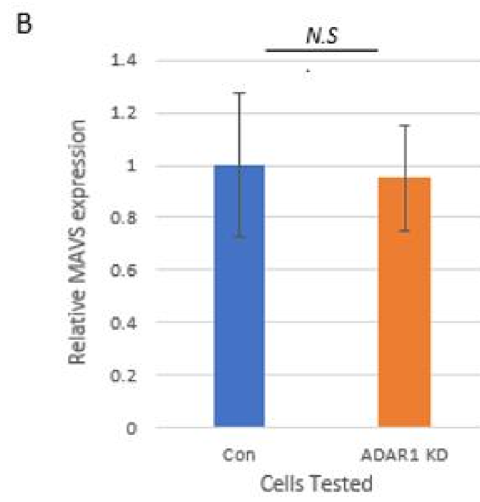

**Figure 3. Downregulation of miRNA-302a-3p by ADAR1 knockdown**

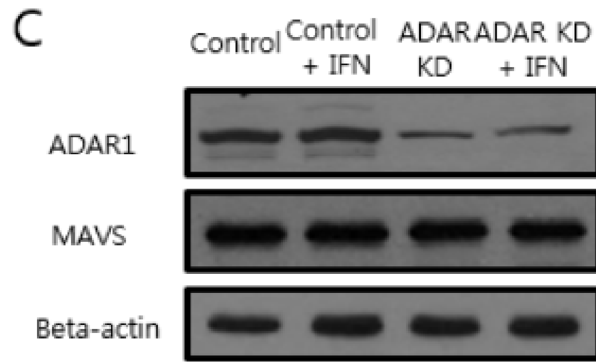
**increases the expression of IRF9.** (A) qRT-PCR analyses of IFN stimulated genes. (B) Alignment image showing the miRNA-302a-3p seed sequence interacting with the 3′-UTR of IRF9 mRNA and miRNA-302a-5p seed sequence interacting with the 3′-UTR of STAT1 mRNA. (C and C1) Luciferase assay result using wild-type IRF9 UTR reporter (WT) or mutant and wild-type STAT1 UTR reporter (WT) or mutant with the seed- binding site of miRNA-302a-3p (MUT) and miRNA-302a-5p(MUT) respectively, along with miRNA-302a-3p and miRNA-302a-5p mimic or scrambled control. (C2 and C3) miRNA-qRT-PCR showed the decreased results of miRNA-302a-3p and miRNA-302a-5p in AGS shADAR1 cell compared to the control group (shGFP). (D) Immunoblot images showing STAT1 and IRF9 level in control (shGFP) or ADAR1 knocked down (shADAR1) AGS cell lines, which were treated with miRNA-302a-3p mimic or scrambled control. (This is the common data with 조정제 선생님)

### *4. MAVS and IFNAR2 3′ UTR are editing targets, but no change in mRNA and protein level*

On the other hand, we also examined whether the editing of MAVS(Mitochondrial antiviral-signaling protein) and IFNAR2(Interferon Alpha And Beta Receptor Subunit 2) can affect the suppression of IFN signaling pathway triggered by ADAR1, since they are in the middle of the IFN signaling pathway and predicted to be edited by ADAR1 in their 3′ UTRs. Indeed, Sanger sequencing of the two edited sites of MAVS (chr20:3850513, chr20:3850514) in AGS cells revealed 21.6% and 16.2% of editing in control cDNA, which was decreased to less than 10% upon ADAR1 knockdown (Figure 4A). However, no detectable changes in expression levels or localization of MAVS were observed by qRT-PCR, immunoblotting, or IF analyses (Figure 4 B-D). We next treated synthetic IFN alpha but found no difference in the editing level. The editing of IFNAR2 at position 34636384 in chr 21, was also confirmed by Sanger sequencing (Figure 4E). However, no detectable change in the localization of IFNAR2 protein was observed in the absence or presence of IFN (Figure 4F).


