

Immunohistochemical Expression and Prognostic Significance of HIF-1 α and VEGF-C in Neuroendocrine Breast Cancer

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Abstract. Aim: To determine the predictive value of HIF-1 α and VEGF-C in primary neuroendocrine breast cancers (NEBC) and their correlation with other clinico-pathological characteristics of NEBC. Materials and Methods: HIF-1 α and VEGF-C were determined immunohistochemically in tissue samples from 31 cases of NEBC. Results: The HIF-1 α expression in NEBC was predominantly negative, with only 5 (16.1%) cases showing strong reaction to HIF-1 α . Eighteen (58.0%) NEBC cases showed moderate-to-strong VEGF-C expression. VEGF-C expression negatively correlated with progesterone receptor positivity ($p=0.014$) and duration of follow-up ($p=0.021$). A multivariate Cox proportional hazard regression analysis showed that HIF-1 α ($p=0.019$) was a significant predictor of disease-free survival, whereas VEGF-C ($p=0.099$) showed no such association. Conclusion: HIF-1 α overexpression indicated unfavourable prognosis and could serve as an additional prognostic factor in NEBC. Moreover, patients with NEBC exhibiting moderate or strong VEGF-C expression could be candidates for a specific VEGF-C antibody therapy.

Breast cancer is the most frequent cancer and the leading cause of death in women, accounting for 23% (1.38 million) of total new cancer cases and 14% (458 400) of total cancer-related deaths in 2008, worldwide (1). According to the American Cancer Society, breast cancer incidence rates of approximately 200 per 100.000 women in USA, have remained stable over the past several years (2). Invasive ductal carcinoma not otherwise specified (NOS) comprises the largest group of invasive breast cancer. On the other hand,

neuroendocrine breast cancer (NEBC) is one of the rarest types and accounts for only 2-5% of all breast cancer cases (3). NEBC exhibits morphological features very similar to those of neuroendocrine tumours of the gastrointestinal tract and lungs, which typically have adverse prognosis. According to the 2003 World Health Organization (WHO) classification of tumours, NEBC is defined as having $\geq 50\%$ of tumour cells expressing neuroendocrine markers (3). Breast cancer-expressing neuroendocrine markers in scattered cells, called 'breast cancer with focal endocrine differentiation', is not included in this group. Recent studies have shown that focal endocrine differentiation has no prognostic value (4). According to the WHO, NEBC comprises of three different subtypes: solid, small/oat cell and large cell NEBC. All subtypes except the small-cell carcinoma have better prognosis than ductal NOS. Sapino *et al.* (5) described five NEBC subtypes, which include solid cohesive, alveolar, small-cell/Merkel cell-like, solid papillary and cellular mucinous carcinomas. Hypoxia-inducible factor-1 alpha (HIF-1 α), a transcription factor involved in tumour growth and metastasis, regulates genes that are involved in response to hypoxia (6), whereas vascular endothelial growth factor-C (VEGF-C) is one of the main inducers of lymphangiogenesis. VEGF-C overexpression is associated with lymphovascular invasion by tumour cells, increased rate of lymph node metastases and adverse prognosis (7). Recent studies have indicated HIF-1 α and VEGF-C expressions to be potential targets for immunotherapy and antitumour treatment (8). The aim of our study was to determine the immunohistochemical expression of HIF-1 α and VEGF-C in NEBC and its correlation with other clinicopathological characteristics of NEBC.

Materials and Methods

Patients. Data were analyzed for the period between January 1, 2001 and December 31, 2005. Among a total of 3,058 cases diagnosed with breast cancer, 31 primary NEBC (sequential archival cases, Institute for Tumours, Sestre Milosrdnice Clinical Hospital Centre)

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Table I. Clinicopathological characteristics of 31 patients with neuroendocrine breast carcinoma.

