ORIGINAL RESEARCH

Immunohistochemical Expression Profile of NF-*k*B in Breast Cancer

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ABSTRACT

Background: Development of large-scale sequencing efforts of cancer genomes has led to identification of newer therapeutic targets. Nuclear Factor- kappaB (NF-*k*B) is one such promising transcription factor and is the subject of much active research as a target for anti- breast cancer therapy. This study was conducted to study the expression patterns of NF-*k*B in breast cancer and to correlate the findings with the histopathological grade of the tumours.

Methods: This study was carried out on 25 samples of breast carcinomas.NF-*k*B was demonstrated immunohistochemically in the malignant epithelial cells of the tissue samples by using ImmPRESS polymerized reporter enzyme staining system which has been shown to have very good sensitivity and signal intensity, low background staining and reduced non- specific binding .This system is based on a new method of polymerizing enzymes and attaching these polymers to antibodies. The specimen was considered to be positive if more than 10% of tumour cells stained. The distribution of staining in the tumour cells was graded as 1= focal (<10%), 2= regional (11–50%), or 3= diffuse (>50%).

Results: Among the 25 cases invasive ductal carcinoma, 12 (48%) cases showed immunoreactivity for NF-*k*B. 67% (8/12) of the cases showed both nuclear and cytoplasmic positivity.7 cases belonged to grade I, 10 cases to grade II and 8 cases to grade III.

Conclusion: NF-*k*B is frequently overexpressed in primary breast cancer and showed increasing trends in tumours of grades I and II. However, discrepancies were seen with grade III tumours. As a result of their widespread expressions in different tumours, and generally low-level expression in normal tissue, NF-*k*B can be considered to be the prospective newer adjuvant target for apoptosis-based chemotherapy. Further studies need to be carried out to investigate the contribution of this protein to various cancer phenotypes and to be used as a potential molecular target for their treatment.

Keywords: NF-*k* B, Breast Cancer, Invasive Ductal Carcinoma, Imm PRESS.

INTRODUCTION

Recently, the discovery of breast cancer-specific therapeutic targets such as ER and erb-B2 (human epidermal growth factor receptor 2) has led to the successful development of therapies that are effective only in patients whose tumours harbour these targets. This paradigm has provided the impetus for large-scale sequencing efforts of cancer genomes to identify additional targets. Nuclear Factor- kappaB (NF-*k*B) is one such promising transcription factor and is the subject of much active research as a target for anti-breast cancer therapy. NF-*k*B stimulates proliferation and blocks programmed cell death (apoptosis) in different cell types, including human breast cancers[1,2,3]. NF-*k*B functions mainly as a regulator of genes that control cell proliferation and cell survival. As such, many different types of human tumors have misregulated NF-*k*B, that is, NF-*k*B is constitutively active. Active NF-*k* B turns on the expression of genes that keep the cell proliferating and protects the cell from conditions that would otherwise cause it to die via apoptosis thereby rendering the cell "immortal" [4]. In addition, NF-*k*B is a key player in the inflammatory response. Methods of inhibiting NF-*k*B signalling has potential therapeutic applications in cancer and inflammatory diseases [5,6]. Hence this study was conducted with the following aims & objectives:

Aims & Objectives

1. To categorise and grade 25 samples of breast carcinomas histomorphologically.

2. To study the expression patterns of NF-*k* B immunohistologically in these tumours.
3. To correlate the immunohistochemical status of NF-*k* B with the histopathological grade of the tumours.

METHODS

Immunohistochemical Detection Of NF-*k* B by Immunoperoxidase Method

NF-*k*B was demonstrated immunohistochemically in the malignant epithelial cells of the tissue samples by using ImmPRESS polymerized reporter enzyme staining system (vector laboratories). The two- step method for antigen detection in tissue sections includes initial binding of the primary antibody to the antigen of interest in the tissue section, followed by detection and visualization by a suitable choice of enzyme- substrate (chromogen) system. The Imm PRESS™ polymerized reporter enzyme staining system is based on a new method of polymerizing enzymes and attaching these polymers to antibodies. The novel approach employed to form enzyme "micropolymers" avoids the intrinsic shortcomings of using large dextrans or other macromolecules as backbones. Attaching a unique "micropolymer" with a high density of very active enzyme to a secondary antibody generates a reagent that overcomes steric interference and provides enhanced accessibility to its target. It is also claimed by the manufacturer that this results in outstanding sensitivity and signal intensity, low background staining and reduced non- specific binding [7].

