Nestin-positive microvessel density is an independent prognostic factor in breast cancer

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Abstract. The process of angiogenesis based on new vessel formation within the tumour area plays a significant role in the progression of breast cancer. Nestin is an intermediate filament protein and participates in the cytoskeleton organization. Nestin expression in the endothelium of blood vessels is mainly limited to newly forming vessels, thus being a more specific marker of angiogenesis than the commonly used vascular antigens. The aim of this study was to determine the prognostic value of nestin-positive microvessel density (Nes^+MVD) in breast cancer patients and to confirm that nestin expression is related to newly forming tumour vessels. In this study, 137 cases of ductal breast carcinoma and 19 cases of non-malignant breast tissue lesions (NBTLs) were examined. Immunohistochemical reactions were performed on paraffin sections using antibodies against nestin, CD34 and CD31 antigens. For each marker, the microvessel density (MVD) was determined. Nestin expression was also examined in human endothelial cell lines (HUVEC-SVT, HMEC-1 and HEPC-CB.1) representing a different level of endothelial cell maturity. HUVEC-SVT and HMEC-1 cells represent the endothelium of mature vessels, whereas HEPC-CB.1 cells represent the early endothelial progenitor cells (EPCs). We have demonstrated that high Nes^+MVD may be associated with a more aggressive course of the disease and a poorer prognosis. We have also found a higher Nes^+MVD in the cases with lymph node metastases, with higher histological grade, with advanced-stage disease and with the triple-negative (TN) breast cancer. In addition, nestin expression in vessels was associated with a shorter overall survival (OS) and earlier relapse, and in the case of OS nestin was an independent prognostic factor. Finally, we further confirmed that nestin expression in endothelial cells reflects a progenitor nature of newly forming vessels.

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Introduction

Breast cancer is the most commonly diagnosed malignant cancer in women and the leading cause of death among women with cancers. The formation of a new vascular network known as tumour angiogenesis plays a key role in the progression of this tumour. The process was first described in 1971 by Folkman, who postulated that the tumour growth and metastasis depends on the number of newly formed blood vessels (1). This hypothesis became the basis for further research demonstrating that the 'angiogenic switch' is an integral part of most cancer progression (2). The administration of adjuvant anti-angiogenic therapy had positive results in the case of colorectal, breast, kidney and pancreatic cancers, and non-small cell lung carcinoma (3,4).

The most commonly used method to quantify intratumoural angiogenesis in histological specimens is the assessment of MVD. The MVD can be assessed by counting immunolabelled vessels stained with antibodies against endothelial cell antigens, e.g., CD31 and CD34 (5). This method was developed by Weidner *et al* who also confirmed that in the case of invasive ductal carcinoma (IDC) high MVD is associated with the incidence of regional lymph node and distant metastases (6). The results of the meta-analysis confirmed the prognostic value of MVD assessment in breast cancer (5). However, the evaluation of tumour angiogenesis with MVD has some limitations. One of them is the fact that commonly used markers are expressed not only in newly forming vessels but also in mature vessels present in body tissues and organs (7).

Nestin is a type VI intermediate filament (IF) protein and participates in the cytoskeleton organization (8). Its expression is typical of neuroepithelial stem cells (8), but it is also present in progenitor (9-11) muscle (9), endothelial (12,13) and cancer cells (14,15). In the cell, nestin expression is transient and following cell differentiation it is downregulated and replaced by tissue-specific IFs (16). It is suggested that nestin

expression in blood vessels is mainly limited to the proliferating and newly forming vessels, which makes nestin a more specific marker of angiogenesis than commonly used CD34 and CD31 (17-20).

Due to nestin progenitor features and proven expression in blood vessels we suspected that it may be a reliable marker for angiogenesis evaluation in IDC patients. Hence, the aim of the present study was to assess nestin-positive microvessel density (Nes^+MVD) and to determine its prognostic value in patients with IDC. We also aimed at determining whether nestin is a marker of newly forming and poorly differentiated blood vessels. Accordingly, we compared Nes^+MVD with the density of newly forming $CD34^+$ vessels ($CD34^+$MVD) and mature $CD31^+$ vessels ($CD31^+$MVD). To confirm whether the expression of nestin is increased in endothelial progenitor cells (EPCs) we developed an *in vitro* model consisting of human endothelial cell lines representing a different level of maturity. HUVEC-SVT and HMEC-1 cells represent the endothelial cells (EC) of mature venous vessels and capillaries, whereas HEPC-CB.1 cells are the early EPCs.