A
gDNA
cDNA control
cDNA ADAR KD
A A A G T G C
A A/G A/G G T G C
A A A A G T G C


B
N.S
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative MAVS expression
Con
ADAR1 KD
Cells Tested


C
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Control
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ADAR
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ADAR KD
+ IFN
ADAR1
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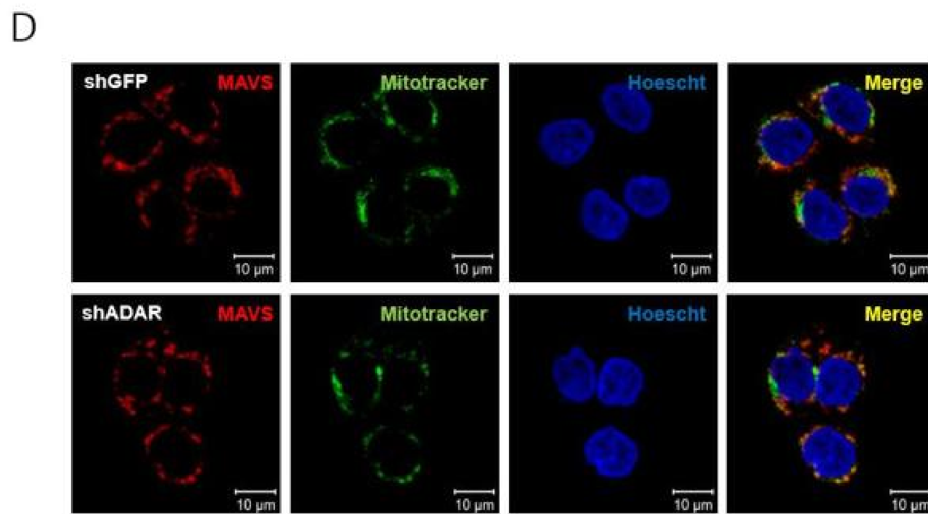
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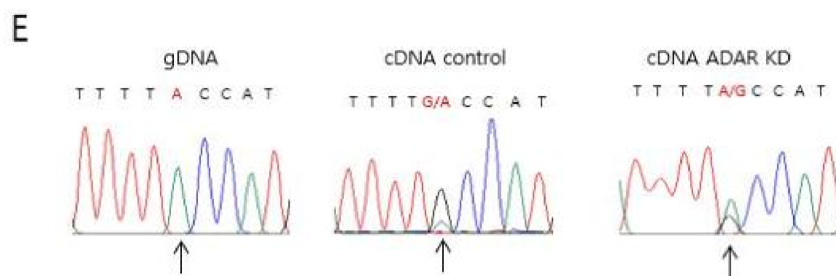
E
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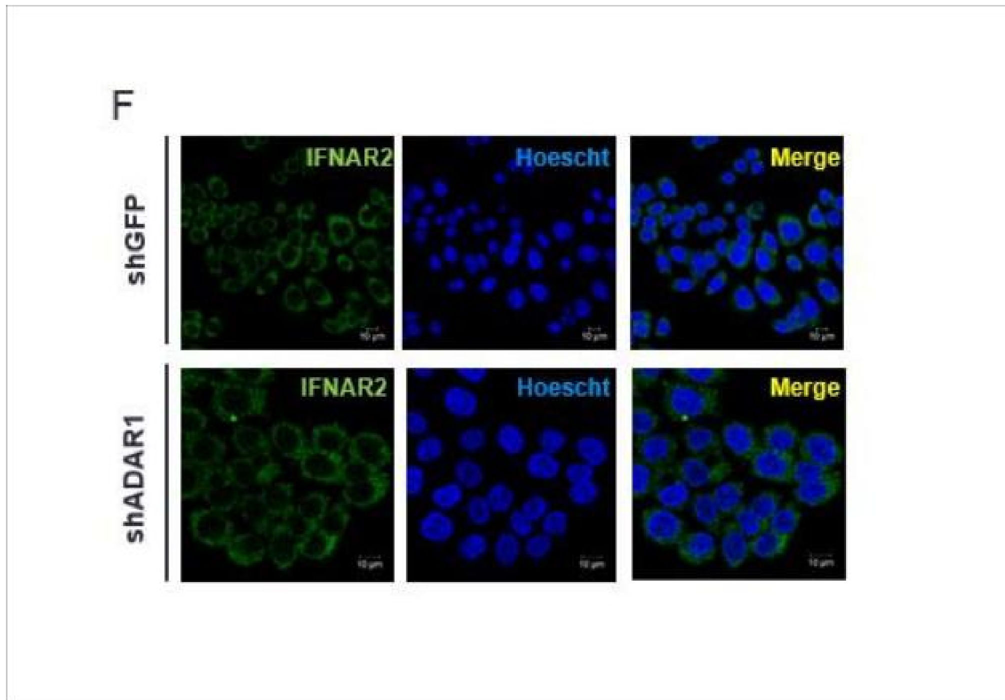


