A Study Protocol on Assessment of Epidermal Growth Factor Receptor (EGFR) Signalling Pathway as a Predictive Molecular Marker in Triple-Negative Invasive Ductal Carcinoma of Breast

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Authors’ contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Background: Breast cancer is the leading cause of cancer-related deaths worldwide. The Indian scenario is no different for the breast cancer mortality. Breast cancers have occupied the prominence not only for their incidence but the pace too. The researchers have studied numerous pathways vastly and widely in the past two decades to ascertain the prognosis and treatment outcome determinants popularly called predictive markers for breast cancer. One such cell signalling pathway that has raised the hope by assessing success for breast cancer is the Epidermal Growth Factor Receptor (EGFR) signalling pathway for a molecular subtype of Triple-Negative Breast Cancer (TNBC), which is challenging to treat therapeutically. This study aims to assess the EGFR pathway and its sub pathways in Triple-negative breast carcinoma (TNBC) and correlate the abnormalities of expressions in the EGFR pathway in TNBC for its therapeutic implications.

Materials and Methods: This will be an observational cross-sectional study. Forty cases of TNBC will be selected. There will be 10 cases each in 31-40 years, 41-50 years, 51-60 years, and 61 years onwards. Cases will undergo histological diagnosis of invasive ductal carcinoma,
Immunohistochemistry for ER, PR, and Her2-neu, IHC for EGFR, BRAF, Kras, and MAP kinase on histological sections, Polymerase chain reaction (PCR) for Kras. Data will be collected and analyzed using appropriate statistical tests.

**Results:** A significant correlation of EGFR, BRAF, KRAS, and MAP kinase is expected in the pathogenesis of triple-negative invasive ductal carcinoma.

**Conclusion:** The results can be put to the commercial advantage for clinical trials of the new drug development targeting anti-EGFR and other antibodies.

**Keywords:** Invasive ductal carcinoma; triple-negative breast cancer; epidermal growth factor receptor; signaling pathway.

1. **INTRODUCTION**

Breast cancer is the leading cause of cancer-related deaths worldwide. The Indian scenario is no different for breast cancer mortality. Breast cancers have occupied the prominence not only for their incidence but the pace too. It has been overtaken its incidence over a one-time killer disease of cancer of the uterine cervix [1,2].

Irrespective of the country's status as developed, underdeveloped, and developing, incidence of breast cancer is rising as published in the report of Globocan 2018 [1]. India bears a heavy burden of breast cancer sensitively affecting women's health for morbidity and mortality.

Breast cancer of various histopathological subtypes is known to arise in 2 clinical situations; i) as a familial disease with evidence of germline mutations and ii) as a sporadic breast cancer [3]. Irrespective of the clinical logic situation, the one that remains constant and every day is multiple interdependent oncogenic drivers. This means the molecular pathway of cell repair assembly, cell cycle proliferation, apoptotic pathways, oncogenic pathways, and tumor suppressor pathways are at play [4,5].

Clinicians and researchers worldwide are engaged in finding novel ways to diagnose breast cancer in its earliest stage by understanding its molecular genetics and probabilities of therapeutics. The research pursued in recent times has identified one area of cell pathology reasoned for the transformation of a normal cell to a malignant one explored through studying cell signaling pathways [4].

Researchers have studied the numerous pathways vastly and widely in the past two decades to ascertain the prognosis and treatment outcome determinants popularly called predictive markers for breast cancer [6].

One such cell signaling pathway that has raised the hope by its assessment at success for breast cancer is the Epidermal Growth Factor Receptor (EGFR) signaling pathway for a molecular subtype of Triple-Negative Breast Cancer (TNBC), which is the difficult one to treat therapeutically (Fig. 1) [7].

![Fig. 1. The EGFR signaling pathway and therapeutic intercceptions](image)
The track truck developing process for the therapeutic interventions of triple-negative breast cancer has chiefly targeted one or the other active proteins of the Epidermal Growth Factor Receptor signaling pathway [8].

As depicted in Fig. 1, RAS is a shared entry molecule that initiates the proliferation pathways not only of MAP Kinase but also JNK and PI3K pathways. Several oncogenic mutations have been described in the Kras gene, resulting in its constitutive activation and autonomous nonregulated proliferation of the transformed cells and their resistance to apoptosis. Anti EGFR therapies rely on the presence of a wild RAS to be effective since oncogenic RAS transmits proliferative and anti-apoptotic signals independently of EGFR activation. For this reason, the separate assessment of Kras status to know its nature of wild or mutant is primarily essential before administration of the anti-EGFR drug. Therefore, the assessment of Kras status requires to be incorporated separately for the detection of its common mutation at exons 12 and 13 by polymerase chain reaction [9,10].

