Loss of membranous VEGFR1 expression is associated with an adverse phenotype and shortened survival in breast cancer

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Abstract. Angiogenesis is a key process in tumor growth and progression, which is controlled by vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs). In order to better understand the prevalence and prognostic value of VEGFR1 expression in breast cancer, a tissue microarray containing >2,100 breast cancer specimens, with clinical follow-up data, was analyzed by immunohistochemistry using an antibody directed against the membrane-bound full-length receptor protein. The results demonstrated that membranous VEGFR1 staining was detected in all (5 of 5) normal breast specimens. In carcinoma specimens, membranous staining was negative in 3.1%, weak in 6.3%, moderate in 10.9%, and strong in 79.7% of the 1,630 interpretable tissues. Strong staining was significantly associated with estrogen receptor and proges-terone receptor expression, but was inversely associated with advanced tumor stage (P=0.0431), high Bloom-Richardson-Ellis Score for Breast Cancer grade and low Ki67 labeling index (both P<0.0001). Cancers with moderate to strong (high) VEGFR1 expression were associated with significantly improved overall survival, as compared with tumors exhibiting negative or weak (low) expression (P=0.0015). This association was also detected in the subset of nodal-positive cancers (P=0.0018), and in the subset of 185 patients who had received tamoxifen as the sole therapy (P=0.001). In conclusion, these data indicated that membrane-bound VEGFR1 is frequently expressed in normal and cancerous breast epithelium. In addition, reduced or lost VEGFR1 expression may serve as a marker for poor prognosis in patients with breast cancer, who might not optimally benefit from endocrine therapy.

Introduction

Breast cancer is the most common malignancy in women. Despite major improvements in early detection through advanced screening and therapy, 522,000 women succumbed to this disease worldwide in 2012 (1). Angiogenesis is a key feature of tumor cells, which is necessary to overcome the hypoxia that is associated with cancer outgrowth. However, previous therapies targeting vascular development have failed to show a clear survival benefit for patients with breast cancer (2). Therefore, it is hypothesized that improved under-standing regarding the role of vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) may help to improve future therapies.

The VEGF family comprises five structurally related factors: VEGF-A, -B, -C, -D and placenta growth factor, which act as the primary activators of angiogenesis by binding to the following tyrosine kinase receptors: VEGFR1, 2 and 3. The roles of VEGFR2 and VEGFR3 as direct stimulators of angiogenesis (VEGFR2) and lymphangiogenesis (VEGFR3) have been thoroughly characterized; however, the function of VEGFR1 is less clear. The VEGFR1 gene (FLT-1) encodes two proteins: Membrane-bound fibroblast growth factor receptor 1 and a soluble form termed sVEGFR1. Both protein forms have been reported to negatively regulate VEGFR2 via high-affinity binding of VEGFs, which consequently become unavailable for VEGFR2 (3). However, it has previously been suggested that VEGFR1 may indirectly promote tumor cell growth by activating monocytes and macrophages, which invade the tumor and produce VEGFs and cytokines, leading

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to angiogenesis and lymphangiogenesis via activation of VEGFR2 and VEGFR3 (4,5).

Previous studies have analyzed the expression patterns and prognostic significance of VEGFR1 in breast cancer and cancer-associated vascular tissues. Positive associations have been reported between high-level VEGFR1 expression and adverse tumor features, including an increased risk for metastasis and relapse in tumors with strongly VEGFR1-positive endothelial cells (6), as well as shortened survival (7) and positive lymph node stage (8) if tumor cells exhibit strong VEGFR1 staining. Conversely, other studies have reported opposite findings, including absence of lymph node metastases in strongly VEGFR1-positive cancers (9), lack of an association with overall survival (6), or generally infrequent positivity of VEGFR1 in breast cancer cells (10). Similar discrepant findings have also been reported in normal breast epithelial cells, with studies describing either no expression (10,11), or uniformly positive staining (12).

Due to these discordant findings, the present study aimed to analyze an existing large breast cancer tissue microarray, including >2,000 breast cancer samples, using an antibody directed specifically against the membrane-bound form of VEGFR1. The results detected an association between reduced membranous expression of VEGFR1 and adverse features of breast cancer.

