Published in final edited form as:

Hum Pathol. 2008 December ; 39(12): 1835–1843. doi:10.1016/j.humpath.2008.06.004.

High levels of vascular endothelial growth factor (VEGF) and its receptors (VEGFR-1, VEGFR-2, neuropilin-1) are associated with worse outcome in breast cancer

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Abstract

Vascular endothelial growth factor has been shown to be upregulated in breast cancers. VEGFR-1 and VEGFR-2 are the principal mediators of its effects. Together with VEGFR-1 and VEGFR-2, neuropilin-1 may act as a co-receptor for VEGF. Although VEGF exerts important effects on endothelial cells, VEGFRs are likely present on tumor cells as well. We used AQUA to analyze tumor-specific expression of VEGF, VEGFR-1, VEGFR-2, and neuropilin-1 on a large cohort breast cancer tissue microarray. Two-fold redundant arrays were constructed from 642 cases of primary breast adenocarcinomas. Automated image analysis with AQUA was then performed to determine a quantitative expression score. Scores from redundant arrays were normalized and averaged. Kaplan-Meier survival analysis showed that high levels of VEGF, VEGFR-1, VEGFR-2, and neuropilin-1 were all significantly associated with survival (Miller Siegmund corrected P value 0.0020, 0.0160, and 0.0320 respectively). In addition, VEGF and neuropilin-1 retained a significant association with survival independent of other standard prognostic factors. VEGF, VEGFR-1 and 2, and neuropilin-1 are expressed to varying degrees in primary breast cancers and have prognostic significance. Further study of the functional significance of this finding is warranted as well as the prognostic value of these biomarkers in other tumor microenvironment-specific compartments (e.g. vessels).

Keywords

VEGF; neuropilin; breast cancer; angiogenesis; automated image analysis

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Introduction

Angiogenesis plays an important role in the growth and spread of cancer. Among the wide array of angiogenic factors described, VEGF (also called VEGF-A) is one of the most potent having key functions in the physiologic and pathophysiologic regulation of endothelial cell (EC) growth and vascular permeability [1,2]. VEGF-A also belongs to a family of related growth factors including VEGF-C, VEGF-D and placenta growth factor that oversees modeling of the vascular system as a whole. VEGF-A will be the primary focus of this study and hereafter will be referred to as VEGF. Alternative splicing of VEGF produces four principal isoforms: VEGF121, VEGF165, VEGF189, and VEGF206. Two tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1, KDR) identified on ECs and on bone marrow derived elements are the principal mediators of VEGF's activities. VEGFR-2 activation leads to endothelial proliferation, survival, and permeability in part through the Raf/Mek/Erk, PI3K/Akt, and PI3K/Akt/nitric oxide pathways respectively. The precise function of VEGFR-1 is not entirely established and some have hypothesized it to potentially play a decoy role for VEGF. Although most studies have localized VEGF expression predominantly to tumors and stromal elements and its receptors to ECs suggesting a paracrine effect, others have demonstrated more ubiquitous expression with receptors present in the tumor as well suggesting a non-angiogenic autocrine loop [3–6].

Neuropilin-1 (NP-1) is a multifunctional non-tyrosine kinase receptor that binds to class 3 semaphorins and was originally identified for its critical role in the developing nervous system [7]. Subsequently, NP-1 was identified as a receptor for VEGF-165, the predominant isoform. In regards to VEGF mediated signaling, NP-1 appears to function by forming coreceptor complexes with VEGFR-1 and with VEGFR-2 [8,9]. Although neuropilin expression has been described in a wide array of normal and developing tissue, its expression and regulation on vascular smooth cells and endothelial cells (e.g. upregulation by ischemia and VEGF) suggest an important role in neoangiogenesis [7]. Furthermore, NP-1 has been shown to be highly expressed in tumor associated vasculature and in a variety of tumor cells *in vitro* and *in situ* [10,11].

Because of VEGF's potent actions on tumor associated angiogenesis, a number of drugs targeting this growth factor and its two principal receptors have been developed for clinical trials, including neutralizing antibodies to VEGF or VEGFR1/2, soluble VEGF/VEGFR hybrids, multi-targeted tyrosine kinase inhibitors, and direct endothelial cell toxins [12]. Although some of these agents such as the monoclonal anti-VEGF antibody bevacizumab have been successful in limited fashion in breast cancer, predictive biomarkers for these agents have not been identified. Although, preclinical studies have pointed to a potential role for NP-1 in tumor growth and angiogenesis and some anti-tumor drugs may indirectly affect NP-1 levels, therapeutic interventions specifically targeting this receptor have not yet been extensively studied in clinical trials.

We have developed an algorithm for quantitatively determining *in situ* protein expression called AQUA® [13]. AQUA is a hybrid of standard IHC and flow cytometry in that it requires antigen retrieval on fixed tissue, application of primary and secondary antibodies, and use of multiplexed fluorescent detection to produce an objective, numeric score. This methodology has been validated with a variety of biomarkers in many different cancers [14–16]. Thus for better quantification of protein levels, we used AQUA to study the correlation of VEGF, VEGFR-1, VEGFR-2, and NP-1 expression on a large historical cohort of breast carcinomas with demographic, pathologic, and survival information.

