

# BigH3 protein expression as a marker for breast cancer

GLORIA M. CALAF<sup>1,2</sup>, C. ECHIBURÚ-CHAU<sup>2</sup>, Y.L. ZHAO<sup>1</sup> and TOM K. HEI<sup>1</sup>

<sup>1</sup>Center for Radiological Research, Columbia University Medical Center, New York, NY, USA;

<sup>2</sup>Instituto de Alta Investigación, Universidad de Tarapacá, Arica, Chile

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**Abstract.** The current hypothesis of tumorigenesis in humans suggests that cancer cells acquire their hallmarks of malignancy through the accumulation of advantageous gene activation and inactivation events over long periods of time. For breast cancer development, this multistep process may manifest itself as a sequence of pathologically defined stages. It is widely held that breast cancer originates at the pre-malignant stage of atypical ductal hyperplasia, progresses to the preinvasive stage of ductal carcinoma *in situ*, and culminates in the potentially lethal stage of invasive ductal carcinoma. Tumor grade has been a highly valuable prognostic factor for breast cancer, and high-grade ductal carcinoma *in situ* lesions are associated with poor clinical outcome. The aim of this work was to investigate the BigH3 protein expression changes associated with various stages of breast cancer progression in comparison to benign specimens using tissue microarray technology. Pathological characteristics of breast tissues ranged from benign lesions to breast cancers either of lobular or ductal carcinomas in origin, and included *in situ* ductal carcinomas, lobular carcinomas, infiltrating ductal carcinomas, carcinomas, scirrhous carcinomas, adenocarcinomas and infiltrating colloid carcinomas. BigH3 protein expression was analyzed by immunohistochemistry in 192 cases of breast tumors. Results indicated a decrease in BigH3 protein expression from benign tissues to *in situ* ductal carcinoma, lobular carcinoma, infiltrating ductal carcinomas, carcinomas, scirrhous carcinoma, adenocarcinomas to infiltrating colloid carcinomas. We observed that the benign tissue had a 23-fold increase in BigH3 protein expression compared to the infiltrating colloid carcinoma which was the most malignant tissue analyzed. In summary, these studies confirmed the suppressor effect of the *BigH3* gene expressed as protein expression in those processes related to the progression of breast tumorigenesis. We conclude that this protein can be used as a marker for breast cancer progression.

## Introduction

It is increasingly apparent that hormonal actions are mediated through the local synthesis of growth factors, including epidermal growth factor (1) transforming growth factor  $\alpha$  (2) and transforming growth factor  $\beta$  (TGF- $\beta$ ) (3), and clinical studies of breast cancer have shown that perturbations in the normal functioning of the TGF- $\beta$  system occur at several distinct stages during disease progression (4). Studies have demonstrated that overexpression of TGF- $\beta$ 1 *in vivo* can markedly suppress mammary tumor development (5).

Currently, there are three mammalian isoforms, TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3 (6). TGF- $\beta$ 1 (BigH3) is a 25-kDa disulfide-linked polypeptide dimer. It has various names such as BigH3, BIGH3, BIGH3,  $\beta$ ig-h3,  $\beta$ taig-h3 and transforming growth factor- $\beta$ -induced (TGFBI) (7). It is an essential constituent of the extracellular matrix and elicits numerous changes in cellular behavior, including the differentiation of epithelial cells (8,9), modification of proliferation in a wide variety of cell types (8-15), inhibition of angiogenesis (16,17), deposition of extracellular matrix components (16-20), alteration of basement-membrane-degrading enzyme production and secretion (16,21-24), and changes in cellular-adhesive properties (17,19,20).

BigH3 was first detected in a human lung adenocarcinoma cell line after stimulation by TGF- $\beta$  (25). BigH3 has been immunohistochemically found in human tissues such as corneal, skin, lung, bone, bladder, and kidney (26). In addition, BigH3 is involved in certain human diseases such as corneal dystrophies (27,28), melorheostosis, osteogenesis (29), diabetic angiopathy, atherothrombosis and restenosis (30).

The current hypothesis of tumorigenesis in humans suggests that cancer cells acquire their hallmarks of malignancy through the accumulation of advantageous gene activation and inactivation events over long periods of time (31). For breast cancer development, this multistep process may manifest itself as a sequence of pathologically defined stages. Tumor grade has been a highly valuable prognostic factor for breast cancer, as poorly differentiated, high-grade ductal carcinoma *in situ* (DCIS) lesions are associated with significantly poorer clinical outcome (32-34).