Materials and methods

Breast cancer tissue microarray. A breast cancer tissue microarray was used in the present study, which has previously been described in detail (13). Briefly, 2,197 formalin-fixed (neutral-buffered aqueous 4% solution), paraffin-embedded tumor samples from patients with a median patient age of 62 years (range, 26-101 years) and a median follow-up time of 68 months (range, 1-176 months) were assembled in a tissue microarray format (Table I). One tissue cylinder per case, with a diameter of 0.6 mm, was obtained from representative tumor areas of a 'donor' tissue block using a homemade semiautomatic robotic precision instrument. Histological grade was determined according to the Bloom-Richardson-Ellis Score for Breast Cancer (BRE score) (14). Several molecular data used in the present study are available from previously published studies. These include amplification data obtained by fluorescence *in situ* hybridization (FISH) for human epidermal growth factor (HER2), MYC and cyclin D1 (CCND1), and expression data obtained by immunohistochemistry (IHC) for estrogen receptor (ER), progesterone receptor (PR) and Ki67 (13,15). All tissue samples included in the present study were double-pseudomized leftover samples from routine pathological diagnoses, which can be used for research purposes without informed consent according to local laws (§12 HmbKHG; Hamburg, Germany). Manufacturing and usage of tissue microarrays for research purposes has been approved by the local institutional review board under protocol #WF-049/09. Control tissue was obtained from tumor patients normal breast tissue.

VEGFR IHC. Standard indirect immunoperoxidase procedures were used for the detection of VEGFR1 (rabbit polyclonal antibody; cat. no. ab2350; 1:450 dilution; Abcam, Cambridge,

UK). Sections were heated in an autoclave at 121˚C for 10 min in Tris-EDTA-Citrate buffer (pH 7.8). Primary antibody was incubated for 60 min at 37˚C. Endogenous peroxidase was blocked with Dako S2023 for 10 min at 20˚C, followed by anti-rabbit peroxidase (Dako real envision detection system K5007; Dako, Glostrup, Denmark) for 30 min at 37˚C. Diaminobenzidine (Dako) was applied for 10 min at 20˚C and sections were counterstained with Mayer's hematoxylin (Sigma-Aldrich). The VEGFR1 staining intensity and the fraction of stained tumor cells were recorded for each tissue specimen (Axiophot Neofluar 20x; Zeiss, Oberkochen, Germany). Staining intensity was estimated using a 4-step scale: 0, no staining; 1+, faint intensity; 2+, moderate intensity; 3+, strongest intensity. The fraction of stained cells was scored according to the following criteria: Score 0, no stained cells; score 1, ≤25% stained cells; score 2, ≤50% stained cells; score 3, ≤75% stained cells; and score 4, >75% stained cells. A final IHC result was generated from these scores: Negative, no staining at all; weak, intensity 1+ in ≤70% of cells, or intensity 2+ in ≤30% of cells; moderate, intensity 1+ in >70% of cells, intensity 2+ in >30% but ≤70% of cells, or intensity 3+ in ≤30% of cells; strong, intensity 2+ in >70% of cells, or intensity 3+ in >30% of cells.

Statistical analysis. Pearson's χ^2 test and Student's t-test were used to study the relationship between VEGFR1 IHC results and clinicopathological or molecular parameters. The effect of VEGFR1 on survival was assessed by Kaplan-Meier curves and log-rank tests. A Cox proportional-hazards model was used to identify independent factors associated with overall survival. Statistical analysis was performed using JMP version 9.0 statistical software package (SAS Institute Software GmbH, Böblingen, Germany). P<0.05 was considered to indicate a statistically significant difference.

Results

Technical issues. A total of 567 of 2,197 (25.8%) tissue specimens were non-informative for VEGFR1 IHC, due to the complete lack of tissue or absence of unequivocal cancer cells.

Association of VEGFR1 IHC results with breast cancer phenotype, cell proliferation, and molecular markers. VEGFR1 immunostaining was located in the membrane, and sometimes also the cytoplasm of luminal epithelial cells. Five samples of normal breast epithelium included in the tissue microarray exhibited strong VEGFR1 staining. Cancer cells typically exhibited strong staining compared with the staining intensity observed in the normal breast samples: Staining was strong in 1,299 (79.7%), moderate in 178 (10.9%), weak in 102 (6.3%) and negative in 51 (3.1%), of the 1,630 interpretable cancers. Representative images of VEGFR1 staining in normal and cancerous breast tissues are presented in Fig. 1. VEGFR1 staining levels were comparable (70-90% with strong staining) in the majority of different histological subtypes, apart from medullary cancers, which exhibited a significantly lower fraction of strongly VEGFR1-positive tumors (50%, P<0.0002), as compared with carcinoma of no special type (NST). In addition, VEGFR1 staining was inversely associated with tumor stage (P=0.0431) and BRE grade (P<0.0001), although

Table I. Association between VEGFR1 IHC results and breast cancer phenotype, ER and PR status, HER2, MYC and CCND1 amplification, and triple negative category.

