

BRIEF REPORT

Localization and hypersecretion of nerve growth factor in breast phyllodes tumors: Evidence from a preliminary study

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Abstract

Background: The pathophysiology of the breast phyllodes tumors is uncertain. Currently, wide surgical removal is the only available treatment option. The histopathological diagnosis of phyllodes tumors is often confused with that of fibroadenomas due to a striking histological resemblance.

Aim: To identify a distinctive biomarker for phyllodes tumors of the breast.

Methods and Results: Fresh human breast tissue was obtained from surgically excised breast phyllodes and fibroadenoma tumors (test), breast cancer (positive control) and normal breast tissue (negative control). Immunohistochemistry and Sandwich ELISA were performed for the detection of nerve growth factor (NGF) in test and control tissues. A marked difference in NGF expression was detected in phyllodes tumors compared to fibroadenomas. The maximum NGF expression was observed in phyllodes tissue followed by cancer tissue, and the least expression in fibroadenomas (3-5 times less than in phyllodes; comparable with normal breast tissue).

Conclusion: NGF secretion by a benign breast tumor is not known in literature. This study reports abundant NGF secretion by breast phyllodes, raising the possibility of its potential role in tumor pathogenesis and progression that can be exploited therapeutically. Additionally, NGF may be used as a distinct biomarker of phyllodes tumors, for differentiating them from fibroadenomas during histopathology.

KEYWORDS

breast cancer, fibroadenoma, histopathology, nerve growth factor, phyllodes tumors

1 | INTRODUCTION

Phyllodes are rare fibro-epithelial tumors of the breast with an incidence of less than 1% of which malignant phyllodes account for 10%-

20% of the tumors.¹ The phyllodes tumors show high potential for growth, recurrence and malignant transformation, being sub-classified into three categories: benign, borderline and malignant.² Lymph node involvement and distant metastasis are rare but known in malignant cases.

Knowledge about phyllodes tumorigenesis is quite patchy and scarce in available literature. No biomarker or drug target has yet been identified for these tumors. This article reports for the first time, new

The preliminary findings of this study were presented to 11th European Breast Cancer Conference (EBCC-11), 2018 and were published as an abstract in the European Journal of Cancer (*Euro J Can.* 2018; 92; S142).

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evidence of hypersecretion of nerve growth factor (NGF) by phyllodes tumors. NGF is known to be secreted exclusively by cancer cells (including breast cancer), and suspected to be involved in their etiogenesis and tumor progression.^{3,4} Excessive secretion by benign breast phyllodes tumors as observed in our study implies NGF involvement in the genesis and progression of these tumors. We also propose a potential diagnostic value for NGF as a biomarker to differentiate benign phyllodes tumors from fibroadenomas. These two benign growths share a very close histological resemblance that leads to frequent errors in histopathological diagnosis.^{5,6}

2 | MATERIALS AND METHODS

2.1 | Materials

The test samples of benign phyllodes and fibroadenoma (2 cases each) breast tumors were obtained from the fresh surgical specimens removed from the clinically and histopathologically diagnosed cases in women. The positive controls (2 cases) and the single negative control were taken from established cases of malignant (intra-ductal carcinoma) and normal breast tissue, respectively. The controls were chosen based on their known NGF secretory status, as reported in the literature.^{3,4}

2.2 | Methods

2.3 | Immunohistochemistry (IHC)

IHC was performed on cryosections (thickness: 12–14 μm) of tissue samples fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 6–8 hours at 4°C, using the standard indirect streptavidin-biotin-peroxidase complex method. The sections were incubated with primary antibodies against NGF (dilution: 1 $\mu\text{g}/\text{mL}$, Abcam Plc., UK, rabbit polyclonal) for 48 hours, followed by incubation in anti-rabbit secondary antibody (dilution: 1 $\mu\text{g}/\text{mL}$) and avidin-biotin peroxidase (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, California). The reactions were developed by treating sections with 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich Corporation, St. Louis, Missouri). The core biopsy tissue of the breast cancer and normal breast tissue were used as external positive and negative controls, respectively. The internal negative control was the tumor tissue without applying primary antibody. Hematoxylin was used as a counter-stain in fibroadenoma cases to see histological details. The images were captured with a digital camera under an optical microscope (Leica DM6000B bright-field microscope) using software (Leica Application Suites, Version 3.4.1; Leica Microsystems, Switzerland).

2.4 | Enzyme-linked immunosorbent assay (ELISA)

ELISA (human beta NGF, sensitivity <14 pg/mL, range 6.86 pg/mL to 5000 pg/mL; catalog number: ab99986, Abcam Plc., UK) was applied

on test and control samples for quantification of NGF. Fresh surgically removed tissue samples were preserved with isopentane at -80°C . The tissues were thawed, weighed and homogenized in homogenization buffer (10 mM Tris, 150 mM NaCl, 0.25% sodium deoxycholate and protease inhibitors). Homogenates were centrifuged at 10000 rpm at 4°C, following which the supernatant was collected for total protein estimation by Bradford method and equilibration using assay buffer. NGF competitive inhibition ELISA was performed using the antibody pre-coated plate, by following the protocol provided with the kit.