Immunohistochemical methods are routinely used in surgical pathology. The tissue microarray technique was invented by Kononen *et al* (35) and is a promising tool in modern pathology, with almost an infinite number of applications (36). BigH3 has been implicated in both mammary development and mammary tumorigenesis (37-41).

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*Correspondence to:* Dr Gloria M. Calaf, Instituto de Alta Investigación, Universidad de Tarapacá, Antofagasta #1520, Arica, Chile  
E-mail: gmc24@columbia.edu

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The aim of this work was to investigate changes in BigH3 protein expression associated with breast cancer progression in comparison to benign specimens by using tissue microarray technology.

## Materials and methods

**Breast cancer specimens in tissue microarray.** BigH3 protein expression was analyzed to determine its value as a marker between benign tissue and malignant breast cancer disease. A commercially available slide with tissue microarray sections was used in this study (Clinomics Biosciences, Frederick, MD) to evaluate 192 patients with breast cancer undergoing primary surgery. Each tissue microarray was retrospectively classified for histological types and tumor grading according to the World Health Organization classification.

**Immunohistochemical techniques.** Protein expression was evaluated by peroxidase immunochemical staining. Studies were performed as described previously (42). The cells were incubated with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min in order to block endogenous peroxidase, washed twice with a buffer solution, and fixed with buffered paraformaldehyde in PBS, pH 7.4, at room temperature. Subsequently, the slide was then covered with normal horse serum for 30 min at room temperature. The tissue microarray was then washed once and incubated with BigH3 primary antibody (kindly provided by Dr Paul C. Billings) at a 1:200 dilution overnight at 4°C. Then sections were incubated with peroxidase-conjugated mouse IgG (Santa Cruz Biotechnology, CA) for 3 h. The protein expression in the tissue microarray was determined by using the avidin-biotin-horseradish immunoperoxidase peroxidase complex (Standard ABC kit; Vector, Burlingame, CA, USA). 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich Chemical Co., Milwaukee, WI) was used as a chromogen for 5 min. For negative controls, duplicate samples were immunostained without exposure to the primary antibody or substituted with pre-immune serum. The tissue microarray was rinsed, dehydrated in ethanol/water baths, rinsed in xylene and mounted.

**Computerized image assessment.** The percentage of tumor cells with definite moderate to intense nuclear immunoreactivity was recorded. To define the BigH3 protein expression level the extent of peroxidase staining was determined in the samples of the tissue microarray. Data generated from this slide were analyzed by using an Olympus CX31 (Rochester, NY, USA) binocular microscope (x40) connected with a Motic MCCamera (2.0 megapixel; MC2001 interface). Motic Image Plus 2.0 ML software was used. Paint Shop™ Pro® was used to measure the BigH3 protein expression level. The freehand selection tool was used to select the sample area to be measured. An arbitrary threshold of 125 was applied to the selected area. Then, this area was inverted to measure the mean of the lightness channel. The value obtained in this procedure was used as a relative grade of luminescence to quantify the samples and to generate the graphics. The data were expressed as the average ± standard error (SE) of the mean of the relative grade of luminescence obtained from each disease.

Table I. Clinical and pathological features of breast specimens present in the tissue microarray.

Parameters	No. of cases (%)
Sex	
Female	192 (100.00)
Age (years)	
≤60	67 (34.90)
61-70	37 (19.27)
71-80	45 (23.44)
81-90	23 (11.98)
Unknown	20 (10.42)
Stage	
0	11 (5.73)
I	46 (23.96)
II	84 (43.75)
III	34 (17.60)
IV	5 (2.60)
Unknown	12 (6.25)
Pathological type	
Benign specimen	12
<i>In situ</i> ductal carcinoma	2
Ductal adenocarcinoma	4
Adenocarcinoma	6
Carcinoma	8
Lobular carcinoma	4
Infiltrating ductal adenocarcinoma	151
Scirrhou carcinoma	3
Infiltrating colloid carcinoma	2

## Results

This study examined BigH3 protein expression in breast tumor specimens found in the tissue microarray in comparison to benign lesions to assess its potential as a marker for breast cancer progression. An immunochemical analysis was performed on slide sections from breast cancers in order to compare differences in BigH3 protein expression between normal and tumor cells and to visualize the cellular localization of this marker. A global survey of BigH3 protein expression levels in the breast was performed on a slide since these formats of tissue analysis allow rapid visualization of protein expression and a wide comparative analysis.