Materials and methods

Tissue samples. The study was conducted on archival paraffin-embedded ductal breast carcinoma samples (n=137) and NBTLs (n=19) collected during resection procedures at The Maria Sklodowska Curie Memorial Cancer Centre and Institute of Oncology in Cracow and at The Lower Silesian Oncology Centre in Wroclaw from 1999 to 2013. From 137 investigated cancer patients, 26 developed pre-invasive *in situ* carcinoma (DCIS), whereas 111 developed invasive cancer (IDC). The investigated cases were categorized into 4 groups according to their invasiveness (Table I): NBTLs, ductal carcinoma *in situ* (DCIS), lymph node-negative invasive ductal carcinoma (IDC N^-) and lymph node-positive invasive ductal carcinoma (IDC N^+). The clinical, pathological and survival data were obtained only for IDC patients (n=111) from the archives of the hospital and are listed in Table II. From these 111 invasive cases, 23 patients died from the disease and 25 patients had recurrence (Table II). The study was approved by the Commission of Bioethics of the Wroclaw Medical University, Poland.

Human endothelial cell lines. To determine the expression level of nestin human immortalized ECs (HUVEC-SVT, HMEC-1) and EPCs (HEPC-CB.1) were selected. The HMEC-1 cells (ATCC, Washington, CO, USA) were cultured in MCDB 131 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA), 10 ng/ml epidermal growth factor (EGF, Invitrogen), 1 μg/ml hydrocortisone (Sigma) and 10 mM L-glutamine (Invitrogen). HUVEC-SVT and HEPC-CB.1 cells (both courtesy of Dr M. Paprocka, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences, Wroclaw, Poland) were grown in Dulbecco's modified Eagle's medium (DMEM, Lonza, Basel, Switzerland) with 4.5 g/l glucose, 25 mM HEPES sodium pyruvate without L-glutamine supplemented with 10% fetal bovine serum (Merck, Millerica, MA, USA), 2 mM L-glutamine and antibiotics (Sigma). All of the studies utilized cell passages 18-26. The investigated cell lines exhibit different phenotypes of endothelial cells. Human umbilical vein endothelial cells (HUVEC-SVT) are immortalised cells isolated from the large vessel representing macrovascular phenotype (21). Immortalised human microvascular endothelial cells (HMEC-1) originate in dermal microvasculature and show the characteristics similar to ECs present in tumour environment (21). HEPC-CB.1 cells are immortalized early human EPCs isolated from the cord blood (22).

Immunohistochemistry (IHC). Immunohistochemical reactions were performed on 4-μm thick paraffin sections using Autostainer Link48 (Dako, Glostrup, Denmark) with a panel of mouse-anti human monoclonal antibodies against: nestin (dilution 1:100, OBT1610, Bio-Rad, Hercules, CA, ISA), CD31 (ready-to-use, IR610, Dako), CD34 (ready-to-use, IR632, Dako) estrogen receptor (ER), clone 1D5 (ready-to-use, IR654; Dako) and progesterone receptor (PR) clone 636 (ready-to-use, IR068; Dako). The sections were boiled in EnVision FLEX Target Retrieval Solution (pH 9.0, 97˚C, 20 min) using Pre-Treatment Link Platform (both from Dako). Activity of endogenous peroxidase was blocked by 5 min incubation with EnVision FLEX Peroxidase-Blocking Reagent (Dako). The samples were incubated with primary antibodies for 20 min at room temperature (RT) and then incubated with EnVision FLEX/HRP for 20 min (Dako). 3,3'-diaminobenzidine (DAB, Dako) was utilized as the peroxidase substrate and the sections were incubated for 10 min. Finally, all slides were counterstained with EnVision FLEX Hematoxylin (Dako) for 5 min. After dehydration in graded ethanol concentrations (70, 96 and 99.8%) and in xylene, slides were closed with coverslips in Dako mounting medium (Dako). Human epidermal growth factor receptor 2 (HER2) expression status was determined using HercepTest and HER2 FISH pharmDx kit (both from Dako), according to the manufacturer's instructions.

Examination of IHC reactions. The IHC reactions were evaluated with a BX-41 light microscope (Olympus, Tokyo, Japan). The MVD was assessed for each investigated antigen i.e., nestin, CD31 and CD34 according to the Weidner method (6). Firstly, the slides were examined under low magnification (x100) to identify three areas with the highest vascular density (hot-spots). Then, under magnification x400 stained vessels were counted. The final score for each slide was presented as a mean number of vessels per mm^2. Any stained EC or ECs clusters were counted as a single microvessel, even in the absence of vessel lumen (6). The status of ER and PR was scored 0-3 points, depending on the percentage of positive cells (0 points, no reactions; 1 point, 1-10%; 2 points, 11-50%; 3 points, 51-100% stained cells) (23). The expression of HER2 was evaluated using a scale that considers both the intensity of the membrane reaction and the percentage of positive tumour cells (24).

Immunocytochemistry (ICC) and immunofluorescence (IF). Investigated endothelial cell lines were grown on glass coverslips for 24 h at 37˚C. After 24 h, cells were washed with phosphate-buffered saline, fixed in 4% paraformaldehyde and permabilised with 0.2% Triton X. The IHC of fixed cells was performed with anti-nestin antibody in Dako Autostainer

Table I. Groups of patients according to tumour invasiveness.

