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Correlation between hepatocyte growth factor receptor and vascular endothelial growth factor-A in breast carcinoma

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Abstract: The aim of the study was to evaluate the prognostic value of the vascular endothelial growth factor A (VEGF-A) and hepatocyte growth factor receptor (HGFR, c-met) expressions in homogenous group of breast cancer patients. Tumor samples were collected from 98 patients with invasive ductal breast carcinoma stage II treated with primary surgery. We have observed a strong correlation between VEGF-A and c-met. No correlations were found between VEGF-A or HGFR expressions and clinical parameters (tumor size, grade, axillary lymph node status, age), 5- and 10-years DFS or OS. Our study did not reveal any prognostic value of c-met or VEGF. In addition they are not useful to separate a patients' subgroup with poor prognosis. Unlike in other authors' studies, our patients' group is very homogenous which might tribute to obtained results.

Key words: c-met, VEGF-A, breast cancer, ductal breast carcinoma, prognostic factor

Introduction

Breast cancer deaths are mostly caused by the systemic disease, representing recurrences after definitive therapy. Recent research efforts have focused on indentifying markers in order to predict risk of cancer outcome [1].

While the patient qualification for further treatment is obvious at extreme (I, III, and IV) stages of breast cancer, the attitude in stage II cases is still conflicting. It was estimated that only the low percentage of stage II patients benefits from aggressive chemotherapy. Consequently, it is of the major importance to define the immunohistochemical features of this group which could make possible patients'stratification and individualization of their treatment.

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Hepatocyte growth factor (HGF) belongs to the plasminogen-prothrombin gene superfamily, which includes macrophage-stimulating protein and plasminogen [2]. The growth promoting activity of HGF requires proteolytic cleavage by extracellular serine proteinases such as urokinase plasminogen activator and tissue-type plasminogen activator [3]. HGF plays a direct role in stimulating blood vessel growth *in vitro* and *in vivo* by signaling through the hepatocyte growth factor receptor – (HGFR, c-met) which is expressed on endothelial cells [4,5]. C-met is a member of the receptor tyrosine kinases family and is involved in the control of proliferation, survival and morphogenesis of the normal and cancer cells. Moreover, the HGF-c-met pathway also contributes to tumor progression by promoting angiogenesis through the recruitment of new vessels [4]. C-met signaling is involved in the regulation of tumor angiogenesis either directly through the proangiogenic activity or indirectly, through the regulated secretion of angiogenic factors such as VEGF-A [6].

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The vascular endothelial growth factors (VEGF) family is a group of growth factors which regulate the growth of endothelial cells [7]. VEGF is a cytokine that selectively induces endothelial cell proliferation and migration, increases the permeability of microvessels, and activates proteolytic enzymes involved in tumor invasiveness [8]. Moreover, it stimulates the growth of vascular endothelial cells derived from arteries, veins and lymphatics. VEGF is also a survival factor for endothelial cells, both *in vitro* and *in vivo* [9]. Recent studies have demonstrated a prognostic significance of VEGF-A expression in malignant tumors arising from several organs [7,10-13]. Furthermore VEGF-A expressions in tumor tissues have been reported to be an independent prognostic factor for breast cancer patients regardless of the nodal status [14,15].

HGFR signaling is implicated in a wide variety of human malignancies, including the following ones: melanoma, colon, gastric, bladder, breast, ovarian, pancreatic, kidney, liver, lung, head and neck, thyroid and prostate cancers [4,16,17,18]. Although there are a number of studies demonstrating the overexpression of c-met in breast cancer, there is no study comparing VEGF-A and HGFR expressions in a very homogenous group of patients with ductal breast carcinomas.

In this study we analyzed HGFR and VEGF expressions, 5 and 10 years overall and disease free survival (OS, DFS) and clinicopathological factors in highly homogenous group of 98 patients with UICC stage II, histological grade 2 and 3 ductal breast carcinomas treated with primary modified radical mastectomy. The aim of the work was to determine whether the expressions of HGFR and VEGF-A in primary tumors were correlated with lymph node metastasis, patients' prognosis and could due to that fact be helpful in defining a subgroup of patients for more or less aggressive treatment.

