RESEARCH ARTICLE

Role of Nuclear Factor-κB in female Breast Cancer: A Study in Indian Patients

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Abstract

Introduction: The nuclear factor κB (NF-κB) is a super family of transcription factors which plays important roles in development and progression of cancer. The present investigation concerns NF-κB /p65 activity in human breast cancers with overexpression of ER, PR, HER-2/neu, as well as the significance of p65 expression with regard to menopausal status, stage, grade, tumor size, nodal status, and NPI of invasive ductal carcinomas in Eastern India. Materials and Methods: In this hospital based study 57 breast cancer patients attending a Breast Clinic of a reputed institute of Eastern India were assessed for p65 protein expression in breast tumor tissue samples by Western blotting. ER, PR and HER-2/neu expression was determined by immunohistochemistry. Results: NF-κB/p65 was significantly associated with advanced stage, large tumor size (≥5 cm), high grade, negative ER, negative PR, and positive HER-2/neu. High NF-κB/p65 expression was more frequent in patients with a high NPI (NPI ≥ 5.4, 84.6%) compared with low NPI (<5.4, 44.4%) and this association was statistically significant (p = 0.002). Conclusion: NF-κB/p65 overexpression was associated with advanced stage, large tumor size, high grade, and high NPI which are poor prognostic factors linked to enhanced aggressiveness of the disease. NF-κB/p65 expression implies aggressive biological behavior of breast cancer and this study validates significant association of NF-κB /p65 overexpression with negative estrogen and progesterone receptor status and overexpression of HER-2/neu oncoprotein. In our good clinical practice, patients with NF-κB positive tumors need to be treated aggressively.

Keywords: Breast cancer - nuclear factor-κB - prognostic marker - Nottingham Prognostic Index - Eastern India

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Introduction

Breast cancer (BC) is the second most common leading female cancer (after cervical cancer) in the world as well as and in India (Ferlay et al., 2000; Ferlay et al., 2008; Debarshi Jana et al., 2012). In eastern India, BC is the most frequently reported cancer (22.7%) in females and the age-specific incidence rate is 25.1 per 100,000 populations (Sen et al., 2002).Breast cancer survival depends on various molecular factors. It becomes important thus, to know about the nature of the disease, so as to ensure optimum adjuvant therapy and predict the course of outcome (Sarkar et al., 2009). Several studies have reported that there are various prognostic markers in survival of BC (Borg et al., 1990; Wülfing et al., 2006; Tovey et al., 2009; Taneja et al., 2010). The nuclear factor-κB (NF-κB) activation of genes are associated with cell proliferation, angiogenesis, metastasis, oncogenesis , survival of BC(Wu et al., 2005; Sethi et al., 2009).The NF-κB/REL family of transcription factors is comprised of a RELA/p65,c-REL,RELB, p105/NF-κB1 and p100/NF-κB2 (Chen et al., 2004).The members of this family are characterized by the presence of a REL homology domain (RHD) in the N-terminus, which is involved in sequence-specific DNA binding and translocation. The C-terminal regions of these proteins have domains responsible for either transcriptional activation (RELA, c-REL and RELB) or the inhibition of REL protein activity (p105 and p100).The p105 and p100 proteins can be processed by proteolytic cleavage into p50 and p52, respectively. These proteins have Glycine rich regions (GRRs) which are important for this processing. The REL family members are capable of forming different combinations of heterodimers and homodimers, the most common being the p65/p50 heterodimer which is often referred to as the NF-κB complex (Senthil Radhakrishnan et al., 2006). NF-κB regulates over 500 genes involved in cellular transformation, survival, proliferation, invasion,

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angiogenesis, metastasis, and inflammation, the NF-κB signaling pathway has become a potential target of therapeutic strategy (Subash Gupta et al., 2010).

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 tumorgenesis and Inflammation and metastatic growth as well as chemo resistance and radio resistance (Florian Greten et al., 2004). 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 (Van Laere et al., 2007). 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.

Activation of the progesterone receptor (PR) can lead to inhibition of NF-κB driven gene expression (Kalkhoven et al., 1996), reducing its DNA binding and transcriptional activity. HER-2 activates NF-κB through the canonical pathway which surprisingly, involves IKKα (Merkhofer et al., 2010). 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. Nottingham Prognostic Index (NPI) is a good prognostic maker as well as survival marker in clinical practice. 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 (Gautam Sethi et al., 2008). There are various prognostic markers in breast cancer with variable sensitivity and specificity. We have to find any association NF-κB/p65 expression with clinical parameters such as menopausal status, stage of the disease, tumor size, grade, lymph node metastasis, NPI, ER, PR, HER-2 /neu. This study aims to validate the role of activation of NF-κB/p65 as a prognostic marker in patients with breast cancer in Indian subcontinent.