Histological type	n
Non malignant breast tissue lesions	19
Ductal carcinoma *in situ*	26
Invasive ductal carcinoma - lymph node negative	62
Invasive ductal carcinoma - lymph node positive	49

Link48 (Dako) according to the procedure described above. The slides were incubated with primary antibodies for 20 min at RT, and then incubated with EnVision FLEX (Dako) to visualize the antigens. For IF the cell lines were cultured and fixed as stated above. All slides were incubated at 4˚C overnight with monoclonal antibody against nestin (Bio-Rad) and resolved in antibody diluent (Dako) in the concentration of 1:100. As a secondary antibody donkey anti-mouse antibody conjugated with rhodamine (1:2,000, polyclonal; 715-025-151 Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was applied. The slides were covered with ProLong® Gold Antifade Mountant mounting medium (Molecular Probes, Eugene, OR, USA) with 4',6-diamidino-2-phenylindole (DAPI) and viewed and imaged with a BX51 fluorescence microscope (Olympus).

RNA isolation and real-time PCR reactions. Total RNA from HUVEC-SVT, HMEC-1 and HEPC-CB.1 cell lines was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To eliminate the genomic DNA, the protocol included on-column DNase digestion. RNA concentration and purity were measured using the NanoDrop1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The absorbance was measured at 260-280 nm. The first-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA, USA). The relative nestin gene (NES) mRNA expression level was determined by quantitative real-time PCR using the 7500 Real-Time PCR system and the iTaq Universal Probes Supermix (Bio-Rad), according to the manufacturer's protocol. We applied the following human Taqman Gene Expression Assays: NES Hs04187831_g1 for nestin and ACTB Hs99999903_m1 for β-actin (Applied Biosystems). Since β-actin is a housekeeping gene, it was used as a reference for determining NES expression in the analyzed human endothelial cell lines. The reactions were carried out in triplicate in the following conditions: initial denaturation at 95˚C for 10 min, followed by 45 cycles of denaturation at 95˚C for 15 sec, and annealing and elongation at 60˚C for 60 sec. The relative mRNA expression level of the NES gene was calculated with the ΔΔCt method (25).

Flow cytometry (FC). For flow cytometric immunophenotyping of intercellular nestin, cells were fixed and permeabilized with BD Cytofix/Cytoperm™ (Becton-Dickinson, CA, USA) according to the manufacturer's instructions. Subsequently, the cells were stained with monoclonal antibody against human nestin (1:100; OBT1610) for 20 min/RT and immunolabelled with sheep anti-mouse secondary antibody conjugated with

Table II. Clinicopathological characteristics of IDC patients.

Parameters	n	%
Age		
≤50	34	30.6
>50	77	69.4
Menopausal status		
Pre-	36	32.4
Post-	73	65.8
No data	2	1.8
Tumour size		
T1	61	55.0
T2	37	33.3
T3	9	8.1
T4	2	1.8
No data	2	1.8
Lymph nodes		
Negative	62	55.9
Positive	49	44.1
Grade		
1	11	9.9
2	60	54.1
3	40	36.0
pTNM		
I + II	92	82.9
III + IV	17	15.3
No data	2	1.8
ER		
Negative	28	25.2
Positive	83	74.8
PR		
Negative	38	34.2
Positive	73	65.8
HER2		
Negative	67	60.4
Positive	44	39.6
Triple-negative		
Yes	17	15.3
No	94	84.7
Overall survival		
Deaths	23	20.7
Alive	86	77.5
No data	2	1.8
Event-free survival		
Recurrence	25	22.5
No recurrence	83	74.8
No data	3	2.7

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor.

