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Expression of basic fibroblastic growth factor (bFGF) in invasive ductal carcinoma of breast and its relation to angiogenesis and other prognostic parameters

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Objective: Tumor growth and metastasis are angiogenesis-dependent processes. In breast cancer, as well as other tumors, a group of angiogenic growth factors are defined and basic fibroblast growth factor (bFGF) is a well characterized potent angiogenic growth factor.

Study design: Expression of bFGF was examined by immunohistochemistry in fifty-eight mastectomy specimens and its relationship with intratumoral microvessel density (MVD) was measured by immunohistochemical staining for anti-CD31 antibody. Association of both parameters were analyzed for prognostic factors, and the clinical and pathologic characteristics in individual patients.

Results: bFGF expression was significantly increased in carcinoma cells compared with normal and hyperplastic ductal epithelial cells. However, bFGF expression was not associated with MVD and other variables, including tumor size, histological grade, axillary node status, estrogen and progesterone receptors, and c-erbB-2 positivity.

Conclusion: bFGF has a role in transformation of normal breast epithelium to malignant form either invasive or non-invasive. Our data suggests that bFGF is not the only growth factor that regulate tumoral growth and angiogenic pathways in invasive ductal carcinoma.

Keywords: basic fibroblastic growth factor, breast cancer, angiogenesis

Introduction

Basic fibroblast growth factor (bFGF; also known as FGF-2) family of cytokines is heparin-binding molecule with potent angiogenic properties and diverse function in cell growth and differentiation in all tissues both normal and malignant.[1,2] Apart from the tumor growth, bFGF has an important role in angiogenesis which is a critical step in invasion of endothelial cells and the metastatic process.[3,4] bFGF up-regulates the proteins that are responsible for the transition from G1 to S phases of cell cycle.[2,5–7]

In normal tissues, bFGF is membrane-bound and present in basement membranes and in the sub endothelial extracellular matrix of blood vessels. In particular, during both wound healing of normal tissues and tumor development, the action of heparan sulphate degrading enzymes activates bFGF, thus mediating the formation of new blood vessels as well as being mutagen for fibroblast cells.[8–10]

In breast morphogenesis, bFGF has been shown to induce formation of bilayered lobuloalveolar structures and accepted that myoepithelial cells are the main source of bFGF. In tissue culture, it does not cause proliferation of myoepithelial cells but is mutagenic for epithelial cells and has paracrine function in controlling the growth of epithelial and myoepithelial cells which are lost in the progression to neoplasia.[2,5,7,11]

The development of new blood vessels in tumors depends on the production of angiogenic factors released both from the tumor and stromal cells.[11–16] This study was undertaken to quantify the expression of the known and one of the most potent angiogenic growth factors bFGF in invasive ductal carcinoma, in non-tumorous breast tissue and in preinvasive stage of the tumor. We also examined the relation between bFGF expression and microvessel count to evaluate the paracrine effects of the endothelial stimuli on neovascularization, and estrogen (ER) and progesterone receptor (PR), c-erbB-2 expression and other prognostic parameters such as tumor grade, tumor size, and axillary lymph node status, in individual patients.

Material and methods

Tissue samples

Fifty-eight radical mastectomy specimens diagnosed as invasive ductal carcinoma with axillary lyphoadenectomy were selected for this study. Paraffin blocks were chosen from the pathology archive that contains invasive carcinoma, ductal carcinoma in situ and normal breast parenchyma tissue. Tumor grading was carried out according to the modified Bloom and Richardson method, and staging system was carried out revisioned AJCC TNM staging system.[17]

Immunohistochemical procedure

Four μm thick sections were deparaffinized and rehydrated using xylene and decreasing ethanol concentrations. Antigen retrieval was performed by microwaving at 75 W for 15 min in 10 mM citrate buffer (pH 6.0) followed by cooling at room temperature for at least 20 minutes. The slides were then incubated for 20 minutes in 1.8% hydrogen peroxide, washed in PBS. Primary antibodies used were: anti-CD31 (Neomarkers, 1:30 dilution), anti-bFGF (Santa Cruz Biotechnology, USA, 1:250 dilution), anti-ER, anti-c-erbB-2, and anti-PR (Neomarkers, 1:50 dilution each). Visualization of antibody binding using a biotinylated secondary antibody and the avidin-biotin complex method was according to the manufacturer's instructions (ABC kit, Labvision, Fremont, USA). Finally, sections were rinsed in deionised water, counterstained by Mayer's Hematoxylin, and mounted in a mounting media.

