Role of NF- κ B as a Prognostic Marker in Breast Cancer : A Pilot Study in Indian Patients

D. K. Sarkar · Debarshi Jana · P. S. Patil ·
K. S. Chaudhari · B. K. Chattopadhyay ·
B. R. Chikkala · S. Mandal · P. Chowdhary

Received: 24 August 2012 / Accepted: 19 March 2013 / Published online: 25 April 2013
© Indian Association of Surgical Oncology 2013

Abstract The nuclear factor κ B (NF- κ B) is a superfamily of transcription factors. It plays an important role in development & progression of cancer. This study was conducted in a tertiary care centre to investigate the significance of NF- κ B as a prognostic marker in breast cancer and study its relation with established prognostic markers such as tumor grade, lymph node status, hormone receptor & HER-2/neu expression. We measured NF- κ B expression of breast cancer tissue as a test sample & from fibroadenoma as a control. Measurement was done by Western Blot Technique using p65 protein of NF- κ B super family of transcription factors. ER,PR and HER-2/neu were measured by immunohistochemistry methods. NF- κ B/p65 is significantly associated with large tumor size (≥ 5 cm), high grade tumors, negative ER, negative PR, positive HER-2/neu and high NPI (≥ 5.4) scores. NF- κ B/p65 expression implies aggressive biological behaviour of breast cancer & this study validates significant association of NF- κ B /p65 overexpression with large tumor size, negative estrogen & progesterone receptor status and overexpression of c-erbB2 oncoprotein.

Keywords Breast cancer · Nuclear factor- κ B (NF- κ B) · NF- κ B /p65 overexpression · Prognostic marker · Nottingham Prognostic Index (NPI) · Immunohistochemistry (IHC)

Introduction

The nuclear factor- κ B (NF- κ B)/REL family of transcription factors is comprised of a RELA/p65,c-REL,RELB, p105/NF- κ B1 and p100/NF- κ B2 [1]. 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

D. K. Sarkar · D. Jana · P. S. Patil · K. S. Chaudhari ·
B. K. Chattopadhyay · B. R. Chikkala · P. Chowdhary
Comprehensive Breast Clinic Service & Breast Cancer Research
Unit, IPGME&R & SSKM Hospital, Kolkata, West Bengal, India

S. Mandal
Department of Epidemiology and Biostatistics, Chittaranjan
National Cancer Institute, Kolkata, West Bengal, India

K. S. Chaudhari (✉)
Type 5, Bldg. No. 5, Flat No. 20, R.C.F. Colony,
Officers' Qtrs., Chembur,
Mumbai 400074 Maharashtra, India
e-mail: kaustubhschaudhari@gmail.com

D. K. Sarkar
396/3,N.S.C.Bose Road,
Kolkata 700047 West Bengal, India

D. Jana
1, Thakurbari Road, P.O.Kalighat,
Kolkata 700026 West Bengal, India

P. S. Patil
Junior Doctors' Hostel, Room No. 335, 242,A.J.C.Bose Road,
Kolkata 700020 West Bengal, India

B. K. Chattopadhyay · P. Chowdhary
Department of General Surgery, IPGME&R and SSKM Hospital,
244, A.J.C.Bose Road,
Kolkata, West Bengal, India

B. R. Chikkala
Junior Doctors' Hostel, Room No. 208, 242, A.J.C.Bose Road,
Kolkata 700020 West Bengal, India

combinations of heterodimers and homodimers, the most common being the p65/p50 heterodimer which is often referred to as the NF- κ B complex [2]. Activation of NF- κ B 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 Inflammation and metastatic growth as well as chemo resistance and radio resistance [3]. Activation NF- κ B 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 [4]. Indeed, the binding of epidermal growth factor (EGF) to its receptor (EGFR) also ultimately activates NF- κ B and most likely contributes to the enhanced activity of this transcription factor in ER negative breast cancer cells [5]. Loss of ER function has been associated with constitutive NF- κ B activity and hyperactive MAPK, because of constitutive secretion of cytokine and growth factors, which ultimately culminates in aggressive, metastatic, hormone-resistant cancers [6]. Activation of the progesterone receptor can lead to inhibition of NF- κ B driven gene expression [7], reducing its DNA binding and transcriptional activity. HER-2 activates NF- κ B through the canonical pathway which surprisingly, involves IKK α [8]. Activation of NF- κ B promotes survival of tumor cells. Several gene products that negatively regulate apoptosis in tumor cells are controlled by NF- κ B activation [9]. Nottingham Prognostic Index (NPI) is a good prognostic marker 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- κ B pathway plays important role in this pathway [9]. There are various prognostic