Parameters	Interpretable (n)	VEGFR1 IHC result (%)				P-value
		Negative	Weak	Moderate	Strong	
All types of cancer	1,630	3.1	6.3	10.9	79.7	
Histology						
No special type	1,146	3.1	6.2	11.6	79.1	
Lobular carcinoma	227	2.2	3.5	6.2	88.1	
Cribriform carcinoma	48	0.0	6.3	6.3	87.5	
Medullary carcinoma	38	13.2	21.1	15.8	50.0	0.0002[b]
Tubular carcinoma	40	0.0	2.5	7.5	90.0	
Papillary carcinoma	24	4.2	4.2	16.7	75.0	
Mucinous carcinoma	46	2.2	10.9	10.9	76.1	
Other rare types[a]	61	4.9	8.2	16.4	70.5	
pT stage						0.0431
pT1	540	1.5	5.2	10.0	83.3	
pT2	784	3.7	7.1	12.1	77.0	
pT3	99	7.1	4.0	8.1	80.8	
pT4	198	3.5	6.6	9.6	80.3	
BRE grade						<0.0001
Grade 1	384	2.9	3.4	6.5	87.2	
Grade 2	627	1.9	5.6	9.7	82.8	
Grade 3	506	5.1	9.7	14.2	70.9	
Nodal stage						0.073
pN0	671	3.4	7.0	12.1	77.5	
pN1	584	2.2	5.8	11.0	81.0	
pN2	93	7.5	11.8	10.8	69.9	
ER/PR status						<0.0001
Negative	313	5.1	11.8	15.7	67.4	
Positive	1,156	2.6	4.6	10.0	82.8	
HER2 FISH						0.0745
No amplification	1,051	3.1	5.8	10.4	80.7	
Amplification	227	0.9	3.5	10.6	85.0	
Triple negative						<0.0001
No	1,021	2.0	4.0	9.7	84.3	
Yes	159	8.2	13.2	17.0	61.6	
CCND1 FISH						0.396
No amplification	1,129	2.6	5.1	10.9	81.4	
Amplification	281	2.1	7.8	10.3	79.7	
MYC FISH						0.1374
No amplification	1,146	2.9	5.8	10.5	80.9	
Amplification	66	6.1	6.1	18.2	69.7	

VEGFR1, vascular endothelial growth factor receptor 1; IHC, immunohistochemistry; pT, primary tumor; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor; CCND1, cyclin D1; FISH, fluorescent *in situ* hybridization. [a]Including adenoid-cystic carcinoma, apocrine carcinoma, atypical medullary carcinoma, carcinosarcoma, clear cell carcinoma, histiocytic carcinoma, lipid-rich carcinoma, lipid-rich or histiocytoic carcinoma, metaplastic carcinoma, neuroendocrine carcinoma, signet ring carcinoma, and small cell carcinoma. [b]Medullary vs. carcinoma of no special type.

the difference in numbers was only small. Staining levels were unrelated to the presence of lymph node metastases.