Table I lists the clinical and pathological features of the breast cancer cases included in the tissue microarray. Such features were sex (all female), age (with a median 64, fluctuating from 33 to 88 years; mean, 63.98) and stage of the disease. Disease stages ranged from 0 to I, II, III and IV, and pathological characteristics of breast tissues ranged from benign lesions to breast cancer considered to be lobular or ductal carcinomas in origin: *in situ* ductal carcinomas, lobular carcinomas, infiltrating ductal carcinomas, carcinomas as well as scirrhou carcinomas, adenocarcinomas and infiltrating colloid carcinomas.

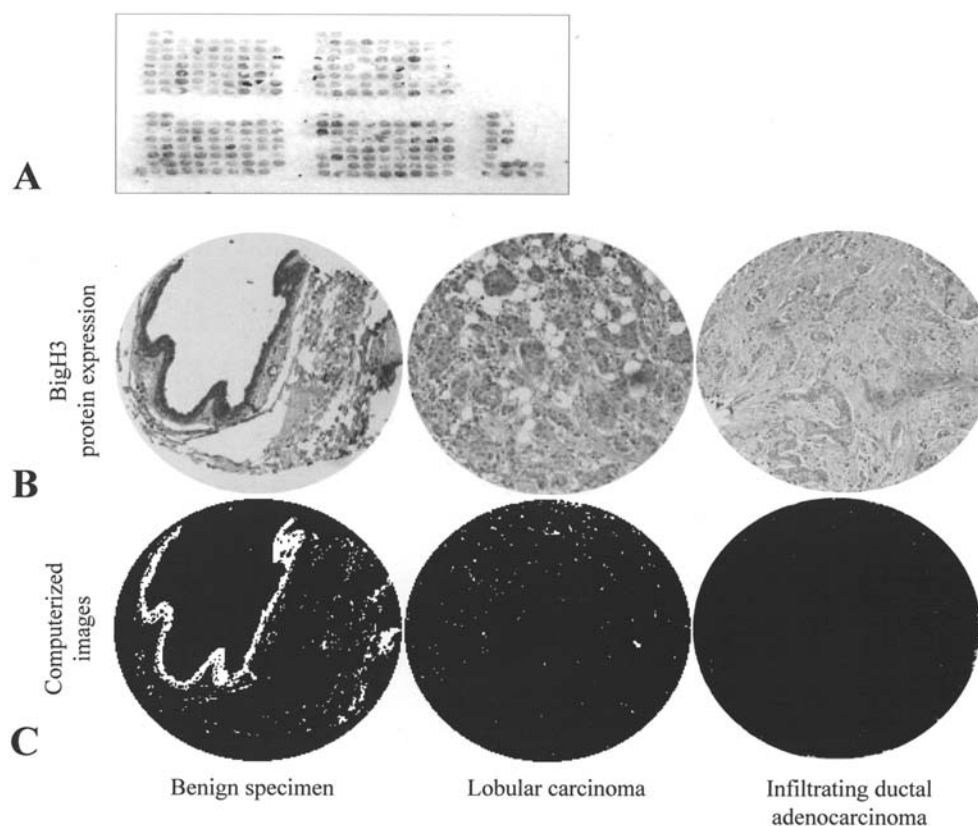


Figure 1. (A) Immunohistochemical images that represent BigH3 protein expression in the tissue microarray containing different histological types of benign and malignant breast tissue specimens. (B) Immunohistochemical images of BigH3 protein expression present in the array of the slide containing benign and malignant breast tumors. (C) Computerized images of BigH3 protein expression which correspond to benign and breast cancer specimens of lobular carcinoma and infiltrating ductal adenocarcinoma.