markers in breast cancer with variable sensitivity and specificity. We have to find any association NF- κ B/p65 expression with clinical parameters such as menopausal status, tumor size, grade, lymph node metastasis, NPI, ER, Progesterone Receptor (PR), HER-2 /neu. This study aims to validate the role of over expression of NF- κ B/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 primary breast carcinoma previous untreated by chemotherapy, radiotherapy, hormone therapy or a combination of any of the modalities who presented to the Comprehensive Breast Clinic Service & Breast Cancer Research Unit, IPGME&R/SSKM Hospital, Kolkata, West Bengal, India between 2008 and 2011 were included in this research work. Fifty four female patients who were histological and clinically fibro adenoma or benign breast disease treated as control group (Group-B).

Tissue Processing The specimens taken intra-operatively from the resected tumor tissue 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 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 (100 mM Tris-Cl, pH 7.4, 300 mM NaCl, 1 % NP-40, and 0.25 %

Table 1 Clinicopathological details according to NF- κ B/p65 status

Table-1		NF- κ B/p65				Comparison using χ^2 (p- value)
		Negative	Percentage	Positive	Percentage	
Menopausal Status	Postmenopausal	11	28.2	28	71.8	0.973
	Premenopausal	5	27.8	13	72.2	
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	
Lymph node metastasis	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*

*Statistically significant

Table 2 Immunohistochemistry parameters according to NF- κ B/p65 status

Table-2		NF- κ B/p65				Comparison using χ^2 (p-value)
		Negative	Percentage	Positive	Percentage	
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	13	65	7	35	<0.001*
	Score 1	2	50	2	50	
	Score 2	0	0	6	100	
	Score 3	1	3.7	26	96.3	
HER-2/neu	Negative, Score 1, Score 2	15	50	15	50	<0.001*

*Statistically significant

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- κ B), 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.

Statistical Analysis

Categorical variables are expressed as Number of patients and percentage of patients and compared across the two groups using Pearson's Chi Square test for Independence of Attributes. Continuous variables are expressed as Mean $\pm$ Standard Deviation and compared across the two 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.

Fig. 1 Immunohistochemically strong ER expression in IDC **Fig. 2** Strong PR expression in IDC

Fig. 3 HER-2/neu strong staining in IDC

Results

NF- κ B was not activated in control group by western blot technique. High level of NF- κ B (71.8 %) activation was showed in postmenopausal status but this was found to be statistically not significant ($p=0.973$, Table 1). The histological grades were measured by Modified Bloom-Richardson Grading Scheme. Activation of NF- κ B was marginally more frequent in high-grade tumors (Grade-III, 86.9 %) compared with low-grade tumors (Grade-I, 37.5 %) and this was statistically significant ($p=0.002$, Table 1). High NF- κ B 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 ($P=0.012$, Table 1). No statistically significant association was found between NF- κ B expression and lymph node metastasis ($P=0.393$, Table 1).

High NF- κ B activation was found in patients with a high NPI (NPI ≥ 5.4 , 84.6 %) compared with low NPI (NPI < 5.4 , 44.4 %) and this association was statistically significant ($P=0.002$, Table 1).

Note : NPI=tumor size $\times 0.2$ +lymph node stage (1=no node, 2=1 to 3 nodes positive, 3=4 or more nodes positive)+grade (1, 2 or 3).

Fig. 4 Represents that NF- κ B/p65 was overexpressed with ER negative tumor with compare that ER positive tumor by western blot methods

Fig. 5 Shows that NF- κ B/p65 was overexpressed in Grade III with compare that Grade II and Grade I by western blot methods

NF- κ B activation was more common in ER-negative tumors (81.8 %) than ER-positive tumors (38.5 %) and this difference was statistically significant ($P=0.002$, Table 2). NF- κ B activation was more common in PR-negative tumors (82.2 %) than PR-positive tumors (33.3 %) and this difference was statistically significant ($P<0.001$, Table 2). Statistically significant association was found between NF- κ B and HER-2/neu expression ($P<0.001$, Table 2). NF- κ B activation was more frequent in HER-2/neu positive tumors (96.3 %) compared with HER-2/neu negative tumors (35 %, Table 2). Statistically significant association was also found between NF- κ B and HER-2/neu positive (Score 3) and HER-2/neu negative (N, Score1, Score2) ($P<0.001$, Table 2).