**Figure 4. MAVS and IFNAR2 are edited in their 3′ UTRs but their protein and/or mRNA levels or localizations are not affected by ADAR1 knockdown.** (A) Sanger sequencing result of two MAVS editing sites (chr20:3850513, chr20:3850514) for genomic DNA, control cDNA and ADAR1 knocked-down cDNA. Arrows indicate A to G editing sites. (B-C) qRT-PCR (B) and immunoblot (C) of MAVS upon ADAR1 knockdown. (D) Immunofluorescence image of MAVS (red on left) upon ADAR1 knockdown. Mitotracker images are shown as green images. (E-F) Sanger sequencing result of IFNAR2 (chr21; 34636384) for genomic DNA, control cDNA and ADAR1 knocked-down cDNA. (E) Arrows indicate A to G editing sites. Immunofluorescence image of IFNAR2 (green on top) upon ADAR1 knockdown. (F) No detectable change in the localization of IFNAR2 protein was observed in the absence or presence of IFN (Data from 조정제 선생님)

### *5. ADAR1 indirectly regulates IRF9 and STAT1 by miRNA-302a-3p, but not by direct RNA editing*

From the results described above, we summarized ADAR1's suppressive role on IFN signaling pathway (Figure 5). In the absence of IFN, ADAR1 suppresses STAT1 and IRF9 level by the interaction of miRNA-302a-3p, and possibly by other components of miRNA-302a-367 cluster. In the presence of IFN, it shows even greater effect. Consequently, ISG expressions are elevated, which in turn contribute to apoptosis and decreased cell proliferation. This suggests a new mechanism by which ADAR1 suppresses IFN signaling pathway, in that the function of ADAR1 is IFN-independent.