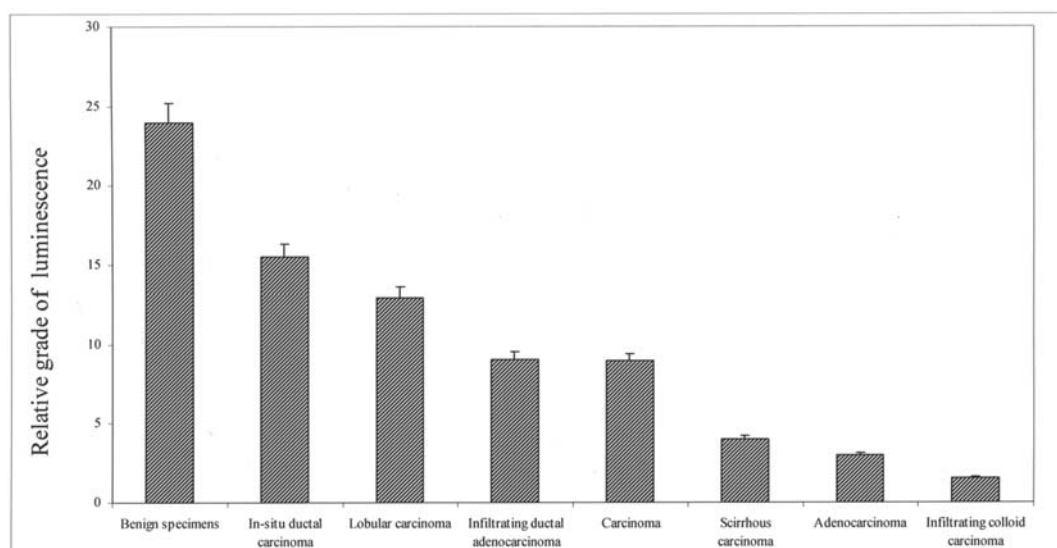


Figure 2. The bars represent the average and standard error of an arbitrary unit of relative luminescence of BigH3 protein expression in benign lesions and breast tumors found in the tissue microarray.

BigH3 protein expression was analyzed by immunohistochemistry in a slide containing 192 cases of breast tumors. Fig. 1 shows the tissue microarray used (A), the BigH3 protein expression (B) and the computerized images where BigH3 protein expression was studied (C) corresponding to benign and breast cancer specimens of

lobular carcinoma and infiltrating ductal adenocarcinoma. Fig. 2 graphically depicts the results of the average and standard error of BigH3 protein expression in the different specimens. The results indicated a significant ( $P < 0.05$ ) decrease in BigH3 protein expression from benign tissues to *in situ* ductal carcinoma, lobular carcinoma, infiltrating