Clinicopathological characteristic	Patients (n=31)
Age (years)	
Median (range)	61.7 (44-86)
Interquartile range	59.0 (54.0-68.0)
Tumour size (cm)	
Median (range)	1.9 (1.0-4.8)
Interquartile range	2.0 (1.0-2.0)
T-Category (n, %)	
T1	12 (38.7)
T2	18 (58.1)
T3	1 (3.2)
T4	0 (0.0)
Lymph node status (n, %)	
N0	16 (51.6)
N1	10 (32.3)
N2	4 (12.9)
N3	1 (3.2)
Histological grade (n, %)	
G1	7 (22.6)
G2	19 (61.3)
G3	5 (16.1)
Oestrogen receptor status (n, %)	
ER +	27 (87.1)
ER –	4 (12.9)
Progesterone receptor status (n, %)	
PR+	23 (74.2)
PR –	8 (25.8)
HER-2/neu status (n, %)	
Negative (0)	30 (96.8)
Overexpressed (+++)	1 (3.2)
Median follow-up time, months range	58.7 (2-144)
Relapse of the disease (n, %)	8 (25.8)
Median time to relapse, months range	34.3 (14.5-54.1)

cases were identified and included in the study (NEBC incidence of 1.01%). All samples diagnosed as NEBC were histologically and immunohistochemically reviewed by an experienced pathologist and all met the strict WHO criteria for diagnosis of NEBC.

The patients were not treated with hormonal therapy, chemotherapy or radiotherapy before surgery. None of the patients had a second primary cancer or distant metastases at the time of the diagnosis and none died of disease during the follow-up (Table I). The TNM system, histological grade and immunohistochemical expression of oestrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (HER2/neu) were interpreted according to the 2003 WHO classification and 2007 the ASCO/CAP Guideline Recommendations (3, 9).

Immunohistochemical methods. Tumours were fixed in 10% buffered formalin for 24 h and cut into 3-7 sections. The specimens were embedded in paraffin, cut at 5 µm and routinely stained with haematoxylin and eosin (HE). For each case, the HE sections were reviewed and the slide with the invasive tumour front was chosen for immunohistochemical analysis. For immunoperoxidase

Table II. Immunohistochemical expression of hypoxia-inducible factor-1 alpha (HIF-1α) and vascular endothelial growth factor-C (VEGF-C) in 31 neuroendocrine breast carcinomas.

Staining intensity	No. of patients (%)	
	HIF-1α	VEGF-C
Negative (–)	17 (54.8)	7 (22.6)
Weak (+)	7 (22.6)	6 (19.4)
Moderate (++)	2 (6.5)	9 (29.0)
Strong (+++)	5 (16.1)	9 (29.0)
Total	31 (100.0)	31 (100.0)

procedures, monoclonal mouse antihuman HIF-1α (1:25 dilution; R&D Systems, Minneapolis, Minnesota USA) and monoclonal mouse antibody against VEGF-C (clone VG1, dilution 1:25; Dako, Glostrup, Denmark), were used. Tissue sections, 3-5 µm thick, were cut from the paraffin-embedded tissue blocks, placed on object slides (Menzel-Glaser, Braunschweig, Germany) and incubated for 20 min in a thermostat at 60°C. The sections were de-paraffinized and incubated for 20 min in Target retrieval solution buffer (pH=9.0 S2367; Dako, Glostrup, Denmark), at 97°C. Immunohistochemical staining was performed in an automated immunostainer (Dako, Glostrup, Denmark) at room temperature. Subsequently, tissue slides were washed with 0.3% hydrogen peroxide for 5 min to block endogenous peroxidase activity (Dako, Glostrup, Denmark). After washing, the slides were incubated with a previously prepared primary antibody solution (1:50 dilution; Dako, Glostrup, Denmark) for 45 min. After 30-min incubation, the antigen antibody complex was visualized by the addition of peroxidase-conjugated universal secondary antibody, which was incorporated in the reagent Dako EnVision (Dako, Glostrup, Denmark). Tissue sections were washed once more in Target retrieval solution buffer, and the chromogen (Dako, Glostrup, Denmark) was added for 5 min. Slides were washed in distilled water, stained with haematoxylin (Dako, Glostrup, Denmark) for 1 min, washed again with water, dehydrated with alcohol (96%), cleared with xylene and mechanically covered. High-grade invasive ductal NOS breast carcinoma and colon carcinoma were used as positive controls for HIF-1α and VEGF-C, respectively. Replacement of the primary antibodies by isotype-matched immunoglobulin was used as a negative control.

