Prognostic Impact of CD133 Immunoexpression in Node-negative Invasive Breast Carcinomas

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Abstract. *Background: Hematopoietic progenitor cells (HPCs) are able to prepare the site for incoming neoplastic cells. Among different markers of HPCs, which one should be considered the most efficient was investigated. Patients and Methods: Five hundred and seventy-nine non-metastatic lymph nodes from 49 patients affected by invasive breast cancer were submitted to an immunohistochemical comparative analysis of hematopoietic (CD34), endothelial (CD133), mesenchymal (CD117) progenitors and vascular endothelial growth factor receptor 1 (VEGFR1, also known as Flt1). The cases with an intensity-distribution score >3 were considered as high HPC expressors. Survival univariate and multivariate analyses were performed. Results: Fifteen out of the 49 patients were recorded as HPC high expressors based on the immunohistochemical VEGFR1 staining. A highly significant relationship was found between high HPC immunoexpression and the development of distant metastasis as well as the occurrence of bone localization ($p<0.001$). By univariate analysis, CD133 showed a highly significant value regarding metastatic localizations in the bone; by multivariate analysis, CD133 emerged as the only independent prognostic variable. Conclusion: CD133 expression shows a potential predictive role, thus representing a helpful tool for the management of breast cancer.*

The molecular and cellular mechanisms underlying selective organ tropism of different cancer types and site-specific metastases are relatively unknown (1). In metastatic spread, breast cancer (BC) displays organ specificity with a particular affinity to target and proliferate in lymph nodes, lung, bone, liver and brain (2-4). Several models have been developed to explain the biological complexity of metastasis in BC (5), but none of the proposed models can yet fully explain the dissemination to distant organs (6-8). At the time of diagnosis, breast tumor cells have often already been disseminated from the primary site and can be detected in the bone marrow (9, 10), where hematopoietic progenitor cells (HPCs) are also resident. BC utilizes the regional lymph nodes as the first step of metastasis (4, 11, 12) and axillary lymph node involvement has been considered one of the major relevant factors in clinical management and is directly correlated with final outcome (4, 13, 14). However, about 20% of patients affected by node-negative BC soon to develop metastases (4, 13, 14). In recent years, employing transplantable syngeneic mouse tumors able to form organ-specific metastases, the earliest steps in this process have been investigated and vascular endothelial growth factor-mediated crosstalk between tumor cells and vascular endothelial growth factor receptor 1 (VEGFR1 also known as Flt-1)-expressing bone marrow-derived HPCs has been suggested (15). The initial interaction leads to the recruitment of HPCs in target organs, potentially sites of metastases, and the recruited HPCs are considered able to prepare a permissive 'nest' for incoming neoplastic cells (15). In order to reveal the cellular clusters that maintain their progenitor cell status at the pre-metastatic sites, VEGFR1 has been utilized a functional marker, even though it has also been detected in other normal cell types (6, 16, 17), as well as in neoplastic cells from melanoma, non-small cell lung carcinoma, prostate carcinoma and BC (18-21). Subsets of $VEGFR1^+$ HPCs have been found to co-express stem/progenitor cell antigens such as hematopoietic (CD34), endothelial (CD133), mesenchymal (CD117) antigens (15). There remains a lack of agreement among pathologists and oncologists as to the best method for identifying HPCs. In order to verify among the different markers of HPCs which should be considered the most effective at identifying the first step of metastatic spread in BC, a large series of axillary negative lymph nodes, taken at surgery from patients affected by invasive carcinomas, were analyzed immunohistochemically.

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Patients and Methods

Five hundred and seventy-nine non-metastatic lymph nodes were obtained from 49 patients (mean age 61.8; age range 41-85 years), surgically treated for invasive BC in the period 1998-2007. Data concerning follow-up were available and causes of death were obtained from city registry offices. Immunocytochemical data concerning sex steroid hormone receptors for estrogen (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2) status as well as growth fraction (Ki-67 labeling index, LI) were also available. All the samples had been fixed in 10% neutral buffered formalin for 12-72 h and embedded in paraffin at 56°C. From each tissue block, 4-μm-thick serial sections were cut and mounted on silane-coated glass, then dewaxed in xylene and rehydrated in graded ethanols. Antigen retrieval, by heating slides placed in 0.01 M citrate buffer pH 6.0 in a microwave oven for 3 cycles × 5 min, was performed before adding primary antibody. For the immunohistochemical study, the sections were treated in a moist chamber: with 0.1% H_2O_2 in methanol to block the intrinsic peroxidase activity (30 min at RT); with normal sheep serum to prevent nonspecific adherence of serum proteins (30 min at RT); with rabbit polyclonal anti-human antisera AC133 (CD133) (Abgent, San Diego, CA, USA; w.d. 1:80), CD117 (Dako, Glostrup, Denmark; diluted 1:500), Flt-1 (VEGFR1) (Santa Cruz Biotechnology, Heidelberg, Germany; diluted 1:400) or with mouse monoclonal anti-human antiserum CD34 (Dako; diluted 1:50) for 16 h at 4˚C; with sheep anti-rabbit or mouse immunoglobulin antiserum (Behring Institute, Scoppito-L'Aquila, Italy; diluted 1:25) for 30 min at RT and with rabbit or mouse anti-horseradish peroxidase-antiperoxidase complexes (Dako; diluted 1:25) for 30 min at RT. For the demonstration of peroxidase activity the sections were incubated in darkness for 10 min with 3-3' diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St Louis, MO, USA) 100 mg in 200 ml 0.03% hydrogen peroxide in phosphate-buffered saline (PBS). The nuclear counterstaining was performed with Mayer's hemalum. Negative controls included omission of the primary antiserum and replacement of the primary antiserum with PBS solution (pH 7.4) or normal horse/goat serum; in each of these conditions, no staining was evident. Two histological sections of capillary hemangioma/hemangioblastoma were utilized as positive controls for each marker such as VEGFR1, CD133 and CD34, while one section of gastrointestinal stromal tumors was used as positive control for CD117 antiserum.

