# Uncovering Gene Mutations that May Interfere with cGAS-STING-IRF3 Pathway Activation in Cancer

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## Abstract

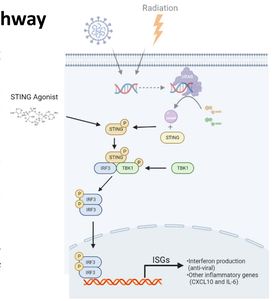
Cancer is one of the leading causes of death. Radiation therapy is a type of treatment that cancer patients undergo with the aim to extinguish tumor cells. Radiation damages the DNA within cancerous cells, killing them. Previous studies have shown that radiation can also induce an immune response to cancer, which can be harnessed to help anti-cancer immunotherapy. The mechanism by which radiation induces an anti-cancer immune response is not well understood. The radiation damaged DNA in cancer cells can leak into the cytoplasm and bind to cGAS, activating the cGAS-STING pathway that ultimately increases the expression of inflammatory genes to generate an immune response against the tumor. After investigating cancer cell line H1299, we noticed that it has all the proteins of the pathway but fails to activate STING. Therefore, we examined all the mutations present in the H1299 cells and hypothesized that a deletion in either its APOBEC3B or SMARCA4 genes was responsible for the pathway dysfunction. In testing the function of the STING pathway, we performed Western Blots to detect pathway activation, an ELISA to quantify the proteins produced by interferon-stimulated genes, and RT-PCR to measure mRNA expression of IRF3 downstream genes. STING agonist activates STING-IRF pathway in THP-1 cancer cells, which has both genes of interest functional. To generate additional evidences, we identified two additional cancer cells (A549, NCI-H1793) that have mutations in APOBEC3B or SMARCA5 genes, and STING-IRF3 pathway was not activated upon exposure of these cells to STING agonist, suggesting that those genes may indeed be responsible for the impairment of STING-IRF3 pathway in these cell lines.[4]

## Introduction

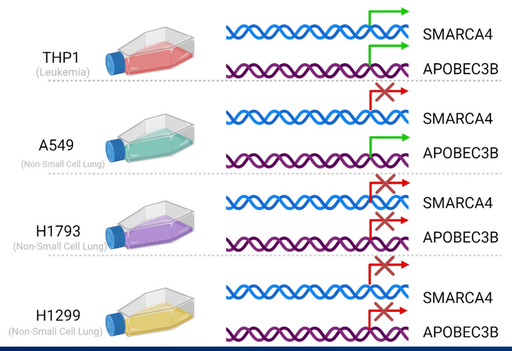
Cancer, a disease caused by the body's cells growing uncontrollably, is induced by certain genetic errors in a cell's DNA. There are more than 100 types of cancer, many of which result in tumors that can spread widely throughout the body[1]. With this aggressive disease, it is projected that there will be approximately 1,918,030 new cancer cases in 2022, resulting in about 609,360 deaths[2] in the US. One of the main treatments used to kill or slow the growth of tumors is radiation therapy, as about half of all cancer patients will undergo this treatment at some point after diagnosis[3]. Radiation therapy targets cells by damaging their DNA; often killing the cell when it attempts to divide. Damaged DNA can leak out of the nucleus and into the cytoplasm and upon entering into the cytoplasm, it binds to cGAS (a sensor of DNA in the cytosol). After this binding, the cGAS/STING pathway, which most likely evolved to defend against viral infection, becomes activated. Then, cGAS catalyzes a chemical reaction that combines GMP and AMP into one cyclic molecule, cGAMP, which attaches to a protein called STING (stimulator of interferon genes). This newly activated protein STING then activates the enzyme TANK-binding kinase 1 (TBK1), which subsequently activates transcription factor interferon regulatory factor 3 (IRF3). Furthermore, IRF3 enters the nucleus and turns on anti-viral and inflammatory genes, such as one that codes for interferon (IFN) proteins that mediate anti-viral responses. Therefore, increasing the expression of inflammatory genes triggers a cascade of immune responses that further attack and kill cancerous cells. Preliminary data from our lab shows that STING-IRF3 pathway in cancer cell line H1299 is not activated upon treatment with STING agonist, despite having all proteins in the pathway present. We then hypothesized that the deleterious mutations in APOBEC3B or SMARCA4 genes in H1299 may be responsible for the impaired STING-IRF3 pathway. Therefore, we tested four cancerous cell lines with different mutations in these genes.