Immunoreactivity for both HIF-1α and VEGF-C in breast cancer tissue was determined by the percentage of positive tumour cells in the entire tissue section. Staining intensity was described as negative (–), weak (+), moderate (++) or strong (+++). All samples were examined independently by two experienced pathologists (FK, DT) and any differences were resolved by joint review.

Statistical analysis. All results are presented in both tabular and graphical forms. Kolmogorov-Smirnov test was used to determine data distribution prior to statistical analysis. Data were analysed using the chi-square test with Yeats correction, Kaplan–Meier test and Cox proportional-hazards regression test. The level of statistical significance was set at $p < 0.05$. Statistical analysis was performed using IBM SPSS Statistical Package 19.0.0.1 (www.spss.com).

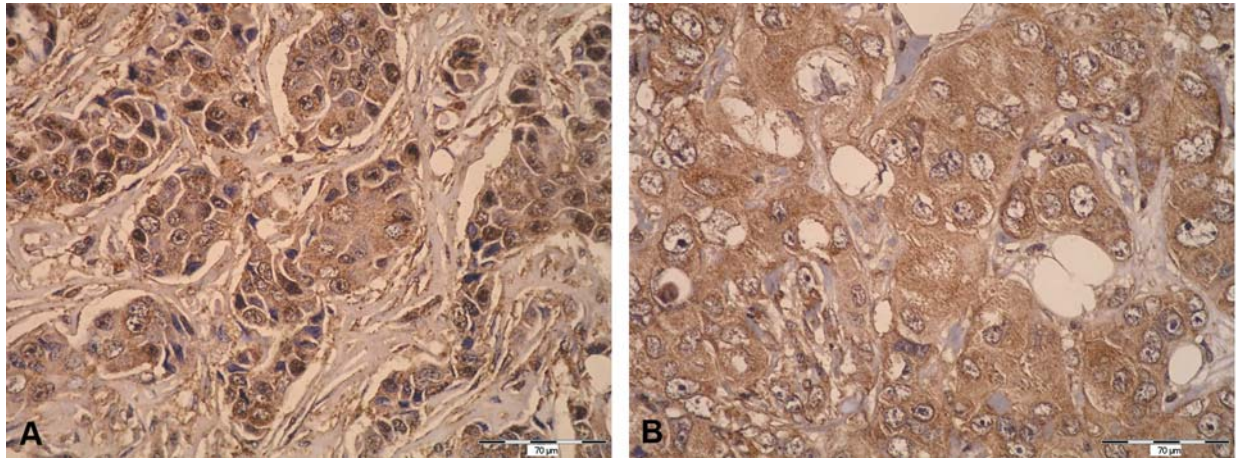


Figure 1. Strong immunohistochemical expression of hypoxia-inducible factor-1 alpha (HIF-1 α) and vascular endothelial growth factor-C (VEGF-C) in neuroendocrine breast carcinoma. A: The expression of HIF-1 α was mostly cytoplasmic, but in some cells, nuclear positivity was also observed ($\times 400$). B: VEGF-C expression was strictly confined to the cytoplasm ($\times 400$).

Results

The immunohistochemical expression of HIF-1 α in NEBC was negative in more than half of the tissue samples (Table II). A strong reaction to HIF-1 α was observed in only 5 out of 31 cases (Figure 1A). VEGF-C expression was predominantly moderate to strong. Eighteen tumours exhibited moderate to strong VEGF-C expression (Figure 1B). Correlation analysis showed that HIF-1 α did not significantly correlate with any of the parameters, whereas VEGF-C negatively correlated with progesterone receptor positivity and duration of follow-up (Table III). In patients with stronger tumoral VEGF-C expression, progesterone receptor expression was significantly lower and follow-up time significantly shorter. Other clinicopathological parameters showed no significant correlation with VEGF-C expression. There was no statically significant correlation between HIF-1 α and VEGF-C expression ($p=0.387$). Kaplan–Meier analysis of disease-specific survival, based on up to 144 months follow-up, showed a borderline statistical prognostic significance of HIF-1 α expression ($p=0.066$) and no prognostic significance for VEGF-C expression ($p=0.405$). Most patients with moderate to strong HIF-1 α expression had a relapse within 34 months. Multivariate Cox proportional hazard regression analysis (overall score: χ^2 value=15.6, df=7, $p=0.030$) showed that HIF-1 α ($p=0.019$) and human epidermal growth factor receptor-2 (HER2/neu) ($p=0.034$) were significant predictors of disease-free survival, whereas VEGF-C ($p=0.099$) progesterone ($p=0.091$) and oestrogen ($p=0.112$) receptor status, age ($p=0.153$) and tumour grade ($p=0.321$) showed no correlation. Patients with higher HIF-1 α expression had a significantly shorter disease-free survival (Figure 2). A single

