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INFLUENCE OF SIRT-1 -NF kB SIGNALLING PATHWAY IN TUMORIGENESIS OF BREAST CANCER: A REVIEW OF PROTOCOL

REVIEW ARTICLE

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Abstract

Objective of the study is to study the influence of SIRT 1- NF kB signalling pathway on tumorigenesis of breast cancer. We also aim to find out whether there is an association between the expressions of sirtuin 1 and P65 of NF kB.

Fifty female patients freshly diagnosed with primary breast carcinoma and fifty female patients with fibro adenoma or benign breast disease as controls will be recruited to the study.The specimen will be taken intra-operatively from the resected tumor tissue.The samples will then be divided into 3 parts and processed for mRNA expression by real-time PCR, protein expression by Western blotting and Immunohistochemistry.

The expression of sirtuin 1 and P65 of NF kB will be compared in cases and controls by using Mann Whitney U test. The association between their expressions will be analyzed by using Chi-square test. Odd’s ratio will be used to analyze the extent of risk of malignancy among cases.

The study may be useful if the expression of sirtuin 1 and p65 of NF kB could be predictors of malignancy of breast, hence may improve patient outcome as well as quality of life in breast cancer patients.

Keywords: breast cancer, SIRT1, NF kB signalling

Introduction

Breast cancer contributes to the second highest incidence of cancer-rated deaths among women worldwide [1].Breast cancer is the most common cancer in Indian females[2] .Development of breast cancer is associated with multiple etiologic factors, including hormonal disorders, inheritance, ionizing radiation, and unhealthy eating habits[3]. Breast cancer is normally treated by a combination of surgery with radiotherapy, endocrine therapy, and/or chemotherapy. Despite improvements in diagnosis and treatment, the 5-year survival rate of patients with breast cancer was still viewed as unsatisfactory in recent decades, which highlights the ongoing need to understand the mechanisms by which it progresses and to explore new therapeutic targets[4].

Sirtuin 1 and Cancer

Sirtuins are nicotinamide adenine dinucleotide (NAD+)–dependent deacetylases that function as intracellular regulators of transcriptional activity. SIRTl is a mammalian homology of yeast Sir2 which deacetylates histones as well as non-histones. It plays important roles in cell survival, signal transduction, and cell apoptosis by deacetylating key cell signaling molecules and apoptotic related proteins, such as NF-kB, p53, Ku70, and HIFs[5] .Various studies have inconclusive reports on the role of SIRT1 in cancer, because of its opposite effects as both a tumor activator or suppressor in various human cancers, including breast cancer. Deng et al found that the expression of SIRT1 was lower in prostate cancer, bladder cancer, ovarian cancer, and glioblastoma when compared with normal tissues[6].On the contrary, it was found that, in the leukemia and lung cancer, SIRT1 was significantly higher[7,8].

This can be explained as follows: SIRT1-mediated deacetylation suppresses the functions of several tumor suppressors including p53, p73, and HIC1, it has been suggested that SIRT1 has a promoting function in tumor development and progression[9]. In contrast, SIRT1 may have a suppressive activity in tumor cell growth by suppressing NF-κB, a transcription factor playing a central role in the regulation of the innate and adaptive immune responses and carcinogenesis, the dysregulation of which leads to the onset of tumorigenesis and tumor malignancy [10].

Here, we aim to further explore the role of SIRT1-NF kB signalling pathway in tumorigenesis of breast as well as its associated mechanisms.

NF-kB and Breast Cancer

The nuclear factor-κB (NF- κB)/REL family of transcription factors is comprised of a RELA/p65,c-REL,RELB, p105/NF- κB1 and p100/NF- κB2 [11].The p105 and p100 proteins can be processed by proteolytic cleavage into p50 and p52, respectively. Activation of NF-κB signaling pathway leads to the induction of target genes that can inhibit the apoptosis, interaction with cell cycle regulation, cell invasion, contribute to tumorigenesis and metastatic invasion[12]. Activation NF-κB in breast cancer is loss of Estrogen Receptor (ER) expression and Human Epidermal Growth Factor Receptor 2 (HER-2) overexpressed via epidermal growth factor receptor (EGFR) and Mitogen Activated Protein Kinase (MAPK) pathway[13]. Indeed, the binding of epidermal growth factor (EGF) to its receptor (EGFR) also ultimately activates NF-κB and most likely contributes to the enhanced activity of this

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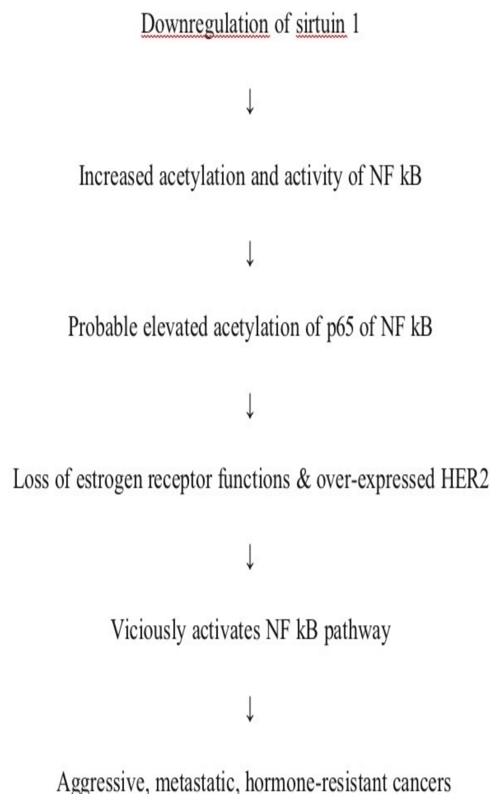
transcription factor in ER negative breast cancer cells[14].

