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GENE EXPRESSION AS A BIOMARKER OF RESPONSIVENESS: A PERSONALIZED THERAPEUTIC APPROACH TOWARDS METASTATIC BREAST CANCER PATIENTS ON CDK4/6 INHIBITOR

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Abstract

Objective of the study is to assess whether gene (mRNA) expression of Retinoblastoma Susceptibility Gene Product (Rb) signature and CCNE1, the gene encoding cyclin E1 can be used as markers of responsiveness to CDK4/6 inhibitors in metastatic breast cancer patients

In this cross-sectional study, eighty metastatic breast cancer patients with positive estrogen and progesterone receptor responses, but negative HER2, on CDK4/6 inhibitors will be recruited. Five ml of EDTA blood samples will be collected. Expression of genes, CCNA2(cyclin 2), MCM7 (mini chromosome maintenance complex component 7) and CCNE1(Gene encoding cyclin E1) will be analyzed by following steps; i.isolation of RNA ii.cDNA synthesis by reverse transcription iii. Quantification by real time PCR.2 ml of blood will be collected in plain vials for Estimation of CCNA2 , MCM7 and CCNE1 levels in serum samples.Chisquare test will be done to find the association of gene expression and serum levels of biomarkers of response (BoR) with the outcome CDK4/6 inhibitor therapy.

Biomarkers for response to CDK4/6 inhibitors in breast cancer patients may be helpful in differentiating responders and non-responders. This may open up a personalized therapeutic approach in breast cancer patients.

Keywords: *breast cancer, metastasis, CDK4/6 inhibitors, biomarkers of responsiveness*

Introduction

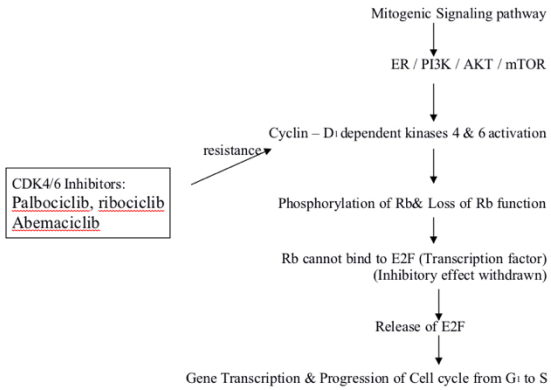
Breast cancer is the most common cancer in Indian females [1]. Incidence of metastatic breast cancer (MBC) has been reported to be approximately 5% to 25% from various centers in India[2,3]. MBC is unlikely to be cured; meaningful improvements in survival have been seen, coincident with the introduction of newer systemic therapies in Western literature[4]. MBC carries a poor prognosis in the Indian subcontinent, 5-year and 10-year overall survival have been reported to be 22% and 5% [5].The data are almost a decade old, and no new studies have been published from India regarding the impact of newer therapies on survival.

CDK4/6 inhibitors And Treatment of metastatic breast cancer

The recent use of cyclin D1-cyclin-dependent kinases 4 and 6 (CDK4/6) inhibitor agents, with an approximate doubling of progression-free survival (PFS) associated with their use in hormone receptor-positive,HER2-negative advanced breast cancer (BC), has radically changed the approach to managing this disease. However, resistance to CDK4/6 inhibitors is considered a near-inevitability in most patients.20% of MBC are intrinsic non-responders to CDK4/6 inhibitors. Biomarkers with the ability to identify early resistance or to predict the likelihood of successful treatment using CDK4/6inhibitors are yet to be identified and it represents an area of unmet clinical need.