patient with HER2/neu overexpression had a relapse 17 months after the initial diagnosis. However, no conclusions about the role of HER2/neu expression in NEBC may be drawn due to the fact that only a single patient in our study group had HER2/neu overexpression, which is insufficient for statistical analysis.

Discussion

Tumour hypoxia correlates with increased malignancy, metastatic potential and adverse prognosis in patients with invasive breast cancer (10). HIF-1 α plays a crucial role in adaptation to hypoxia and is frequently activated in tumours. The activation of HIF-1 α is considered to support the process of tumour growth through the activation of anaerobic metabolism and induction of angiogenesis that partly results from increased *VEGF* gene transcription (11). Direct correlations between HIF-1, VEGF and tumour angiogenesis have been demonstrated, but not entirely clarified (12). A significant association between HIF-1 α overexpression and patient mortality has been shown for cervical, ovarian, brain, head and neck, oropharyngeal, oesophageal, nasopharyngeal and non-small cell lung cancer (13–20). Bos *et al.* (21) showed that the HIF-1 α level was a strong and independent prognostic factor in patients with invasive breast cancer, especially in those with poorly-differentiated breast lesions and negative lymph nodes. Therefore, they proposed the use of immunohistochemical assessment of HIF-1 α as a new predictor of poor outcome in this group of patients (21). VEGF-C is considered the main inducer of lymphangiogenesis and its overexpression was associated with lymphovascular invasion by tumour cells, increased rate of lymph node

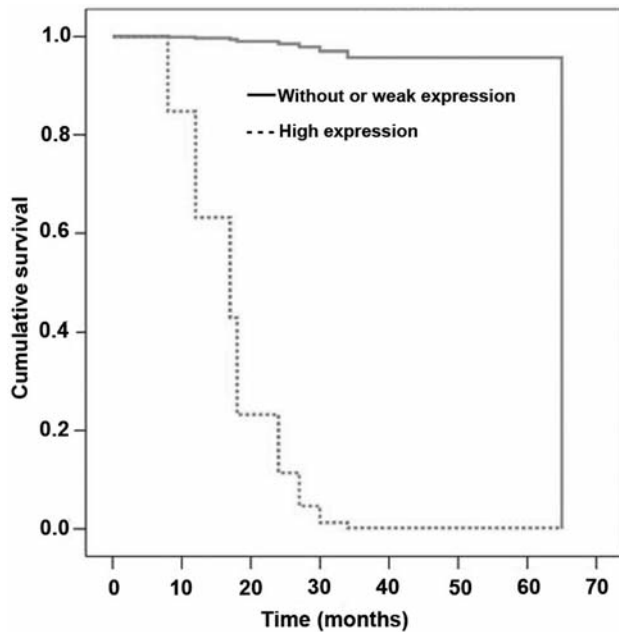


Figure 2. Cox proportional hazard regression analysis showed that the intensity of hypoxia-inducible factor-1 alpha (HIF-1 α) expression could serve as an independent prognostic factor for disease-free survival in patients with neuroendocrine breast carcinoma (odds ratio=145.92, 95% confidence interval=2.22-9188.54, $p=0.019$).

