

## HISTOLOGICAL AND IMMUNOHISTOLOGICAL CHANGES DURING THE EXPRESSION OF FOXO3A IN HUMAN BREAST CANCER TISSUES

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### **Abstract**

**Background & Objectives:** Breast cancer is an aggressive cancer in females, worldwide and is the major cancer among Indian women. The cause of breast cancer includes environmental, genetic and hormonal factors. The present study is to analyse the expression of FOXO3a in Infiltrating Ductal carcinomas of breast cancer patients and corresponding normal tissues.

**Materials & methods:** Formalin fixed paraffin embedded tissues of breast cancer and corresponding normal tissues of women, were included in this study. Expression of FOXO3a was analysed by IHC in relation to histology.

**Results:** In Immunohistochemistry study FOXO3a was found to be expressed in breast cancer and its expression correlated with clinical and pathological parameters.

**Conclusions:** FOXO3a expression showed a significant alteration in breast cancer patients.

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## INTRODUCTION

Breast cancer is one of the most common cancers among the women population and it is second leading cause of cancer death. It is estimated that one in eight women will develop breast cancer during her lifetime.<sup>[1]</sup> Worldwide, it is estimated that 1.4 million women are diagnosed with the disease per year, which accounts for about 23% of all cancers. Around 5,00,000 breast cancer related deaths are reported annually.<sup>[2]</sup> According to National Cancer Registry Programme, the number of breast cancer deaths in India will increase to 1,06,124 in 2015 and to 1,23,634 in 2020.<sup>[3]</sup> Present trend is that one in four women will develop breast cancer during her life-time. It is more common in Indian women and the average age (< 30 years) of developing a cancer underwent a significant change over last few decades.

Breast cancer is a heterogeneous disease, caused by multiple risk factors like hereditary factors and carcinogenic elements, shorter duration of breast feeding, obesity, early menarche, late menopause, use of oral contraceptives, hormone replacement therapy and family history.<sup>[4-10]</sup> More than 95% of breast cancers arise from the breast epithelial cells. The histological diversity of adenocarcinoma of the breast has long been fascination to pathologists, who have identified specific morphological and cytological patterns that were consistently associated with distinctive clinical features and outcomes. These patterns are called 'histological types' that are classified into different subgroups according to microscopic appearance and histological grade.<sup>[11]</sup> The commonest types of breast carcinoma are in situ carcinomas and invasive (sometimes called infiltrating carcinomas).<sup>[12]</sup> The gold standard method in routine use is the TNM staging system.<sup>[13]</sup> One of the most useful indices of prognosis of breast cancer is the Nottingham Prognostic Index (NPI).<sup>[14]</sup> The tumour markers are the routinely

employed tool to evaluate the breast cancer based on the levels of estrogen, progesterone and HER/neu. The most commonly employed technique at present, to evaluate the hormone receptor status of breast tumours, is immunohistochemistry (IHC) which relies on the recognition of the receptor protein by specific antibodies.

Breast cancer heterogeneity comprises inter and intra tumours. Inter tumor heterogeneity is recognized by histopathologists who have their microscopic observations, of identified 17 different histological subtypes with different clinical behavior.<sup>[15]</sup> Intra-tumor heterogeneity has long been observed by pathologists as an area with different morphology and staining patterns within a tumor sample.<sup>[16]</sup> Increased understanding of the molecular heterogeneity that is intrinsic to the various subtypes of breast cancer will likely to shape the future of breast cancer prognosis, diagnosis and treatment. Two recent advances in the breast cancer research field have led to paradigm shift: first, the identification of intrinsic breast tumor subtypes, second, the recent characterization of cancer stem cells (CSCs), which are suspected to be responsible for tumor initiation, recurrence and resistance to therapy.<sup>[17]</sup> There are over 150 expression genes that have been correlated with breast cancer development and progression. However, as gene expression studies evolve, further sub classification of breast tumors into new molecular entities is expected to occur. In 2007, a new molecular subtype was identified and referred to as claudin-low, using 13 samples.<sup>[18]</sup>

Forkhead box O (FOXO) transcription factors are involved in multiple signaling pathways that play critical roles in a number of physiological and pathological processes of diseases including that of cancer. The importance of involvement of FOXO factors in the regulation of phosphorylation, acetylation, ubiquitination and protein-protein interactions has been already