3 | DATA ANALYSIS

IHC images were analyzed using ImageJ software (NIH) for assessment of staining intensity in specific tissue components and overall scoring (Nil [No expression detected, score = 0], + [weak expression, score = 1], ++ [moderate expression, score = 2], +++ [intense expression, score = 3]). ELISA test was performed in duplicates (2X1). The readings of the test were taken at 450 nm in an ELISA plate reader and the data analyzed for final NGF concentration in the samples, using the standard curve values derived from the recombinant NGF provided with the kit (concentration was expressed in ng/50 μL). IHC staining intensity scores and ELISA NGF concentration scores were matched for the cases and their mean values were compared between test and control groups.

4 | RESULTS

4.1 | Immunohistochemistry (IHC)

Intense NGF expression was observed in test samples obtained from cases of benign phyllodes, comparable with that seen in breast tissue collected from cases of intra-ductal carcinoma (IDC, positive control). NGF staining was observed in all tissue components of phyllodes tumors (ductal layers, stroma, vessels) (Figure 1, Table 1).

However, NGF expression was almost absent in test samples obtained from cases of fibroadenoma (Figure 1, Table 1). No significant NGF expression was detectable in the components of the normal breast tissue (external negative control). No tissue staining was found in internal negative control (without applying primary antibody) (Figure S1a and b).

4.2 | Enzyme-linked immunosorbent assay (ELISA)

Quantification of NGF secretion gave readings that matched with IHC scores for all the test and control samples. The highest NGF levels were observed in phyllodes tumors, followed by breast carcinoma (CA). The levels were low and quite comparable in tissue specimens obtained from fibroadenomas and normal breast (Figure 2).

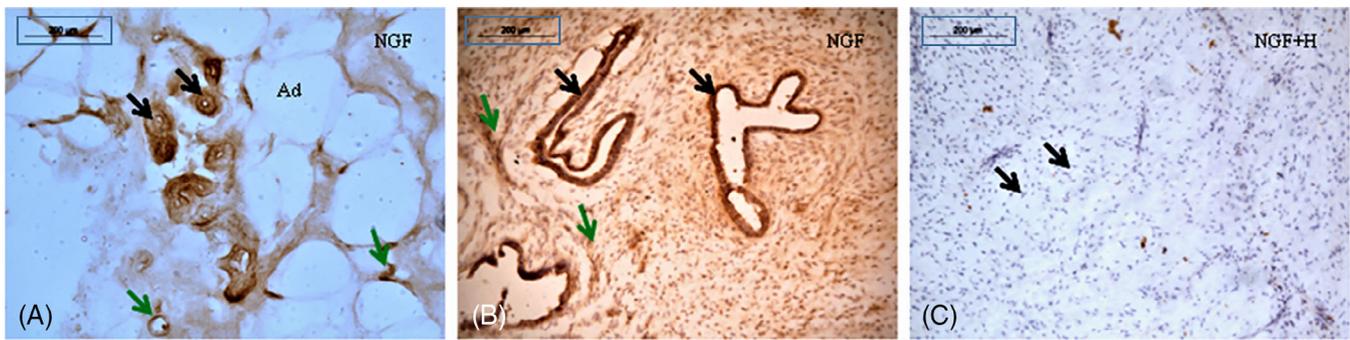


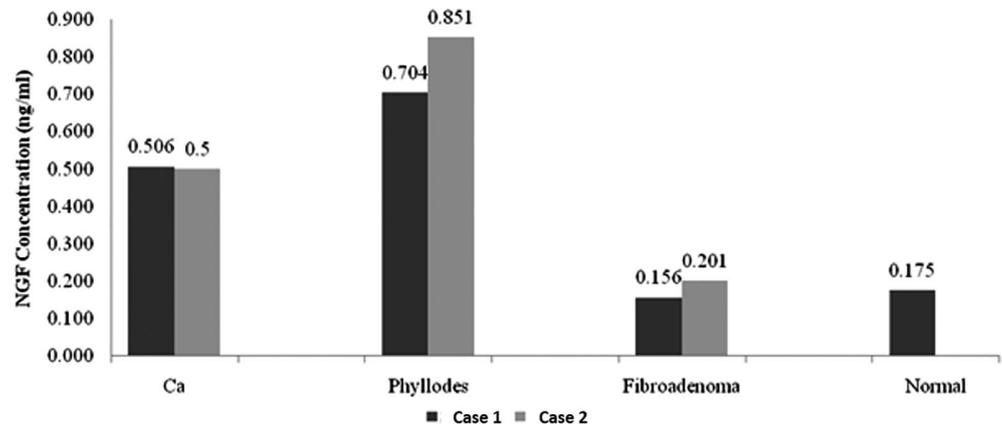
FIGURE 1 Immunohistochemical localization of NGF in breast tumors. A, CA breast (IDC), intense NGF expression in ductal epithelium (black arrows) and small vessels (green arrows), Ad—adipocyte. B, Phyllodes tumor, intense expression in ductal epithelium (black arrows) and stroma (green arrows). C, Fibroadenoma, negligible expression, tissue counterstained with hematoxylin (H) to show nuclei of fibrocytes, characteristic of fibroadenoma (black arrows). (Scale bar, resolution 200 μ m, is marked by bracket at the upper left corner of images)

