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Role of Vascular Endothelial Growth Factor Expression in Sub-typing of Breast Carcinoma

***Ahmad M. Ragab Shalaby**

*Oncologic Pathology Department-Damanhour National Medical Institute

The detection of subtypes of breast cancer based on their molecular features has brought new scope in breast cancer work. Some key regulators of angiogenesis and tumor infiltration were evaluated in breast carcinomas of basal phenotype (cytokeratin [CK] 5+). Immunohistochemical analysis with 14 primary antibodies was performed in 200 formalin-fixed, paraffin-embedded samples of invasive ductal carcinomas. CK5 correlated with indicators of poor outcome, including precocious age, high histological grade, lymph node positivity, advanced pathologic stage, negativity for hormonal receptors, and a high proliferative rate (Ki-67 labeling index). CK5 also correlated with vascular endothelial growth factor (VEGF) expression. Considering that VEGF-over-expressing neoplastic mammary cells display increased proliferative activity in vitro regardless of the angiogenic effect of VEGF, the differential expression of VEGF might contribute to the more aggressive behavior of these neoplasms. CK5 correlated with tissue inhibitor of metalloproteinase (TIMP)-1, but not matrix metalloproteinase (MMP)-1, MMP-2, extracellular matrix metalloproteinase inducer, TIMP-2, or plasminogen activator inhibitor, indicating that antiproteolytic stimuli might be preponderant in these neoplasms.

**** Key words:** Breast Cancer, Immuno-histochemistry.

Introduction

Breast carcinoma causes some 20% of cancer deaths among females and has been called "the foremost cancer" in women. Despite all efforts, the age-adjusted death rate from breast cancer in females in the United States has virtually remained stable over the past 30 years, now being about 27 per 100,000.[1-3]

According to the molecular profile, sporadic breast carcinomas can be categorized into at least 5 subtypes: luminal subtype A, luminal subtype B, normal breast-like subtype, HER-2-overexpressing subtype, and basal-like subtype.[1-4] There is a highly significant difference in overall survival and relapse-free survival between these subtypes, with the basal-like and HER-2+ subtypes associated with the poorest prognoses.[2] The basal-like subtype is characterized by expression of the high-weight cytokeratins (CKs) such as CK5.[2, 5] This phenotype is found in 17% to 37% of breast carcinomas[3, 4, 6-9], however, the biologic significance of the differential expression of CK polypeptides in breast carcinomas is unclear.[10]

Tumor progression followed by invasion and metastases occur owing to a complex interaction among several factors produced by the tumor and the response of the host. This process depends overall on the formation of new blood vessels (tumoral angiogenesis).[11] The main stimulus for angiogenesis is the interaction between the vascular endothelial growth factor (VEGF) and its receptor.[12]

Tumor cells must produce some proteinases, particularly; cathepsin D, plasminogen activator factor, and metalloproteinases 1, 2, 3, and 9 to allow the neoformed vessels to infiltrate the stroma.[13] Moreover, there is increasing evidence that these proteinases are activated by a cell surface glycoprotein known as extracellular matrix metalloproteinase inducer (EMMPRIN).[14, 15] In addition to these proteinases, tumor cells also might produce inhibitory proteins, including tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor (PAI).[16] The ability of the neoformed vessels to infiltrate the tissues depends on which proteinases

Correspondence:
Ahmad M. Ragab Shalaby
Oncologic Pathology Department
Address: 1 Ismail Sedky St., Damanhour, El-Behera, Egypt
العنوان: أرض الميرى - بجوار نقابة المحامين معمل د/ أحمد شلبى - دمنهور
Tel: +20453333664, +20101076294, +20453350758
E-mail: ahmad_shalaby20@yahoo.com
ahmad_shalaby20@hotmail.com

or their inhibitors predominate.[14, 15]

Aim of the Work

The aim of this work is to study the relationship between the expression of CK5 and the key regulators of angiogenesis and tumor infiltration in human breast carcinomas and compare these data with the clinicopathologic features of prognostic significance in cases attended to Damanhour National Medical institute, Egypt and recorded in the files of the pathology department during the period from April 2001 to September 2006.