Requisite solutions & reagents

Primary Antibody used for immunohistochemical staining-

- p- NF-*k*B p65 (Ser276): sc101749- is a rabbit polyclonal antibody(IgG) raised against a short amino acid sequence containing phosphorylated Ser276 of NF- kB p65 of human origin; purchased from Santa Cruz Biotechnology Inc., CA, USA.

1. Bovine serum albumin- used in a concentration of 1.5 % to suppress non- specific binding of the primary antibody
2. Phosphate buffer saline - stock(pH 7.4) -
   Solution a 0.15M NaH2PO4. 2 H2O 23.4g/l
   Solution b 0.15M Na2HPO4 21.2g/l
   A working solution was prepared by adding 18ml of solution 'a' and 82ml of solution 'b' and this was stored at 4° C. This was used to wash the slides after each staining step.
3. Trisodium citrate buffer(pH 6- 6.2) –
   0.001M solution - 2.941g trisodium citrate dihydrate was dissolved in 1000ml of deionised water.
4. DAB (3, 3' Diaminobenzidine Tetrahydrochloride) substrate buffer: consists of Tris buffer, peroxide, DAB and stabilizers.
5. The Imm PRESS™ polymerized reporter enzyme staining system ready- to- use kit: The kit comprises the following reagents:
   - ImmPRESS Universal reagent - contains a "micropolymer" of a very active peroxidase coupled to a mixture of affinity – purified anti- mouse IgG (H+L) and anti – rabbit IgG (H+L) secondary antibodies.
   - 2.5 % normal horse serum blocking solution – universal protein blocking solution to reduce non- specific staining.
   - Peroxide block – 3% hydrogen peroxide in deionised water, to quench the endogenous peroxidase.
6. APES (3- aminopropyl triethoxysilane) – permanent section adhesive.
7. Lillie – Mayer's haematoxylin –used as counterstain.
8. DPX- permanent mounting medium.

Procedure for immunohistochemical staining of NF-*k* B: (Table 1)

1. 3µm sections of breast carcinomas were deparaffinised in xylene, hydrated in graded alcohols and washed in water.
2. Sections were then transferred to citrate buffer (pH 6) for 10 minutes.
3. Slides were subjected to heat - induced epitope retrieval (antigen unmasking) by pressure cooking for 10 minutes. Then, the slides were immediately removed from the pressure cooker and allowed to cool for 20 minutes in the same citrate buffer at room temperature.
4. Slides were transferred to phosphate buffer saline (PBS) and incubated for 10 minutes.
5. Slides were laid flat in a humidifying chamber and treated with 3 % hydrogen peroxide solution for 5 minutes.
6. The sections were treated with 2.5 % normal horse serum blocking solution for 10 minutes, after brief (5 minutes) immersion in PBS.
7. The sections were incubated with the primary antibody, after draining the excessive blocking solution and wiping it carefully off the sections.
8. Sections were incubated with the primary antibody for 1 hour at room temperature and also overnight at 4°C.
9. Sections were thoroughly washed with PBS for 5 minutes.
10. Subsequent incubation with ImmPRESS Universal reagent of the sections was done for 30 minutes.
11. After washing the slides in PBS solution for 5 minutes, freshly prepared DAB chromogen solution was applied to the sections and incubated for 10 minutes.
12. Sections were then transferred to PBS solution for 5 minutes and subsequent washing with tap water for 5 minutes.

13. Sections were counterstained with Lillie - Mayer's haematoxylin for 5 seconds and washed with tap water.
14. Slides were finally mounted with DPX after thorough dehydration & clearing of the sections. Throughout the staining procedure, the sections were not allowed to dry. After PBS washes, the slides were carefully wiped around the sections without damaging them. Positive and negative controls were used along with the test cases to ensure the quality of staining and to evaluate non-specific binding of the reagents to the specimen. Brown- coloured staining in the nucleus and/ or in the cytoplasm of the cells, indicated the end product.

Marker	Technique	Primary Antibody Dilution	Primary Antibody Incubation Time	Micropolymerised Enzyme - Attached Secondary Antibody	Secondary Antibody Time	Chromogen	Haematoxylin
NF – *k*B Polyclonal	Immuno-peroxidase Method	1 : 50	1 Hour AT 37° C + Overnight AT 4°C	Peroxidase Enzyme Attached to Anti-Mouse IgG and Anti – Rabbit IgG Secondary Antibodies	30 Minutes	DAB	Lillie – Mayer's Haematoxylin

Table 1: Protocol for NF–k B IHC Staining

Interpretation of the results

The intensity of expression of the stained sections were done by two observers independently using standardized methods.