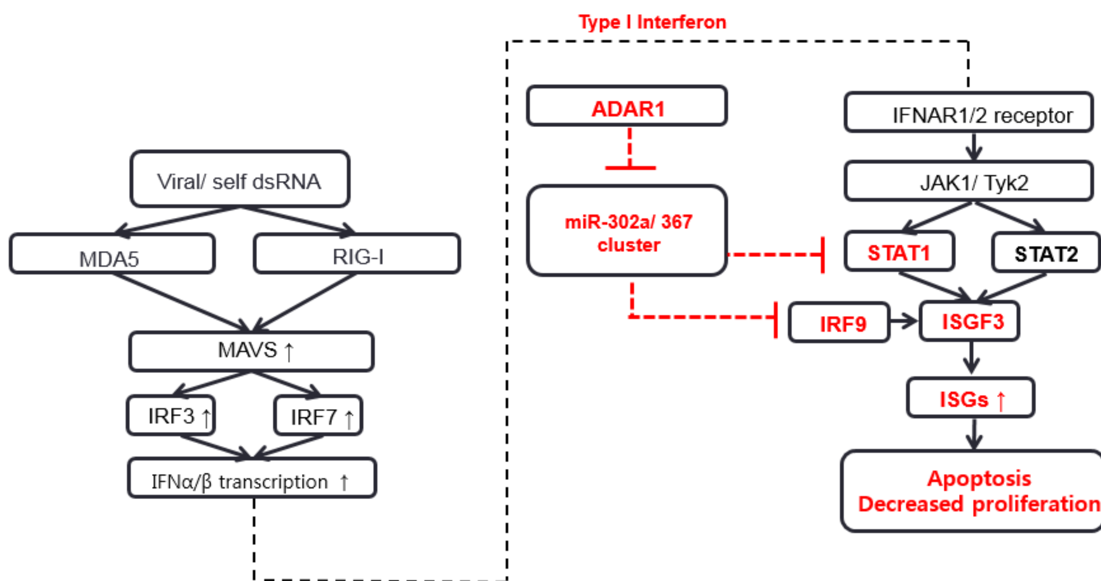


**Figure 5. Schematic diagram of IFN signaling pathway suppressed by ADAR1. (Data from 조정제 선생님)** Under normal circumstances,

when cellular external factors such as viruses, bacterial dsRNA enter the cells or the intracellular dsRNA are formed, the cells will produce a series of reactions that eventually lead to produce interferon factors such as IFN-alpha and IFN-beta, which in turn act on downstream counterparts IFNAR1/2 receptors. The downstream of IFN receptor activates JAK1/Tyk2, STAT1/STAT2 and IRF9, which subsequently form the ISGF3 complex and finally activates the interferon-related genes in the nucleus, thereby enhancing the cellular immunity and related cellular functions. However, when the ADAR1 gene is knocked down, it can reduce the expression of miRNA-302a-3p (from miR-302/367 cluster), which causes increased expression of STAT1 and IRF9. Therefore, ADAR1 suppresses interferon signaling pathway by regulating the expression of miRNA-302/367 cluster, which in turn inhibit the expression of STAT1/IRF9.

**6. *ADAR1 did not result in the mutation of phenylalanine 172 of STAT1 in AGS cell line.***

It has been known that the phenylalanine 172 to serine (F172S) in the coiled-coil domain of STAT1 decreased the protein expression without decreasing mRNA expression in human fibroblast cell line [50]. Different nucleotide substitutions for serine residue also exerted identical effects[50]. Proteasome inhibitor failed to increase F172S expression. These results suggest that the F172S mutation affects Stat1 expression by translational/post-translational mechanism independent of proteasome machinery. However, the best characterized type of RNA editing event is conversion of adenosine to inosine (A-to-I editing), so we speculate whether ADAR1 editing function made the phenylalanine 172 to serine (F172S) in the coiled-coil domain in STAT1 or not. We checked the STAT1 cDNA sequence, but the result showed no mutation on the cDNA coding phenylalanine 172 (Figure 6).

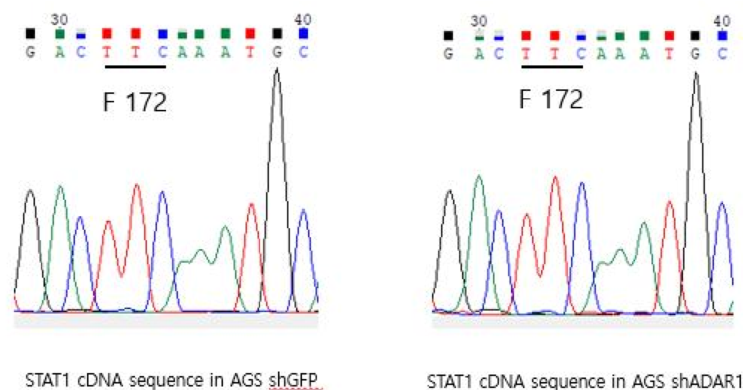

**Figure 6. STAT1 cDNA sequence in AGS shGFP and AGS shADAR1 cell.** STAT1 cDNA sequence did not show any change for phenylalanine in AGS shGFP and AGS shADAR1 cell.