Materials and Methods

The material of this study included a total of 200 cases of breast cancer diagnosed between during the period from April 2001 to September 2006. All were admitted to the Damanhour national medical institute hospital for the sake of pathological diagnosis and treatment. The criterion for selection was based on the histopathologic diagnosis. The cases were selected randomly to represent the 3 histologic grades of not otherwise specified invasive ductal carcinomas according to the Scarff-Bloom and Richardson grading system modified by Elston and Ellis.[17-19] The histologic types of invasive ductal carcinoma selected were as follows: grade I, 64 cases; grade II, 78 cases; and grade III, 58 cases. None of the patients had received treatment before the biopsy procedure. Clinical data were obtained from the medical files. Conventional clinical features were evaluated, including age, menstrual status, tumor size, lymph node metastasis, and pathologic grading.

Immunohistochemical Analysis

Briefly, 3-µm-thick sections were cut from paraffin blocks containing representative tumor samples. Paraffin sections were dewaxed in xylene, rehydrated through a graded alcohol series, placed in 10 mmol/L of citrate buffer, and submitted to heat retrieval using a vapor lock for 40 minutes. After heating, the slides were allowed to cool to room temperature and briefly washed with tris (hydroxymethyl) aminomethane-buffered saline. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 5 minutes. Normal serum was used for 30 minutes to block nonspecific immunoassaying.

Immunohistochemical staining was performed using a standard avidin-biotin peroxidase system (Novostain Super ABC kit, Novocastra, England). The primary antibodies were incubated overnight at room temperature. Following washes in phosphate-buffered saline, biotinylated universal secondary antibody was applied for 30 minutes. The sections then were incubated with the avidin-biotin complex reagent for 30 minutes and developed with 3, 3'-diaminobenzidine tetrahydrochloride in phosphate-buffered saline, pH 7.5, containing 0.036% hydrogen peroxide for 5 minutes. Light Mayer hematoxylin was applied as a counterstain. The slides then were dehydrated in a series of ethanols and mounted with Permount.

Tumors were considered positive for CK5 when at least 10% of the neoplastic cells displayed a distinct brown cytoplasmic staining. Expression was graded in a binary manner for ER (positive, at least 10% of neoplastic cells with nuclear staining), p53 (positive, at least 10% of neoplastic cells with nuclear expression), PR (positive, at least 10% of neoplastic cells with nuclear expression), and VEGF (positive, at least 10% of neoplastic cells with cytoplasmic staining).[20, 21] C-erb-B2, Ki-67, and p27 were scored as described elsewhere.[22-24] EMMPRIN, MMP-1, MMP-2, PAI, TIMP-1, and TIMP-2 were scored as 0 (negative), fewer than 20% of neoplastic cells with cytoplasmic staining, 20% to 50% of neoplastic cells with cytoplasmic staining, and more than 50% of neoplastic cells with cytoplasmic staining.[13]

To quantify the peritumoral microvascular density, capillaries and small venules were highlighted by staining endothelial cells for CD34. Single brown-stained endothelial or clusters of endothelial cells, were counted as individual microvessels. The number of microvessels along the border between cancer nests and the stroma was recorded. The average number of microvessels in the fields scanned was calculated and given as the final microvessel count.[25]

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism software, version 4 (San Diego, CA). Association between CK5 expression and other pathologic variables was determined by using the Fisher exact test (2 groups) or the χ^2 test (≥33 groups). All tests were 2-tailed, and a *P* value of less than .05 was considered significant.

Results

The median age of patients was 54.2 years (range, 25-85 years). Of the 200 patients, 68 were premenopausal, 128 were postmenopausal, and 4 had undergone hysterectomy for gynecological reasons. Thirty two patients had tumors measuring less than 2 cm, while 168 patients had tumors that were 2 cm or larger. The tumors in 58 cases were lymph node-

negative; in 56 cases, 1 to 3 nodes were positive; and in 86 cases, more than 3 nodal metastases were present. In 16 cases, the disease was stage I; in 88 cases, stage II; in 82 cases, stage III; and in 14 cases, stage IV.