Evaluation of immunohistochemical staining, assessment of microvessel density and statistical analysis

The degree of immunopositivity was evaluated semiquantitatively. Immunoreactive cells were assessed and expressed as a percentage. The scoring system for bFGF was as follows; 0-5%:negative; 5-25%:low positivity; 25-50%:moderate positivity; >50%:high positivity. To assess the effects of bFGF overexpression on tumor-associated neo-vascularization, we stained intratumoral vessels with CD31/PECAM-1-specific antibodies. Average micro-vessel density (MVD) was performed by counting CD31 positive blood vessel areas of high microvessel density (vascular "hot spots") pointed by scanning the entire tumor at 100X magnification. The mean microvessel counts from five hot spot fields were calculated on each slide at 200X magnification (0.78 mm^2). Analysis of the cases was performed in a blinded fashion.

Statistical analysis of clinicopathological parameters was evaluated using Pearson's Chi^2 test. Spearman's correlation coefficient was used to investigate the relationship between prognostic parameters and bFGF expression. The data of immunohistochemical evaluation were statistically analyzed using computer software (SPSS 10.0, Chicago, IL, U.S.A). P values of less than 0.05 were considered to be significant.

Results

Patients and tumor variables are listed in Table 1. Specific bFGF immunostaining was mainly confined to the cytoplasm, but occasional cells demonstrated faint nuclear positivity. bFGF expression was determined both in invasive and preinvasive tumor cells and in normal ductal, acinar epithelial and myoepithelial cells adjacent to the tumor tissue (Figure 1). However, bFGF staining was significantly higher in carcinoma cells in both invasive and preinvasive stage compared with normal breast tissue that showed very weak staining in normal ductal and acinar

compartments (p=0.001). We noticed only scattered bFGF expression in occasional capillary endothelial cells and fibroblasts in the stroma.

Table 1. Clinicopathologic characteristics of patients.

Features	Number of patients (%)
Patients enrolled	58
Age (mean)	50±11.36
Tumor size	
T1	10 (17.2)
T2	36 (62.1)
T3	12 (20.7)
Histopathologic grade	
I	20 (34.5)
II	27 (46.6)
III	11 (18.9)
Lymph-node status	
Node-negative	26 (44.8)
Node positive	32 (55.2)
bFGF staining (TSS)	
Low TSS	45 (77.6)
High TSS	13 (22.4)
Estrogen receptor	
Positive	17 (29.3)
Negative	41 (70.7)
Progesterone receptor	
Positive	19 (32.8)
Negative	39 (67.2)
c-erbB-2	
Negative	8 (13.8)
Positive	50 (86.2)

In tumor tissue the immunopositive areas varied greatly in the different tumors, even in the same tumor from a scattered weak positivity to dense positivity over the whole tumor area, rare cells showed nuclear reactivity as well. Strongest staining was prominent at the edge of invasive tumor (Figure 2). But, the majority of cases (45/58, 77.6%) showed low to moderate intensity of staining; whereas only in 13 of cases (22.4%) we were able to show dense immunostaining. Although, no significant association was found between the presence of bFGF immunostaining and tumor size, axillary lymph node involvement, ER, PR, and c-erbB-2 positivity, but bFGF expression was stronger in larger tumors.

As expected, CD31 immunostaining was restricted to endothelial cells. CD31 positive microvessels were observed throughout the tissue

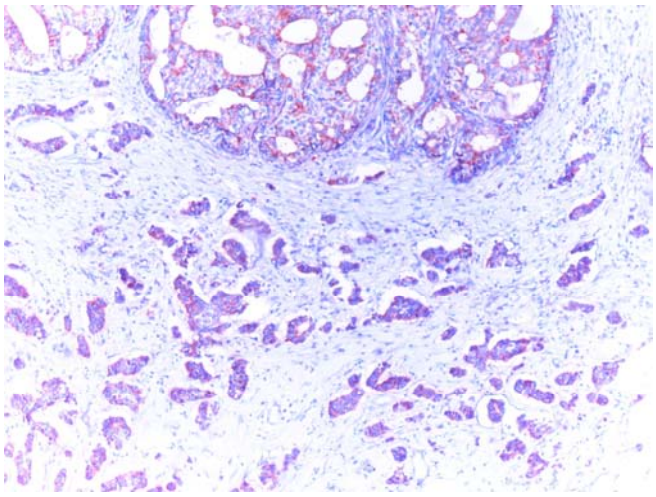

Figure 1. Immunopositivity of bFGF in ductal carcinoma of breast, both in invasive and preinvasive stages.