### *7. ADAR1 did not result in the changes for PKR in AGS cell line.*

The double-stranded RNA-dependent protein kinase (PKR) is a serine/threonine kinase, which is expressed constitutively in mammalian cells. The most-studied role of PKR in cells is the regulation of translation. In response to viral double-stranded RNA, PKR phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF-2α). Phosphorylated eIF-2α leads to the inhibition of the translation of mRNA and protein synthesis [27)]. PKR plays a critical role in the antiviral defense of the host cells by inhibiting protein synthesis in virally infected cells [28, 29)].

Recent studies have demonstrated that PKR plays pivotal roles in the signal transduction. It is reported that in addition to double-stranded RNA stress, various growth factors, cytokines, and cellular stresses activate PKR [29–32)]. In response to these stimuli, PKR is activated through several signal transduction pathways, such as Toll-like receptors (TLRs), mitogen-activated protein kinase (MAPK), and nuclear factor of κB (NF-κB) [33–35)]. It is also reported that PKR is involved in cell cycle progression, cell proliferation, cell differentiation, apoptosis, and tumorigenesis [36–39)]. Recently some research showed that the expression and degradation of STAT1 protein were regulated by PKR in a SLIM-dependent pathway.

So we tested whether ADAR1 can edit PKR and cause the STAT1 degradation in AGS cell line. We checked the PKR whole cDNA sequence, but did not showed any PKR editing. Also, the analysis of

PKR and eIF-2α protein by western blotting did not show any changes in AGS (Figure 7).

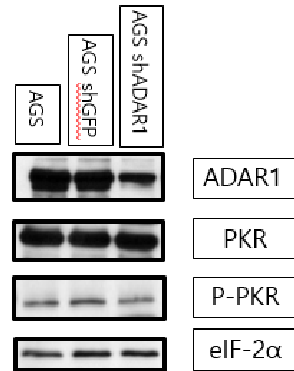


**Figure 7. Western blotting results of PKR, P-PKR and eIF-2α in AGS, AGS shGFP and AGS shADAR1 cell.** Western blotting did not show marked changes in the levels of protein PKR, P-PKR and eIF-2α in AGS and AGS shGFP and AGS shADAR1 cells.

# DISCUSSION

Signal transducer and activator of transcription 1 (STAT1), a key mediator of interferon (IFN) signaling, regulates a variety of cellular activities, such as apoptosis, proliferation and differentiation[40]. In response to extracellular stimuli, such as IFN-γ, the activation of STAT1 is achieved by Janus kinase mediated phosphorylation of its conserved tyrosine and serine residues, present in the C-terminal transactivation domain, that result in STAT1 dimerization, nuclear translocation, DNA binding and eventually modulation of expression of its target genes[41]. In a number of models, STAT1 has been shown to possess tumor suppressor functions, and the evidences can be summarized as follows: (1) pro-apoptotic effects are largely mediated through STAT1 signaling[41]; (2) constitutively active STAT1 can effectively induce apoptosis and inhibit cell growth[42]; (3) STAT1 is frequently downregulated in various human cancers, including breast cancer, head and neck cancer, multiple myeloma and leukemia[43,44]. Some studies have reported that STAT1 is an important tumor suppressor in esophageal squamous cell carcinoma (ESCC), where the loss of STAT1 contributes to the pathogenesis of these tumors and correlates with a worse clinical outcome[45,46]. Our results showed a significant STAT1 upregulation in the ADAR1 knockdown AGS cell line, which means that STAT1 maybe plays an important role in the pathogenesis and treatment strategies of gastric cancer. Although the mechanism of cytokine-induced activation of Stat1, transcriptional regulation of Stat1

