Analysis of TNF-α regulated metabolic reprogramming in ER/PR +ve and –ve breast cancer cells

Pre-Synopsis Submission

Submitted by

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INTRODUCTION

Breast Cancer

Breast cancer is the second most cause of death in women worldwide[1][2].The heterogeneity of breast cancer makes them a challenging solid tumor to diagnose and treat[3]. Based on the presence and absence of steroid hormone receptors, and growth receptor HER2, breast tumors are originally categorized into ER/PR/HER2 positive and ER/PR/HER2 negative (triple negative), a characteristic of primary and metastatic breast cancer respectively [4]. Breast cancer not only consists of neoplastic cells but also develops alterations in tumor microenvironment.

Tumor microenvironment is complex and constitute many cell types including immune cells [5]. The tumor heterogeneity observed is due to interactions between tumor cells and their microenvironment. The tumor microenvironment includes stromal cells, blood vessels and the immune system via paracrine factors which contributes to tumor progression.

Chronic inflammation, a hallmark of solid tumor, is intricately associated with tumor microenvironment and promotes metastatic progression of breast cancer [6][7]. Tumor necrosis factor-α (TNF-α) is a potent immunostimulatory cytokine which contributes to metastatic progression by promoting the generation of a proinflammatory microenvironment[7][12]. In breast cancer, TNF-α is found consistently high in the tumor microenvironment. The increased levels of TNF-α results in elevated levels of ROS generation and chronic activation of inflammatory pathway such as NF-kB, however the mechanisms are not well understood [8]. NFKB pathway is known to regulate metabolism and mitochondrial functions in cells [13][14][17].

Mitochondria acts as a metabolic hub and provides essential energy and building blocks for cancer cell proliferation [9]. Mitochondrial proteins effect the mitochondrial forms and functions and are critical for cellular metabolism [15][16][19]. The functional adaptations of mitochondria in presence of TNF-α may alter the cancer cell metabolism however the mechanism underlying the process is not well understood. The previous reports had shown that TNF-α regulates mitochondrial functions and identified several regulators of innate immune pathway which also alters mitochondrial functions [10][11]. Its differential role in regulation of mitochondrial functions in ER/PR $_{+}$ve and ER/PR –ve essential for metabolic adaption for the breast cancer cells needs to be understood. These evidences clearly suggest that mitochondria had been placed such that it links inflammation and metabolism to support tumor cell growth.

This study will help to understand the linkages between mitochondria, inflammation and cancer and to identify novel modulators of TNF-α induced signaling and inflammatory pathways for future therapeutic interventions.

KEY QUESTION:

The tumor microenvironment of breast cancer creates an inflammatory state which contributes to the metastatic progression. The presence of TNF-α in tumor microenvironment may regulate the cell metabolism and progression of cancer. Here we would like to investigate the role of proinflammatory cytokine TNF-α and inflammatory pathways like NFKB in altering the mitochondrial functions and metabolic needs of the ER/PR +ve and -ve cells residing in tumor microenvironment. The needs to the basal and luminal breast cancer cells may alter and dictate their fate in tumor progression.

AIM OF THE STUDY:

The aim of the study will be to identify the differentially regulated mitochondrial proteins and their role in mitochondrial functions in presence of TNF-α in ER/PR +ve and -ve breast cancer cells. This study will also aim in understanding the metabolites in presence of TNF-a and the alterations and regulation of metabolic needs of basal and luminal breast cancer cells. This study will identify molecular regulators in mitochondrial complexes and their role in ER/PR negative breast cancer cell survival advantage. This may also help to provide therapeutic target along with other chemotherapeutic agents to prevent the progression of aggressive breast cancer cells.

RELEVANCE OF THE STUDY:

The tumor microenvironment (TME) consists of various types of cells like stromal and immune cells. The inflammatory state created by the presence of cytokines in the TME is known to drive different phases of tumor progression. TNF-α, a proinflammatory cytokine is found to be high in tumors from different origin and known to contribute to the inflammatory state. This may affect the form and progression of ER/PR +ve and -ve cells and lead to different adaptations for survival.