Discussion

In our study NF- κ B/p65 (71.9 %) was activated in case of human invasive ductal breast carcinoma analysed by western blotting, whereas NF- κ B was undetectable in control group patients breast tissue. In the present study, activation of NF- κ B was significantly correlated with high grade, large tumour size, high NPI value, ER negativity, PR negativity and HER-

Fig. 6 Represents that NF- κ B/p65 was overexpressed with HER-2/neu positive tumor with compare that HER-2/neu negative tumor by western blot methods

2/neu positivity in breast cancer patients. C. Montagut et al. [10] found that there is no significant correlation was found between cytoplasmic or nuclear NF- κ B 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. Yamei Zhou et al. [11] demonstrated that activation of NF- κ B identifies a high-risk subset of hormone-dependent breast cancers. Debajit K. Biswas et al. [12] suggested that epidermal growth factor-induced NF- κ B activation: A major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. So NF- κ B controls cell-cycle progression by modulating action of cell-cycle regulatory genes. Low level of ER and PR are activated the NF- κ B [5]. Harikrishna Nakshatri et al. [13] showed that Constitutive Activation of NF- κ B during Progression of Breast Cancer to Hormone-Independent Growth. NF- κ B activation is linked to loss of ER expression and activation in IBC and in breast cancer in general. The inverse correlation between NF- κ B activation and ER activation is due to EGFR and/or ErbB2 overexpression, resulting in NF- κ B activation and ER down regulation [4]. The clinical significance was found between activation of NF- κ B transcription factor and overexpression of HER-2/neu oncoprotein [14] (Figs. 1, 2, 3, 4, 5, and 6).

We found that 89.3 % of tumours more than 5 cm in size activated p65 (Table 1) and this was statistically significant ($p=0.012$). 86.9 % of high grade (Grade III) tumours activated p65 (Table 1) and this was statistically significant ($p=0.002$). This implies NF- κ B is associated with aggressive tumour behaviour such as large tumour size, high grade and poor differentiation. Our study showed that, p65 activated 50 %, 72.7 %, 84.6 %, 66.7 % (Table 1) in breast cancer patients no node, 1–3, 4–9, <9 lymph node metastasis respectively but no significant association was found between lymph node metastasis and p65 activation ($p=0.393$). NPI is a reliable index to predict overall survival of breast cancer patients over 5 years. Low NPI (NPI<5.4) is associated with good prognosis (about 70 % survival over 5 years) while high NPI (NPI $\geq$ 5.4) has less than 50 % 5 year survival rate. Our study showed that 84.6 % of tumours expressing p65 had high NPI value (Table 1) & this was statistically significant ($p=0.002$). Our study also showed that p65 expressing tumours were ER negative and this was statistically significant (Table 2, $p=0.002$ and also showed that p65 expressing tumours were PR negative and this was statistically significant ($p=0.001$). We demonstrated that relation of HER-2/neu with p65 as shown in Table 2. We found that 96.3 % tumours over expressing HER-2/neu oncoprotein were p65 positive & this association was statistically significant ($p<0.001$) which was poor prognosis.

EC Merkhofer et.al suggested that Activation of NF- κ B and PI3K pathways downstream of HER-2, leading to changes in invasion and proliferation of breast cancer cell.

This study also demonstrated that IKK α has a larger role than IKK β in activation of NF- κ B in HER-2 breast cancer cell, including the phosphorylation of the p65 subunit at serine 536 [7]. So activation of NF- κ B is the major role of metastasis via IKK α 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- κ B are signaling [15]. NF- κ B stimulates proliferation and blocks programmed cell death (apoptosis) in different cell types, including human breast cancers [12, 15, 16]. Various study reported that activated NF- κ B is detected in ER-negative human breast cancer cells harboring overexpressed ErbB1 [12, 17, 18]. Activation of NF- κ B is the major role in cell proliferation and apoptosis to use an ER-negative and ErbB2-positive expression [19].