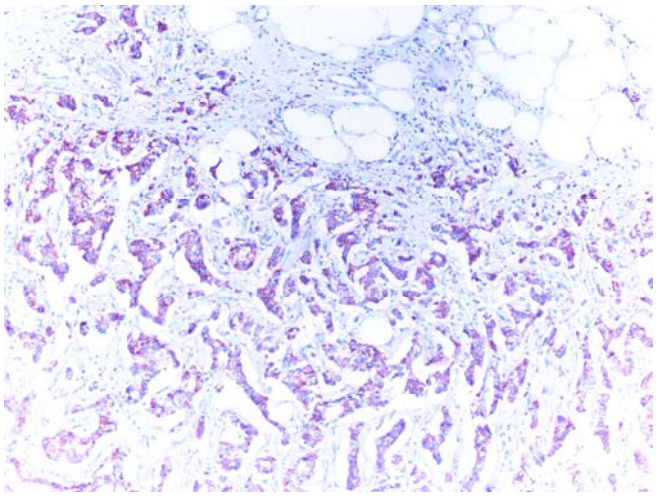

Figure 2. Invasive edge of the same tumor, showing dense immunostaining with bFGF.

sections. MVD (range: 20-76; mean: 36±11.3; median:34) was significantly higher in invasive tumor than in neighbouring normal breast tissue (p=0.001). Highest MVD was found at the infiltrating lateral border of tumor (Figure 3). Median microvessel counts in 200X magnification in low, moderate and high grade tumors were 32, 38 and 42 respectively. There was a significant association between MVD and tumor grade between grade 3 to grade 1 (p=0.03), however no relation was found between grade 1 to 2 and 2 to 3. Although there was not any significant difference between MVD and bFGF expression, MVD was higher (42 in 200X magnification) in cases with stronger bFGF expression (34 in 200 X magnification). Additionally, we found borderline significance

between MVD and with lymph node involvement (p=0.06).

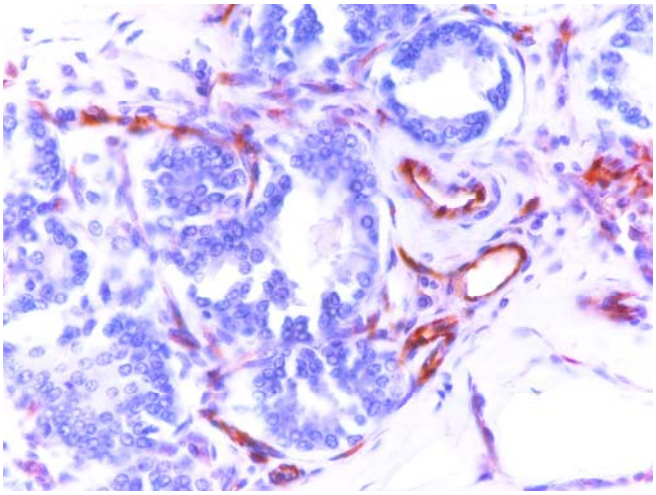

Figure 3. Increased number of microvessels in tumor tissue at the infiltrating border of tumor.

Discussion

In this report, we have shown that bFGF expression in preinvasive and invasive ductal carcinoma is significantly increased in comparison to normal breast tissue. Interestingly, there was a tendency to stronger bFGF expression in higher grade intraductal carcinoma, but strongest expression was found in invasive ductal carcinoma. This shows that bFGF seems to have a role, both in the normal breast development and in tumorogenesis with progressively increasing intensity transition from epithelial hyperplasia to ductal carcinoma in situ and to invasive ductal carcinoma. Lord *et al* found similar results in esophageal adenocarcinoma compared with normal esophagus and Barrett esophagus.[12] Additionally, Wakulich *et al* demonstrated progressive increasing intensity of bFGF expression through the dysplasia to squamous cell carcinoma in oral cavity.[15] Our data also supports the possibility that progressive accumulation of bFGF is conductive to tumor growth, invasion and progression directly or indirectly. But, in invasive ductal carcinoma bFGF expression did not yield significant difference in different grades of tumor. It is not clear yet at what stage during malign transformation of breast epithelium bFGF is stimulated and what is the exact stimulator.