The positivity rates for ER, PR, and p53 were 52%, 46%, and 35%, respectively. Of the 200 Ductal carcinomas (figure 1, 2, 3, 4), 54 were positive for c-erb-B2. Staining for Ki-67 was as follows: staining in less than 10% of neoplastic cells, 106; staining in 10% to 50% of neoplastic cells, 60; and staining in more than 50% of neoplastic cells, 34.

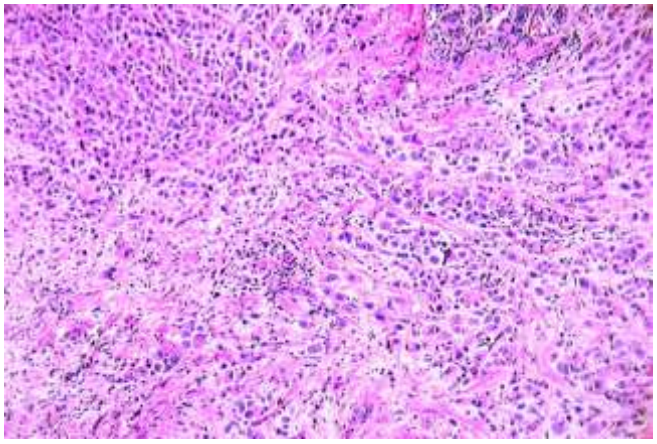

Figure-1: Infiltrating Ductal Carcinoma (H & E x 100)

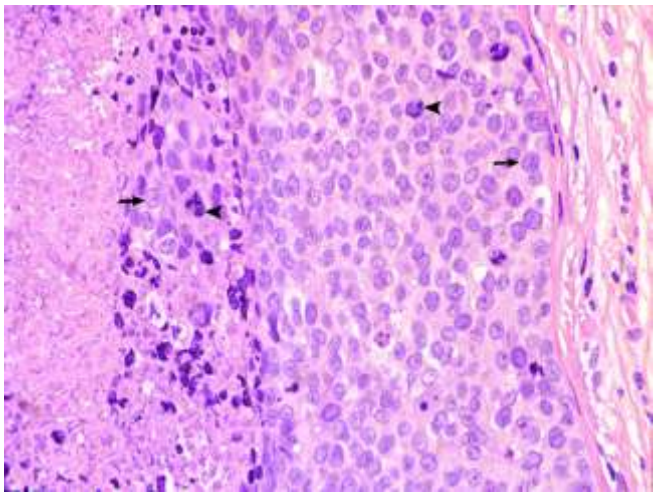

Figure 2: Infiltrating Duct Carcinoma, with Comedo-pattern, Note the Mitotic Figures (H & E x 200)

In breast carcinomas, 38 of 200 cases were positive for CK5 (Figure 5). CK5 expression correlated with age (P = 0.0022), histologic grading (P < 0.0001), nodal status (P = 0.0002), pathologic staging (P = 0.0349), ER status (P < 0.0001), PR status (P < 0.0001), and Ki-67 labeling index (P = 0.0011). There was no statistical relationship between CK5 expression and menstrual status, tumor size, or expression of p53 and c-erb-B2. The relationship of statistical significance between CK5 expression and the clinico-pathologic features is summarized in Table 1.