Two pathologists using a double-headed microscope performed the assessment of immunostained sections on a consensus basis, blinded to the clinicopathological data. Immunostained sections were estimated by light microscopy using a ×40 objective lens and ×10 eyepiece. The quantification of immunostained HPC aggregates (2-5 cells) was performed for each marker on three different areas: a value of 1 or 2 was assigned to the case with <3 or ≥3 HPC aggregates respectively, the intensity of immunostaining was also taken into consideration (weak=1; moderate=2; strong=3). An intensity-distribution score (ID score) was calculated for each antiserum, by multiplying the value of the HPC aggregates and the staining intensity; cases with an ID score >3 were considered as high expressors.

The sensitivity, specificity, positive and negative predictive values and efficiency (expressed as a result as percentage of what ideally could be expected, hence with 100% as ideal case) of each immunohistochemical marker of HPC were evaluated using dichotomous values (high, low) taking the metastatic status of the patients as the reference.

Survival analysis was performed by the Kaplan-Meier method utilizing HPC immunoexpression as a strata variable; in this way, disease-free survival (DFS) was evaluated. To compare the survival curves of different groups of patients, the Mantel-Cox log-rank test was applied. A multivariate analysis (Cox regression model) was utilized to determine the independent effect of each resulting variable on DFS. In particular, a forward stepwise procedure and likelihood ratio tests were used to select the variables included in the final model. An estimation of the relative risk with 95% confidence interval was computed. A *p*-value less than 0.05 was considered statistically significant. Statistical analysis was carried out using the SPSS package (SPSS Inc., Chicago, IL, USA).

Results

The follow-up time of patients ranged from 6 to 136 months (mean 64.3 months); twenty patients showed progression of disease with the appearance of bone metastases in 14 cases. Twenty-nine patients were alive without disease or censored.

Adequate immunostaining quality was evident in all the lymph node samples, although the immunohistochemical expression of the utilized antisera was heterogeneous. Generally, the stained cells present in the lymph nodes were arranged in small groups (2-5 cells) with some isolated ones (Figure 1).

Utilizing the VEGFR1 antiserum, 15/49 (30.6%) patients with BC were recorded as HPC high expressors having an ID score more than 3. With CD133, CD34 and CD117 17/49 (34.7%), 24/49 (49.0%) and 23/49 (46.9%), respectively were high expressors. For each antiserum utilized, a highly significant relationship was found between high HPC immunoexpression and the development of distant metastases, as well as the occurrence of bone localization ($p<0.001$).

Among the HPC markers, VEGFR1 and CD133 exhibited the greatest specificity (both 93.1%), while CD34 was characterized by highest sensitivity (85.0%). The highest positive predictive value was obtained by CD133 immunostaining (88.2%); the highest negative predictive value was attributable to CD34 (88.0%). CD 133 showed the highest efficiency value (85.7%). Table I shows the analytical data concerning each immunomarker.

The univariate analysis relative to DFS in the BC patients for all the considered markers able to identify the HPCs are shown in Table II; a highly significant *p*-value was obtained for each immunomarker. However, by Cox multivariate analysis, only CD133 emerged as an independent prognostic variable for the BC patients (Table III).