gene expression and post-transcriptional regulation of Stat1 protein have been established, ADAR1-dependent regulation of Stat1 protein is not shown by far. We have checked all kinds of ADAR1 editing regulation on STAT1 gene expression, such as ADAR1 editing for MAVS and IFNAR2 which are the important components of IFN signaling pathway, and PKR protein which can mediate the degradation of STAT1, but we still could not find the real reason that can result in the STAT1 increase in the AGS cell line by ADAR1 knockdown. However, recently, some studies showed that STAT1β can enhance STAT1 function by protecting STAT1α from degradation in esophageal squamous cell carcinoma. STAT1β, a naturally spliced isoform of STAT1, lacks a 38-amino acid segment that includes the conserved STAT1 S727 phosphorylation site and most of the C-terminal transactivation domain. STAT1β has not been extensively studied, although one report has described that STAT1β in human B-cells is transcriptionally inactive and exerts a dominant-negative effect on STAT1α, the full-length STAT1 isoform[47)]. Specifically, overexpression of STAT1β was found to inhibit the phosphorylation, DNA binding and transcriptional activity of STAT1 in human B-cells. However, in another study using B-cells, STAT1β was reported to induce cell death via a mechanism that is independent of p53 and STAT1α[48)]. In a more recently published paper, STAT1β was found to be transcriptionally active and capable of eliciting IFN-γ-dependent immunity against infection in vivo[49)]. In our experiments, we found that both of the STAT1β and STAT1α showed high expression in the ADAR1 knockdown AGS cell lines. (Figure 8)

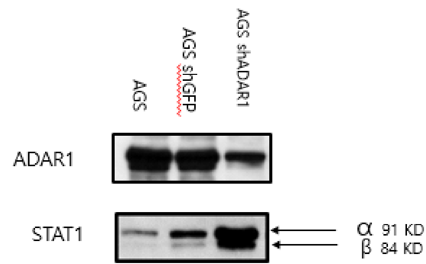


**Figure 8: Western blotting results of STAT1α and STAT1β in AGS, AGS shGFP and AGS shADAR1 cells.** Western blotting showed marked STAT1α and STAT1β high expression in AGS shADAR1 cells compared to the AGS and AGS shGFP cells.

So, we need to examine if there is the biological function and clinical significance of STAT1β and if the STAT1β possesses tumor suppressor activity in human gastric cancer.

Moreover, we checked the sequence of the whole STAT1 CDNA and found one A-to-G editing in AGS cell which is reversed in the ADAR1 knockdown AGS cells (Figure 9).

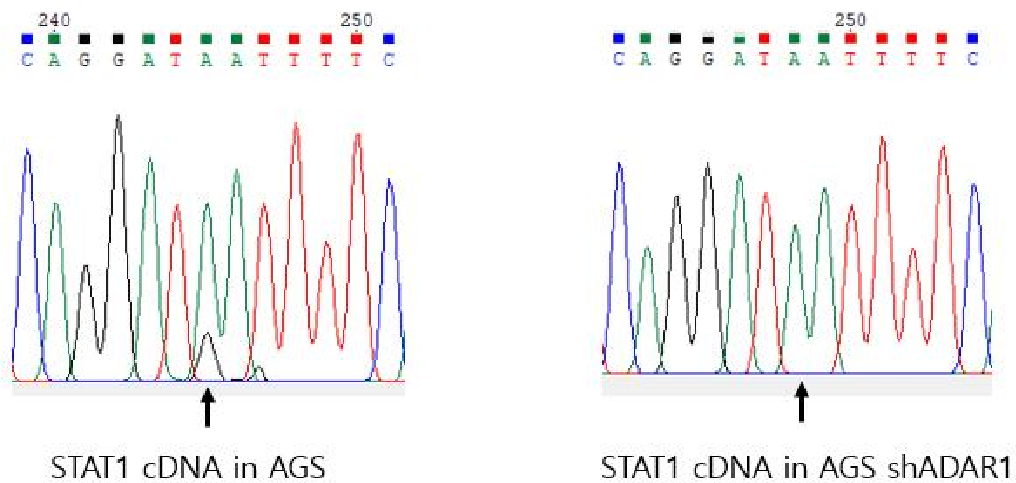


**Figure 9: STAT1 editing (A-to-G) situation by ADAR1 in AGS and AGS shADAR1 cells.** STAT1 cDNA sequencing showed significant RNA editing (A-to-G) in AGS cell and the same editing point is obliterated in AGS shADAR1 cell.