Loss of Retinoblastoma Susceptibility Gene Product (Rb) Function the main target of CDK4/6, has been implicated by multiple preclinical studies in being a driver of resistance to CDK4/6 inhibitors[7-9]. RBsig, a gene expression signature of Rb loss-of-function, has been validated in identifying between palbociclib-sensitive and resistant BC cell lines[10]. CCNE1, the gene that encodes cyclin E1, is upregulated in models with resistance to CDK4/6 inhibitors[11,12]. Concurrent over-expression of CCNE1 and down-regulation of Rb may play a major role in resistance to CDK4/6 inhibitors in MBC patients. This may demand a change of drug or an add on drug. The large inter individual variability may reflect functional consequence of down regulation or up regulation of gene expressions, encoding mitogenic pathway. The concept of "individualized medicine" is evolving and there has been a paradigm shift from the concept of "one drug fits all" to "right drug for the right patient at the right dose and time." Hence it is very important to investigate the possible mechanism of drug resistance as well as to establish biomarkers of responsiveness to CDK4/6 inhibitors in breast cancer patients. There are hardly any studies which assess Rb Signature and CCNE1 gene expression as markers of responsiveness to CDK4/6 inhibitors in Ca breast patients to the best of our knowledge.

Fig 1:Proposed mechanism of resistance to CDK4/6 inhibitors:



Objectives

- To assess whether gene (mRNA) expression of Retinoblastoma Susceptibility Gene Product (Rb) signature and CCNE1, the gene encoding cyclin E1 can be used as markers of responsiveness to CDK4/6 inhibitors in metastatic breast cancer patients
- To establish biomarkers of responsiveness to the treatment with CDK4/6 inhibitors in metastatic breast cancer patients

Novelty/Innovation

The study results may produce personalized "Biomarkers of Response (BoR)" to readily differentiate Responders from Non-responders .It may help in personalizing the treatment of breast cancer patients with CDK4/6 inhibitors.

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Methods

Study setting: The study will be conducted in the Molecular division of Central Research Laboratory of K.S. Hegde Medical academy and Department of Oncology, Justice K.S. Hegde Charitable Hospital of Nitte University, Mangaluru, Karnataka, India.

Type of study: Prospective Cross sectional

Study subjects: Eighty metastatic breast cancer patients

Inclusion criteria:

- Post-menopausal metastatic breast cancer patients (stage IV)
- Hormone Receptor positive (ER+ or PR+)
- HER2 negative
- On first or second line therapy with any of the following agents or combinations:
  - CDK inhibitor (palbociclib, ribociclib or abemaciclib) with/without Aromatase inhibitors letrozole , anastrozole Exemestane
  - CDK inhibitor with fulvestrant

Exclusion criteria: breast cancer patients in early stages of malignancy

Ethical Issue: Institutional Ethics committee approval will be obtained and Written Informed Consent will be taken from patients or first degree relatives of patients.

Sample Size Calculation- Considering the incidence of polymorphisms to be 5.0%, we would require a sample size of 80 patients to design a study with 4% absolute precision and 95% confidence.

A pre-tested semi-structured questionnaire containing socio-demographic details of patients like age, place of residence etc will be collected after formal written consent maintaining confidentiality of identity.

Sampling:

- 5mL of whole blood will be collected at the following time points: time of diagnosis (baseline/pre-dose), 2 months-post treatment (while on drug), at time of regression.
- Sample will be stored at stored at -80°C
- Clinical documentation of patient demographics, ER/PR & HER2 status, treatments, responses and survival will be done
- Clinical documentation of pattern of metastases (bone only vs. visceral)

To test our hypothesis that a signature of functional Rb loss would be predictive of resistance to CDK4 and 6 inhibitors in metastatic breast cancer patients, a signature (RBsig) from the The Cancer Genome Atlas (TCGA) data set is selected[13]. The signature included a set of 87 genes out which following three genes are selected which have a role in G1/S transition in cell cycle.

CCNA2: cyclin 2 gene, controls both G1/S and G2/M transition phase of cell cycle

MCM7: mini chromosome maintenance complex component 7, has a role in G1/S phase and CDK-mediated phosphorylation and removal of Cdc6.