NF-*k*B staining profile

The intensity and distribution of cytoplasmic staining were considered in the semiquantitative assessment of the immunohistochemical results of NF-*k*B. Additionally, nuclear immunoreactivity for NF-*k*B was scored independently. The intensity of cytoplasmic and/or nuclear staining was subjectively graded as 1= weak, 2= moderate, or 3= intense. The distribution of staining in the tumour cells was graded as 1= focal (<10%), 2= regional (11–50%), or 3= diffuse (>50%). Cases in which the cytoplasmic staining patterns were categorized as intense diffuse, intense regional, and moderate diffuse were considered overexpression of the protein, whereas cases with any pattern of nuclear staining were considered positive for nuclear NF-*k*B. In each tissue section, the NF- *k*B stained hotspots were searched for and within these hotspots, a total number of 100 nuclei were counted at x 400 magnification and if more than 10% of tumour cells stained, the specimen was considered to be positive [8].

RESULTS

Our study material included 25 cases of breast carcinomas from women belonging to different age groups and they were categorised based on their histomorphology in haematoxylin and eosin stained sections. Among the 25 cases of breast cancers of invasive ductal carcinoma – not otherwise specified type, 7 cases belonged to grade I, 10 cases to grade II cases, 8 cases to grade III. The frequencies and patterns of expressions of NF–*k*B were evaluated using standardised methodologies and the results are tabulated in Table 1.

Evaluation of NF-*k*B Expression in Breast Carcinoma:(Table 2)

The immunostaining pattern for NF- *k*B was both cytoplasmic and nuclear. Totally 12/25 (48%) cases showed immunoreactivity for NF- *k*B. Overall, 67% (8/12) of the cases showed both nuclear and cytoplasmic positivity. (Figures 1 ,2) 43% (3/7) of the grade I tumours were positive for this protein with most of them showing diffuse weak to moderate expression of NF- *k*B. Majority (67%) of the cases showed diffuse weak nuclear and cytoplasmic positivity. One case (33%) showed only diffuse weak cytoplasmic expression. There is a definite correlation of the observed expression profile of this protein under study, with the grade of the tumour. (Figure 3) The positivity rate for grade II tumours was 60% (6/10). The immunoreactivity profile was predominantly diffuse moderate nuclear staining which was found to be the sole pattern of expression in 2 (20%) cases. However, one of the cases (10%) showed diffuse intense nuclear staining .Combined cytoplasmic and nuclear over expression were seen in 20% of the cases. Focal moderate nuclear staining was observed in 10% (1/10) cases. Overall, the immunostaining pattern studied in the 10 cases, is in accordance with the grade of the tumour. (Figure 4) Out of the 8 cases under grade III category, three (38%) cases showed increased expression of this protein. Overall, a diffuse moderate combined (both nuclear and cytoplasmic) pattern of staining was observed. More than 50% of grade III tumours were negative for this protein. There was no correlation of the immunoreactivity pattern of NF- *k*B in this category with the advanced grade (III) of the tumours. (Figure 5)

Interestingly, benign stromal cells adjacent to the tumour also showed moderate to intense immunoreactivity to this protein.
(Figure 6)

S.No.	Grade	NF - kB Expression Score	
		Cytoplasmic	Nuclear
1.	I	Diffuse moderate	Diffuse weak
2.	I	Diffuse weak	Diffuse weak
3.	I	-	-
4.	I	-	-
5.	I	-	-
6.	I	-	-
7.	I	Diffuse weak	-
8.	II	-	Diffuse moderate
9.	II	-	Diffuse moderate
10.	II	Diffuse moderate	Diffuse moderate
11.	II	-	Diffuse intense
12.	II	-	-
13.	II	-	-
14.	II	Diffuse moderate	Diffuse moderate
15.	II	-	-
16.	II	Diffuse weak	Focal moderate
17.	II	-	-
18.	III	-	-
19.	III	Diffuse moderate	Focal moderate
20.	III	-	-
21.	III	Diffuse weak	Diffuse moderate
22.	III	-	-
23.	III	-	-
24.	III	-	-
25.	III	Diffuse moderate	Diffuse moderate