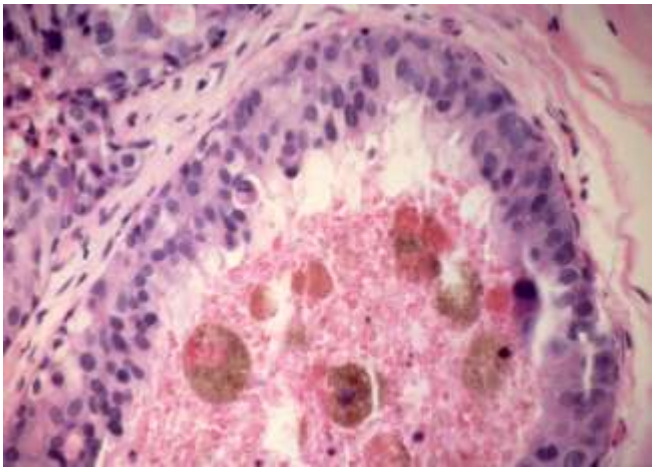

Figure 3: Previous Case of Duct Carcinoma, with Comedo-Pattern (H & E x 400)

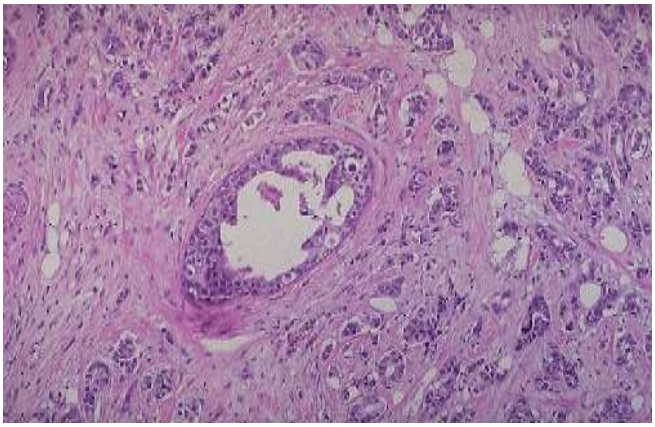

Figure 4: Infiltrating Duct Carcinoma, with Comedo-type (H & E x 100)

The average number of microvessels by CK5 expression is specified in Table 2 (Figure 6). Of the 200 carcinomas, 4, 22, 8, 14, 128, and 88 were positive in more than 20% of neoplastic cells for EMMPRIN, MMP-1, MMP-2, PAI, TIMP-1, and TIMP-2, respectively (Figure 7), and 108 were positive for

VEGF (Figure 8). The relationship of statistical significance between markers and CK5 expression is shown in Table 2.

Table 1: Relationship between Cytokeratin 5 Expression and Clinicopathologic Variables of Prognostic Significance

Feature	Cytokeratin 5		P
	Negative (n=162)	Positive (n=38)	
Age (y)			0.0022
<30	0	6	
30-50	46	14	
50-70	84	12	
>70	32	6	
Histologic grading (Bloom and Richardson)			<0.0001
I	62	2	
II	72	6	
III	28	30	
Lymph nodes			0.0002
Negative	58	0	
1-3	50	6	
>3	54	32	
Pathologic staging			0.0349
I	16	0	
II	72	16	
III	68	14	
IV	6	8	
Estrogen receptor			<0.0001
Negative	62	34	
Positive	100	4	
Progesterone receptor			<0.0001
Negative	72	36	
Positive	90	2	
Ki-67			0.0011
<10%	98	8	
10%-50%	46	14	
>50%	18	16	

Table 2: Peritumoral Blood Vessel Counting and Expression of VEGF, Metalloproteinases, and Their Inhibitors According to Cytokeratin 5 Expression

Feature	Cytokeratin 5		P
	Negative (n=162)	Positive (n=38)	
CD34 (No. of peritumoral blood vessels)			0.4586
<10	4	0	
10-20	24	0	
21-30	54	14	
31-40	44	10	
41-50	26	10	
>50	10	4	
VEGF			0.0207
Negative	84	8	
Positive	78	30	
MMP-1			0.8491
0%	90	18	
<20%	56	14	
21%-50%	12	4	
>50%	4	2	
MMP-2			0.7291
0%	130		
<20%	48	8	
21%-50%	8	0	
>50%	8	0	
TIMP-1			0.0135
0%	44	0	
<20%	20	8	
21%-50%	22	14	
>50%	76	16	
TIMP-2			0.1395

0%	70	16	
<20%	16	10	
21%-50%	28	8	
>50%	48	4	
Plasminogen activator inhibitor			0.4408
0%	136	34	
<20%	14	2	
21%-50%	10	0	
>50%	2	2	

EMMPRIN, extracellular matrix metalloproteinase inducer; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; VEGF, vascular endothelial growth factor.