The understanding of oncsignalingnalling in tumorigenesis has simplified molecular cross talks’ nuances. This is the advantage that has enabled altering the treatment options of breast cancer. The results of such treatments have been sequentially published in breast cancer-related literature. Understanding this pathway has opened a new vista of nanotherapeutics through siRNA in breast cancer which is resistant to chemotherapy, especially in a molecular subtype of TNBC [8,11,12].

Indian medical literature published over the subject lacks definitive observations on this aspect of carcinogenesis, molecular drivers, treatment interventions, and assessments for the molecular subtype of triple-negative breast cancer [13].

Breast cancer marred by the heterogeneity of the tumor cells with regards to the clinical setting of familiar or sporadic cancer for its numerous underlying molecular defects, has one thing, one pathway in common, that is EGFR signaling pathway offering autonomy to the malignant tumor cells, in other words, the immortality. Breast cancer belongs to both the categories of tumors i) familial (through BRCA I and BRCA II) ii) sporadic (through BRAF). The molecular defects in both have co helper pathways that contribute to the cell’s autonomy of proliferation and potential for invasion and metastasis [4].

Her 2/neu (Her2) is an epidermal growth factor which is expressed by one of the molecular subtype of breast cancer plays an important role at decision makings of the treatment. Similarly the expression of ER and PR or all negative expression of breast cancer cells determines the treatment choices or deferment of few treatment protocols. This aspect of molecular subtype of breast cancer have been studied by the researchers but in isolation [14,15,16]. Recent years the pathologic staging of breast cancer has been upgraded by addition of molecular subtype.

Till date, the correlation studies of the abnormalities between EGFR pathway and various molecular subtypes of breast cancer have been sporadic [14-20].

The PRISMA search through identified data bases by four phase flow recovered 3221 articles published world over with the keywords selected from the title for the present post doctoral research work through web search by Google engine in data base of PubMed. It shares that the Indian medical literature publish over the topic is minuscule (12) from year 2013 to 2020.

This prompted to a research gap which requires to be entertained and learnt through undertaking the research over the topic in context to breast cancer scenario in India for prognostic and predictive interventions of triple negative breast cancer especially for the situations from pathologic anatomic staging to pathologic prognostic staging which is revolutionary for the purposes of new treatment protocols of breast cancer.

The articles in recent years published certain interesting data over a work up of epidermal growth factor signalling pathway and triple negative breast cancer to open the new vistas of monoclonal antibody therapy for these patients which otherwise is prognostically in poor category of breast cancer.

Inoue et al. [5], Bk Banin Hirata et al. [6], Nur Izyani et al. [8], Maennling et al. [11], Ogden et al. [13] have observed that the breast cancer stands for long disease free survival if recurrences of it are prohibited through the treatment by monoclonal antibodies against EGFR or other pathway proteins by arresting the mitosis of the tumour cells.
Research gap and Implications of the study:

Presently undertaken study will provide the answers to the following questions in assertive case:

1) The role of Epidermal Growth Factor Receptor cell signalling pathway in oncogenesis of breast cancer.
2) Its relationship with the molecular subtype of triple negative breast cancers.
3) The opening of options of treatment modalities in context to TNBC which are otherwise poorly responsive to established chemotherapy protocols.
4) Create a basal new data for further research and therapeutic interventions for assessment of EGFR signalling pathway for TNBC.

This implicated research gap is related to the primary and secondary objectives negotiated for the present study.

1.1 Research Question

Do the knowledge of status of EGFR signalling pathway by molecular methods in triple negative breast cancers alter the therapeutic treatment plan?

1.2 Aim

The aim of this study is to evaluate Epidermal Growth Factor Receptor signalling pathway in the molecular subtypes of the Triple Negative Invasive Ductal Carcinoma in pursuit of assessment as a predictive marker.

1.3 Objectives

The objectives of this study are as follows

1) To classify Invasive ductal carcinoma (NST) for its molecular subtypes and prognostic pathologic stage.
2) The stratified assessment of Epidermal Growth Factor Receptor signalling pathway with molecular milestones as
   1. Epidermal Growth Factor Receptor (EGFR)
   2. Kirsten Rat Sarcoma viral oncogene homolog (kras)
   3. BRAF( v RAF murin sarcome viral oncogene homolog B)
   4. Mitogen activated Protein Kinase by Immunohistochemistry.