The relationships between the clinico-pathological characteristics as well as the biomolecular data of BC and HPC status for each antiserum are reported in Table IV. A significant correlation was recorded between Ki-67 LI as well as HER2 status and the immunohistochemical data concerning the VEGFR1, CD133 and CD117 antisera

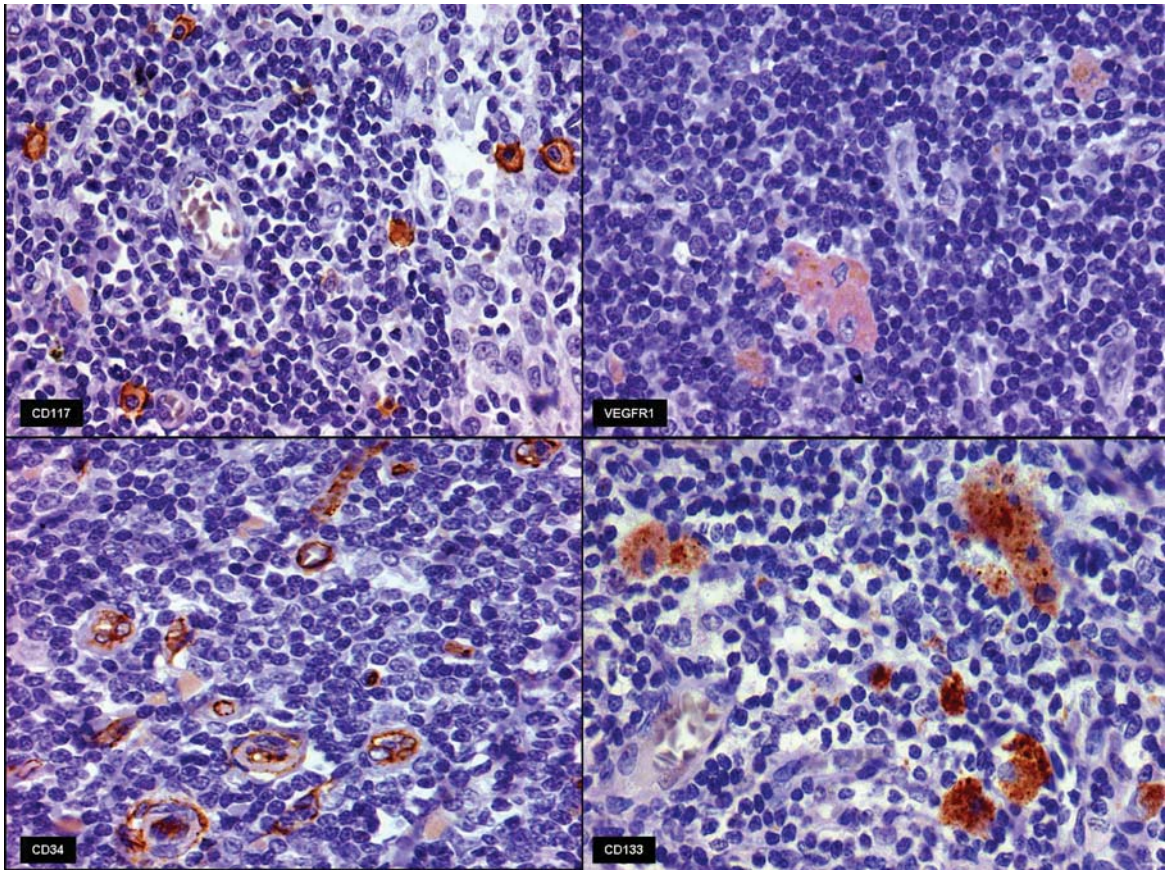

Figure 1. *Scattered or small group HPC elements immunohistochemically revealed in lymph nodes by CD117, VEGFR-1, CD34 and CD133 staining (immunoperoxidase, Mayer's hemalum counterstain, ×400).*

Table I. *Sensitivity, specificity, positive and negative predictive values, and efficiency of each immunohistochemical marker of HPCs evaluated taking as reference the presence of distant metastases.*

	VEGFR1	CD133	CD34	CD117
Sensitivity	65.0%	75.0%	85.0%	80.0%
Specificity	93.1%	93.1%	75.9%	75.9%
Positive predictive value	86.7%	88.2%	70.8%	69.6%
Negative predictive value	79.4%	84.4%	88.0%	84.6%
Efficiency	81.6%	85.7%	79.6%	77.6%

Table II. *Immunohistochemical markers of HPCs in patients with invasive BC: a univariate analysis of disease-free survival by Mantel-Cox log-rank test.*

Parameter	χ^2	df	*P*-value
VEGFR1	18.62	1	0.0000
CD133	21.73	1	0.0000
CD34	16.52	1	0.0000
CD117	13.18	1	0.0003

df: Degrees of freedom.

values, while a tendency towards a statistically significant *p*-value was obtained with the CD34 antiserum. No significant relationships were found between the HPC immunohistochemical data and age, histotype, tumor grade or stage and ER data, although a significant correlation between PR data and immunoexpression of VEGFR1 and CD133 was found.