established. FOXOs are actively involved in promoting apoptosis in a mitochondria-independent and dependent manner by inducing the expression of death receptor ligands, including Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand, and Bcl-2 family members, such as Bim, bNIP3 and Bcl-XL, respectively. FOXO1, FOXO3a, FOXO4, FOXO6 are the members of FOXO subfamily. In proliferating cells, the transcriptional activity of FOXO1, FOXO3a, and FOXO4 is under the control of signalling pathways initiated by the growth factors, such as insulin and insulin-like growth factor 1 (IGF-1), which culminate in the phosphorylation of FOXOs.<sup>[19]</sup> Although FOXO transcription factors are known to be regulated by oxidative stress and serum deprivation, their role in modulating cellular responses to such stresses is incompletely understood.<sup>[20]</sup> Down-regulation of FOXO3a activity is often seen in cancers and ERK- or inhibitor kappa B kinase (I $\kappa$ B)-mediated inhibition of FOXO3a has been shown to promote tumorigenesis.<sup>[21]</sup> This evidence highlights FOXO3a as a potentially important target by cytotoxic drugs, especially in receptor-negative cells. An understanding of FOXO proteins and their biology would provide new opportunities for developing more effective therapeutic approaches to treat cancer. The objective of the present study is to investigate the histological and immunohistological expression pattern of FOXO3a gene in human breast cancer tissue.

## **MATERIALS AND METHODS:**

### **SAMPLE COLLECTION**

Formalin- fixed paraffin-embedded (FFPE) tissues of infiltrating ductal breast carcinoma and the corresponding normal tissues of women, were collected from the database of Department of Pathology, Government Mohan Kumaramangalam Medical College & Hospital (GMKMCH) in Salem, Tamil Nadu. This study was approved by Clinical Research Ethics

Committee (Ref. No. 569MEI/2011) of GMKMCH, Salem.

### **HISTOLOGY OF BREAST TISSUES**

Histological examinations were done for both breast cancer and normal tissues using routine Haematoxylin and Eosin (H&E) stains.<sup>[22]</sup> Briefly, tissues were hydrated, then dehydrated in graded alcohol series (50%, 70%, 90% and 100%), briefly cleared in chloroform, xylene and then embedded in paraffin wax. Tissues were sectioned at 5  $\mu$ m thickness using Rotary microtome and kept at 37 °C overnight. Then the sections were deparaffinized, rehydrated through descending alcohol series (100%, 90%, 70% & 50%) followed by distilled water. These sections were stained with Haematoxylin and Eosin and then rapidly carried through ascending alcohol series (50%, 70%, 90% & 100%). The sections were cleared in three changes of xylene and mounted in DPX. Finally, the sections were observed under light microscope with appropriate settings and photographed (Fig: 1).

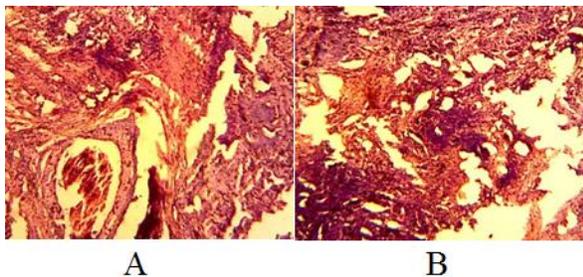
### **IMMUNOHISTOCHEMICAL ANALYSIS**

Immunohistochemical analysis was carried out through standard procedure using the DAB universal staining kit (Merck Genie, Bengaluru, India).<sup>[23]</sup> The sections were deparaffinized in xylene and dehydrated in ethanol. After washing with PBS, slides were incubated with 3% H<sub>2</sub>O<sub>2</sub> at room temperature for 15 min to quench endogenous peroxidase activity. Antigen retrieval was done by incubating at 90 °C for 15 min in 10 mM citrate buffer, pH 6.0. The slides were incubated with blocking solution (10% normal goat serum) for 5 min at room temperature. Then, the sections were incubated overnight with primary antibody (FOXO3a (D19A7), Cell Signalling Technology). Subsequently, the sections were incubated with HRP secondary link antibody for 30 min at room temperature, washed with PBS, treated with secondary antibody for 30 min at 37 °C and washed. Then, the sections were treated with DAB chromogen for 15 min. Finally,

the sections were washed with deionised water, counter stained with haematoxylin and mounted. Photographs were taken using Nikon microscope (Fig: 2).

## RESULTS & DISCUSSION: HISTOLOGY OF BREAST

Breast composed of epithelial and stromal components. The ductolobular system is composed of a dual layer of epithelia resting on the basement membrane and enveloped by stroma. A variety of benign and metaplastic changes were seen in luminal and myoepithelial cells. In the present study histology shows that no vascular or lymphatic lesions seen in normal histology, and hence no evidence of tumour infiltration (Figure 1). The normal breast tissue have peripheral layer of monolayer epithelial cells with basement membrane.