### Figure 1. Overview of the cGAS-STING-IRF3 pathway

- STING agonist is a drug that acts as the natural occurring cGAMP.
- cGAS (a sensor for DNA) is activated either by DNA released in cytosol by phagocytosed bacteria or by DNA released from nucleus after being damaged by radiation therapy.
- Activated cGAS uses ATP and GTP as substrates to catalyze the formation of the second messenger, cGAMP, which binds to STING.
- The binding of cGAMP to STING promotes STING translocation to the Golgi apparatus.
- During the translocation, STING recruits and activates TBK1, which in turn catalyzes the phosphorylation and nuclear translocation of IRF3 leading to increased synthesis of interferon beta (IFN-β) and other inflammatory genes.

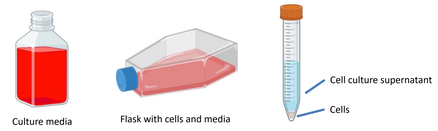



### Figure 2. Cancer cell lines and their status for the genes of interest[4]



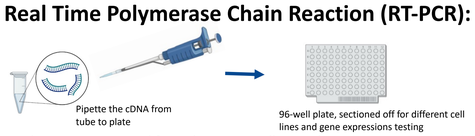

## Methods

### Cell culture:



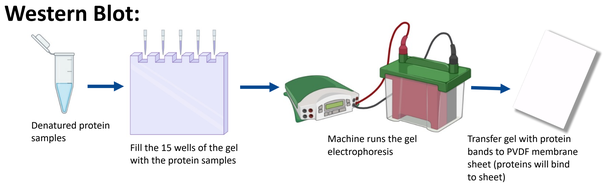

1 x $10^6$ cancer cells were plated in 25 $cm^2$ flasks with 5 mL corresponding culture media. Next day the media was removed, and the cells were treated with fresh media containing STING agonist (ADU-S100, Chemietek, USA) at 50 μM concentration. Samples (cells and cell culture supernatant) were collected at 1, 4, and 24 hours post-treatment for further investigation. As a control, untreated cells (only media without drug) were used.

### Real Time Polymerase Chain Reaction (RT-PCR):



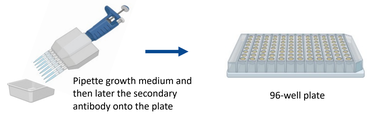

Total RNA was extracted from the cancer cells samples using miRNeasy Mini Kit (Qiagen, Germany) following kit manufacturer's protocol. SSIII First-Strand System for RT-PCR kit (Invitrogen, USA) was used to prepare cDNA from 1-3 mg extracted total RNA. RT-PCR was performed using cDNA in iTaq Universal SYBR Green Supermix (Biorad, USA) to study mRNA expression levels in a Biorad CFX96 thermocycler.

### Western Blot:




Whole cell protein lysates were prepared from the cancer cells samples as mentioned in the cell culture section with or without treatment using 1X RIPA lysis buffer containing protease and phosphatase inhibitors. Equal volumes of 2X laemmli sample buffer (Biorad, USA) and protein lysate were mixed and boiled to denature the proteins in the lysates. Denatured protein samples were subject to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels and separated protein bands were transferred on to PVDF membranes (Biorad, USA) at constant voltage conditions. Protein bands were detected using specific antibodies for the proteins of interest in STING-IRF3 pathway (CellSignaling, USA).

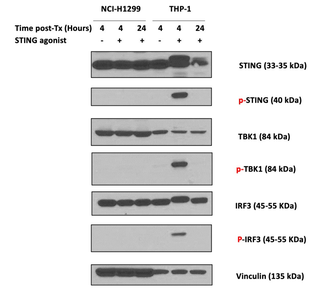
### Sandwich enzyme-linked immunosorbent assay (ELISA):




This was performed for detection of CXCL10, IFN-β, and IL-10 protein expression in cell culture supernatant and was performed following the manufacturer's instruction (R&D Systems, DuoSet, Minneapolis, MN).