Mitochondrial proteome changes may be altered in order to meet the metabolic needs of tumor and effect its growth. The functional adaptations of mitochondria in presence of TNF-α may alter the cancer cell metabolism however the mechanism underlying the process is not well understood.

This study will lead to identification of mitochondrial proteins that are differentially and dynamically regulated during TNF-α mediated metabolic reprogramming in breast tumors. These proteins may act as biomarkers for early detection and diagnosis of breast cancer in individual patients and designing therapeutics for aggressive breast cancer.

HYPOTHESIS:

TNF-α is found to be high in several tumor microenvironments including the breast cancer. It is very well known that mitochondria is one of the central organelle where several cytokines including TNF-α act and regulate its functions to meet the anabolic demands of the cancer cells. The role of TNF-α in regulating the metabolic regulation in cancer cells is not well understood. TNF-α mediated metabolic reprogramming in ER (+ve) and ER (-ve) in human breast tumors is not well understood.

The current study aims to understand the role of TNF-α regulated metabolic reprogramming in human breast cancer cells. Analysis of mitochondria-mediated inflammatory signaling and its role in maintaining bioenergetic status will be studied by analyzing mitochondrial proteome and metabolome in ER/PR +ve and -ve cell lines.

OBJECTIVES:

Objective 1: Study the role of TNF-α -regulated oxidative phosphorylation and aerobic glycolysis in ER (+ve) and ER (-ve) breast cancer cells.

Objective 2: Analysis of mitochondrial proteome and mitochondrial respiratory complexes assembly in ER (+ve) and ER (-ve) breast cancer cells in presence and absence of TNF-α.

Objective 3: Functional characterization of differentially abundant proteins (TNF treated vs Untreated), their role in mitochondrial functions and effect on tumorigenicity in ER/PR +ve/-ve cells.

EXPERIMENTAL DESIGN:

MCF-7 will be used as ER/PR +ve (Luminal) and MDA-MB-231 as ER/PR -ve (Basal) breast cancer cells for the study.

Objective 1: (Completed)

Study the role of TNF-α -regulated oxidative phosphorylation and aerobic glycolysis in ER (+ve) and ER (-ve) breast cancer cells.

- ✓ To understand the effect of TNF-α on mitochondrial functions in ER/PR +ve (Luminal) and MDA-MB-231 as ER/PR -ve (Basal) breast cancer cells the levels of TNF induced

ATP production and ROS generation was checked. This lead to understand the differentially regulated mitochondrial functions in both cells. The results showed that mitochondrial and cellular ATP production was decreased in MDA-MB-231 cells in comparison to MCF-7.

- ✓ Activity of mitochondrial complexes in TNF-α treated ER/PR +ve and -ve cells was monitored by spectrophotometric assay of mitochondrial complexes. Complex I and Complex IV activity was decreased in MDA-MB-231 in comparison to MCF-7. This very well corelated with the decreased ATP and enhanced ROS production in MDA-MB-231 cells.

- ✓ Effect of TNF-α induced assembly of mitochondrial complexes was also monitored. TNF-α decreased the assembly of mitochondrial complex I, Complex III and complex IV in MDA-MB-231 cells in comparison to MCF-7. This concluded that there is defect in the mitochondrial complex assembly and mitochondrial functions in MDA-MB-231 cells in presence of TNF-α.

- ✓ To understand the effect of TNF-α on tumorigenic potential and migration ability of these cells wound scratch assay and clonogenic assays were performed in presence and absence of TNF. Surprisingly TNF-α enhanced the migration and clone forming ability of MDA-MD-231 in comparison to MCF-7.

Objective 2: **(Completed)**

Analysis of mitochondrial proteome and mitochondrial respiratory complexes assembly in ER (+ve) and ER (-ve) breast cancer cells in presence and absence of TNF-α.

- ✓ Mitochondrial proteome from ER/PR +ve and -ve breast cancer cells was measured from isolated mitochondrial fractions from TNF-α treated ER (+ve) and ER (-ve) breast cancer cells by high-resolution mass spectrometry. Both the breast cancer cells showed differential expression of mitochondrial proteins and strengthened the hypothesis of differential mitochondrial metabolic regulation in these cells. Proteins related to different complexes were analyzed and their abundance was measured in both cells.