Cell proliferation was previously determined immunohistochemically using the Ki67 labeling index (LI) (13). An inverse
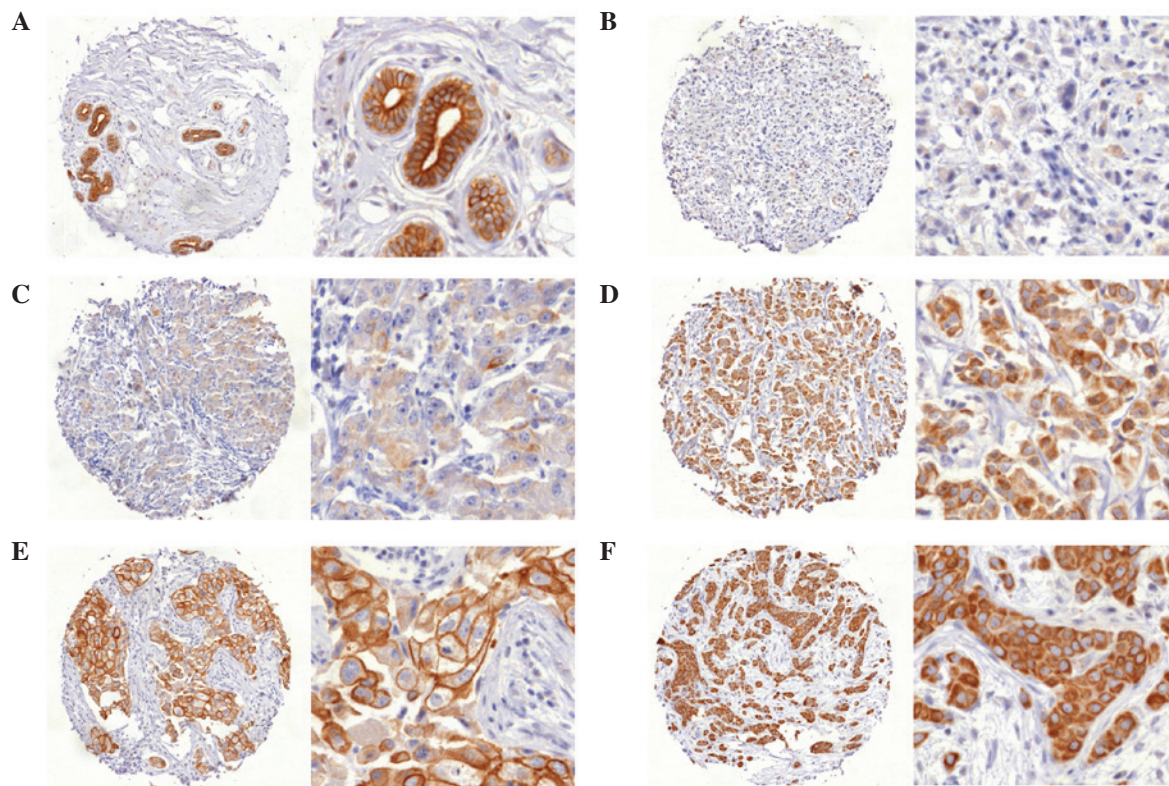
Figure 1. Representative images of vascular endothelial growth factor receptor 1 immunostaining. (A) Normal breast tissue, (B-F) breast cancer tissue with (B) negative, (C) weak, and (D and E) moderate to strong staining at 10x and 40x magnification. Staining localization was either (D) mainly cytoplasmic, (E) mainly membranous, (F) or both cytoplasmic and membranous.

association was detected between VEGFR1 staining and cell proliferation: The average Ki67LI increased from 26.5% in 1,135 cancer specimens with strong VEGFR1 staining to 33.1% in 46 tumors lacking detectable VEGFR1 staining (P<0.0001; Fig. 2). Strong VEGFR1 immunostaining was also associated with positive ER and PR status and the triple negative category (all P<0.0001); however, VEGFR staining was unrelated to amplifications in HER2, CCND1 or MYC. The results are summarized in Table I. A multivariate analysis of overall survival demonstrated that VEGFR1 staining had non-significant impact on hazard ratio (Table II).

Association with patient survival and response to tamoxifen treatment. To exclude a potential influence of the histological subtype on patient prognosis, survival analysis was limited to the largest subset of 1,144 carcinomas of NST with interpretable VEGFR1 IHC data. Since tumors with moderate or strong staining exhibited improved overall survival compared with tumors with negative or weak staining (P=0.0054; Fig. 3A), all NST specimens were grouped into subsets with low (i.e. negative or weak) or high (i.e. moderate or strong) staining for further survival analyses. According to these groups, tumors with high VEGFR1 staining exhibited superior overall survival in all subsets of NST (P=0.0015; Fig. 3B). This association was also true for subsets of nodal-negative NST (pN0, P=0.0256; Fig. 3C) and nodal-positive NST (pN1-2, P=0.0018; Fig. 3D). Additional analysis in a subset of 185 patients with breast cancer who had received tamoxifen monotherapy revealed a significant association between high levels of VEGFR1 and prolonged survival after treatment (P=0.0010; Fig. 3E).