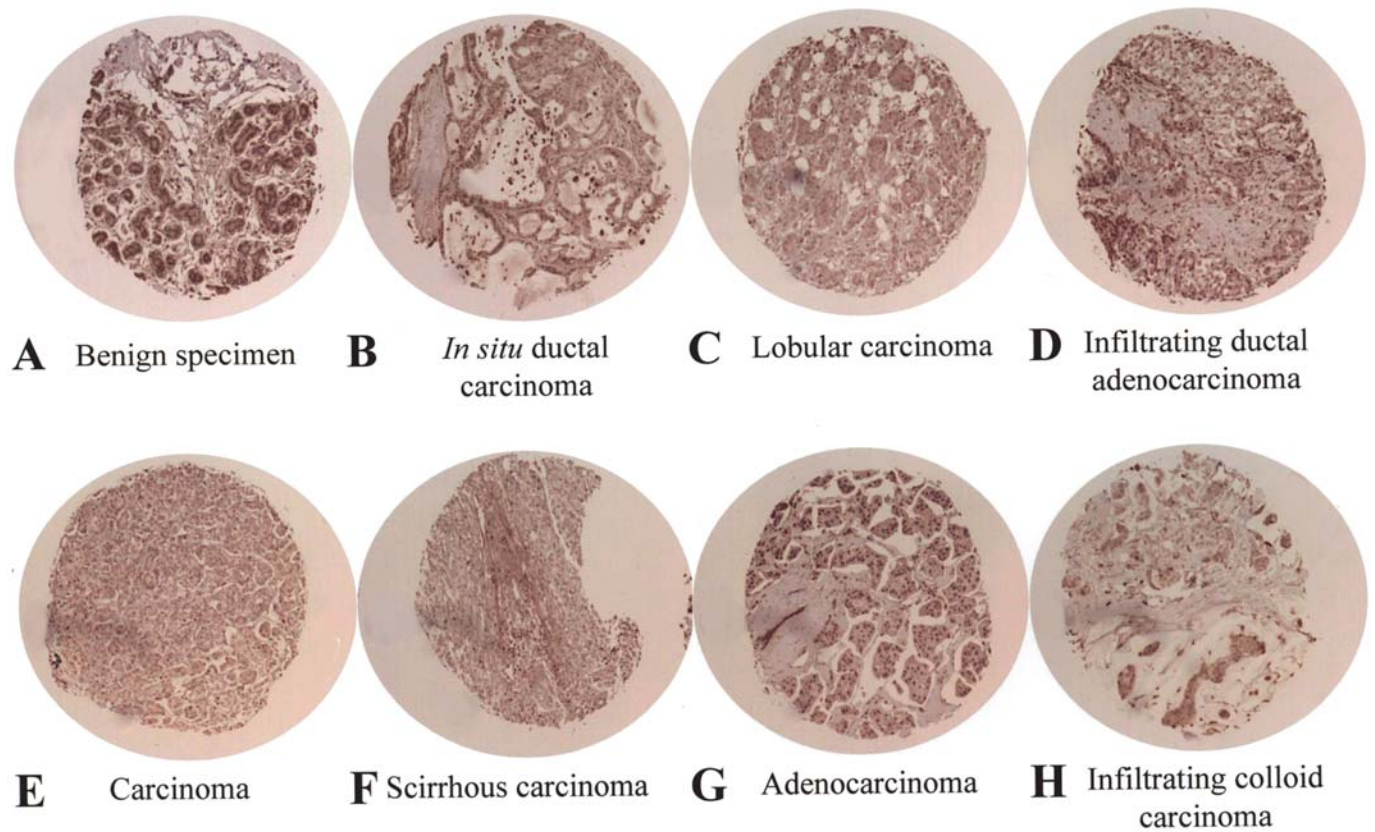


Figure 3. Representative immunohistochemical images of BigH3 protein expression in the human breast tissue microarray for different histologic types of (A) benign lesions, to breast cancers considered to be lobular or ductal carcinomas in origin: (B) *in situ* ductal carcinoma, (C) lobular carcinoma, (D) infiltrating ductal adenocarcinoma, (E) carcinoma, (F) scirrhous carcinoma, (G) adenocarcinoma and (H) infiltrating colloid carcinoma.