Materials and methods

Patients and tumor samples. The present study includes archival tumor samples from 98 patients of Lower Silesian Oncology Center (Wroclaw, Poland) treated for stage II ductal breast cancer in 1993-1994. The study was approved by a regional Institutional Review Board. The median age of the patients was 56, range from 29 to 86 years. All the patients underwent surgery (radical modified Patey-Madden mastectomy) with or without standard adjuvant treatment. Information about the patients' clinical histories and clinical and pathological variables was obtained from patients' medical records and during follow-up visits. The size of primary tumor was evaluated from the surgical specimen. Lymph node status was determined by lymphadenectomy of axiliary lymph nodes and by proving histological evidence of metastatic breast carcinoma. Overall survivals (OS, in weeks) and disease-free survivals (DFS, in weeks) were established for all the patients. Follow-up period amounted 5 years (261 weeks) and then 10 years (522 weeks) in all patients. Microscopic studies were performed on formalin-fixed, paraffin-embedded cancer tissues, obtained during surgery and stained routinely with haematoxylin and eosin. Histopathological type according to the World Health Organization [19] (ductal breast cancer in all the cases), grade (only Bloom 2 and 3 were qualified to the experiment) and stage II according to the TNM classification were determined during microscopic examination. Tumor grade was estimated according to Bloom-Richardson in the Elston and Ellis modification. The detailed characteristics of patients are shown in Table 2.

Immunohistochemistry. HGFR: Formalin-fixed paraffin embedded, freshly cut 4 µm tissue sections were mounted on Superfrost slides (Menzel Glaeser, Germany), dewaxed with xylene, and gradually rehydrated. Sections were incubated with citrate buffer at 98°C to unmask the epitopes and treated with 1% hydrogen peroxide (H_2O_2) for 10 min to block endogenous peroxidase. The sections were then incubated with human hepatocyte growth factor receptor mouse monoclonal antibody (from Novocastra Laboratories Ltd). The sections were further incubated with biotin-labeled secondary antibody and streptavidin-biotin-peroxidase, for 20 min each. Tissues were stained for 5 min with 0.05% 3,3'-Diaminobenzidine tetrahydrochloride (DAB), counterstained with haematoxylin, dehydrated and mounted (Figs. 1 and 2) [20].

VEGF-A: Formalin-fixed paraffin embedded, freshly cut 4 µm tissue sections were mounted on Superfrost slides (Menzel Glaeser, Germany), dewaxed with xylene, and gradually rehydrated. Sections were incubated with citrate buffer at 98°C to unmask the epitopes and treated with 1% hydrogen peroxide (H_2O_2) for 10 min to block endogenous peroxidase. Then in the next step sections were then incubated overnight in monoclonal anti-VEGF165 antibody (from Novocastra Laboratories Ltd). The sections were further incubated with biotin-labeled secondary antibody and streptavidin-biotin-peroxidase, for 20 min each. Tissue was stained for 5 min with 0.05% 3,3'-Diaminobenzidine tetrahydrochloride (DAB), counterstained with haematoxylin, dehydrated and mounted (Figs. 3 and 4). Results of immunohistochemical reactions were estimated independently by two pathologists using the semi-quantitative score scale based on the percentage of positive stained cells as follows: 0 = none, 1 level – if up to 33% cells in tumor were positive, 2 level – 33-66% positive cells and 3 level if more than 66% cells were positive [21]. In cases of controversy, a reevaluation was performed with the use of a double-headed microscope.