Table 2- Evaluation of NF- kB Expression

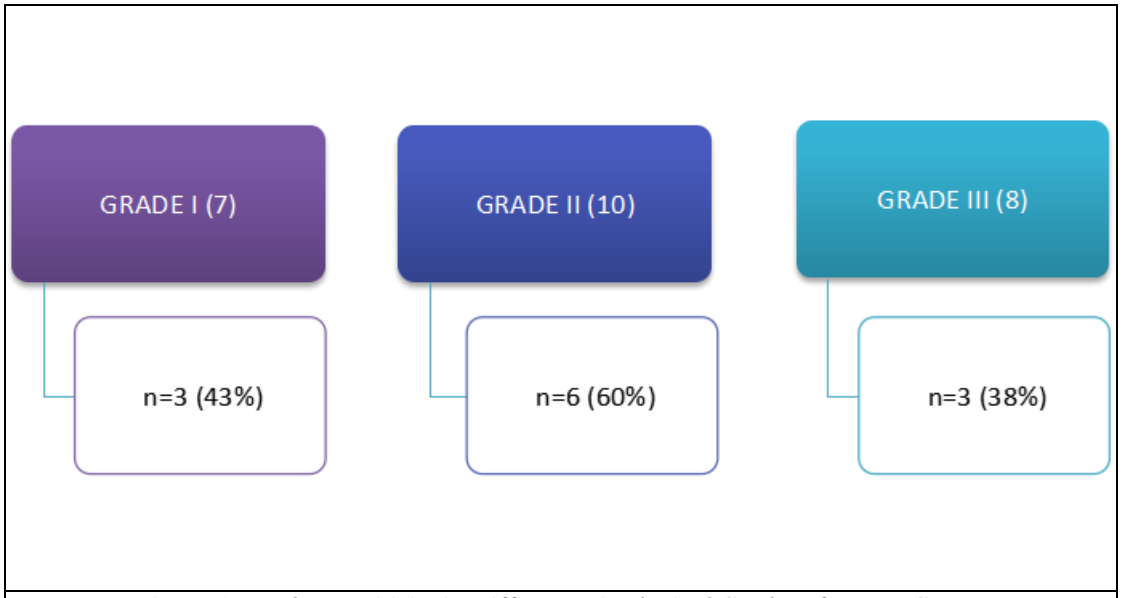

Figure 1: NF-k B Positivity in Different Histological Grades of Breast Cancer

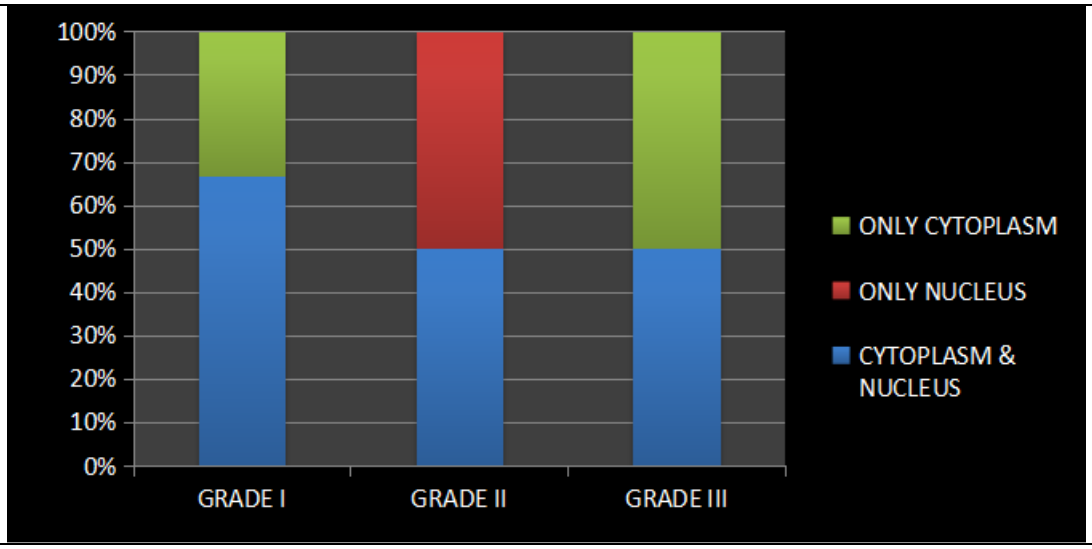

Figure 2: Grade- Wise Analysis of NF-kB Expression Pattern

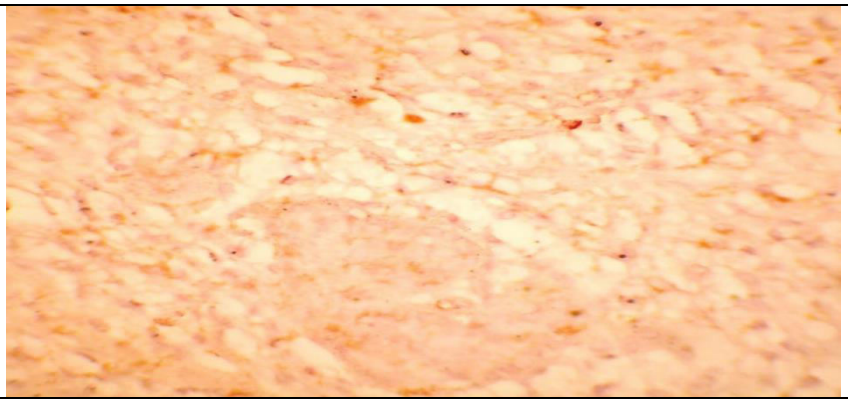

Figure 3: Invasive Ductal Carcinoma (NOS), Histological Grade I, cells showing Diffuse Moderate Cytoplasmic and Diffuse Weak Nuclear NF-kB Positivity, x 400 Magnification