3) To study the kras for its wild or mutant forms by amplification study by polymerase chain reaction in the triple negative breast cancers.
4) The statistical analysis of the abnormalities incurred with Epidermal Growth Factor Receptor signalling pathway in triple negative breast cancers for its suitability to the eligible criteria's of monoclonal anti-EGFR antibody therapy.

2. MATERIALS AND METHODS

The study will be conducted with the following materials and methods:

Data Collection: The details of the patient for their name, age, medical record department details, address, unit, department, consultant in charge and any other details related to the study in study suitable Performa.

Place of Study: Department of Pathology, Jawaharlal Nehru Medical College, Datta Meghe Institute of Medical Sciences deemed to be university, Sawangi (Meghe), Wardha.

Study Design: It is an observational cross sectional study.

Sample Size: The following formula was adopted for calculation of sample size with prevalence rate of breast cancer of 25.8%.
Sample size formula with desired error of margin:

\[ n = \left( \frac{Z_{\alpha/2}^2 \times P \times (1-P)}{d^2} \right) \]

where;

- \( Z_{\alpha/2} \) is the level of significance of \( s \); i.e. 95% confidence interval = 1.96.
- \( P \) = Prevalence = 25.8% / lakh population = 0.058/1000 population = 0.0258
- \( d \) = desired error of margin = 5% = 0.05

\[ n = \left( \frac{1.96^2 \times 0.0258 \times (1-0.0258)}{0.05^2} \right) = 38.62 \]

n = 40 patients needed in the study.

Estimated sample size therefore in the recruitment of 40 patients who have undergone mastectomy indicated for Invasive Ductal Carcinoma. Randomisation of the samples over the variable of age will be given for the study.

**Samples Randomised:** Study population meeting to inclusion criteria are randomised over the age for its distribution as below:

- 10 cases each in the following age groups: 31-40 years, 41-50 years, 51-60 years and 61 years onwards.

**Sample Types:** Retrospective and prospective samples of 40 patients of triple negative breast cancer.

Controls: The mastectomy specimen tissue blocks that show normal terminal duct lobular units at histology (40 cases) for evaluation of epidermal growth factor receptor signalling pathway.

**Study Subject Characteristics:** The following inclusions and exclusions criteria’s were used for inclusions and exclusions for the study.

**Inclusion Criteria:**

i) Female mastectomy specimens

ii) The histopathological diagnosis of Invasive Ductal Carcinoma irrespective of Bloom Richardson Grade and TNM stage.

iii) Specimens reported triple negative (ER (-), PR(-), Her2(-) ) breast cancer on molecular subtype on immunohistochemistry.

iv) The patients who are in follow up for at least 3 months in the tumour board of Acharya Vinoba Bhave Rural Hospital, Sawangi (Meghe), Wardha.

**Exclusion Criteria:**

i) Male breast mastectomy specimens.

ii) Females who have undergone neoadjuvant chemotherapy followed by mastectomy.

iii) The tumour tissue with wide areas of necrosis with small isolated tumour islands which would provide inappropriate results at Immunohistochemistry for EGFR proteins.

**Methods of Mastectomy Specimen Examination:** The specimens received to the Division of Surgical pathology, Department of Pathology will undergo the gross examination of specimen and sectioning as per the protocol of American College of Pathology [21].

**Histopathological Examination of the Mastectomy Specimen and Reporting:** Tissue sections will be processed as usual by histokinette followed by paraffin embedding microtomy and H & E staining by established methods. The histopathological examination of breast specimen for diagnosis, grade and pTNM staging will be performed by standard reporting protocol meant for mastectomy specimen as per the College of American Pathologists [22].

**Laboratory Intervention on Tissue Specimen:** Special investigations over the tumours of mastectomy specimens (study core investigation).

**Molecular Sub Typing of Invasive Ductal Carcinoma by Immunohistochemical Methods as Below:** Major molecular subtypes of breast cancer [23,24].

Molecular classification of Invasive Ductal Carcinoma (Table 1).