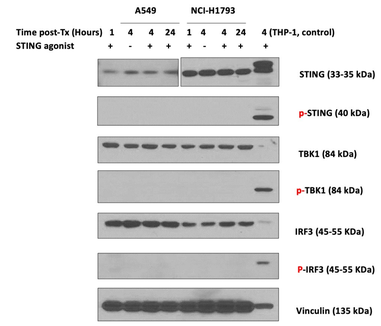
## Results

### Figure 3A. Effect of STING agonist on STING-IRF3 pathway in NCI-H1299 and THP-1 cells



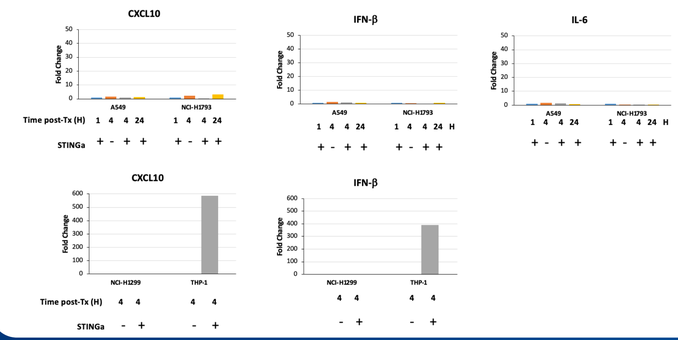

- STING agonist does not activate STING-IRF3 pathway in NCI-H1299 cells. Therefore, the downstream proteins STIING, TBK1 and IRF3 are not phosphorylated.
- THP-1, which has both genes of interest APOBEC3A and SMARCA4 normal, treated with STING agonist shows the phosphorylation of STING and downstream proteins TBK1 and IRF3. Signal is lost quickly due to the consumption of phosphorylated proteins within 24 hours post-treatment.

### Figure 3B. Effect of STING agonist on STING-IRF3 pathway in A549 and NCI-H1793 cells




- Neither NCI-H1793 nor A549 have a functional STING-IRF3 pathway. Upon treatment with STING agonist, no downstream phosphorylation of STING TBK1 and IRF3 was observed.
- Positive control functions properly confirming the integrity of the assay.

### Figure 4. RT-PCR results showing fold changes in the expression of CXCL10, IFN-β, and IL-6 genes upon treatment with STING agonist



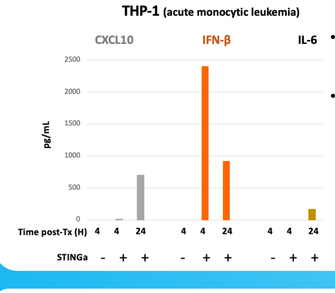

- THP-1 shows strong upregulation of CXCL10 and IFN-β genes after treatment with STING agonist.
- A549, NCI-H1299, and NCI-H1793 have no significant changes in gene expression after STING agonist treatment, confirming the impairment of STING-IRF3 pathway function.

## Results

### Figure 5. Bar graph displaying ELISA results on CXCL10, IFN-β and IL-6 cell supernatant levels (pg/mL) in cancer cells treated with STING agonist

| Features | Human Cancer Cell Line | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | A549 (lung) | | | | NCI-H1793 (lung) | | | | NCI-H1299 (lung) | | |
| Time point (Hours post-treatment) | 1 | 4 | 4 | 24 | 1 | 4 | 4 | 24 | 4 | 4 | 24 |
| STING agonist (ADU-S100 50 μM) | YES | NO | YES | YES | YES | NO | YES | YES | NO | YES | YES |
| CXCL10 (pg/mL) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| IFN-β (pg/mL) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| IL-6 (pg/mL) | ND | ND | ND | ND | ND | ND | 18 | 19 | ND | ND | ND |

ND – not detectable (below the detectable limit in ELISA)




- THP-1 secreted large quantities of CXCL10, IFN-β, and IL-6 after STING agonist treatment.
- A549, NCI-H1793, and NCI-H1299 cells did not secrete any detectable levels of CXCL10, IL-6, and IFN-β after STING agonist treatment, confirming the impairment of STING-IRF3 pathway. Only NCI-H1793 cells secreted very low amount of IL-6 upon STING agonist treatment.

## Conclusions

- NCI-H1299, NCI-H1793, and A549 cell lines do not respond to STING agonist as measured by STING-IRF3 pathway activation, strongly suggesting the role of SMARCA4 and/or APOBEC3 in the functionality of this pathway.
- THP-1 cell line, which had both genes SMARCA4 and APOBEC3B normal, has a functional STING-IRF3 pathway.

## Future Directions

- Individual knock out of APOBEC3A and SMARCA4 genes in THP-1 cells to demonstrate the impact of these genes (proteins) on functional STING-IRF3 pathway.
- Upon identification of the gene responsible for dysfunctional STING-IRF3 pathway, both NCI-H1299 and NCI-H1793 cells will be knock-in with the gene of interest to demonstrate the restoring of STING-IRF3 pathway function.

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