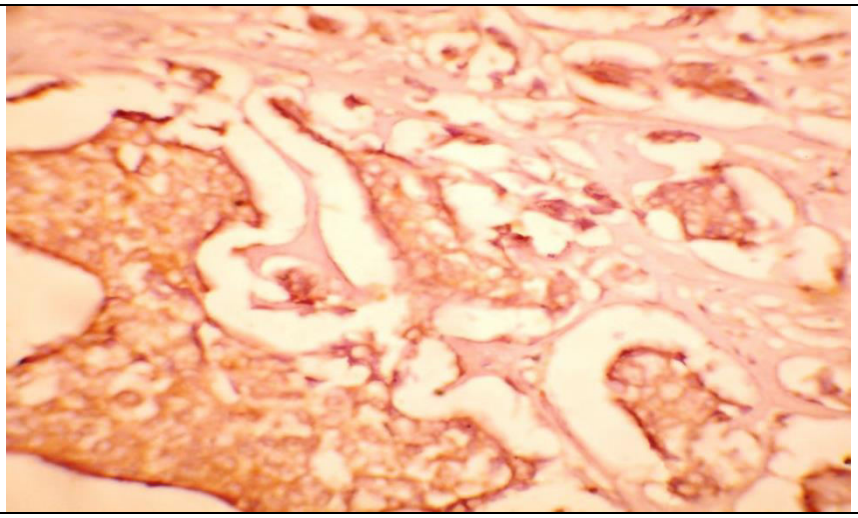

Figure 4: Invasive ductal Carcinoma (NOS), Histological Grade II, Cells Showing Diffuse Moderate Cytoplasmic NF-kB Positivity, x 400 Magnification

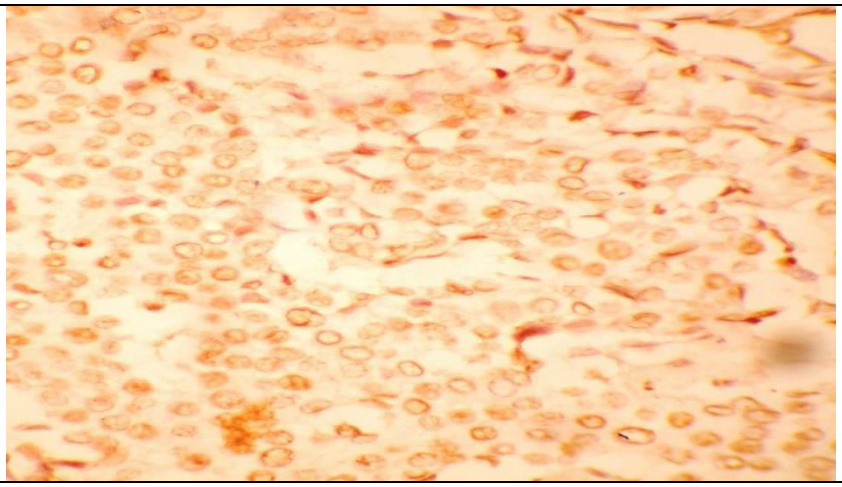

Figure 5: Invasive Ductal Carcinoma (NOS), Histological Grade II, Cells Showing Diffuse Moderate Nuclear NF-kB Positivity, x 400 Magnification