CCNE1: Gene encoding cyclin E1

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

a. RNA extraction

Five milli litres of whole blood will be collected intotwo2.5 mLPAX gene blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) and will be stored at –80°Cuntil RNA isolation. RNA will be isolated from blood, using the PAX gene system and following the manufacturer's instructions. Briefly, PAX gene Blood RNA tubes will be centrifuged, and the pellets will be washed and resuspended in buffer. Using the PAX gene Blood RNA Kit (Qiagen, Valencia, California, USA), lysis buffer will be applied to the resuspended pellets, and RNA purification and extraction will be performed using the columns. The extracted RNA will be stored at –80°C until further analysis.

b. cDNA Synthesis

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

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will be converted into cDNA by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermofischer scientific, USA). The gene-specific suitable oligonucleotide primers (Integrated DNA Technologies) will be used.

c.Real time PCR(qPCR)

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).

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–ΔCtmethod. The gene expression data will be represented in arbitrary units. The following genes will be analyzed:

CCNA 2: cyclin 2 gene

MCM7: mini chromosome maintenance complex component 7

CCNE1: cyclin E1

B. Quantification of proteins coded by the genes of interest

2 ml of blood will be collected in plain vials for Estimation of CCNA2, MCM7 and CCNE1 levels in serum samples. The assay will be carried out using ELISA kits.

C. Outcome Assessment

Therapeutic outcome will be assessed by health-related quality of life (HRQoL) and measurement of patient-reported outcomes (PROs).PROs comprise various aspects of the subjectively perceived state of health from the patients' point of view such as HRQoL, satisfaction with care, and drug adherence[14-16].

The data collection will be performed in 4 parts. The first part will be focused on patients socioeconomic variables. The second part comprises the FACT-B questionnaire, consisting of 37 questions with responses required on a 5-point Likert scale that constitute 5 dimensions[17-20]. While in the third part of the assessment, patients were asked about preexisting technical skills, their willingness to use PRO, and potential barriers in relation to their health status[21], the fourth part concerned with the patients' evaluation of the PRO tool (manuscript in preparation).

Statistical Analysis/Results

The values will be expressed as mean ± S.D/SEM for parametric data and median (interquartile range) for non parametric data and will be analyzed using statistical package for social sciences (SPSS), version 19.0 software. For non parametric data, Chisquare test will be done to find the association of gene expression and serum levels of biomarkers of response (BoR) with the outcome CDK4/6 inhibitor therapy. Statistical significance will be presumed if a null hypothesis could be rejected at a p value of ≤ 0.05.

Discussion

Biomarkers for response to CDK4/6 inhibitors in breast cancer patients may be helpful in differentiating responders and non-responders. This may open up a personalized therapeutic approach in Ca breast patients. The screening of patients with these markers may be helpful in minimizing the adverse drug reactions and cost of illness as well as may have a role in improving therapeutic outcome.

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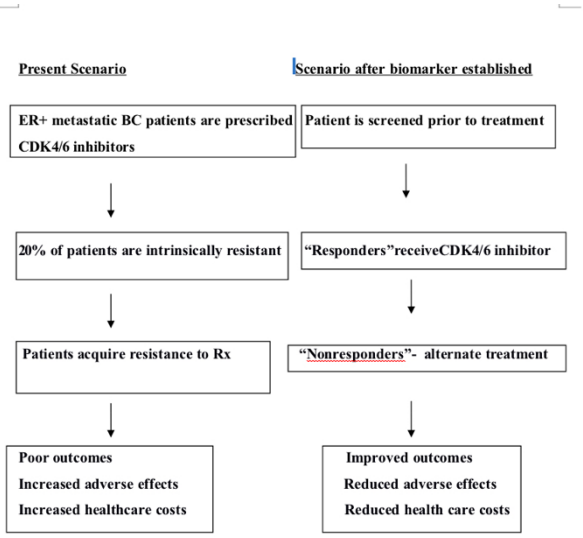
Fig 2:Representation of expected outcome



Conflicts of interest: None

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