Statistical analysis. The univariate significance of differences in studied markers expressions was assessed by the chi-square test for binary or categorical covariates, by Pearson test and by the Spearman rank correlation for ordered covariates. Cancer specific overall survival (OS) and disease free survival (DFS) were estimated using the Kaplan-Meier method, and comparison between study groups was performed with log-rank test. The survival time was measured from date of diagnosis to date of death or last follow up. In all tests, the significance level was set at 0.05 and all were two-sided tests. Statistical analyses were performed using the Software StatSoft Inc. STATISTICA for Windows ver. 7.0 A, Tulsa, OK, USA.

Table 1. HGFR and VEGF expression in breast cancer tissue

	HGFR		VEGF	
	n	%	n	%
0 – none	36	37	30	31
1 level	7	7	15	15
2 level	49	50	39	40
3 level	6	6	14	14

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Table 2. Distribution of breast ductal cancer patients according to HGFR and VEGF expression in tumor tissue

Patient characteristics		n (%)	HGFR 0-1	HGFR 2-3	p value	VEGF 0-1	VEGF 2-3	p value
Total		98 (100%)	43	55		45	53	
Age	>median	49 (50%)	21	28	1.0	19	30	0.22
	<median	49 (50%)	22	27		26	23	
Nodal status	Negative	57 (58%)	23	34	0.42	25	32	0.69
	Positive	41 (42%)	20	21		20	21	
Tumor size	1	12 (12.%)	5	7	0.75	6	6	0.64
	2	86 (88%)	38	48		39	47	
UICC stage	2a	39 (40%)	14	25	0.22	18	21	1.0
	2b	59(60%)	29	30		27	32	
Grade (Bloom)	2	67 (68.%)	30	37	0.83	29	38	0.51
	3	31 (32%)	13	18		16	15	

Results

HGFR expression in breast cancer tissue

In breast cancer cells, expressions of c-met protein were observed in the cytoplasm. According to the criteria accepted for c-met immunostaining level evaluation, c-met protein positive were 63% (62 of 98 specimens) of the breast cancer patient's tumor tissue samples (Table 1).

VEGF-A expression in breast cancer tissue

In breast cancer cells, expression of VEGF-A was observed in the cytoplasm (Table 1). According to the criteria accepted for VEGF-A immunostaining level evaluation, positive breast tissue samples were 69% (68 of 98 specimens).

Correlation between HGFR and VEGF-A

We observed significant correlation between those two markers: in Pearson's test p=0.0176 and in Spearman's rank correlation p=0.0205.

Association between c-met and VEGF-A expression and clinicopathological factors

No associations were observed between c-met and VEGF-A expression and patients' age, tumor size, tumor grade, ER status nor axillary lymph node metastases (Table 2).

Prognostic value of c-met and VEGF-A in breast carcinoma patients

We examined whether c-met and VEGF-A expression might be associated with poor prognosis in breast carcinoma patients. Five and 10 years OS and DFS were analyzed in groups with different HGFR and VEGF-A expression levels. With the time horizon of five and ten years in both tests there was no significant difference found in overall and disease- free survival (Fig. 1 and 2).

Discussion

The importance of the HGF regulatory system in development of cancer is still not fully understood. Up to date HGF is known to be a multifunctional cytokine which induces cell proliferation, motility and angiogenesis in a wide variety of neoplastic cells [22].

In our study we have shown c-met expression in 63% of breast cancer patient's tumor tissue samples. Masuya *et al*. [23] observed lower percentage of c-met positive cells – 40.9% in non-small-cell lung cancer and Endo *et al*. [24] noticed 42% in squamous cell cancer of tongue. Significantly higher percentage of c-met immunopositivity was shown by Lo Muzio [25] – 82.2% and Yucel [3] – 83% in head and neck squamous cell cancer. In Ghoussoub *et al*. [26] studies only 20 of the 91 cases (22%) showed strong positive expression of the c-met protein. The expression of VEGF-A in our study was observed in 69% of breast cancer patients. Yang *et al.* [27] observed expression of VEGF-A in cytoplasmic staining in the majority of breast carcinoma cells, whereas only weak expression was found in nonmalignant breast tissue samples. Other authors assessed only VEGF A levels in cytosol of breast cancer tissues, without quantification, therefore we could not compare those results with ours [8,28].