- ✓ Metabolomic analysis of major rate limiting metabolites of TCA cycle and glycolysis was performed using LC-MS platform to understand the metabolic regulation in MCF-7 and MDA-MB-231 cells in presence and absence of TNF-α. The differential abundance of

metabolites related to glycolytic and TCA pathways were analyzed. These results suggested defects in the mitochondrial OXPHOS in MDA-MB-231 cells in comparison to MCF-7. The alteration in the metabolic pathways in presence of TNF-α acts as survival advantages for the cancer cells to meet the metabolic and energy demands.

OBJECTIVE 3: (Completed)

Functional characterization of differentially abundant proteins (TNF-α treated vs Untreated), their role in mitochondrial functions and effect on tumorigenicity in ER/PR +ve/-ve cells.

The effect of TNF-α induced NFκB in regulating the mitochondrial proteins will be checked in further objectives.

The role of mitochondrial proteins in altering the mitochondrial functions and metabolism acts as a basis of many diseases including cancer. Such changes in the mitochondrial proteome in the presence of inflammatory conditions may lead to changes in disease progression.

Effect of NFKB on mitochondrial proteins in breast cancer cells.

- ✓ To understand the effect of NFKB pathway on changes in mitochondrial proteins we designed sgRNA for P65 which is main subunit of NFKB and checked for CRISPR based knockdown of the P65 gene. We established the knockdown at protein level using western blotting in breast cancer cells.
- ✓ Further we checked for the turnover pIKBα levels by western blotting in presence and absence of MG132 and nuclear translocation of P65-GFP in presence and absence of curcumin to further establish the decreased activation of NFKB pathway in breast cancer cells.
- ✓ We selected the differentially altered mitochondrial protein LYRM7 for our further studies. LYRM7 is a mitochondrial complex III assembly factor and regulates the assembly of the complex at crucial steps. The protein levels were checked in presence of P65sgRNA and vector condition and we observed a decrease in the LYRM7 protein levels in decreased P65 condition. We also observed a decrease in presence of curcumin in both the cell lines.
- ✓ The results were consistent with the realtime PCR under P65 knockdown condition. The results suggest that LYRM7 may be regulated by the TNF-α induced NFKB pathway.

Effect of mitochondrial protein knockdown on mitochondrial functions.

- ✓ Further we analyzed the role of LYRM7 on regulating the mitochondrial functions in presence and absence of TNF-α. We observed that knocking down LYRM7 increased the

mitochondrial ROS which further increased in presence of TNF-α in highly metastatic MDA-MB-231 cells. The knockdown also affected the mitochondrial membrane potential which decreased in the LYRM7 knockdown condition.

- ✓ The LYRM7 knockdown further effected the ATP levels which decreased in MDA-MB-231 and MCF-7 cells. It also affected the mitochondrial complex assembly and complex activities of both the cells in presence and absence of TNF-α.

Effect of mitochondrial protein on breast cancer cells and patients' survival

- ✓ To elucidate the effect of altered mitochondria protein on survival and migration potential of these cells we transfected the cells with LYRM7 sgRNA and observed reduced migration in knockdown condition compared to control in presence of TNF-α in MDA-MB-231 cells as compared to MCF-7. The clone forming ability was also reduced in MDA-MB-231 and MCF-7 cells. These results suggested altering mitochondrial protein in inflammatory conditions can determine the mitochondrial functions and survival ability of breast cancer cells.
- ✓ We further analyzed the expression of LYRM7 in normal vs tumor tissue of breast cancer patients and observed a decrease in the expression of LYRM7 in tumor. The expression also varied in different subtypes of breast cancer. The KM plot analysis showed reduced survival in patients with low LYRM7 expression compared to high expression.