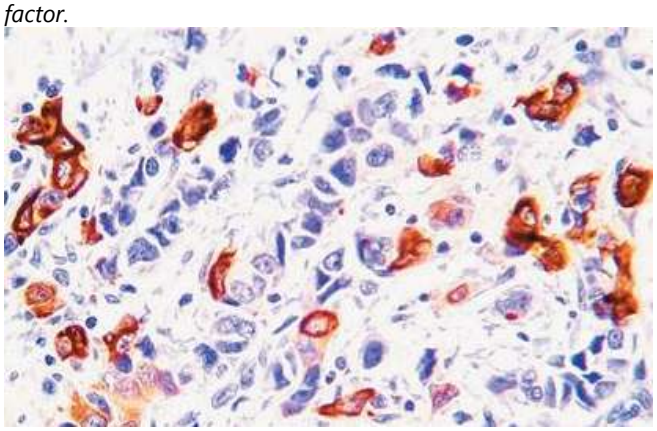

Figure 5: Positive Cytoplasmic Staining for Cytokeratin 5 (×400)

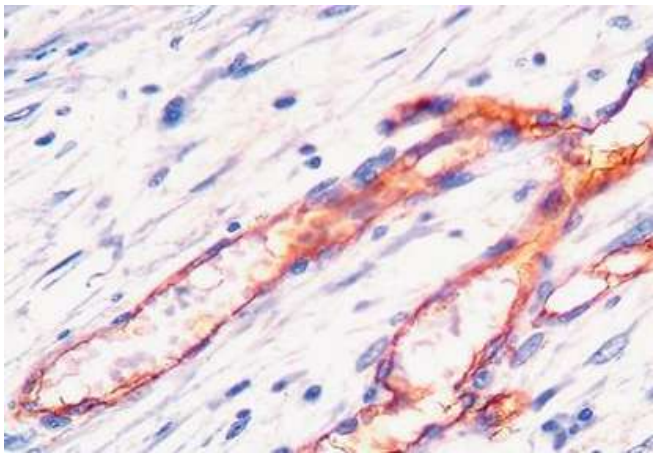

Figure 6: Blood Vessels with Positive Cytoplasmic Staining for CD34 (×400)

Discussion

Nowadays, cancer is considered a multistep disease in which the accumulation of several genetic changes leads to an uncontrolled cellular growth.[26] The understanding of these changes in CK5+ breast carcinomas might provide new insight for the management of these tumors.

In the present work, the expression of CK5 correlated with several indicators of poor outcome, including precocious age, high histologic grade, lymph node positivity, advanced pathologic stage, negativity for hormonal receptors, and a high proliferative rate, as assessed by the Ki-67 labeling index. These findings are in accordance with the current literature and indicate that CK5+ breast carcinomas are aggressive neoplasms.[10, 27, 28]

There was a statistically significant correlation between CK5 and VEGF expression (P = 0.0207). Because VEGF is a potent and specific endothelial cell mitogenic factor[21], it might be expected that microvessel density would be augmented in CK5+ carcinomas, a fact not observed in the present study. These data suggest that other factors besides VEGF might be important for angiogenesis. Indeed, there is increasing evidence that the overall angiogenesis phenotype is determined by the balance between proangiogenic and antiangiogenic molecules.[30] In that way, not all tumors expressing VEGF are highly angiogenic.[31] Although the prognostic significance of VEGF expression in breast cancer is controversial[12, 32, 33], VEGF-overexpressing neoplastic mammary cell lines display increased proliferative activity in vitro, suggesting that VEGF might act as an autocrine growth factor regardless of its angiogenic effect.[34] According to this assertion, the differential