We observed significant correlations between expression of HGFR and VEGF-A ($p<0.05$) but we

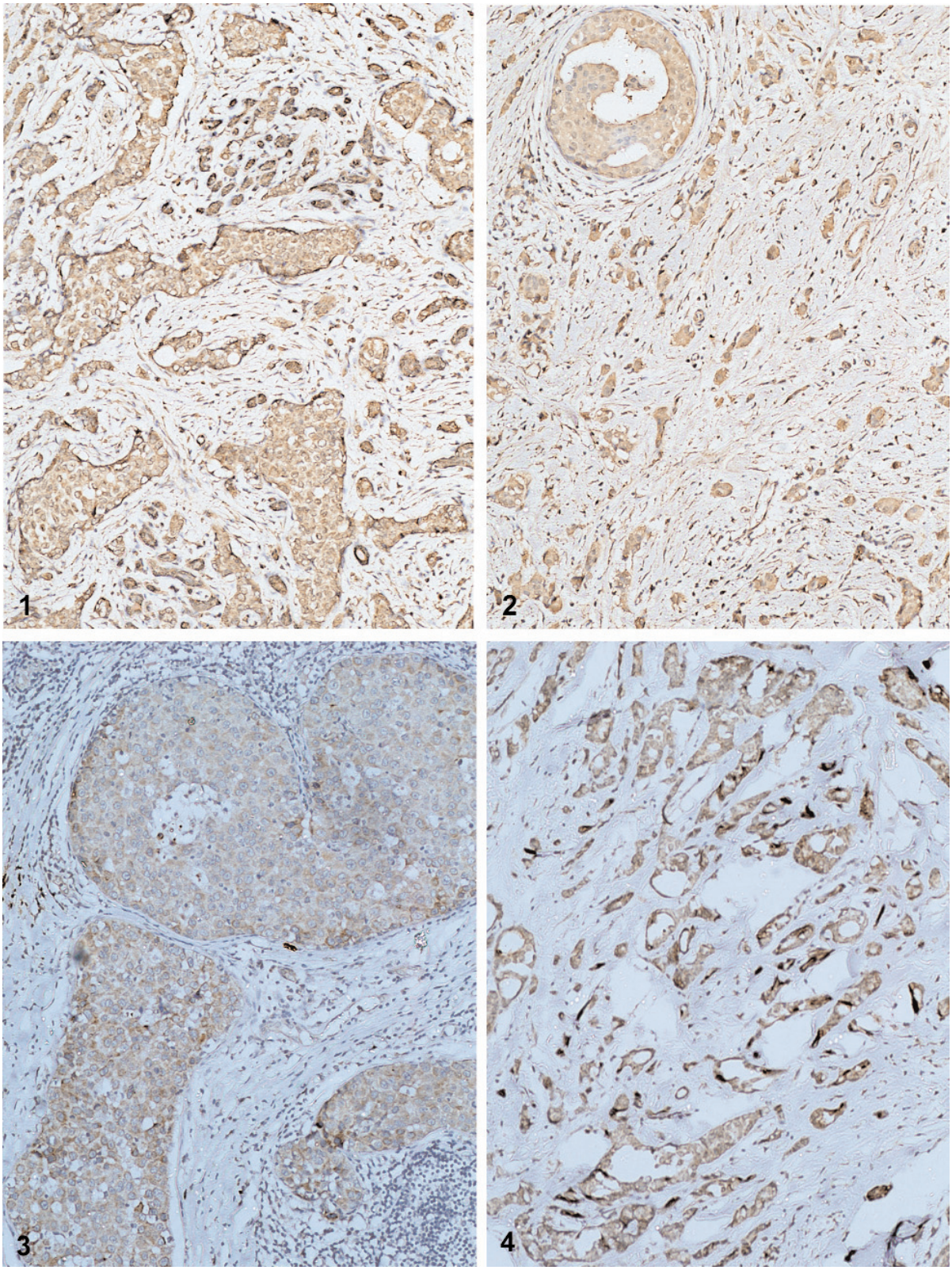

Fig. 1. Invasive ductal carcinoma. Immunohistochemical reaction with anti-c-met antibody. Strong, diffuse reaction within neoplastic cells (original magnification ×100). **Fig. 2.** DCIS and invasive ductal carcinoma. Immunohistochemical reaction with anti-c-met antibody. Strong, diffuse reaction within intraductal component and dispersed neoplastic cells (original magnification ×100). **Fig. 3.** Invasive ductal carcinoma, intraductal component. Immunohistochemical reaction with anti-VEGF antibody. Moderate, diffuse cytoplasmatic reaction within neoplastic cells (original magnification ×100). **Fig. 4.** Invasive ductal carcinoma. Immunohistochemical reaction with anti- VEGF antibody. Strong, diffuse cytoplasmatic reaction within dispersed neoplastic glands and cells (original magnification ×200).

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could not compare those results with data from other authors because there is no available literature on this subject. This correlation seems to be very interesting because both markers play an important role in tumor progression through angiostatin cooperation. Angiostatin is a 38-kD peptid of plasminogen [29], which interacts with vascular endothelial growth factor by a selective inhibitory activity on sprouting angiogenesis. This peptid counteracting with VEGF-A induced migration of primary human microvascular endothelial cells but without affecting intracellular signaling pathways known to regulate endothelial cell migration and proliferation [30]. Thus, angiostatin may induce an antiangiogenic cascade, and therefore control tumor angiogenesis by supresing expression of VEGF [31].

Wajih and Sane [32] reported that recombinant angiostatin kringles 1-3 inhibited HGF- induced phosphorylation of c-met and also inhibited downstream signaling mediators in human endothelial cells *in vitro*. Futhermore, HGF inhibited the binding of angiostatin to human endothelial cells, and angiostatin inhibited HGF-induced proliferation of these endothelial cells. The ones, discovered in laboratories on tumors cell lines relations may be an explanation of correlation between expression of VEGF-A and c-met in our patients.