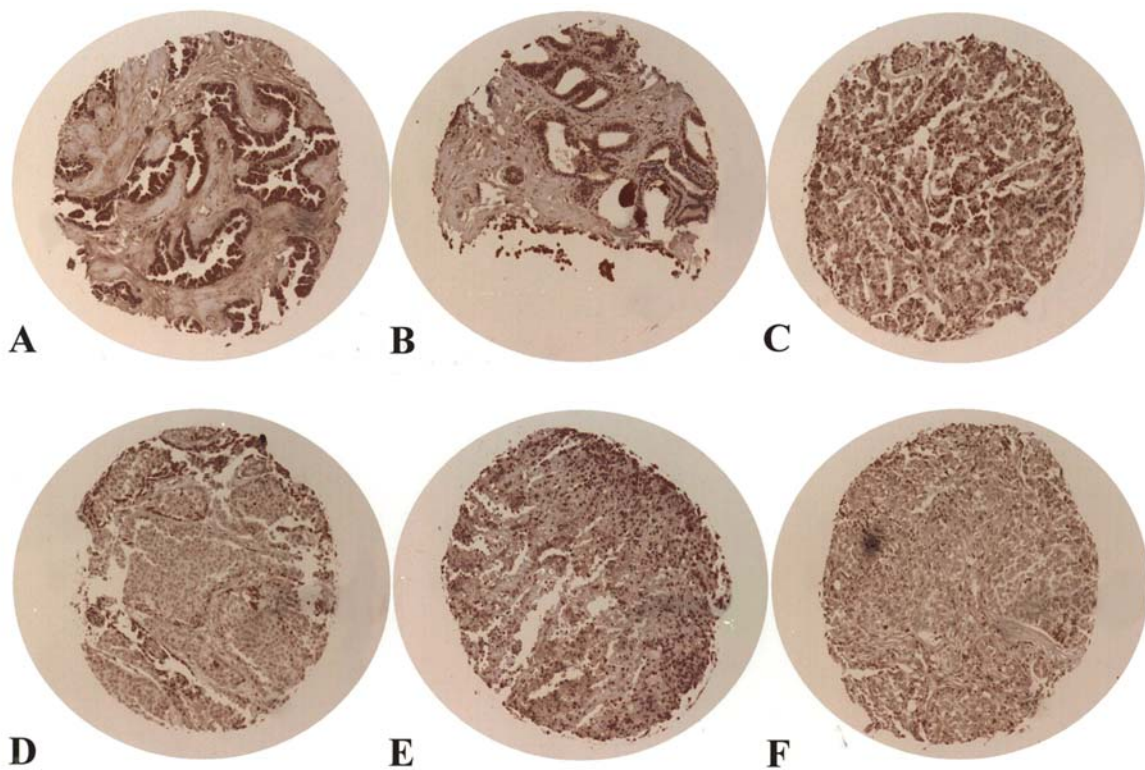


Figure 4. Representative immunohistochemical images of BigH3 protein expression among various infiltrating ductal carcinoma samples in the human breast tissue microarray (A-F).

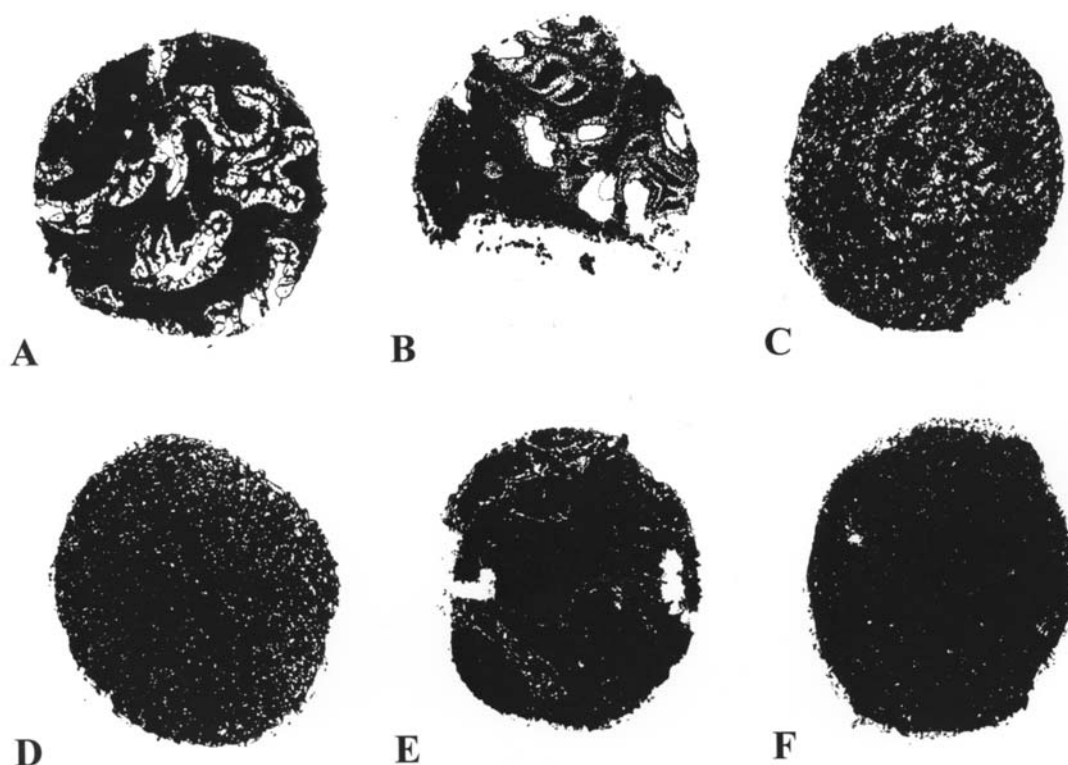


Figure 5. (A-F) Computerized immunohistochemical images of BigH3 protein expression in the human breast tissue microarray of the progression of breast cancer in relation to infiltrating ductal adenocarcinoma. The values indicated by the program fluctuated from 51 to 5: A=51; B=40; C=30; D=19; E=11; and F=5.

ductal carcinomas, carcinomas, scirrhous carcinoma, adenocarcinomas to infiltrating colloid carcinomas. It was observed that the benign tissue had a 23-, 15-, 13-, 9-, 9-, 4- and 3-fold increase in BigH3 protein expression when compared with infiltrating colloid carcinoma. Representative images of BigH3 protein expression in the above breast samples are shown in Fig. 3 where protein expression intensity can be observed.