**Molecular Methods of EGFR Signalling Pathway Assessment on Tumour Tissue of Triple Negative Invasive Ductal Cancer:** The following proteins will undergo detection by Immunohistochemistry.

i) Epidermal Growth Factor Receptor

ii) Kirsten Rat Sarcoma viral oncogene homolog (Kras)

iii) BRAF( v RAF murin sarcome viral oncogene homolog B)

iv) Mitogen activated Protein Kinase.
Table 1.

| Molecular Subtype | Luminal A | Luminal B | HER2/neu | Basal like<br>^a |
|-------------------|-----------|-----------|----------|------------------|
| Gene expression pattern | Expression of luminal (low molecular weight) cytokeratins, high expression of hormone receptors and related genes | Expression of luminal (low molecular weight) cytokeratins, moderate-low expression of hormone receptors and related genes | High expression of HER2/neu, low expression of ER and related genes | High expression of basal epithelial genes and basal cytokeratins, low expression of ER and related genes, low expression of HER2/neu |
| Clinical and biologic properties | 50% of invasive breast cancer, ER/PR positive, HER2/neu negative | 20% of invasive breast cancer, ER/PR positive, HER2/neu expression variable, higher proliferation than Luminal A, higher histologic grade than Luminal A | 15% of invasive breast cancer, ER/PR negative, HER2/neu positive, high proliferation, diffuse TP53 mutation, high histologic grade and nodal positivity | ~15% of invasive breast cancer, most ER/PR/HER2/neu negative (triple negative), high proliferation, diffuse TP53 mutation, BRCA1 dysfunction (germ line, sporadic) |
| Histologic correlation | Tubular carcinoma, Cribriform carcinoma, Low grade invasive ductal carcinoma, NOS, Classic lobular carcinoma<br>^b | Invasive ductal carcinoma, NOS Micro papillary carcinoma | High grade invasive ductal carcinoma, NOS | High grade invasive ductal carcinoma, NOS Metaplastic carcinoma, Medullary carcinoma |
| Response to treatment and prognosis | Response to endocrine therapy | Response to endocrine therapy (tamoxifene and aromatase inhibitors) not as good as Luminal A | Response to trastuzumab (Herceptin) | No response to endocrine therapy or trastuzumab |
| Variable response to chemotherapy Good prognosis | Variable response to chemotherapy (better than Luminal A) Prognosis not as good as Luminal A | Response to chemotherapy with anthracyclins Usually unfavorable prognosis | Sensitive to platinum group chemotherapy and PARP inhibitors Not all, but usually worse prognosis | |

PARP: poly-adenosinedi phosphate ribose polymerase

^aBasal like tumor group includes a low-grade group with low proliferation but expression of basal type (high molecular weight) cytokeratin and triple negative phenotype (like adenoid cystic carcinoma, secretur carcinoma)

^bClassical lobular carcinoma generally exhibits luminal A properties, while pleomorphic lobular carcinoma usually shows features of other molecular subtypes

The methodology of evaluation and assessment of above proteins by immunohistochemistry will be carried out by simple steps as described below:

i) Paraffin embedded tumour tissue blocks of triple negative breast cancer will be taken.
ii) Sectioning is done at 4µm thickness by the instrument microtome.
iii) Deparaffinisation with xylene will be done.
iv) Rehydration will be done with 100% alcohol, 90% alcohol, 70% alcohol and 50% alcohol.
v) Antigen retrieval in citrate buffer by microwave processing at 60 watts will be carried out.
vi) Endogenous peroxidase blocking is carried out with 3% hydrogen peroxide and methanol for 30 mins.
vii) Incubation with commercially available primary antibody in the dilution suggested by manufacturer.

viii) This is followed by multiple washings for 3 times, 5 minutes each.

ix) Biotin Streptavidin complimentary secondary antibody application will be done.

x) Addition of 3,3’ Diaminobenzidine for colour development will be carried out.

xi) Light microscopic assessment for presence and absence of brown discoulouration to be reported for positive and negative presence of protein of interest.

xii) Scoring.

xiii) Interpretation of the results.

Note: Each tumour tissue block will be cut into four sections independently to be stained for EGFR, RAS, BRAF, MAP Kinase independently in separate section.

Cumulative assessment score to the finding of Immunohistochemistry for the case.

**Molecular methods of PCR for Kras:**

i) DNA extraction from tissue of paraffin embedded section by commercially available kits.

ii) Kras target sequence of interest

iii) Amplification of Kras in detection of abnormalities at exon 12 and 13 by commercially available primers.

iv) The standard amplification technique and steps.

v) Recording the graph and findings.

vi) Interpretation of the results in expression of kras and its abnormalities.