So whether this RNA editing (A-to-G) point can lead to the function change of STAT1 protein and further lead to the STAT1 degradation in AGS cell? We will examine it in the future study plan again.

IRF9 is well-known to be a component of ISGF3 complex, along with phosphorylated STAT1 and STAT2, which moves into the nucleus upon stimulation by IFN, binds to ISRE to promote transcription of more than 100 ISGs[21)]. In addition, IRF9 was previously proven as a target of miRNA-302d[22)]. Especially, the seed sequence of IRF9 for miRNA-302d coincides with that of miRNA-302a-3p, the target of our study. In fact, these miRNAs constitute miRNA-302a cluster, which consists of nine different miRNAs co-transcribed in a polycistronic manner. miRNA-302a cluster is known to be highly expressed in embryonic stem cell (ESC)s, and not in differentiated cells as well as somatic stem cells[23)]. In accordance, well-known ESC related transcription factors-Nanog, Oct3/4, Sox2, and Rex1 –101 regulate the activity of miRNA-302a clusters, which may be responsible for its turn-off in development[24)]. It is interesting that AGS cell line can produce conceivable amount of miRNA-302a-3p even though it is a cancer cell line derived from fully differentiated gastric epithelial cells. This phenomenon has been reproduced by other researchers as well. Two hypotheses can be drawn from the phenomenon: 1) Dedifferentiation of fully differentiated cancer cell may have occurred during carcinogenesis, which reactivated miRNA-302a cluster activity. 2) A proportion of cancer stem cells may

gain ability to transcribe miRNA-302a cluster, probably due to production of Oct4 or Sox2, which is known to bind to promotor region of miR-302a[25]. In contrast, several references showed that miR-302c and miR-302d were detectable in cancer cell lines, and these candidates may be important to drive suppression of STAT1 and IRF9, and should also be further investigated. It has been reported that in H9 human ESC, miRNA-302s expression decreases during differentiation[26]. However, when ADAR1 is knocked down in embryonic stem cell, the degree of decrease in miRNA-302a cluster level is smaller than when ample amount of ADAR1 is present[26]. Which is contradictory to our result showing positive correlation of ADAR1 and miRNA-302a-3p. The opposite effect of ADAR1 in cancer cell line may be its inherent property and is point of investigation. Finally, it is more tempting to find out that inherent IFN suppressive function of ADAR1 involves miRNA-302a-3p, which is related to its another function - self renewal. It has been suggested that there may be a hidden pathway that does not involve IFN signaling regarding late STAT1 phosphorylation. We can also postulate endogenous presence or absence of ADAR1 level as a regulating factor of late STAT1 phosphorylation response.

# CONCLUSION

This study has confirmed that protein amount of STAT1 and IRF9 was increased upon ADAR1 knockdown even in the absence of type I or type II IFN in AGS cell.

We found miRNA-302a-3p, the level of which decreases upon ADAR1 knockdown, binds to the seed sequence of IRF9. And IRF9 UTR reporter level was decreased upon the addition of miRNA 302a-3p, which was ameliorated when seed sequence was mutated. Moreover, treatment of miRNA-302a-3p mimic to ADAR1 knocked-down AGS cell reversed IRF9 or STAT1 protein level to that of control AGS cells. And synthetic IFN treatment further increased both un-phosphorylated and phosphorylated STAT1 as well as STAT2 level, the degree of which was lower when ADAR1 was present.

Above results suggest that ADAR1 plays a role in causative role in gastric cancer through suppression of STAT1 and IRF9 via miRNA302a-3p, independently from activation or editing of known IFN signaling pathway.

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