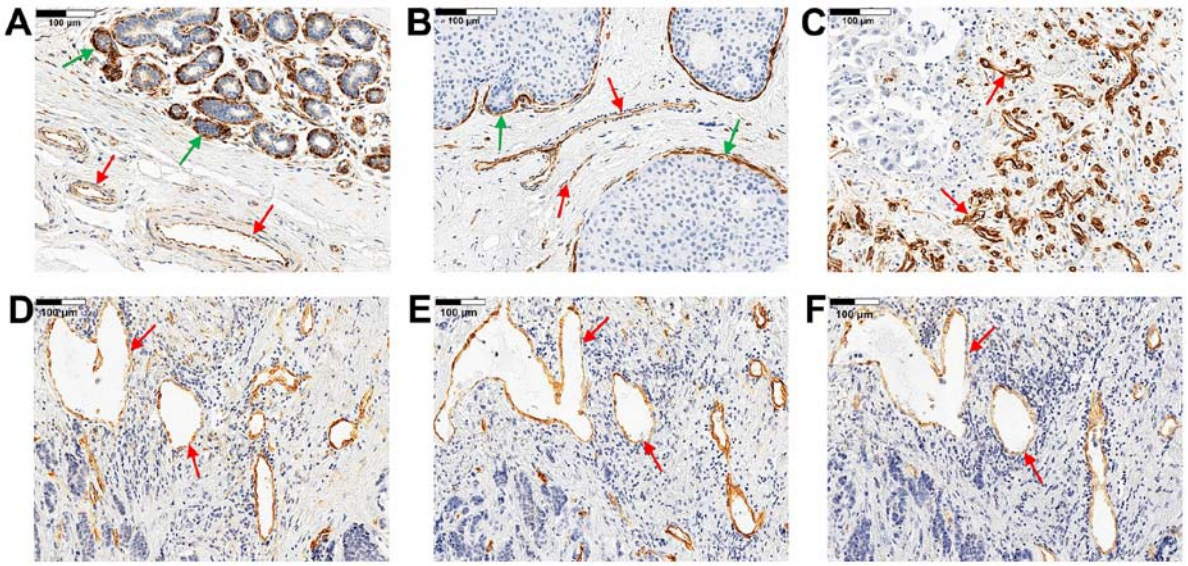

Figure 1. Nestin expression (red arrows) in blood vessels in NBTLs [(A) x300], DCIS [(B) x300] and IDC [(C) x300]. Nestin expression in myoepithelial cells is also observed [(A and B) green arrows]. Nestin [(D) x250], CD34 [(E) x250] and CD31 [(F) x250)] expression in blood vessels (red arrows, serial sections).

FITC (1:200, polyclonal; P8547 Sigma). The cells were analyzed by flow cytometry using FACSCalibur (Becton-Dickinson) equipped with a 488-nm laser and filter for FITC analysis (530 BP). As single labelling was performed, no compensation setting was required. Data were recorded for 10,000 events using CellQuest version 3.3 software (Becton-Dickinson), analyzed on the ungated population (except for debris) and presented without any transformation as histograms using WINMDI 2.7 (Scripps Institute, CA, USA) software. Mean fluorescence intensity (MFI) for nestin is shown as a difference between MFI for samples incubated with primary and secondary antibodies and MFI for isotype control.

Statistical analysis. The data were analyzed with Prism 5.0 (GraphPad, La Jolla, CA, USA) software. The Kolmogorov-Smirnov test was applied to determine whether sample data are normally distributed. To evaluate the relationships and correlations between the examined markers and with clinicopathological factors, Student's t-test and Spearman rank correlation test were utilized. The Kaplan-Meier method and the Mantel-Cox test were used to determine the significance of patient OS and event-free survival (EFS). A Cox proportional hazards model with forward stepwise selection was used to calculate univariate and multivariate hazard ratio for the study variables. Differences were considered statistically significant at $p<0.05$.

Results

IHC. Nestin expression was observed in the cytoplasm of ECs in all study cases (Fig. 1A-D). In addition, nestin expression was observed in myoepithelial cells of the ducts and lobular acinar units (Fig. 1A and B) and in some cases in tumour cells (data not shown). A significantly higher Nes^+MVD was observed in both groups of IDC patients i.e., IDC N^- and IDC N^+ in relation to the control group comprising NBTLs (Fig. 2A; 72.32±25.01; 86.12±31.60 vs. 33.85±14.83; $p<0.0001$, $p<0.0001$, respectively, Student's t-test). It was also demonstrated that Nes^+MVD was higher in both groups with IDC (IDC N^+ and IDC N^-) as compared to the group with DCIS (Fig. 2A, 86.12±31.60; 72.32±25.01 vs. 43.52±18.21; $p<0.0001$, $p<0.0001$, Student's t-test). We also showed that Nes^+MVD was significantly higher in the group of patients with IDC N^+ than with IDC N^- (Fig. 2A, 86.12±31.60 vs. 72.32±25.01; $p=0.0132$, Student's t-test). Additionally, in the group of IDC, Nes^+MVD was significantly higher in the case of G3 and G2 tumours than in G1 tumours (Fig. 2B, 86.63±32.96; 78.84±28.88 vs. 57.14±20.87; $p=0.0072$, $p=0.0203$, Student's t-test). A lower value of Nes^+MVD was found in patients with early-stage disease (I and II) than in patients with advanced-stage disease (III and IV; Fig. 2C, 75.99±27.62 vs. 92.02±35.05; $p=0.0377$, Student's t-test).

Among patients with IDC, a high Nes^+MVD was also related to the TN phenotype of breast cancer characterized by a lack of ER, PR and HER2 expression (Fig. 2D, 76.91±28.97 vs. 93.83±39.58; $p=0.0357$ Student's t-test). The analysis of survival data in the group of IDC patients showed that a high value of Nes^+MVD was associated with shorter OS (Fig. 3A, $p=0.0013$, Mantel-Cox) and shorter EFS (Fig. 3B, $p=0.0091$; median 75.76; Mantel-Cox). Moreover, in the case of OS, nestin turned out to be an independent prognostic factor (Table III, $p=0.007$, multivariate Cox analysis). Additionally, the correlation analysis showed that Nes^+MVD in IDC correlates with the density of $CD34^+$ vessels (Fig. 4A, $r=0.3280$; $p=0.0032$, Spearman rank test) whereas no correlation was noted between Nes^+MVD and the density of $CD31^+$ vessels (Fig. 4B, $r=0.1563$; $p=0.1304$, Spearman rank test).