**Statistics:** The statistics of correlation will be carried out in SPSS software for the following parameters.

i) Spearman coefficient

ii) Pearson coefficient of confidence intervals.

iii) Multivariate analysis

iv) Fisher exact test and Chi square test for clinicopathological parameters and molecular subtypes of breast cancers.
3. RESULTS

The expected outcome of the study in a molecular class of triple negative invasive ductal carcinoma as per the objectives set in change of increase expression of EGFR, KRAS and MAP kinase. Other expected outcome is the amplification of KRAS studied by PCR significantly.

The statistical analysis will probably show the relationship of EGFR, BRAF, KRAS and MAP kinase and its role in pathogenesis of triple negative invasive ductal carcinoma.

4. DISCUSSION

A number of studies related to Breast carcinoma were reviewed [25-31]. The results of the study will be compared with the following major studies carried out on this topic as below:

Zakaria et al. [15] conducted a study for EGFR expression in the triple negative breast cancers. The study was conducted to determine the possibility of using anti EGFR combinational therapy for the prophylaxis of triple negative breast cancer patients. The study comprised of 58 cases of breast cancer whose immunohistochemistry revealed negative staining for ER, PR and Her2. The age range of patients was from 26 to 89 years and all belong to female gender. The tumour grade of these triple negative breast cancers were as follows: Grade 1 (4 cases), Grade 2 (19 cases), Grade 3 (35 cases). Of the 58 cases, 18 specimens revealed lymph node metastasis while 17 had lymph nodes but were negative for metastasis. 23 cases did not reveal the lymph nodes in their specimen. The anatomical stage of these 58 cases of triple negative breast cancers had a following distribution: T1 (9 cases), T2 (37 cases), T3(11 cases) and T4 (1 case). The assay for detection of EGFR protein over expression was observed in all 58 samples (100%). The correlation between EGFR protein expression was of low gene copy in 49 cases, high in 6 cases and amplified in 3 cases. The authors observed a positive correlation between EGFR protein expression to be low in 48 samples and high in 10 samples. The study concluded that the demonstration of EGFR alteration and expression in triple negative breast cancers provide a treatment alternative for anti EGFR antibody therapy for triple negative breast cancer cases.

Sanchez- Munoz et al. [9] carried out a study to know oncogenic mutations in kras in triple negative breast cancers. The study was conducted to evaluate recently known and establish research finding that mutational analysis of kras is complementary in vitro diagnostic tool for the identification of patients with colorectal carcinoma who will not benefit from anti epidermal growth factor receptor (EGFR therapies). The study comprised of 35 patients whose 35 formalin fixed paraffin embedded triple negative breast tumour samples were obtained for DNA studies by Real time polymerase chain reaction using primers specific for the detection of wild type kras and mutated kras (exon 12 and 13).

Prior to the inclusions of patient to the study, immunohistochemistry of ER, PR and Her 2 were carried out. The sections of the tissue of triple negative breast cancers were submitted for DNA extraction carried out by commercially available DNA extraction kits. The usual steps of PCR were followed to know the results of amplification of wild and mutated kras. The results of PCR showed that no evidence of kras oncogenic mutation is constantly associated with triple negative breast cancer tumour tissue. The study concluded that kras mutation are very infrequent in triple negative breast tumours and EGFR inhibitors may be of potential benefit in the treatment of basal like breast tumours which express EGFR.

Hoadley et al. [16-18] studied EGFR associated expression profile of breast tumour subtypes based on immunohistochemistry. EGFR associated signatures were evaluated in 241 primary breast tumors. The authors concluded that basal like cell lines of breast carcinomas are sensitive to EGFR inhibitors and drugs like carboplatin. Therefore, the assessment of EGFR, RAS, BRAF, MAP Kinase is of prognostic value in tumour subtype of triple negative breast cancers [32-45].

5. CONCLUSION AND TRANSLATORY COMPONENT

The results of the present study will be translated for following:

i) It will segregate a class of TNBCs which is prognostically poor but can undergo alternative therapy of anti EGFR monoclonal antibody therapy.
i) The results can be put to the commercial advantage for clinical trials of the new drug development targeting anti-EGFR and other antibodies.

ii) It will create the database for Indian cancer registry which will be shared for further research on cell-signalling pathway and applied treatment modality.

CONSENT

The consents for the histopathological examination of surgically resected specimens is informal. These specimens are routinely received to the Department of Pathology (post surgery). Still the guidelines for the consent from template for use of samples in the study as laid down by WHO were followed [25].

ETHICAL APPROVAL

The present study does not involve any conflicts or the human or animal ethics that would cause physical, mental or any form of injury.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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