Summary of work done:

In tumor microenvironment of breast cancer, the levels of TNF-α, a pleiotropic cytokine is high. The effect of TNF-α on survival and metastatic potential of breast cancer cells was studied. The work here concluded that TNF-α alters the mitochondrial proteome and metabolites of breast cancer cells. It altered mitochondrial proteins may have role in regulating the mitochondrial

functions and cellular metabolism which effects the breast cancer cell survival and migration potential. Such alterations of mitochondrial proteins and their outcome can be markers for therapeutics and help in designing strategies for reducing the progression of diseases.

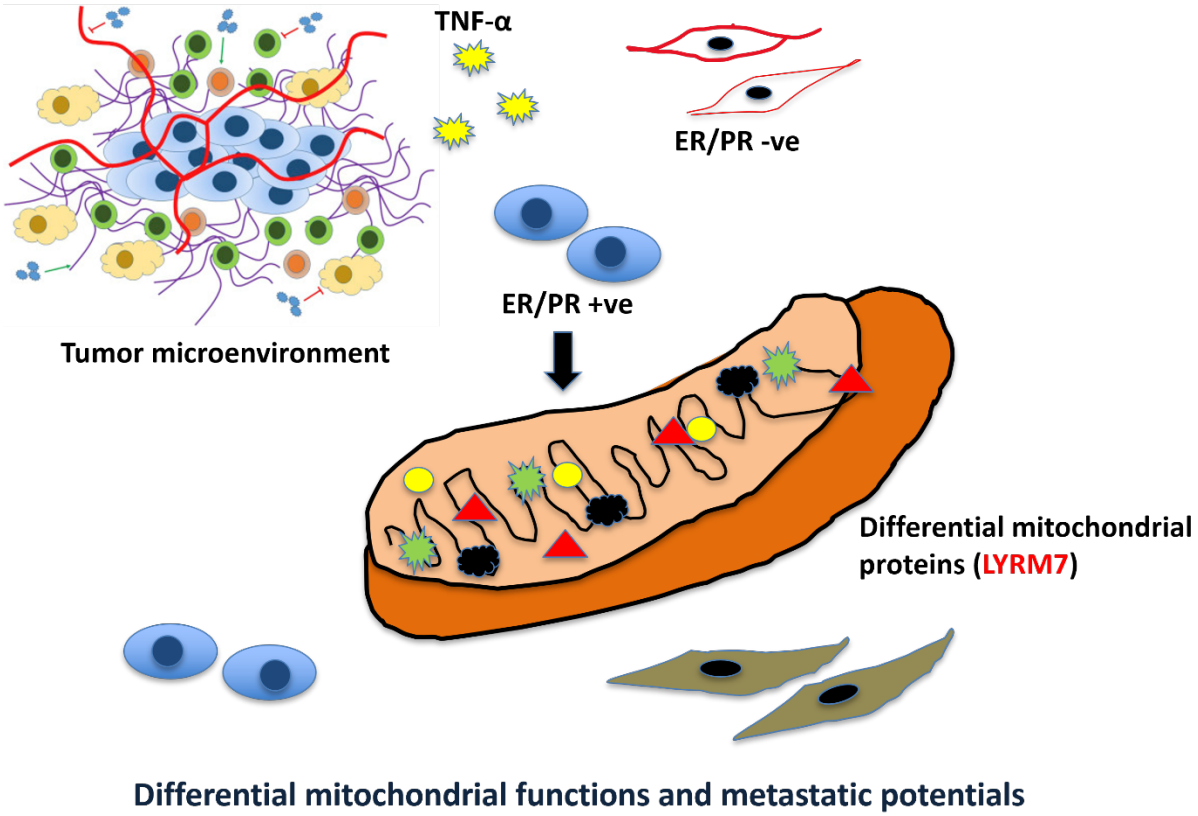

Figure: Schematic representation of altered mitochondrial proteins in TME and their role regulating metastatic potentials and survival of ER/PR +ve and -ve breast cancer cells

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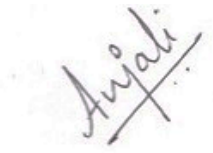
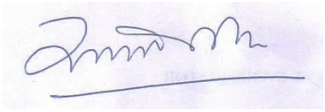


Signature of Candidate



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