TABLE 1 NGF expression in breast tumors (as detected by IHC)

Tissue components	CA Breast	Phyllodes	Fibroadenoma	Normal breast tissue
Duct	+++	+++	nil	nil
Stroma	++	+++	nil	nil
Adipocytes	++	+++	nil	nil
Small vessels	+++	+++	nil	nil
Total score	10	12	0	0

Note: Nil (No expression detected, score = 0), + (weak expression, score = 1), ++ (moderate expression, score = 2), +++ (intense expression, score = 3), CA (carcinoma), IHC (Immunohistochemistry).

FIGURE 2 NGF expression in breast tumors (as detected by ELISA). Ca—breast cancer, Case 1 and 2 refers to the tissue samples collected from separate cases



5 | DISCUSSION

NGF is known for its growth-inducing potential and has been implicated for tumor progression in breast CA.⁴ Although NGF has not been directly implicated in the pathogenesis of phyllodes tumors of the breast so far, a thorough literature survey revealed that certain molecules and pathways reported for these tumors can be structurally or functionally linked with NGF signalling.⁷⁻¹⁰ We observed a generalized over-expression on IHC in all tissue components of phyllodes tumors (ductal layers, stromal components, vessels) and negligible expression in those of fibroadenoma (Figure 1, Table 1). Quantitative estimation by ELISA revealed NGF levels in benign phyllodes tumors

to be approximately 1.5 times those observed in CA breast and 3-5 times those seen in fibroadenoma. This singular NGF over-expression by benign phyllodes tumors of the breast that has been noted in this study, points towards its possible role in tumorigenesis and tumor progression, and suggests the use of this molecule as a potential drug target.¹¹

Jardim et al. reported activating mutations for N-RAS oncogene and intense expression of Protein Kinase B (Akt) and mammalian target of rapamycin (mTOR) in malignant phyllodes with a concomitant over-expression of phosphoinositide 3-kinase (PI3K).⁷ Tyrosine kinase A (TrkA) is a high affinity receptor for NGF that mediates growth and proliferation. N-RAS is an oncogene which is thought to be an



essential molecule for executing NGF-TrkA mediated PI3K/Akt/mTOR activation and NGF mediated differentiation of PC12 cells.⁸ Coincidentally, PI3K/Akt/mTOR is the chief pathway mediating NGF signaling involved in growth and proliferation.⁹ Furthermore, N-RAS has the chromosomal location 1p13.2 (Gene ID: 4893, NCBI, 2016) very close to that of NGF which is 1p13.1 (Gene ID: 4803, NCBI, 2016), suggesting the possibility of a functional linkage between the two genes.¹⁰ N-RAS mutations are common in various tumors and a point mutation of the N-RAS gene may cause constitutive activation of the N-RAS dependent signaling pathways.¹²

Several researchers have also found a huge genomic instability in phyllodes tumors which includes loci of 1p and 1q among other chromosomes.^{7,13} TrkA, the high affinity NGF receptor that mediates growth and proliferation is located on chromosome 1q 21-22. Moreover, keeping in mind the above-mentioned proximity of gene loci for NGF (1p13.1) and N-RAS (1p13.2), the instability of either of these loci may influence NGF expression and signaling in phyllodes tumors. Hence the role of this proximity of loci and concomitant genomic instability in the pathogenesis of phyllodes tumors needs to be examined.

5.1 | Differentiation of phyllodes from fibroadenomas

Phyllodes tumors are difficult to differentiate histologically from fibroadenomas.^{2,6} Although pronounced stromal cell activation is characteristic of phyllodes, sometimes it becomes truly puzzling for the pathologist to make an accurate diagnosis in cases where the tumors have intermediate features.^{5,6} The unambiguous NGF overexpression by phyllodes (3-5 times more than that by fibroadenoma) that was observed in the present study, makes NGF a highly likely candidate for a suitable biomarker of breast phyllodes tumors.

5.2 | Limitations and further considerations

The sample size of this study being small, further studies with adequate sample size are necessary to validate the results. Future research may elucidate the dynamic interaction of NGF signaling pathway molecules in breast phyllodes tumors. It would be particularly relevant and rewarding to establish beyond doubt, the diagnostic efficacy and specificity of NGF as a potential biomarker for phyllodes tumors of the breast.

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ETHICAL STATEMENT

The ethical clearance was received from the institutional human ethics committee of All India Institute of Medical Sciences (AIIMS), New Delhi (IEC No: NP-325/2013RP03/2013), and consents of the patients were sought as per rules, prior to conducting this study.

CONFLICT OF INTEREST

Authors declared "no conflict of interest."

AUTHOR CONTRIBUTIONS

Khursheed Raza: Data curation; formal analysis. Tapas C. Nag: Data curation; formal analysis; supervision; writing-review and editing. Anurag Srivastava: Data curation; supervision. Ritu Sehgal: Funding acquisition; supervision; writing-review and editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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