Materials and Methods

Patient selection

The patients were divided into two groups, first group (Group-A) comprised of 57 female patients with invasive ductal carcinoma (IDC) previous untreated by chemotherapy, radiotherapy, hormone therapy or a combination of any of the modalities who presented to the Comprehensive Breast Clinic Service and Breast Cancer Research Unit, IPGME and R/SSKM Hospital, Kolkata, West Bengal, India between 2008 and 2011 were included in this research work. 23 female patients who were histological and clinically fibro adenoma or benign breast disease treated as control group (Group-B).

Tissue Processing

The specimens were washed with phosphate buffered saline (PBS), cut into small pieces and immersed in collagenase at 37°C for 4-6 h. Collagenase incubated tissue was minced and treated with 0.125% trypsin-EDTA for 10 min. Total protein was extracted by homogenizing cells in ripa : lysis buffer mixture (1:3) at 4°C and measured spectrophotometrically by Lowry's method.

Western Blot analysis

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

Histology and Immunohistochemistry

Breast carcinoma tumors were fixed in 10% neutral-buffered formalin for 24 h, measured the tumor size, nodal status, grade and embedded in paraffin, and sectioned. For immunohistochemistry, paraffin sections of tumors were 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 mM KCl). Antigen retrieval was accomplished with diluted antigen retrieval buffer (DAKO Corp.) Endogenous peroxidase was blocked with 3% hydrogen peroxide. Subsequently, slides were 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 incubated overnight at 4°C. The slides were then incubated with biotinylated secondary antibody for 45 min, followed by ABC reagent and diaminobenzidine.

Counterstaining was done with hematoxylin. Sections were dehydrated by washing sequentially with 95% ethanol, 100% ethanol, and xylene. Coverslips were mounted on slides using Paramount. Digital images of stained and unstained cells were obtained using an Olympus microscope equipped with a SPOT digital camera (Debarshi Jana et al., 2012).

Statistical analysis

Categorical variables are expressed as Number of patients and percentage of patients and compared across the 2 groups using Pearson's Chi Square test for Independence of Attributes. Continuous variables are expressed as Mean ± Standard Deviation and compared across the 2 groups using unpaired t test. The statistical software SPSS version 16 has been used for the analysis. An alpha level of 5% has been taken, i.e. if any p value is less than 0.05 it has been considered as significant.

Results

NF-κB/p65 was not activated in control group by western blot technique. The relationship between NF-κB expression and clinicopathological parameters was shown in table-1. The relationship between NF-κB and immunohistochemistry parameters was shown in table-2. As per table-1, there was equal NF-κB expression of premenopausal and postmenopausal status (p = 0.973). According to clinical stage, 7 (35.0%) patients in stage I, 2(50.0%) patients in stage II, 6 (100.0%) patients in stage III and 26(96.3%) patients in stage IV were NF-κB positive tumors. The significant association was found between NF-κB and stage of the disease (p < 0.001).

The histological grades were measured by Modified Bloom-Richardson Grading Scheme. In grade I out of 8 patients 3(37.5%), in grade II out 11 patients 5(45.5%), grade III out of 38 patients 33(86.9%) were NF-κB positive and this was statistically significant (p = 0.002).

High NF-κB activation was associated with size of the tumor, being more frequently observed in large (≥5 cm) tumors (89.3%) than small (<2 cm) tumors (42.9%), and this association was statistical significance (p = 0.012).

Patients with lymph node negative tumors 4(50.0%), 1-3 lymph nodes 24(72.7%), 4-9 lymph nodes 11(84.6%) and >9 lymph nodes metastasis 2(66.7%) were found with NF-κB positive tumors (p=0.393).

High NF-κB activation was found in patients with a high NPI (NPI ≥ 5.4) 33 (84.6%), compared with low NPI (<5.4) 8 (44.4%) and this association was statistically significant (p = 0.002). NPI = tumor size x 0.2 + lymph node stage (1=no node, 2=1 to 3 nodes positive, 3=4 or more nodes positive) + grade (1, 2 or 3).

As per Table-2, NF-κB activation was more common in ER-negative tumors (81.8%) than ER-positive tumors (38.5%) and this difference was statistically significant (p = 0.002). NF-κB expression was more common in PR-negative tumors (82.2%) than PR-positive tumors (33.3%) and this difference was statistically significant (p < 0.001). Statistically significant association was found between NF-κB and HER-2/neu expression (p < 0.001). NF-κB activation was more frequent in HER-2/neu positive tumors (96.3%) compared with HER-2/neu negative tumors (35.0%).