Fig. 4 shows the BigH3 protein expression in the progression of breast cancer in relation to infiltrating ductal adenocarcinoma. Representative images of BigH3 protein expression indicate a decrease in the intensity of this marker in this type of cancer (A-F) when the malignancy increased. Fig. 5 shows the computerized images of these samples. The values indicated by the program fluctuated from 51 to 5 (A=51; B=40; C=30; D=19; E=11; F=5). The intensity of the BigH3 protein expression in infiltrating ductal carcinomas decreased from stage I to stage IV. In stages I to IV, the respective values of 12, 9, 7 and 4 were found in relation to the relative grade of luminescence.

## Discussion

This study analyzed BigH3 protein expression in breast tumor specimens found in the tissue microarray to assess its potential value as a marker for breast cancer progression. An immunochemical analysis was performed to visualize this marker. Pathological characteristics of breast tissues ranged from benign lesions to breast cancer considered to be lobular or ductal carcinomas in origin, and were *in situ* ductal carcinomas, lobular carcinomas, infiltrating ductal carcinomas,

carcinomas as well as scirrhous carcinomas, adenocarcinomas and infiltrating colloid carcinomas.

It is well established that the overall development of the mammary gland is regulated by a complex interplay of ovarian, adrenocortical and pituitary hormones (4). However, it is becoming increasingly apparent that many of the diverse effects of these hormones are mediated through the local synthesis of growth factors, including epidermal growth factor (1), transforming growth factor  $\alpha$  (2), fibroblast growth factor (1,44), transforming growth factor  $\beta$  (TGF- $\beta$ ) (3), insulin-like growth factors (45) and colony-stimulating factor 1 (46).

Studies have demonstrated that the protein TGF- $\beta$ 1 (TGF- $\beta$ -induced gene-human clone 3) is secreted in a latent form in mouse mammary gland development (47). It shows different patterns of expression in the mouse mammary gland (48), suggesting that this peptide plays an important role. Direct delivery of exogenous TGF- $\beta$ 1, and their isoforms TGF- $\beta$ 2 and TGF- $\beta$ 3 into mammary glands, inhibits proliferation of end-bud cells and ductal elongation (48,49). However, their administration does not inhibit proliferation of alveolar cells during pregnancy (5).

BigH3 protein expression was analyzed by immunohistochemistry in 192 cases of breast tumors. The results indicated a decrease in BigH3 protein expression from benign tissues to *in situ* ductal carcinoma, lobular carcinoma, infiltrating ductal carcinomas, carcinomas, scirrhous carcinoma, adenocarcinomas to infiltrating colloid carcinomas. The benign tissue had a 23-fold increase in BigH3 protein expression compared to the infiltrating colloid carcinoma which was the most malignant tissue analyzed. The intensity



of the BigH3 protein expression in infiltrating ductal carcinomas decreased from stage I to stage IV. In stages I to IV, the respective values of 12, 9, 7 and 4 were found in relation to the relative grade of luminescence.

It is widely known that breast cancer originates at the premalignant stage of atypical ductal hyperplasia (ADH), progresses to the preinvasive stage of ductal carcinoma *in situ* (DCIS), and culminates in the potentially lethal stage of invasive ductal carcinoma (IDC) (50). This linear model of breast cancer progression has been the rationale for the use of detection methods such as mammography in the hope of diagnosing and treating breast cancer at earlier clinical stages (51). However, the stages of DCIS and IDC are heterogeneous with respect to mitotic activity and cellular differentiation both within a tumor and among individual tumors. To further characterize DCIS and IDC with respect to this heterogeneity, several tumor grading systems have been created. Such systems are clinically used to subtype the stages of DCIS and IDC into three tumor grades in which grade I, II, and III lesions correspond to well, moderately and poorly differentiated breast tumors, respectively (32,33).

The present observations demonstrated a downregulation of BigH3 protein expression in breast cancer. It is known that the *BigH3* gene is ubiquitously expressed in various normal human tissues, with the exception of the brain, where there is little or no expression. The results here indicated a progressive decrease in BigH3 protein expression from benign tissues to *in situ* ductal carcinoma, lobular carcinoma, infiltrating ductal carcinoma, carcinoma, scirrhous carcinoma, adenocarcinoma to infiltrating colloid carcinoma suggesting that this protein can be used as a marker for advanced stages in breast cancer.