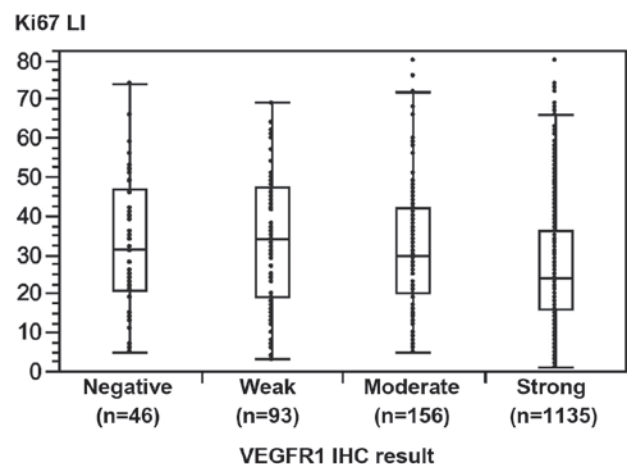Figure 2. Association between VEGFR1) immunostaining levels and cell proliferation, as determined using the Ki67 labeling index (LI) (P<0.0001). Box plots show 1st, 2nd (median) and 3rd quartile. Whiskers indicate 3rd quartile + 1.5x interquartile range or 1st quartile - 1.5x interquartile range. VEGFR1, vascular endothelial growth factor receptor 1; IHC, immunohistochemistry.

Discussion

The present study successfully analyzed >1,600 breast cancer specimens using an antibody directed against membrane-bound VEGFR1. The results suggested that high-level immunostaining of VEGFR1 is a common feature of normal and cancerous breast epithelial cells, and that lost

Table II. Multivariate analysis of overall survival in 824 patients, including tumor stage, BRE grade, nodal stage, ER and PR status, HER2 amplification, triple negative category, Ki67 labeling index and VEGFR1 staining.

Clinicopathological parameter	Hazard ratio	95% Confidence interval	P-value
pT stage			
pT2 vs. pT1	1.4	1.02-2.04	0.0357
pT3 vs. pT2	1.0	0.61-1.46	0.8753
pT4 vs. pT3	1.7	1.08-2.80	0.021
BRE grade			
Grade 2 vs. grade 1	1.5	0.97-2.29	0.0707
Grade 3 vs. grade 1	1.9	1.38-2.8	<0.0001
Nodal stage			
pN1 vs. pN0	2.7	2.03-3.73	<0.0001
pN2 vs. pN1	1.9	1.34-2.8	0.0007
ER status			
Negative vs. positive	1.6	0.25-5.12	0.5677
PR status			
Negative vs. positive	1.5	1.07-2.02	0.018
ER/PR status			
Negative vs. positive	0.4	0.1-2.38	0.2485
HER2 FISH			
No vs. amplification	0.5	0.36-0.78	0.0021
Triple negative			
Yes vs. no	2.7	1.51-4.92	0.0009
Ki67 labeling index			
Per unit change	1.0	0.99-1.01	0.4404
VEGFR1			
Weak vs. negative	0.9	0.47-1.93	0.843
Moderate vs. weak	0.6	0.33-1.08	0.087
High vs. moderate	1.2	0.82-1.92	0.3337

pT, primary tumor; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor; FISH, fluorescent *in situ* hybridization; VEGFR1, vascular endothelial growth factor receptor 1.

or reduced expression of VEGFR1 is associated with tumor progression, rapid cell proliferation and shortened survival.

All normal breast tissues (5/5) and 90% of cancer tissues exhibited moderate to strong VEGFR1 immunostaining in the present study, indicating that high levels of VEGFR1 represent a physiological situation. These data thus corroborate the hypothesis that full-length VEGFR1 physiologically regulates VEGFR2 activity by trapping free VEGF (3,16). In addition, these results are consistent with the findings of a previous study, which suggested that VEGFR1 expression is indicative of better prognosis in breast cancer (9). Based on the known function of VEGFR2 as an activator of cell growth (17,18), the adverse features of breast cancers with low-level VEGFR1 expression may be a consequence of unregulated VEGFR2 activation in the absence of sufficient levels of VEGFR1.