Table 1. Clinicopathological Details According to NF-κB/ p65 Status

		NF-κB/p65				
		Ve -	%	Ve +	%	p- value
Menopausal Status	Postmenopausal	11	28.2	28	71.8	0.973
	Premenopausal	5	27.8	13	72.2	
Stage	I	13	65	7	35	<0.001*
	II	2	50	2	50	
	III	0	0	6	100	
	IV	1	3.7	26	96.3	
Grade	I	5	62.5	3	37.5	0.002*
	II	6	54.5	5	45.5	
	III	5	13.1	33	86.9	
Tumor Size (cm)	<2	4	57.1	3	42.9	0.012*
	2 – 4.99	9	40.9	13	59.1	
	≥ 5	3	10.7	25	89.3	
Nodal status	No Node	4	50	4	50	0.393
	1 – 3 Node	9	27.3	24	72.7	
	4 – 9 Node	2	15.4	11	84.6	
	>9 node	1	33.3	2	66.7	
NPI	< 5.4	10	55.6	8	44.4	0.002*
	≥ 5.4	6	15.4	33	84.6	

*statistically significant

Table 2. Immunohistochemistry Parameters According to NF-κB/p65 Status

		NF-κB/p65				
		Ve -	%	Ve +	%	p- value
ER	Negative	8	18.2	36	81.8	0.002*
	Positive	8	61.5	5	38.5	
PR	Negative	8	17.8	37	82.2	0.001*
	Positive	8	66.7	4	33.3	
HER-2/neu	Negative	15	50.0	15	50.0	<0.001*
	Positive	1	3.7	26	96.3	

*Statistically Significant

Figure-1A, Figure-1B and Figure-1C illustrate the ER, PR and HER-2/neu positive staining by immunohistochemistry method.

Figure -2A represents that p65 was more overexpressed with ER negative tumor with compare that ER positive tumor by western blot method. Figure-2B shows that p65 was more overexpressed in Grade III with compare that Grade II and Grade I by western blot method. Figure-2C represents that p65 was more overexpressed with HER-2/neu positive tumor with compare that HER-2/neu negative tumor by western blot method.

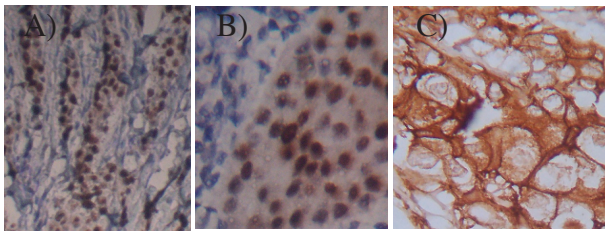

Figure 1. A) Strong ER Expression; B) Strong PR Expression; C) HER-2/neu Strong Staining

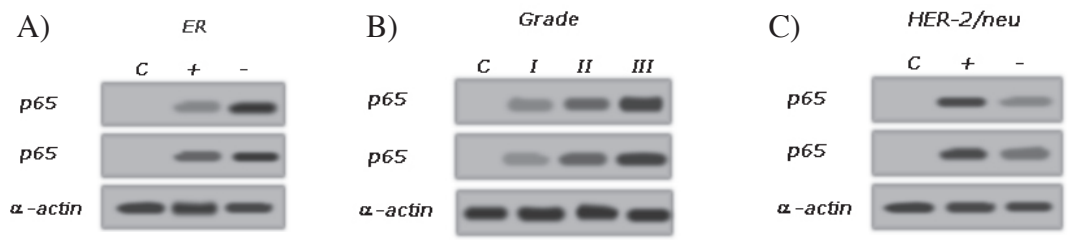

Figure 2. NF-κB/p65 Expression by Western Blotting. A) With reference to ER expression; B) With reference to grade; C) With reference to HER-2/neu