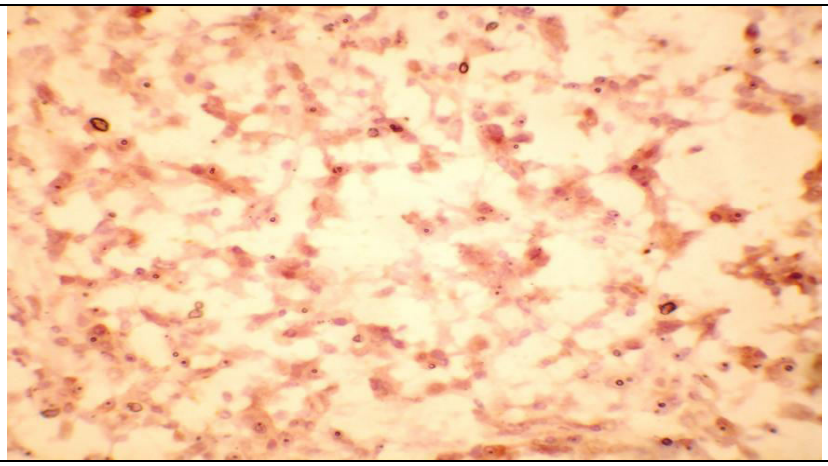

Figure 6: Invasive Ductal Carcinoma (NOS), Histological Grade III, Cells Showing Diffuse Moderate Cytoplasmic and Nuclear NF-kB Positivity, x 400 Magnification

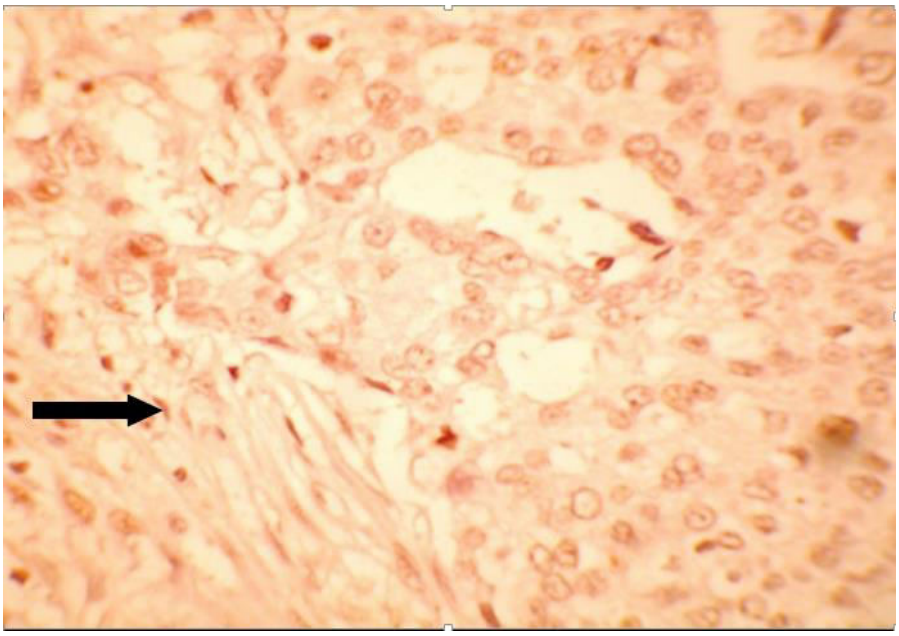

Figure 7: Stromal Cells Adjacent to the Tumour Showing Moderate Nuclear NF-kB Positivity, x 400 Magnification