Loss of ER function has been associated with constitutive NF-κB activity and hyperactive MAPK, because of constitutive secretion of cytokine and growth factors, which ultimately culminates in aggressive, metastatic, hormone-resistant cancers[15]. Activation of the progesterone receptor can lead to inhibition of NF-κB driven gene expression[16], reducing its DNA binding and transcriptional activity. HER-2 activates NF-κB through the canonical pathway which surprisingly, involves IKKα[17]. Activation of NF-κB promotes survival of tumor cells. Several gene products that negatively regulate apoptosis in tumor cells are controlled by NF-κB activation. Estrogen plays an important role in breast cancer initiation and progression. Breast cancer over time acquires different mutations and the proportion of estrogen receptor negative cells in tumor increases. This transformation confers aggressive biological characteristics to breast cancer such as rapid growth, poor differentiation, and poor response to hormone therapy. NF-κB pathway plays important role in this pathway.

The study aimed to explore the influence of SIRT 1-NF kB signalling pathway on the tumor formation in breast cancer. It is important to investigate whether SIRT 1-NF kB signalling pathway could be a novel therapeutic target in breast cancer.

Rationale of the study

Fig1:Probable SIRT1 pathway



There is a scarcity of literature which explores this pathway in the pathogenesis of breast cancer to the best of our knowledge.

Novelty/Innovation:

Role of Sirtuin 1-NF kB signalling pathway has been least studied in breast cancer. If expression of sirtuin -1 and p65 subunit of NF kB could be used as novel markers for the prediction of malignant transformation of breast cells , early prediction of may help in personalizing the therapy which may lead to better prognosis. Early detection and treatment will definitely improve quality of life of breast cancer patients.

Objectives of the Study:
Aims of our study are to

i) study the influence of SIRT 1- NF kB signalling pathway on tumorigenesis of breast cancer

ii) compare the expressions of sirtuin 1 and p65 subunit of NF kB in malignant versus benign tumors of breast

find the association between the expressions of sirtuin 1 and p65 subunit of NF kB in tumor cells of breast

Methods

i. Study design: Prospective

Study type: Case-control

ii. Project implementation Plan

Study site: Molecular division of Central research laboratory, KSHEMA

The study will be collaborative between Department of Biochemistry and Department of Oncology, Justice K S Hegde charitable Hospital, Mangalore.

The study will consist of two groups. Patients fulfilling all below mentioned criteria will be included in the study;

Inclusion Criteria:

Cases

Fifty female patients with primary breast carcinoma,freshly diagnosed by histopathology, untreated by chemotherapy, radiotherapy, hormone therapy or a combination of any of the modalities who will be consulting the Dept of Surgery/Oncology for treatment

Controls

Fifty female patients who present to the Dept of Surgery/Oncology , diagnosed to be histologically and clinically fibro adenoma or benign breast disease

Exclusion Criteria for both cases and controls: Patients with associated illnesses like diabetes mellitus, congestive heart disease, inflammatory and autoimmune disorders

Ethics Review

- NITTE University Central Ethics Committee approval will be obtained prior to the study
- Written Informed Consent will be taken from the parents/care takers and assent will be taken from children

Gene expressions to be studied:

- Sirtuin 1
- P65 of NF kB

Sample collection and Tissue Processing

The specimen will be taken intra-operatively from the resected tumor tissue.Tissue will be washed with phosphate buffered saline (PBS), cut into small pieces and immersed in collagenase at 37 °C for 4–6 h. Collagenase incubated tissue will be minced and treated with 0.125 % trypsin-EDTA for 10 min. Total protein will be extracted by homogenizing cells in RIPA: lysis buffer mixture (1:3) at 4 °C and will be measured spectrophotometrically by Lowry's method.

The samples will then be divided into 3 parts and processed for mRNA expression by real-time PCR, protein expression by Western blotting and Immunohistochemistry.