The high rate of cancers with high-level VEGFR1 expression in the present study is consistent with the results of a recent study reporting 100% positivity in 25 normal breast samples and 90% positivity in 96 invasive breast cancer samples (12). Notably, in the previous study, the same antibody was used as

in the present analysis. According to the manufacturer's datasheet, this particular polyclonal antibody (ab2350; Abcam) detects a 180 kD protein sequence, which is not present in sVEGFR, and does also not detect the phosphorylated form. Other studies using different antibodies have reported largely variable results, including complete lack of staining in normal breast epithelium (10), and a broad range of positivity in cancer cells ranging from 16-91% (7,9,10,19). Furthermore, compared with the findings of the present study, several of these studies also detected associations between strong VEGFR1 staining and adverse features of breast cancer, including early recurrence, reduced overall survival and metastasis (6-8). Such discrepant findings are most likely attributed to the different antibodies used in these studies. Notably, the majority of studies described cytoplasmic staining (6-8), which is unexpected given that full-length VEGFR1 is a membrane-bound receptor, and that at least some membranous staining would be expected. However, high homology exists between the full-length membranous VEGFR1, sVEGFR1 lacking the transmembrane and intracellular domains, and the five
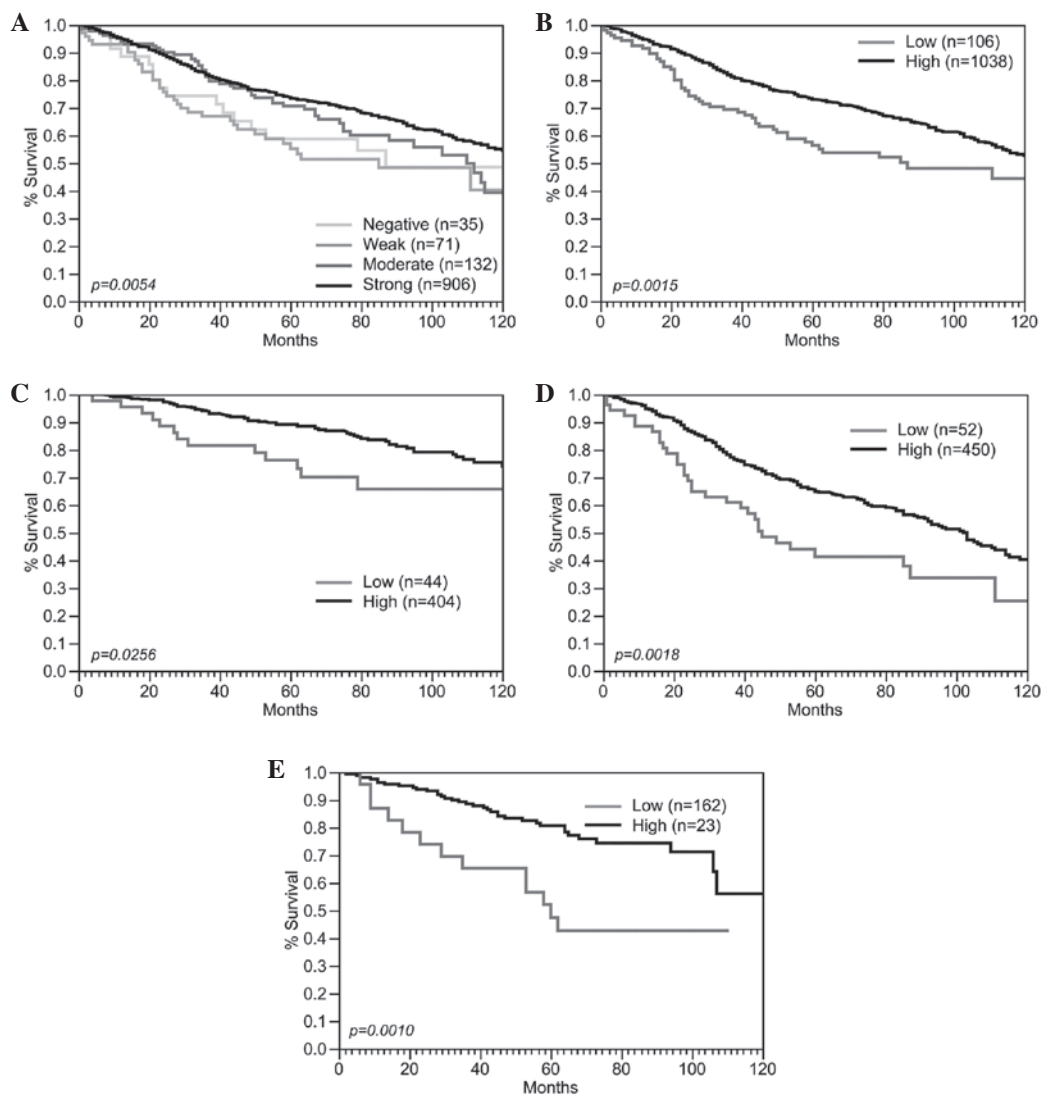
Figure 3. Prognostic significance of vascular endothelial growth factor receptor 1 staining in (A) all cancers of no special type (NST), (B) in all NST cancers grouped according to low (negative and weak) and high (moderate and strong) staining, (C) in the subset of nodal-negative NST cancers, (D) in the subset of nodal-positive NST cancers, and (E) in the subset of 185 patients with breast cancer that received tamoxifen monotherapy.