Real-time PCR. The real-time PCR was performed to evaluate NES expression level in human endothelial cell lines. The relative *NES* expression was assessed in relation to the HUVEC-SVT cell line. The analysis showed significant differences in NES expression between all the investigated cell lines i.e., HUVEC-SVT, HMEC-1 and HEPC-CB.1 (Fig. 5, $p<0.0001$, Student's t-test). The highest *NES* expression

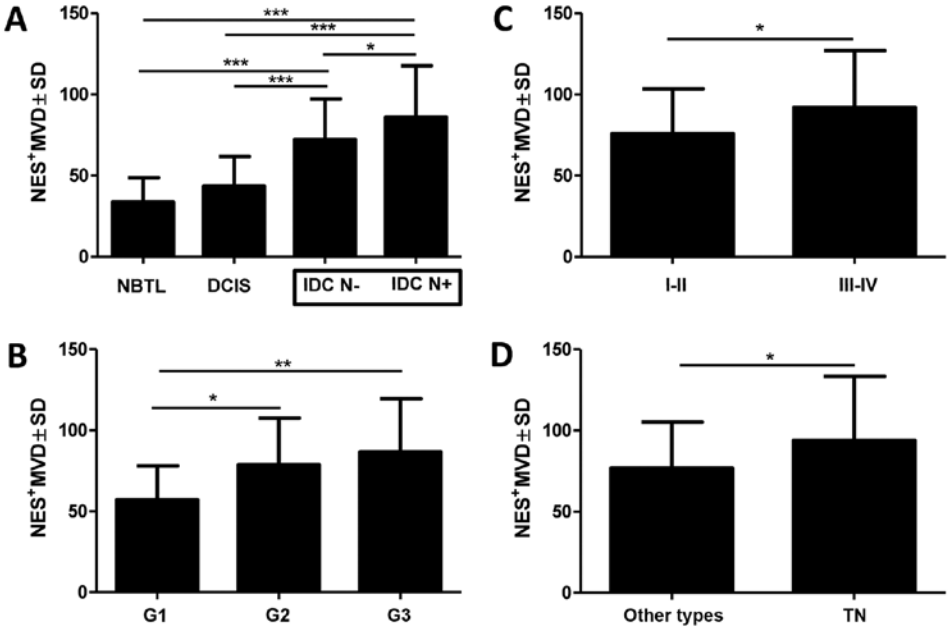

Figure 2. Nes$^+$MVD increases with the increase in invasiveness of lesions in the breast [(A) Student's t-test]. Nes$^+$MVD is higher in lesions with higher histological grade [(B) Student's t-test], in advanced-stage disease patients [(C) Student's t-test] and in TN breast cancers [(D) Student's t-test]; *p<0.05, **p<0.01, ***p<0.001.

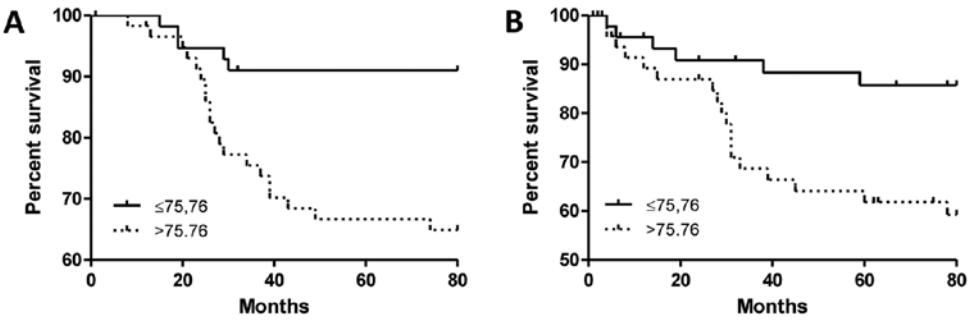

Figure 3. Nes$^+$MVD is related to a shorter OS of patients [(A) p=0.0013, Mantel-Cox] and EFS [(B) p=0.0091, Mantel-Cox)].

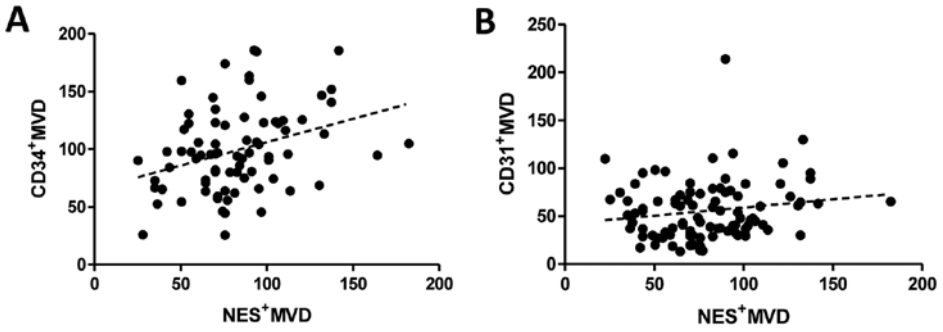

Figure 4. Nes$^+$MVD correlates with the density of newly forming CD34$^+$ vessels [(A) r=0.3280; p=0.0032, Spearman rank test], but not with the density of CD31$^+$ mature vessels [(B) r=0.1563; p=0.1304, Spearman rank test].

was observed in the progenitor HEPC-CB.1 cells isolated from human umbilical cord blood, and a trace expression in the HMEC-1 line isolated from dermal microvessels.

