Synopsis of thesis on

Study of expression of cyclic-GMP-AMP synthase (cGAS) and Stimulator of interferon genes (STING) in Breast cancer and its potential for anti cancer therapy

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Introduction

Developing and underdeveloped countries are majorly affected by different types of cancers yearly. There have been increasing incidences of cancer even though there have been notable advancements in treatment and diagnosis. It is estimated that19.3 million new cancer cases (18.1 million excluding non-melanoma skin cancers) and death 10.0 million cancers (9.9 million excluding non-melanoma skin cancers) occurred in 2020. In females, breast cancer is the most diagnosed cancer with 2.3 million (11.7%) new cases, followed by lung (11.4%), colorectal (10.0 %), prostate (7.3%), and stomach (5.6%) cancer. Increasing the ratio of relapse due to drug resistance and metastasis in various organs with primary tumors leads to more than 90 % mortality in cancer [2]. There remains a challenge for the advancing research and development in diagnosis and treatment options. However, the different physiological conditions, origins, metastasis and re-occurrences of breast cancer in female is still not understood.

The heterogeneity of breast cancer is the major challenge for diagnosis and therapeutics. The ER-PR positive cancer having standard care of therapy availed as ER inhibitors, anti-hormone therapy and for the HER2 subtype having trastuzumab like receptor blocked therapy. Highly metastatic breast (triple-negative) cancer subtypes have no standard care of treatment available like for the TNBC (tipple negative type of breast cancer). Radiation and chemotherapy are the only available therapy for such cancer subtypes [3]. Due to the high rate of occurrence and mortality in breast cancer; there is further need for understanding the pathogenesis of breast cancer. There is a complex signaling mechanism in the tumor microenvironment, between tumor cells and other cells. The analysis of chemokines, cytokines suggests a pattern of cytokines in the breast cancer tumor microenvironment which are involved in the communication and may provide signals for survival and tumor immunity [4]. A necrotic cell in the tumor activates many damages associated molecular patterns (DAMPs). This cascade of signaling events the results in activation of cytokines like interferon. Some cytokines support cell growth and proliferation while others have an opposite effect [5]. Type-1 immune response majorly regulates the cytokines levels and majorly interferon in the tumor microenvironment. Pathogen-associated molecular patterns (PAMPs) and Damage associated molecular patterns (DAMPs) activate the type-1 immune response. Generally during neurodegeneration is inflammation and DMAPS like nuclear DNA released from damaged cells and pathogens can activate type-1 immune response [6]. Similarly, mitochondrial DNA and RAN are known to be immunogenic and can activate type-I IFN; DNA being the major genetic material

that is released from damaged cells or intracellular pathogens. Various intracellular DNA sensors like IFI16 and cGAS are found in the cytoplasm that senses cytoplasmic DNA and activates immune response [7]. The enzyme Cyclic GMP AMP synthase (cGAS) binds to double-stranded DNA and forms 2'3' cGAMP. The catalytic activity of cGAS involves the addition of a phosphate bond between AMP and ATP. 2'3'cGAMP is a natural ligand that activates STING (Stimulator of Interferon genes); localized on ER. There, it further phosphorylates TBK1 (Tank binding kinase-1) has two sites of phosphorylation and it activates p65 and IRF-3 via phosphorylation. The activation of p65 and IRF-3 causes dimerization and trans-locates to the nucleus and activates inflammatory genes like IFN-β, IFN-α, IL-6, TNF-α [7].

the cGAS-STING pathway is activated via different mechanisms. cGAS can be activated through viruses and intracellular microorganisms that release their DNA into the cytoplasm. The cGAS can also be activated by the DNA fragments due to DNA damaging agents; which may be and mitochondrial DNA from mitochondria [8]. Interestingly pathogenic bacteria also produce di-nucleotide which is capable of activating STING [9]. C-di AMP derived from *listeria monocytogens* also and gram-positive bacteria like *Mycobacterium* also activate STING [10].

Several reports suggest that the activation of the cGAS-STING pathway plays a critical role in tumor inhibition. Reduced levels ofcGAS-STING in tumor shows high variability of tumor aggression. cGAS interacts with the chromatin during mitosis and regulates the cellular division; loss of cGAS induces abundant cell growth. Highly proliferative cell-like TNBC breast cancer cells show high chromosomal instability which also induces the formation of micronuclei. which further activates STING and shows the non-canonical activation which leads to aggressiveness and metastasis of the tumor. Activation of STING in tumors leads to the production of the IFN pathway that attracts CD8+ cells in the tumor site. Infiltrated CD8+ cells into the tumor are considered hot tumors and decreased CD8+ cells are called cold tumors. Activated CD8+ cells kill the tumor cells as a form of cell-mediated immunity. These reports suggest that the expression and activation of the cGAS-STING pathway in breast cancer can be developed as a therapeutic target. In the current work, we hypothesize that cGAS and STING may activate cell survival in highly proliferative triple-negative breast cancer cells however its modulation and for regulated IFN release and NF-kB may be exploited for combinatorial therapy along with DNA damage and immunotherapy. Considering all the emerging pieces of evidence the following objectives have been proposed:

Specific objectives:

Major Objectives of the present study are:

1. To study the expression of cGAS and STING in different breast cancer cell lines as well as patients' tissues
2. To study the crosstalk between cGAS and STING and its effect on tumorigenesis of breast cancer
3. To study the role of cGAS and STING in context of anti-tumor activity
4. Results:

Objective-1: To study the expression of cGAS and STING in different breast cancer cell lines as well as patients' tissues

- STING expression was predominant in ER-negative cell line as MX-1 as progesterone positive cell line but ER-negative cell line. B474, BT549 as the HER2 positive and ER-negative cell line.MDA-MB-231 and MDA-MB-468, triple-negative breast cancer cell lines (ER/PR/Her2 negative) showed no detectable and low level found by both methods RT-PCR and Western blot.
- cGAS was found ubiquitously present in all cell lines tested via Western blot as well RT-PCR
- Further, the expression of STING was negatively correlated with Estrogen receptor expression that was analyzed via TCGA data

- **Objective-2: To study the crosstalk between cGAS and STING and its effect on tumorigenesis of breast cancer**
- DNA damage includes NF-kB activation in STING positive triple-negative breast cancer cells
- cGAS having no role in NF-kB activation during DNA Damage conditions
- STING mediated NF-kB activation induces IL-6 expression
- The knockdown of STING sensitizes breast cancer cells to genotoxic stress
- STING up-regulates IL-6 mediated PD-L1 expression during DNA damage

- STAT3 inhibitors combination with genotoxic drug show synergetic effect
- STING expression positive correlates with IL-6 and PD-L1 expression and high STING expression in chemotherapy shows the poor outcome

5. Objective -3: To study the role of cGAS and STING in context of anti-tumor activity

1. c-di AMP induces STING mediated IFN pathway in breast cancer cell lines.
2. c-di-AMP binds to STING.
3. c-di- AMP activates cell death in ER/PR negative breast cancer cell lines.
4. STING is essential for c-di-AMP induced cell death.
5. IRF-3 is indispensable for c-di-AMP induced apoptosis in ER-negative breast cancer cells.
6. c-di-AMP induces IRF-3 translocation to mitochondria and induces the mitochondrial-mediated intrinsic pathway of apoptosis.

Conclusion: The current study indicates that highly proliferating and metastatic cells which are triple-negative breast cancer cells show STING expression. Genotoxic drugs treatment to triple-negative breast cancer activates NF-kB and IFN pathway in the triple-negative breast as compared to positive leads to more insensitiveness via IL-6 and STAT3 pathway. Thus, the expression of STING remains significant in the TNBC cell line and also TCGA data shows correlation; which means that the expression of STING was significantly higher and shows drug resistance. One of the ways to treat this type of condition would be combinations therapy like STAT3 inhibitor and Doxorubicin as we have shown in our data. On the other end, the higher expression itself becomes the target, hence as STING agonist, c-di-AMP, can activate the IFN pathway and long-term treatment can induce apoptosis. Overall, the cGAS-STING pathway can be modulated for therapeutic purposes to develop combinatorial therapy with DNA damaging agents for highly proliferative breast cancer cells. Further study also suggests that the IL-6 pathway stimulated by STING can activate STAT3/JNK pathway and also inhibited specific inhibitors and immune evasion of these cells can be inhibited. The present study had opened immense possibilities to develop combinatorial therapy for breast cancer however similar strategies can be used for other cancer.

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- **Vasiyani H**, et al. **"**DNA damage induces STING mediated IL-6-STAT3 survival pathway in triple-negative breast cancer cells(Manuscript under communication)
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Publications as co-author:

1. Shinde, A., Jung, H., Lee, **Vasiyani H**. et al. TNF-α differentially modulates subunit levels of respiratory electron transport complexes of ER/PR +ve/−ve breast cancer cells to regulate mitochondrial complex activity and tumorigenic potential. Cancer Metab 9, 19 (2021). https://doi.org/10.1186/s40170-021-00254-9

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Achievements:

1. Completed Diploma certificate course for "Principles of kinetics theory, Biacore kinetics assay development and different models for kinetics evaluation" held at Mumbai by GE Healthcare in 2016.
2. Attended 4th International conference 'Drug discovery india2016' at Bangalore.
3. 6. Completed workshop on Cellinsight CX7 high content screening (HCS) platform and Attune NXT Flow Cytometer in 2018