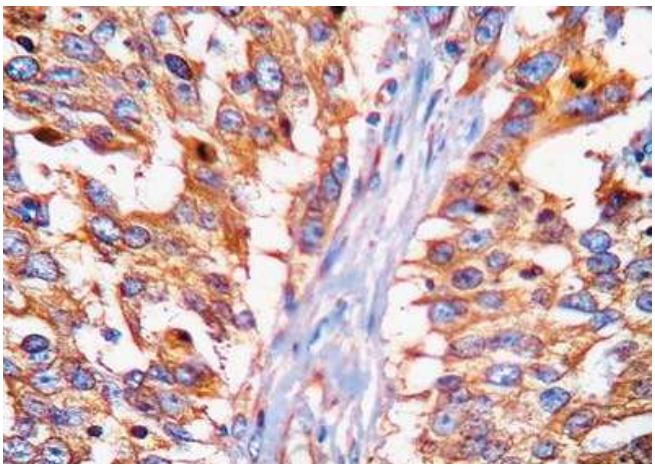

Figure 7: Positive Cytoplasmic Staining for

Tissue Inhibitor of Matrix Metalloproteinase 1 (×400)

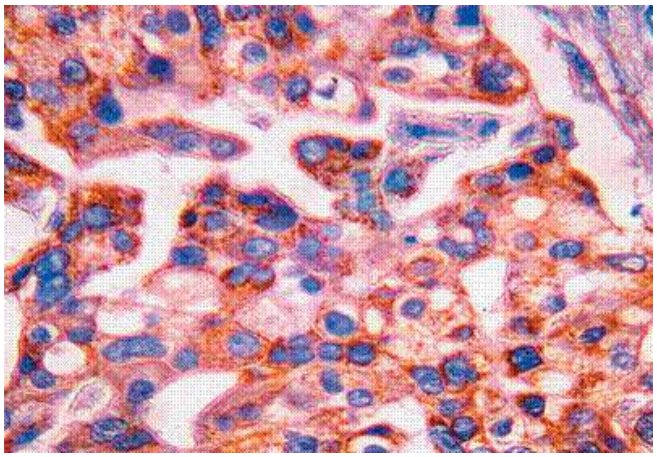

Figure 8: Positive Cytoplasmic Staining for Vascular Endothelial Growth Factor (×400)

expression of VEGF observed in CK5+ carcinomas might contribute to the more aggressive behavior of these neoplasms.

The ability of extracellular matrix degradation by neoplastic cells is related directly to invasiveness and the metastatic potential of the carcinomas.[13] The degree of this degradation is regulated by the abnormal balance between the proteinases and their inhibitors. MMPs are zinc-dependent proteolytic enzymes capable of breaking down basement membranes and most extracellular matrix components.[35] They have pivotal roles in promoting tumor disease progression, including tumor angiogenesis. MMP expression is regulated partially by EMMPRIN.[36, 38] Normally, protease activity is controlled tightly by TIMPs and PAIs. The balance between active proteases and inhibitors is thought to determine the occurrence of proteolysis in vivo.[37, 38]

In this study, we assessed the expression of MMP-1, MMP-2, EMMPRIN, TIMP-1, TIMP-2, and PAI in invasive breast carcinomas. Curiously, in the present study, CK5 expression correlated only with TIMP-1, which is an inhibitor of MMP-1. These data might indicate that the antiproteolytic stimuli are preponderant in CK5+ breast carcinomas. Considering the aggressiveness of CK5+ carcinomas, this finding is an apparent paradox. However, this apparent discrepancy might be related to the assertion that the differential expression of the proteinases and their inhibitors might be activated at different stages of tumor progression.[13]

Conclusion

We conclude from work that, the overall angiogenesis phenotype is determined by the balance between pro-angiogenic and anti-angiogenic molecules and the balance in the concentrations of activated proteinases and their inhibitors might change during tumor progression. In that way and from this viewpoint, we can say that a single factor could not justify cancer progression in vivo.

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