Conclusions

NF- κ B over expression implies aggressive tumour biology in breast cancer & it can predict tumours likely to have poor prognosis. Patients with NF- κ B positive tumours need to be treated aggressively. We detected a positive correlation between NF- κ B and HER-2/neu when studied according to score evaluation. NF- κ B 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- κ B 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- κ B overexpression may decrease tumour progression in patients and may block breast carcinogenesis, reducing the incidence of breast carcinoma in patients at high risk.

References

1. Chen LF, Greene WC (2004) Shaping the nuclear action of NF- κ B. *Nat Rev Mol Cell Biol* 5:392–401
2. Radhakrishnan SK, Kamalakaran S (2006) Pro-apoptotic role of NF- κ B: implications for cancer therapy. *Biochim Biophys Acta* 1766:53–62
3. Greten FR, Karin M (2004) The IKK/NF- κ B activation pathway—a target for prevention. *Cancer Lett* 206:193–199
4. Van Laere SJ, Van der Auwera I, Van den Eynden GG, van Dam P, Van Marck EA, Vermeulen PB, Dirix LY (2007) NF- κ B activation in inflammatory breast cancer is associated with oestrogen receptor downregulation, secondary to EGFR and/or ErbB2 overexpression and MAPK hyperactivation. *Br J Cancer* 97:659–669
5. Shostak K, Chariot A (2011) *Breast Cancer Res* 13:214

6. Ali S, Coombes C (2002) *Nat Rev Mol Cell Biol* 2:101–115
7. Kalkhoven E, Wissink S, van der Saag PT, van der Burg B (1996) *J Biol Chem* 271:6217–6224
8. Merkhofer EC, Cogswell P, Baldwin AS (2010) Her2 activates NF- κ B and induces invasion through the canonical pathway involving IKK α . *Oncogene* 29:1238–1248
9. Sethi G, Sung B, Aggarwal BB (2008) Nuclear factor- κ B activation: from bench to bedside. *Exp Biol Med* 233:21–31
10. Zhou Y, Eppenberger-Castori S, Eppenberger U, Benz CC (2005) The NF- κ B pathway and endocrine-resistant breast cancer. *Endocr Relat Cancer* 12:S37–S46
11. Zhou Y, Eppenberger-Castori S, Marxa C, Yaua C, Scotta GK, Eppenberger U, Benz CC (2005) Activation of nuclear factor- κ B (NF- κ B) identifies a high-risk subset of hormone-dependent breast cancers. *Int J Biochem Cell Biol* 37:1130–1144
12. Biswas DK, Cruz AP, Gansberger E, Pardee AB (2000) Epidermal growth factor-induced nuclear factor κ B activation: a major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. *PNAS* 97(15):8542–8547
13. Nakshatri H, Bhat-Nakshatri P, Martin DA, Gouet RJ Jr, Sledge GW Jr (1997) Constitutive activation of NF- κ B during progression of breast cancer to hormone-independent growth. *Mol Cell Biol* 17(7):3629–3639
14. Hou M-F, Lin S-B, Yuan S-SF, Tsai S-M, Wu S-H, Ou-Yang F, Hsieh J-S, Tsai K-B, Huang T-J, Tsai L-Y (2003) The clinical significance between activation of nuclear factor kappa B transcription factor and overexpression of HER-2/neu oncoprotein in Taiwanese patients with breast cancer. *Clin Chim Acta* 334:137–144
15. Balkwill F, Mantovani A (2001) Inflammation and cancer: back to Virchow? *Lancet* 357(9255):539–545
16. Karin M, Lin A (2002) NF-kappaB at the crossroads of life and death. *Nat Immunol* 3:221–227
17. Biswas DK, Martin KJ, McAlister C, Cruz AP, Graner E, Dai SC, Pardee AB (2003) Apoptosis caused by chemotherapeutic inhibition of nuclear factor-kappaB activation. *Cancer Res* 63:290–295
18. Biswas DK, Dai S-C, Cruz A, Weiser B, Graner E, Pardee AB (2001) The nuclear factor kappa B (NF- κ B): a potential therapeutic target for estrogen receptor negative breast cancers. *PNAS* 98:10386–10391
19. Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ Jr, Sledge GW Jr (1997) Constitutive activation of NFkappaB during progression of breast cancer to hormone-independent growth. *Mol Cell Biol* 17:3629–3639