different intracellular forms (iVEGFR1, possessing only one or more of the intracellular domains) that have recently been described (20). Therefore, it appears possible that some antibodies may cross-react with several forms of VEGFR1. This would provide an explanation for some of the discrepant findings, in particular since numerous studies have demonstrated that sVEGFR1 and iVEGFR1 have opposite implications for tumor biology and patient prognosis (21,22).

In the present study, low levels of VEGFR1 expression were associated with reduced ER and PR positivity in all cancer specimens, as well as shortened survival in the subset of ER-positive patients receiving endocrine (tamoxifen) therapy. These results provide additional support for the concept that VEGFR1 acts as a negative regulator of VEGFR2 signaling, since several studies have reported that activation of VEGFR2 is associated with high tumor stage and grade, metastatic growth, negative ER status and early recurrence in patients with breast cancer following tamoxifen therapy (17,23,24). Furthermore, the findings of the present study suggest that VEGFR1, possibly in combination with

VEGFR2, may be a suitable marker to stratify patients for endocrine therapy.

In 2008, the Food and Drug Administration approved the VEGF neutralizing antibody bevacizumab for the treatment of advanced and metastatic breast cancer. However, subsequent large clinical phase III trials (e.g. E2100, AVADO, RIBBON-1 and -2) (25-27) have failed to detect a clear survival benefit, resulting in withdrawal of the approval in 2011. The reasons for the lack of a therapeutic benefit remain poorly understood, although the pivotal role of angiogenesis in cancer growth is undisputed (28). Since 90% of cancers in the present study, including 70-80% high grade, advanced and metastatic tumors, exhibited high-level VEGFR1 staining, it seems obvious that the vast majority of breast cancers are capable of regulating growth signaling via an intact VEGF/VEGFR1 loop. It would be interesting to study the effects of VEGF inhibitors in breast cancers with various levels of VEGFR1. Theoretically, it may be speculated that VEGF inhibition could be more effective in tumor cells lacking VEGFR1 expression, than in those with physiologically high protein levels, and that VEGFR1

levels could be of potential value in selecting patients for anti-angiogenic therapies.

A tissue microarray with a single 0.6 mm spot per cancer was used in the present study. A limitation of the present study is that this approach is not suitable for the detection of possible intratumoral heterogeneity of VEGFR expression. Therefore, it is possible that some cancers with heterogenous VEGFR expression were overlooked in the present analysis. It has previously been suggested that analysis of multiple spots per tumor may increase the representativeness of microarray studies (29,30). However, this approach bears the disadvantage that not all tissue spots of one cancer are interpretable. Given that the likelihood of finding a positive result increases with the number of interpretable tissue spots, analysis of various amounts of cancer spots per patient may introduce a statistical bias to the analysis (31). Our previous study demonstrated that microarrays consisting of a single spot per cancer are superior for detecting clinically relevant associations between molecular markers and breast cancer phenotype (32). In addition, previous tissue microarray studies using a single spot per tumor have been able to reproduce known associations between molecular markers and cancer phenotype, or patient prognosis in breast cancer (13,32,33).

A limitation of the present study is that only one (VEGFR1) of many angiogenic molecules, including VEGFR2, VEGFR3, neuropilin (NRP)1 or NRP2, was analyzed (34). Given the complex biological interactions of these receptors and their corresponding growth factors it would be interesting to study their co-expression patterns, provided that antibodies suitable for formalin-fixed tissues become available, in order to obtain a comprehensive picture of angiogenic factors in breast cancer.

In conclusion, the results of the present study suggested that reduced or lost expression of full-length and membrane-bound VEGFR1 identifies a small but clinically relevant subset of breast cancers that are characterized by adverse tumor features and shortened survival, which may not respond optimally to endocrine therapy. Furthermore, the choice of antibody may have a serious impact on the outcome of VEGFR1 expression analyses.

Acknowledgements

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