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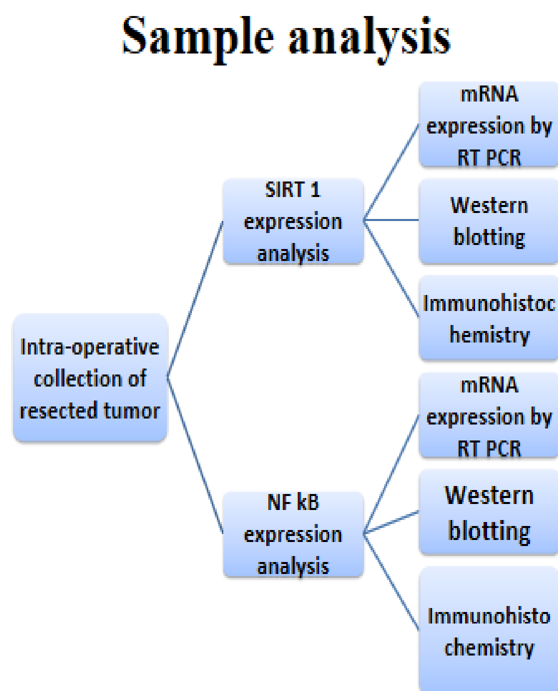
Fig 2: Scheme of sample collection and analysis



A. Gene (mRNA) Expression By Real Time PCR (qPCR) Analysis

The tumor samples will be chopped into fine pieces and quickly immersed in Trizol solution. The samples will be then stored at -80oC till analysis. The tissue will be then homogenized using Trizol for further processing.

RNA Extraction, cDNA Synthesis and Real time PCR

RNA will be isolated from tumor tissues by High pure Tissue RNA isolation kit (Roche, Indianapolis, IN). Purity and RNA concentration will be assessed by measuring the absorbance at 260 and 280nm using Nanodrop 2000 (Thermo Scientific, United States). 1 ug of RNA will be converted into cDNA by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermofischer scientific, USA). The gene-specific oligonucleotide primers (Integrated DNA Technologies) will be used. CFX96 Real-Time PCR Detection System will be used for evaluating the gene expression levels by using SYBR green and probe master mix (Roche, Indianapolis, IN). Thermal conditions used for reactions in real time PCR . In order to confirm the presence of a single PCR product in PCR reaction, melting curve analysis will be performed. Relative fold change will be calculated by using $2{-}\Delta Ct$ method . The gene expression data were represented in arbitrary units.

B. Western Blot Analysis

For whole cell lysates, cells will be resuspended and homogenized in buffer (100 mMTrisCl, pH 7.4, 300 mMNaCl , 1 % NP-40, and 0.25 %sodium-deoxycholate). All the buffers will be supplemented with protease and phosphatase inhibitor mixtures. For direct Western blot analysis, the cell lysates or the particular fractions will be separated by SDSPAGE, transferred to nitrocellulose membrane (Amersham Hybond-P, GE Healthcare) and will be probed with specific antibodies, e.g.anti-Sirtuin and anti –p65(NF-κB), produced from Santa Cruz , thereafter the immunoblots will be visualized by chemiluminescence or alkaline phosphatase method. Equal protein loading will be confirmed with α- actin antibody (Santa Cruz).

C. Histology and Immunohistochemistry

Breast tumors will be fixed in 10 % neutral-buffered formalin for 24 h, measured the tumor

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size, nodal status, grade and embedded in paraffin, and sectioned. For immunohistochemistry, paraffin sections of tumors will be deparaffinized and hydrated by successive washes with xylene, 100 % ethanol, and a phosphate buffer [10 mM (pH 7.4) and 0.138 M saline containing 2.7 mMKCl).

Antigen retrieval will be accomplished with diluted antigen retrieval buffer (DAKO Corp.) Endogenous peroxidase will be blocked with 3 % hydrogen peroxide. Subsequently, slides will be washed in PBS/KCl, incubated with 10 % normal horse serum followed by the primary antibody (rabbit anti-ER antibody or rabbit anti-PR antibody rabbit anti-c-erbB2; HER-2/neu) and will be incubated overnight at 4 °C. The slides will then be incubated with biotinylated secondary antibody for 45 min, followed by ABC reagent and diaminobenzidine. Counterstaining will be done with hematoxylin. Sections will be dehydrated by washing sequentially with 95 % ethanol, 100 % ethanol, and xylene. Cover slips will be mounted on slides using Paramount. Digital images of stained and unstained cells will be obtained using an Olympus microscope equipped with a SPOT digital camera.

Statistical analysis

The values will be expressed as mean ± S.D/SEM for parametric data and median (interquartile range) for nonparametric data and will be analyzed using statistical package for social sciences (SPSS), version 23.0 software. Chi-square test will be used to find the association of SIRT-1 -NF kB signalling pathway expression and breast cancer. Mann Whitney U test will be used to compare levels sirtuin -1 and p65 of NF kB among cases and controls. Odd's ratio will be used to find the extent of risk of malignancy with SIRT1 and NF kB expression.

Expected Outcome

- This study may reveal the role of SIRT 1 -NF kB signalling pathway in tumorigenesis of breast.
- This study might reveal that expression of sirtuin 1 and p65 of NF kBmay be a predictor of malignancy of breast
- This study may also suggest that SIRT 1- NF kB as potential therapeutic target to treat hormone resistant breast cancers

Limitations of the study:
Small sample size is the limitation of the study

Conclusion

Influence of SIRT 1-NF kB signalling pathway is the least studied pathway in the tumorigenesis of breast cancer. If the study establishes the role of this pathway in the pathogenesis of carcinoma of breast, this could be a potential therapeutic target for breast cancer.

Conflicts of interest: None

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