It was previously found that BigH3 protein expression was markedly decreased in asbestos-induced tumorigenic cells when using the human papillomavirus immortalized human bronchial epithelial (BEP2D) cells. Such results strongly suggested that loss of BigH3 expression is a frequent event in human cancer and causally related to acquisition of the tumorigenic phenotype in asbestos-treated BEP2D cells (52). Furthermore, ectopic expression of the *BigH3* gene in asbestos-induced tumorigenic cells inhibited cell growth *in vitro*, the anchorage-independent phenotype, as well as tumorigenicity in nude mice.

It has previously been shown that downregulation of the *BigH3* gene was also involved in the cellular transformation of human bronchial epithelial cells induced by radiation. Recovery of the *BigH3* gene expression in H522 lung cancer cells lacking endogenous BigH3 protein significantly suppressed their *in vitro* cellular growth and *in vivo* tumorigenicity. The downregulation of BigH3 expression was causally linked to the tumorigenic phenotype of BEP2D cells treated with high-LET  $\alpha$ -particle radiation. A BEP2D cell culture system, a radiation-induced transformation model was established by a single 60-cGy dose of  $^{56}\text{Fe}$  heavy-ion radiation. The high-energy (HZE) heavy-ion radiation when compared to low-LET radiation was more effective in inducing gene mutation, chromosomal aberrations and neoplastic transformation than high-LET  $\alpha$ -particle radiation. The *BigH3* gene was found to be involved in  $^{56}\text{Fe}$  ion-induced tumorigenesis, since the expression levels of the *BigH3* gene in tumorigenic cell lines increased the ability

*in vivo* of tumor suppression through the re-introduction of the *BigH3* gene in tumorigenic cells. Fusion of tumorigenic and control BEP2D cells resulted in the recovery of *BigH3* gene expression to the control level and loss of the tumorigenic phenotype. The expression level of this gene was markedly decreased in three tumorigenic cell lines ( $^{56}\text{FeT1}$ - $^{56}\text{FeT3}$ ) when compared with parental BEP2D cells. Although biologically active TGF- $\beta$ 1 was elevated in two of three tumorigenic cell lines, all these cell lines were resistant to the induction of BigH3 expression when BigH3 expression was analyzed. Such data strongly suggest that downregulation of BigH3 expression resulted from the defect in the TGF- $\beta$ 1 signalling pathway and played a pivotal role in the tumorigenic process induced by  $^{56}\text{Fe}$  heavy-ion radiation (53).

Other studies (53,54) have indicated that the expression of the *BigH3* gene was downregulated or lost in a variety of tumor cell lines derived from lung, prostate, mammary, and kidney tumors. The results showed that the *BigH3* gene was expressed at a relatively high level in normal and immortalized cell lines, whereas it was downregulated in most of the tumor cell lines. Notably, *BigH3* expression was undetectable in three lung cancer cell lines (H522, H810 and H1417) and in one kidney cell line 293T. Gene silencing by CpG island methylation in the promoter region is one of the mechanisms by which tumor suppressor genes are inactivated in human cancers. To unravel the underlying molecular mechanism for this phenomenon, DNA methylation patterns of the *BigH3* CpG island were examined in normal, immortalized, and cancer cell lines derived from lung, prostate, mammary, and kidney tumors. A good correlation was observed between promoter hypermethylation and lost expression of the *BigH3* gene, which was supported by data that indicated demethylation of the promoter by 5-aza-2'-deoxycytidine reactivated *BigH3* and restored its expression in *BigH3*-silenced tumor cell lines (54).

BigH3 is a secreted protein induced by transforming growth factor which has been suggested to modulate tumor formation. Previously, BigH3 expression was studied by immunohistochemistry in 130 primary human lung carcinomas. BigH3 protein was absent or reduced by >2-fold in 45 of 130 primary lung carcinomas relative to the normal lung tissues examined. Several observations demonstrated that downregulation of the *BigH3* gene is a frequent event and related to tumor progression in human lung cancer (54).

In summary, these studies confirmed the suppressor effect of the *BigH3* gene expressed as protein expression in those processes related to the progression of breast tumorigenesis. We conclude that this protein can be used as a marker for breast cancer progression.

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