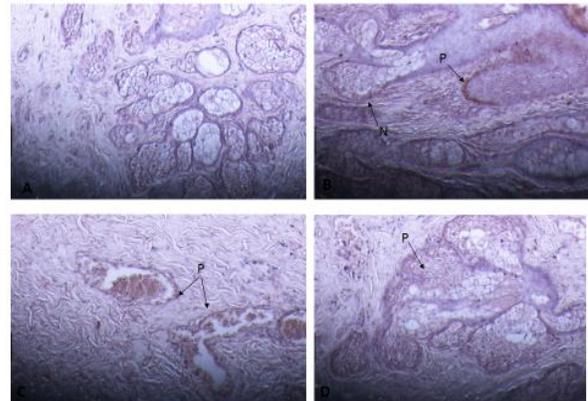
**Fig: 1** Histology of FOXO3a in breast and Normal Tissues

Haematoxylin and Eosin stains of Human breast cancer tissue and corresponding normal tissue (40X magnification).

## IMMUNOHISTOCHEMISTRY

In Immunohistochemistry study FOXO3a was found to be overexpressed in breast cancer and its overexpression was correlated with clinical and pathological parameters. One of the clinical samples shows distraction of intralobular but did not show any FOXO3a gene expression (Figure 2a) FOXO3a gene expression was found in the regions of stroma (Figure 2b), intralobular (Figure 2c) and extralobular duct system (Figure 2d). The Immunohistochemical analysis shows an outer layer of myoepithelial cells but the inner

cuboidal to columnar epithelial cells are not reactive for this antigen.



**Fig: 2** Immunohistochemistry of FOXO3a

Immunohistochemical analysis of FOXO3a expression in breast cancer tissue A. No protein expression is observed in the normal luminal epithelial cells as well as in stromal cells surrounding but distraction of intralobular B. FOXO3a gene expressed in the region of stroma C. intralobular and D. extralobular duct system. P-Positive, N-Negative.

## DISCUSSION:

Breast cancer is a genetically and clinically diverse disease. [24] In order to bring together this heterogeneity, breast cancer classification systems have been developed. This classification scheme developed after many decades of experiments, is now used as an important tool for prognosis and treatment. The normal breast tissue composed of three cell types which express different subsets of proteins like luminal, basal and myoepithelial. The most commonly employed technique at present, to evaluate the clinical diagnosis and receptor status of breast tumors, is immunohistochemistry (IHC) which relies on recognition of the receptor protein by specific antibodies. Although technically easy to perform and cost effective, this method is subjective and time consuming.

For routine histological diagnosis of surgically removed tumors, formalin-fixed paraffin-embedded (FFPE) tissue is normally used and thus a wide range of FFPE tumor

samples are available for gene expression analytical study. The importance of gene expression profiling technology to FFPE tissues so as to analyse the breast epithelial and stromal compartments in the context of tumour progressions is increasingly felt. However, gene expression profiling of archival FFPE samples remains extremely challenging.<sup>[25]</sup> The fixation and embedding have a detrimental effect on nucleic acids, resulting in fragmentation and chemical modifications and so making it of less use for expression analysis.<sup>[26]</sup>

FOXO3a are family of fork-head transcriptional factors characterized by a distinct DNA-binding domain. Members of FOXO subfamily, regulate PI3/AKT pathway.<sup>[27]</sup> FOXO family are functionally active and involved in a various biological processes.<sup>[28]</sup> Gene expression analysis of tumor samples are generally performed on whole tumour homogenates and thereby represent a pattern that reflects the expression from all cell types present in the tumour. The tumour microenvironment, comprising a large variety of cells, such as fibroblasts, immune cells, and endothelial cells, can constitute a significant part of the tumor and substantially contribute to observed expression patterns.

Immunohistochemistry (IHC) is considered to be an important tool in the modern pathology of breast disease study. Immunohistochemical markers are useful for estimating prognosis of cancer and predicting therapy response. IHC was performed on paraffin wax embedded sections from patients with infiltrating type of breast cancer to identify the expression of FOXO3a. Immunohistochemistry is used to characterize intracellular proteins of various cell surfaces in all tissues. Most infiltrating ductal carcinoma are readily distinguished on hematoxylin and eosin stained sections. The dynamic interaction between the epithelium and stroma plays an important role in governing the behaviour of a

tumour.<sup>[29]</sup> During the progression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) both epithelial and stromal compartments are played major role and the majority of expression changes during this progression occurred within the epithelial cell compartments with relatively little changes occurring in the stroma.<sup>[30]</sup>

#### CONCLUSION:

In the present study, FOXO3a are prominently expressed in stroma, inter and intra lobular region of clinical isolates of breast cancer tissues. The expression of genes was up regulated in stroma of cancer patients corresponding to normal stroma. In extra lobular duct underwent more gene expression changes during progression. Gene expression changes in the cancer-associated stroma have proven to be important in predicting clinical outcome of breast cancer patients.<sup>[31]</sup> Hence, it is best approach to adopt the use of Immunohistochemical markers coupled with standard hematoxylin-eosin histology for the prognosis of cancers.

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