In our study, 98 cases of invasive ductal breast cancer in II stage were analyzed for immunohistochemical expression of c-met and VEGF-A in order to evaluate the biological significance of this protein by testing their associations with clinicopathological features. No associations were observed between both markers expression and patients age, tumor size, tumor grade, ER status nor axillary lymph node metastases. The same results for c-met observed Nakopoulou *et al*. [33] in breast carcinoma and Osada *et al*. [34] in hepatocellular carcinoma. Other studies also did not find any correlations between VEGF-A expression and clinicopathologic variables in breast cancer [8,27] and in ovarian carcinoma [10].

On the contrary, Greenberg *et al.* [35] found that positive c-met assays correlated with increased tumor size, grade and lymph node metastases in breast cancer tissues. Lo Muzio *et al*. [25] found correlations with c-met expression and tumor stage in head and neck squamous cell carcinoma. Manders *et al*. [28] showed that in group of node-negative breast cancer patients a high cytosolic levels of vascular endothelial growth factor-A were associated with advanced age and tumor size.

We did not find any significant differences in overall- and disease- free survival in patients groups with different HGFR and VEGF-A expression levels in the time horizon of 5 and 10 years. Our data is similar to Nagy's *et al.* study concerning c-met [36] as well as Toi *et al*. [37] and Byrnes *et al*. [1] studies concerning VEGF-A in breast cancer patients. On the other way opposite results concerning c-met [26,38-40] and VEGF [41,42] expressions in patients with breast cancer were obtained in several studies. However in any of above mentined experiments, ten years follow-up was reported and similarly homogenous patients group was assessed. Conflicting conclusions concerning prognostic significance of c-met and VEGF-A expressions suggest conducting study in larger homogenous groups of patients with breast carcinoma.

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