It is known that tumor is unable to grow beyond 1–2 milimeters without neovascularization. Angiogenesis is a complex multi-step biologic process that is necessary but not sufficient for tumor growth and molecular mechanism is not totally known. As might be expected, only one angiogenic growth factor is insufficient to initiate angiogenesis. In a group of studies, it was demonstrated that malignant tumors express multiple pro-angiogenic growth factors that has an important role in both tumor growth and angiogenesis.[18–20] Additionally, angiogenesis is regulated at least in part with genetical factors and altered local environmental conditions, such as hypoxia.[18,21] But, we also know that there is a balance between angiogenic growth factors and endogen inhibitors to control angiogenesis. The mechanisms leading to the alteration of the balance between positive and negative modulators of angiogenesis are only partially known.[19,22,23] We were unable to find direct involvement between bFGF expression and angiogenesis, additionally no significant association was found between bFGF expression and tumor size, tumor grade and MVD. But we have shown that MVD is increased in poorly differentiated tumor. Additionally in our study vascular hot spot areas and stronger bFGF staining were closer or overlapped at the peripheral infiltrating border tumors. Verhoeven *et al* supported our study that higher proliferative activity using Ki-67 were at the periphery of invasive tumor.[24] This may be candidate that bFGF have a potent role for both angiogenesis and tumor proliferation demonstrated at the growing edge of tumors.

In a group of studies, increased vasculogenesis was found in the preinvasive stage of tumor, even very early in the process of transformation potentially before histopathologically changes have occurred and certainly by the preinvasive stage of disease even in usual hyperplasia.[3,12,15,22,25–29] But future investigations are needed to explain at what stage during malignant transformation of breast epithelium begins to express bFGF to stimulate tumor growth. Perhaps some genetical changes occur and angiogenic growth factor expression is upregulated in cancer cells at preinvasive dysplastic stage of breast carcinoma, and continues to expression in the invasive stage of tumor growth and metastasis.

Apart from the reports that support growth stimulation and angiogenetic effect of bFGF,

discordantly in a group of studies it was revealed that non-malignant breast cells expressed higher levels of FGF mRNA compared to.epithelial cells with malignant transformation which expressed no or lower levels of bFGF status.[4,31,32] Additionally, Yiangou *et al* showed that reduced mRNA expression in breast carcinoma was associated with poor prognosis suggesting loss of bFGF staining may be related greater liability possibly due to lack of binding to protegycans.[32]

In our study, it is also worthy of note that bFGF expression in tumor cells was not closely related to established prognostic parameters. It seems that angiogenesis is independent of ER, PR status and appears to be regulated by nonendocrin pathways as reported previously.[20] Various signalling pathways may regulate ER expression in breast cancers. c-erbB-2 is an epidermal growth factor receptor family with tyrosine kinase activity, and determined to be a negative prognostic factor for breast carcinoma.[32,33] Linderholm *et al* found in their report that expression of c-erbB-2 was related with lower expression of bFGF and have shown over expression of c-erbB-2 to be related to be a negative prognostic factor in lymph node positive patients.[32] Although we failed to show any significant relation between c-erbB-2 expression and other prognostic parameters, but found higher c-erbB-2 positivity in stronger bFGF expressed tumors.

In conclusion, we have shown that the content of bFGF in invasive ductal carcinoma is markedly increased compared to normal tissue implying an involvement of bFGF in breast carcinogenesis, and we have found that poorly differentiated cancers have increased MVD, which would enhance their response to bFGF but bFGF expression does not related with the differentiation of tumor. But lack of correlation between bFGF and MVD suggest that bFGF alone is not a key regulator of angiogenesis. Although the presence of increased growth factor concentrations in the cancers is consistent with paracrine stimulation of growth and invasion, it does not prove that such stimulation is essential for tumor progression.

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