metastasis and adverse prognosis (22). Although most authors emphasize the direct association between VEGF-C and lymphangiogenesis, this has not been consistently confirmed. Several studies did not show any prognostic significance of VEGF-C in breast cancer, nor correlation between the angiogenic and lymphangiogenic microvessel density, VEGF-C expression and tumour diameter, grade, progression and patient survival (23, 24). In our study, the expression of HIF-1 α in NEBC was predominantly weak, whereas VEGF-C was moderately or strongly expressed in more than half of the samples. High expression of HIF-1 α was associated with adverse prognosis and earlier relapse of the disease. These findings are comparable with those of Bos *et al.* (21). However, the expression of VEGF-C failed to show any prognostic value, which supports previous findings by Gisterek *et al.* (23) and Al-Mowallad *et al.* (24). The expression of HIF-1 α and VEGF-C in neuroendocrine tumours has rarely been investigated at the same time. To the best of our knowledge, the present study is the first that investigated these two markers in NEBC. Partanen *et al.* (25) studied VEGF-C and VEGF-D expression in various neuroendocrine cells, such as alpha cells of the islets of Langerhans, prolactin-secreting cells in the anterior pituitary gland, adrenal medullary cells, and dispersed neuroendocrine cells in the gastrointestinal tract. Their results suggest that

Table III. Correlation coefficients between hypoxia-inducible factor-1 alpha (HIF-1 α) and vascular endothelial growth factor-C (VEGF-C) expression and clinicopathological parameters in neuroendocrine breast carcinoma: Spearman correlation coefficients (for nominal correlation Kendall's tau_b).

		HIF-1 α	VEGF-C
Age (years)	Correlation coefficient	0.241	0.070
	p -value	0.192	0.707
T-stage*	Correlation coefficient	-0.044	-0.236
	p -value	0.806	0.174
N-stage*	Correlation coefficient	0.152	-0.133
	p -value	0.350	0.403
Oestrogen receptor (%)	Correlation coefficient	-0.253	0.067
	p -value	0.171	0.721
Progesterone receptor (%)	Correlation coefficient	0.081	-0.438
	p -value	0.664	0.014
HER-2/neu status	Correlation coefficient	-0.297	0.147
	p -value	0.105	0.429
Relapse time (months)*	Correlation coefficient	0.278	0.109
	p -value	0.105	0.513
Metastasis*	Correlation coefficient	0.225	0.180
	p -value	0.199	0.291
Follow-up (months)	Correlation coefficient	-0.234	-0.412
	p -value	0.205	0.021
Tumour grade	Correlation coefficient	0.116	-0.020
	p -value	0.533	0.915

*Kendall's tau_b.

these factors could have a paracrine function and a possible role in peptide release from secretory granules from some neuroendocrine cells into the surrounding capillaries. Non-small cell lung carcinoma exhibits VEGF overexpression and its suppression might contribute, at least, to partial tumour regression (26). Monsef *et al.* (27) analyzed the expression of HIF-1 α and HIF-2 α and VEGF in neuroendocrine cells of both benign and malignant prostate tumours and found that levels of VEGF were increased in androgen receptor-negative malignant neuroendocrine cells. *In situ*-hybridization indicated that HIF-1 α mRNA levels were not higher in neuroendocrine prostate cancer cells than in corresponding non-neuroendocrine tumour cells (27). In our study, a similar correlation between progesterone receptor and VEGF-C was found. Progesterone receptor expression negatively correlated with VEGF-C expression, and tumour with low progesterone receptor expression had a significantly higher VEGF-C expression. Several new anticancer therapies are based on targeting VEGF. Bevacizumab, a humanized monoclonal antibody that inhibits VEGF-A, has significantly changed the treatment of patients with metastatic colorectal cancer after being approved by the US Food and Drug Administration (FDA) in 2004 (28). It is administered to patients with metastatic non-small lung cancer, pancreatic cancer, renal cancer, glioblastoma and advanced epithelial ovarian cancer

(31-35). However, reports for its use in breast cancer treatment remained controversial and the FDA revoked the approval of the drug for this indication in 2011. Recently, unique HIF-1 α antibody-conjugated nanomicelles filled with paclitaxel have been shown to be successful in the selective killing of gastric cancer cells with HIF-1 α overexpression, thus pointing to HIF-1 α as new potential target for specific antitumour therapy (36). In conclusion, we found both HIF-1 α and VEGF-C to be expressed in NEBC. Since HIF-1 α overexpression indicated an unfavourable prognosis, it could serve as an additional prognostic factor. Taking into account that more than half of the tumours exhibited moderate or strong VEGF-C expression, we may assume that patients with NEBC could be candidates for specific VEGF-C antibody therapy. Further larger studies on HIF-1 α and VEGF-C expression in NEBC are needed to more accurately determine their potential prognostic and therapeutic benefits in NEBC.

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