Table III. *Multivariate survival analysis by Cox regression model in invasive BC.*

Variable	β	SE	Exp(β)	*P*-value
CD133	2.065	0.527	7.882	0.0001

β=Regression coefficient; SE=standard error; Exp(β)=ratio of risk.

Table IV. *Clinicopathological and biomolecular data with corresponding immunohistochemical markers of HPCs in patients with invasive BC.*

Parameter	No.	VEGFR1	CD133	CD34	CD117
Histological type					
Ductal	43	14 (33%)	16 (37%)	23 (53%)	22 (51%)
Lobular	5	1 (20%)	1 (20%)	1 (20%)	1 (20%)
Medullary	1	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Histopathological grade					
G1	6	0 (0%)	1 (17%)	3 (50%)	2 (33%)
G2	24	8 (33%)	8 (33%)	12 (50%)	12 (50%)
G3	18	7 (39%)	8 (44%)	9 (50%)	9 (50%)
Stage					
I	23	6 (26%)	6 (26%)	11 (48%)	11 (48%)
II	26	9 (35%)	11 (42%)	13 (50%)	12 (46%)
ER					
Negative	23	10 (43%)	11 (48%)	14 (61%)	14 (61%)
Positive	26	5 (19%)	6 (23%)	10 (38%)	9 (35%)
PR					
Negative	25	12 (48%)	12 (48%)	14 (56%)	14 (56%)
Positive	24	3 (13%)	5 (21%)	10 (42%)	9 (38%)
Ki-67					
Low	27	4 (15%)	5 (19%)	10 (37%)	9 (33%)
High	22	11 (50%)	12 (55%)	14 (64%)	14 (64%)
HER2					
Not amplified	40	8 (20%)	10 (25%)	17 (43%)	16 (40%)
Amplified	9	7 (78%)	7 (78%)	7 (78%)	7 (78%)

Discussion

VEGFR1 has been revealed on subsets of epithelial tumor cells in invasive ductal carcinomas, as well as on tumor vascular endothelium and myoepithelial cells, suggesting an immunohistochemical profile indicative of malignant phenotype in breast carcinoma (23). Although VEGFR1 exhibited high specificity for HPC, in the present study it was not the most efficient marker.

Normal non-hematopoietic human tissues such as breast epithelia, parotid and dermal sweat glands, melanocytes, central nervous system, placenta, interstitial cells of the testes and ovaries express CD117 (*c-KIT*) (23-25); furthermore, CD117 has also been documented in small cell lung cancer, breast carcinoma and melanoma (26-28). In general, the expression of CD117 during hematopoietic development is highest at the early stages and then diminishing with maturation (25); moreover, up to 60-70% of $CD34^+$ bone-marrow progenitor cells co-express CD-117 (25, 29).

CD34 has been widely used as a marker of vascular endothelial cells and hematopoietic stem and progenitor cells (30-32). Although more recently CD34 has been considered a useful tool for stem-progenitor cell characterization (33), its function has not yet been definitively determined. It has been proposed that CD34 promotes proliferation and blocks differentiation of stem or progenitor cells (34). Moreover, by flow cytometry, Mehra *et al*. (35) showed that hematopoietic marker CD34 was co-expressed in 85% of cells which exhibited a positivity for CD133, already considered as a cell surface marker of adult stem cells (36). In hematopoietic lineages in man, *CD133 antigen expression is restricted to* $CD34^+$ cells, although CD133 transcripts have been found in many human cell lines and differentiated cells (37, 38). Furthermore, CD133 expression has been found to correlated with patient survival in early colorectal carcinoma (39, 40), may play an important role in the evolution of gastric carcinoma, and should be considered as a potential prognostic marker (41, 42).

CD133 showed the greatest efficiency in the present study, it may be considered the most useful immunomorphological indicator for identifying HPCs, showing a potentially positive predictive role for analyzing the risk of metastasis in BC. Additionally, in the multivariate analysis, CD133 emerged as the exclusive independent prognostic value in pN0 BC patients. Bone involvement has also been related to high *CD133* levels in peripheral blood and in patients with bone metastases, high CD133 mRNA expression was significantly and independently related to survival (35). However, the present data concerning the CD133 immunoexpression in pN0 BC patients showed a highly significant value regarding bone metastasis only by univariate analysis. Finally, in the present study, no significant relationship was found between the HPC immunohistochemical data and age, histotype, tumor grade

or stage and hormone receptor expression. On the other hand, a significant correlation was recorded between Ki-67 LI, as well as HER2 status and CD133 immunoexpression, and well as VGFR1 and CD117 antisera, revealing an interesting relationship between well-known predictive parameters of poor clinical course in BC and the detection of HPCs. Therefore, we contend that the identification of HPCs in pN0 BC, in particular as recognized by CD133 for its intrinsic independent prognostic relevance, may represent an additional useful tool to predict the clinical behavior as well as indicating the management of breast carcinomas.

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