IF and ICC. Experiments using ICC and IF showed a cytoplasmic expression of nestin in all the examined cell lines (Fig. 6). The most intense reaction was observed in the progenitor HEPC-CB.1 cells, mildly intense in the HMEC-1 cells and the weakest in the HUVEC-SVT cells.

FC. To confirm the different expression level of nestin in the examined cell lines, we used flow cytometric assay. Measurements of the MFI showed a lack of nestin expression in the HUVEC-SVT cells and a very weak nestin expression

Table III. Uni- and multivariate Cox analysis of overall survival.

	Univariate Cox analysis p-value				Multivariate Cox analysis HR			
Characteristics	p-value	HR	95% CI lower	95% CI upper	p-value	HR	95% CI lower	95% CI upper
Age ≤50 vs >50	0.714	0.978	0.869	1.101				
Menopausal status Pre vs post	0.687	0.832	0.340	2.034				
G	**0.032**	2.220	1.073	4.595	**0.042**	2.417	1.034	5.649
Stage I-II vs III-IV	**0.0001**	7.675	3.357	17.549	**0.004**	6.971	1.865	26.063
pT	**0.0001**	3.787	2.281	6.288	**0.041**	2.045	1.029	4.062
pN N0 vs N1-3	0.129	1.015	0.995	1.035				
ER Negative vs positive	**0.0118**	0.346	0.152	0.790	0.446	0.582	0.145	2.338
PR Negative vs positive	**0.039**	0.423	0.187	0.959	0.14	0.384	0.108	1.368
HER2 Negative vs positive	0.209	1.816	0.716	4.609				
Triple-negative	0.069	2.376	0.936	6.031	**0.03**	0.182	0.039	0.849
$CD34^+$MVD <95.44 vs >95.44	0.315	1.655	0.62	4.421				
$CD31^+$MVD <48.42 vs >48.42	0.244	1.718	0.691	4.272				
Nes^+MVD <75.76 vs >75.76	**0.002**	4.960	1.84	13.37	**0.007**	4.303	1.478	12.52

HR, hazard ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor; MVD, microvessel density. Bold, statistically significant.

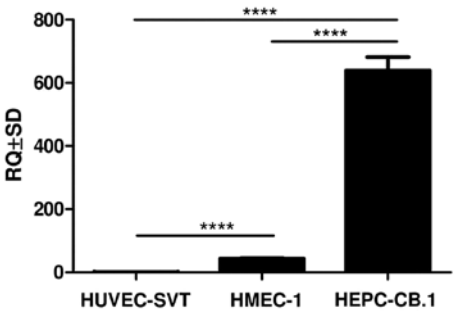

Figure 5. Expression of *NES* mRNA in human endothelial cell lines (Student's t-test, ****p<0.0001).

in HMEC-1 cells (Table IV and Fig. 6). A high MFI of nestin was observed in the progenitor HEPC-CB.1 cells (Table IV and Fig. 6).

Table IV. Mean values of fluorescence for isotype control and nestin in HUVEC-SVT, HMEC-1 and HEPC-CB.1 cell lines.

Cell line	Isotype control	I+II antibody	Nestin
HUVEC-SVT	6.08	7.20	1.12
HMEC-1	3.93	9.86	5.93
HEPC-CB.1	7.10	51.20	44.10

Discussion

Initially, nestin expression in the proliferating endothelium of blood vessels in human tumours was reported in the tumours of the central nervous system (26) and rhabdomyosarcoma (27). Further studies on animal models showed that nestin expression