Discussion

In our study NF-κB/p65 (71.9%) was activated in case of human invasive ductal breast carcinoma analysed by western blotting, where as NF-κB was undetectable in control group patients breast tissue. In the present study, activation of NF-κB was significantly correlated with advance stage, high grade, large tumor size, high NPI value, ER negativity, PR negativity and HER-2/neu positivity in breast cancer patients. C.Montagut et al (Montagut et al., 2006) found that there is no significant correlation was found between cytoplasmic or nuclear NF-κB expression and clinical pathological characteristics including tumour size, nodal status, grade, histological type, ER and HER-2/neu but activation of p65 is linked to resistance to neoadjuvant chemotherapy. We found that NF-κB activation is dependent on stage, grade, tumor size, NPI, lymph node involvement. We observed an inverse relationship between NF-κB and ER expression which was poor prognostic outcome. Zhou et al (2005) demonstrated that activation of NF-κB identifies a high-risk subset of hormone-dependent breast cancers. Biswas et.al (2000) suggested that HER-2/neu induced NF-κB activation: a major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. So NF-κB controls cell-cycle progression by modulating action of cell-cycle regulatory genes. Low level of ER and PR are activated the NF-κB (Ali et al., 2002). Nakshatri et al (1997) showed that Constitutive Activation of NF-κB during Progression of BC to Hormone-Independent Growth. NF-κB activation is linked to loss of ER expression and activation in IBC and in breast cancer in general. The inverse correlation between NF-κB activation and ER activation is due to EGFR and/or ErbB2 overexpression, resulting in NF-κB activation and ER down regulation (Van Laere et al., 2007). The clinical significance was found between activation of NF-κB transcription factor and overexpression of HER-2/neu oncoprotein (Ming-Feng Houa et al., 2003). Donald Earl Henson et al demonstrated that breast cancer survival depends on some prognostic factor such as stage of the disease, histological grade, tumor size and nodal status (Carter et al., 1989; Henson et al., 1991). In this study also prognosis is dependent on stage, grade, tumor size, NPI, lymph node involvement. Both ER and PR were associated with better prognosis replicating western results. Almasri et al (2005) suggested that HER-2 overexpression was associated with young age presentation, larger tumor size, more auxiliary lymph node metastases and was inversely related to ER and PR expression. Overexpression of HER-2/neu was strong prognostic and predictive marker in survival (Cooke et al., 2001; Yamashita et al., 2004). Merkhofer et.al (2010) suggested that Activation of NF-κB and PI3K pathways downstream of HER-2, leading to changes in invasion and proliferation of breast cancer cell. This study also demononstrated that IKKα has a larger role than IKKβ in activation of NF-κB in HER-2 breast cancer cell, including the phosphorylation of the p65 subunit at serine 536 (Kalkhoven et al., 1996). So activation of NF-κB is the major role of metastasis via IKKα and HER-2/neu activation. Formation of new blood vessels is essential for tumor progression, as the growing tumor mass quickly exceeds the capacity of the native blood supply. Many of the signals that orchestrate angiogenesis are elaborated by tumor-associated macrophages (TAMs), most of which dependent on NF-κB are signaling (Balkwill et al., 2001). NF-κB stimulates proliferation and blocks programmed cell death (apoptosis) in different cell types, including human breast cancers (Nakshatri et al., 1997; Karin et al., 2002; Biswas et al., 2003). Various study reported that activated NF-κB is detected in ER-negative human breast cancer cells harboring overexpressed ErbB1 (Biswas et al., 2000; 2001; 2003). Activation of NF-κB is the major role in cell proliferation and apoptosis to use an ER-negative and ErbB2-positive expression (Nakshatri et al., 1997). In our study for large tumor size (≥5 cm) was poor prognosis and larger proportion of NF-κB positivity had been observed. There was strong association between HER-2/neu positive NF-κB activation. For NF-κB positive tumor showed higher number of lymph node metastasis.

Albergaria et al found that NPI is good predictor of survival tool in breast cancer (Almasri et al., 2005). NPI is a reliable index to predict overall survival of breast cancer patients over five years. Low NPI (< 5.4) is associated with good prognosis (about 70% survival over 10 years) while (NPI ≥ 5.4) has less than 50% ten year survival rate. There is a positive correlation of NPI with NF-κB expression, again replicating western data. NF-κB positivity had been observed in advance stage like Stage-III and Stage-IV. Activation of NF-κB was inversely related with ER and PR negative tumor. NF-κB expression was directly related with HER-2/neu positive tumor. This implies that NF-κB is associated with aggressive tumour behaviour such as large tumour size, high grade and poor differentiation.

In conclusion, NF-κB over expression implies aggressive tumour biology in breast cancer and it can predict tumours likely to have poor prognosis. Patients with NF-κB positive tumours need to be treated aggressively. We detected a positive correlation between NF-κB and HER-2/neu expression. NF-κB expression is directly correlated with ER negative and also associated with higher NPI value which is poor prognostic outcome. In conclusion these data support the oncogenic role of NF-κB in invasive ductal breast carcinoma and highlight its correlation with higher NPI value which is poor outcome for the patients. We also conclude that inhibition NF-κB overexpression may decrease tumour progression in patients and may block breast carcinogenesis, reducing the incidence of breast carcinoma in patients at high risk. To evaluate this fact further study with large sample size is to be contemplated.

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