DISCUSSION

Cellular homeostasis in higher organisms is maintained by fine - tuning positive and negative signal-induced cellular regulatory mechanisms responsible for cell proliferation and death, which are apparently disturbed in tumour cells[9]. Inappropriate gene expression and function, leading to uncontrolled cell proliferation and loss of regulated cell death, contributes significantly to the tumor cell phenotype. The proliferative and antiapoptotic properties of activated NF-*k*B qualify this transcription factor as a key cellular regulatory molecule, potentially contributing to both normal and neoplastic phenotypes [10,11]. Recent evidence indicates that NF-*k*B and the signaling pathways that are involved in its inactivation are important for breast cancer development. Hence, it has been the focus of extensive research in this regard. To date, NF-*k*B has been studied extensively in breast cancer preclinical and experimental models, but not as a clinical prognostic factor. Interestingly, NF-*k*B activation in breast cancer cell lines has been linked to the expression of both cyclin D1[12] and HER-2/neu[13], two well-known adverse outcome predictors in breast cancer[14,15].The major emphasis of these studies has been in the activation of NF–*k*B and its related pathways[16]and its essential role in the development of resistance to chemotherapeutic drugs designed to treat these tumours. Elevated levels of this transcription factor in breast tumors have been reported (Weinstein, 2000).[17] NF-*k*B is seen overexpressed in 12/25 (48%) of cases of breast cancer in our study. Other studies showed in the range of 71% (12 of 17) of cases (JeffreyRoss, Bhaskar et al, 2004).[18] Studies also show that phospho-NF-*k*B p65 antibody, both a nuclear and cytoplasmic staining pattern was observed[18].Our study with p65 RelA clone showed both nuclear and cytoplasmic pattern of immunoexpression. An important observation was the fact that not only tumor cells but also stromal cells stained using this antibody and the tumour cell immunoreactivity was less than or equal to the immunoreactivity of the benign stromal cells as seen in the study by Steven Van Laere et al[19] and Biswas, Shi et al.[20] In our study, a diffuse weak to moderate expression of NF- *k*B positivity was displayed by 43% (3/7) of the grade I tumours were positive for this protein of which 2/7 (67%) of the cases showed diffuse weak nuclear and cytoplasmic positivity and 1/7 (33%) cases showed only diffuse weak cytoplasmic expression. There is a definite correlation of the observed expression profile of this protein under study, with the grade of the tumour. Also, predominantly diffuse moderate nuclear staining was found to be the sole pattern of expression in 2 (20%) cases of grade II tumours in our study. However, one of the cases (10%) showed diffuse intense nuclear staining. Combined cytoplasmic and nuclear overexpression was seen in 20% of the cases of moderately differentiated tumours. Focal moderate nuclear staining was observed in 10% (1/10) cases. Overall, the immunostaining pattern studied in the 10 cases is in accordance with the grade of the tumour. This feature exhibited by both grade I and II tumours is in accordance with results of Fernandez Val, Losada et al.[21] Under grade III category in our study, 3/8(38%) cases showed diffuse moderate both nuclear and cytoplasmic pattern of expression. The intensity of expression was same as grade II tumours and many (>50%) of the grade III tumours were negative for NF-*k*B. There was no correlation of the immunoreactivity pattern of NF-*k*B in this category with the advanced grade (III) of the tumours probably owing to the small size of the study group. This was observed in contrast to few other solid tumours like prostatic and oesophageal adenocarcinomas [22]. Disparity was also seen with other studies in female breast carcinomas where EGFR over-expression[23,24,25] in aggressive and metastatic grade III breast carcinomas, also reported over- expressed NF-*k* B (Biswas, Shi 2004)[26], which was also linked to unfavorable prognosis by promoting tumor metastasis and inhibiting apoptosis [27,28]. Several studies of NF- *k*B in inflammatory breast carcinomas suggest a distinct role of this transcription factor in its unique clinical behavior.

CONCLUSION

Being a heterogeneous group of tumours, breast cancers vary in morphology, clinical presentation and behaviour. The morbidity and mortality from breast cancer remains high, despite significant advances in our understanding and management over the last several decades. Gene expression profiling studies of individual tumours reveal a gamut of molecular alterations/ markers based on which diagnostic classifications and subclassifications are designed. These would help in devising personalised treatment plans. With this standpoint, we embarked on a novel marker, NF–*k*B, the propitious contender in breast cancer prognostication and targeted therapy. We have showed, through our study, the significance of expression patterns of NF–*k*B and its implications in treatment of this perplexing group of tumours. Our results showed that NF–*k*B is frequently overexpressed in primary breast cancer and showed increasing trends in tumours of grades I and II .However, discrepancies were seen with grade III tumours. As a result of their widespread expressions in different tumours, and generally low-level expression in normal tissue, NF–*k*B can be considered to be the prospective newer adjuvant target for apoptosis-based chemotherapy. Further studies need to be carried out to investigate the contribution of this protein to various cancer phenotypes and to be used as a potential molecular target for their treatment.

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