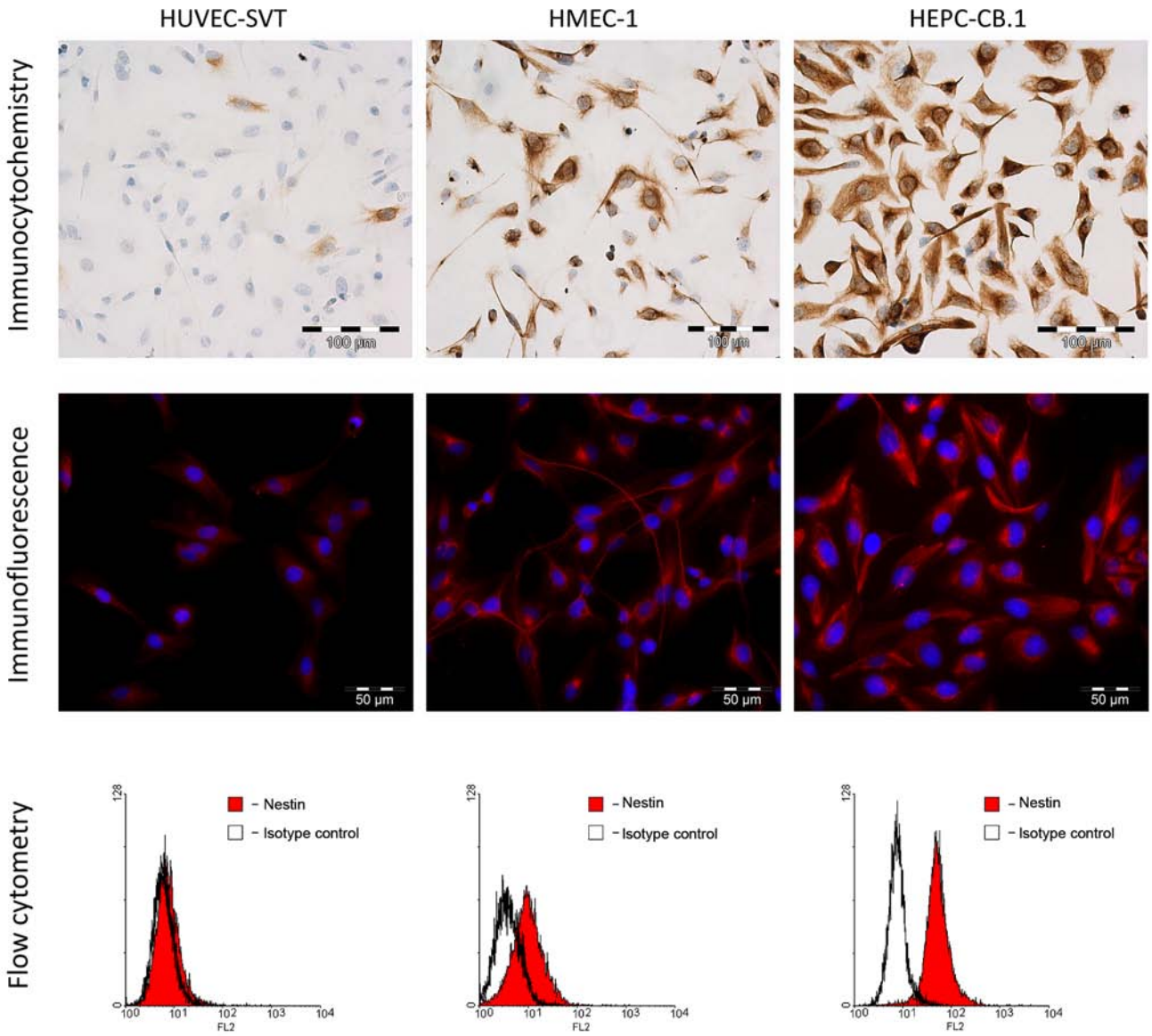

Figure 6. Cytoplasmic nestin expression in HUVEC-SVT, HMEC-1 and HEPC-CB.1 cell lines examined with the use of ICC, IF and FC.

in cerebellar blood vessels can be induced by pro-angiogenic factors (28). Then Mokry *et al* confirmed that nestin undergoes expression in blood vessels in many human tissues and organs in which angiogenesis occurs (13). The first attempt at determining the density of Nes$^+$ vessels and comparing it with the density of CD34$^+$ vessels was assessed in patients with gastric adenocarcinoma (17). However, this study demonstrated no prognostic value for either antigen, but in the case of large tumours nestin showed some prognostic value. Further studies revealed that the high density of proliferating microvessels co-expressing nestin and Ki-67 antigen was associated with a worse prognosis in patients with prostate (29) and breast cancers (30). Previous studies on nestin expression in breast cancer tumour cells revealed that nestin correlates with TN subtype and worse prognosis (15,31,32). However, Kruger *et al* demonstrated in a large population-based study that nestin expression in tumour cells strongly correlates with basal-like molecular subtype of TN breast cancers, with BRCA1-related breast cancer and with reduced survival (33) In our study, we also noted nestin expression in some of tumour cells (data not shown).

In the study, we assessed the prognostic value of Nes$^+$MVD in patients with ductal breast carcinoma. Our results suggest that in ductal breast carcinoma Nes$^+$MVD increases with tumour invasiveness. The lowest value of Nes$^+$MVD was observed in non-malignant lesions and in *in situ* carcinomas, while in invasive cancers it was significantly higher. These results are consistent with the Folkman hypothesis, according to which increased tumour vascularity is necessary for its transition from *in situ* to invasive cancer (1). When tumour cells become invasive, they start to migrate in the extracellular matrix and invade blood and lymphatic vessels, thus resulting in the formation of metastases. Increased blood and lymphatic vessel density within the tumour area increases the probability of vessel invasion, and thus the probability of invading lymph nodes and developing metastases (34). In this study, we demonstrated that Nes$^+$MVD in IDC was higher in patients with lymph node metastases than in cases without lymph node invasion. Similar results for MVD, but for different endothelial antigens (i.e., CD31 and CD34) were obtained by Weidner *et al* (6), Popiela *et al* (35) and Xie *et al* (36).

Furthermore, we demonstrated that Nes$^+$MVD increases with the histological grade of IDC and achieves the highest value in the G3 and the lowest in G1 tumours. Similar results, but with the use of Nes$^+$Ki-67$^+$ vascular proliferation index (VPI) were reported in breast cancers by Kruger *et al* (30). In their study, VPI was calculated as the ratio between the number of Nes$^+$ microvessels containing Ki-67$^+$ proliferating endothelial cells (Nes$^+$Ki-67$^+$MVD) and the total number of Nes$^+$ microvessels (Nes$^+$MVD) expressed as a percentage (Nes$^+$Ki-67$^+$MVD/Nes$^+$MVD). They shown that high value of VPI but not Nes$^+$MVD itself, correlates with aggressive features and poor outcome of breast cancer (30). Additionally, we found significantly higher Nes$^+$MVD values in patients with the advanced-stage disease as compared to patients with the early-stage disease. Interestingly, we also noted that the number of Nes$^+$ vessels was associated with molecular subtype of breast cancer. A high Nes$^+$MVD was observed in patients with TN breast cancer. TN is an extremely aggressive cancer subtype with a poor prognosis. Similarly to our results, Kruger *et al* demonstrated that the highest MVD of both Nes$^+$ and Nes$^+$Ki-67$^+$ vessels and a higher VPI were noted in TN and basal-like cancers (30). Furthermore, in contrast to their results, we found that in IDC Nes$^+$MVD is an independent prognostic factor. In our study, high Nes$^+$MVD was associated with a shorter OS and earlier relapse. On the contrary, Kruger *et al* (30) did not demonstrate statistically significant relationship between Nes$^+$MVD and patient survival. This might be due to the use of a more restrictive cut-off values determining high Nes$^+$MVD values and that they obtained higher median value of Nes$^+$ microvessels (84.3 >75.8 v/mm^2). However, the same authors showed that in the case of nestin VPI might be a valuable prognostic factor (30). The different results might be due to the fact, that in our study we took into account only ductal carcinoma cases (from 0.4 to 8 cm in diameter), whereas Kruger *et al* (30) selected both ductal and lobular histological types and tumours oscillating ~2 cm in diameter during screening mammography. Finally, we are the first to report that the number of Nes$^+$ microvessels is noticeably higher in invasive tumours than in pre-invasive lesions and that Nes$^+$MVD correlates with immature CD34$^+$ vessels but not with mature CD31$^+$ vessels.

To date, CD34 and CD31 antigens have been commonly used for the assessment of angiogenesis. However, these markers are not selective for newly forming vessels and they do not constitute a reliable reflection of tumour angiogenesis. During EC differentiation, cells initially express CD34 antigen whereas the expression of CD31 occurs at later stages of EC development (22). The findings suggest that the expression of CD31 antigen is typical of more mature vessels, while CD34 expression is related to a more primary vascular phenotype (37-40). Our results demonstrated that Nes$^+$MVD correlates with the density of newly forming CD34$^+$ vessels, whereas no correlation was found in the case of CD31$^+$ mature vessels. It may indicate that nestin expression reflects a more progenitor nature of vessels and that it is mainly limited to those undifferentiated and newly forming ones. To confirm the obtained results, we developed an *in vitro* model consisting of three endothelial cell lines isolated from different types of vessels. Examinations of nestin expression showed that the highest expression occurs in the HEPC-CB.1 cell line, originating from human umbilical cord blood. This line was characterized as early EPCs and shows the expression of both stem cell (e.g., CD133) and endothelial antigens (e.g., VEGFR2, nitric oxide synthase) (22). We noted a lower nestin expression in the HMEC-1 cells isolated from dermal microvessels and the HUVEC-SVT cells isolated from the umbilical vein, respectively. HUVEC-SVT and HMEC-1 cell lines are derived from different blood vessel types, but both represent mature and differentiated phenotypes of vessels. Similarly, Suzuki *et al* demonstrated that in the bone marrow nestin is expressed exclusively in proliferating progenitor ECs, but not in mature ECs (20). Moreover, the literature data indicate that nestin undergoes expression not only in EPCs, but also in mesenchymal stem cells (MSCs) which may differentiate into pericytes and vascular smooth muscle cells (41). Thus, Nes$^+$ cells may participate not only in the formation of the endothelium but also in the stabilization of the entire vessel wall.

In conclusion, we assume that nestin might be a reliable marker for angiogenesis evaluation in IDC and higher Nes$^+$MVD may be related to a more aggressive course of the disease and a poorer prognosis. Additionally, nestin seems to be a selective marker for newly forming vessels and its expression may reflect the process of tumour angiogenesis.

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