Materials and Methods

Patient Selection

Our cohort consisted of 642 formalin-fixed, paraffin-embedded blocks of primary breast cancer specimens (Table 1). The median follow up time was 8.9 years. Clinicopathologic data were extracted from the Yale and Connecticut Tumor Registries and all data were collected in accordance with the Yale Human Investigations Committee.

Tissue Microarray

The blocks were retrieved from the archives of the Yale University Department of Pathology in accordance with the Yale Human Investigations Committee. Representative areas of invasive tumor were identified and two 0.6 mm diameter cores were placed into separate recipient blocks using a precision arraying instrument (Beecher Instruments, Silver Spring, MD) and representing the two-fold redundant blocks. Five μ m sections were affixed to adhesive slides using a UV cross-linkable tape transfer system (Instrumedics Inc., Hackensack, NJ), then coated in paraffin and stored in a nitrogen chamber prior to staining to prevent antigen oxidation and degeneration [17]. One slide from each of the two-fold redundant TMA recipient blocks was analyzed: Block 1 and Block 2.

Immunohistochemistry

Staining tissue microarray slides for AQUA has been previously described [13] [13]. Briefly, slides were deparaffinized in xylene, rinsed in ethanol, and rehydrated. Antigen retrieval was performed by pressure cooking for 15 minutes in 6.5mM sodium citrate buffer. Endogenous peroxidase was quenched by immersing the array in a 2.5% methanol/hydrogen peroxide buffer for 30 minutes. Non-specific background staining was further minimized by pre-incubating the array with 0.3% bovine serum albumin in 0.1M tris-buffered saline (pH 8.0) for 1 hour. Primary antibodies used were anti-VEGF, a polyclonal antibody against the amino terminus, anti-VEGFR-1, a polyclonal antibody against the carboxy terminus, anti-VEGFR-2, a monoclonal antibody also against the carboxy terminus, and anti-neuropilin-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For VEGFR-1 and VEGFR-2, cell blocks and western blots of HUVEC cell lines were used as controls. Similarly for NP-1, MDA-MB-231, a breast cancer line known to have high levels NP-1, was used as controls. Slides were also incubated in the absence of primary antibody or with control immunoglobulin as negative controls. For purposes of our automated analysis, tumor cells were also differentiated from stroma with an anti-pancytokeratin antibody (DAKO, Carpinteria, CA). The primary antibody cocktail (VEGF and cytokeratin or VEGFR-1 and cytokeratin or VEGFR-2 and cytokeratin or NP-1 and cytokeratin) was incubated overnight at 4°C in a humidity chamber. Goat anti-mouse or rabbit antibody conjugated to a horseradish peroxidase-decorated dextran polymer backbone (Envision; DAKO Corp.) was used as a secondary reagent to detect the bound primary target (e.g. VEGF) and Cy5-tyramide was used to visualize the amplified signal. Cy-5 (red) was used because its emission peak is well outside the green-orange spectrum of tissue autofluorescence. The cytokeratin was visualized with a Cy3-conjugated secondary antibody and the array was then counterstained with 4',6-diamidino-2-phenylindole (DAPI) to localize nuclei.

Image Collection and AQUA Analysis

Image acquisition and automated analysis have also been described extensively in our previous work. Images of each histospot are automatically acquired with a high-resolution monochromatic camera using filter cubes specific to the emission/excitation spectra of DAPI, Cy5, and Cy3. Then, using this stack of uncompressed images, the AQUA software then allows one to distinguish between areas of tumor and stromal elements using the cytokeratin stain, resulting in a unique binary cytokeratin tumor mask for each spot. Furthermore, the

cytokeratin and DAPI stains are used to assign each pixel under the tumor mask into non-overlapping membrane/cytoplasmic (non-nuclear) and nuclear locales. AQUA scores for the target are then calculated that correspond to the average signal intensity divided by locale area. For the case of VEGF/VEGFR-1/VEGFR-2/NP-1, the signal was quantified in the entire tumor mask locale. The AQUA score is thus proportional to the protein concentration in this region of the cell averaged across all of the cells within the keratin staining mask. This information can then be exported in a format suitable for analysis by X-tile (see below) or by standard statistical software packages. Raw AQUA scores for the two-fold redundant arrays were placed into a linear regression model to normalize the raw scores to each other. Subsequently, the mean of the normalized scores were then used for further statistical analysis. The data was then natural log transformed to minimize skewness and maximize normality.

Statistical Analysis

AQUA scores represent expression of a target protein on a continuous scale from 1–255. It is often useful to categorize continuous variables in order to stratify patients into high versus low categories. Several methods exist to determine a cutpoint, including biological determination, splitting at the median or into quartiles, and cutpoint which maximizes difference between groups. If the last method (the so-called “optimal p-value” approach) is used, a dramatic inflation of type-I error rates can result [18]. Our program, X-Tile, allows determination of an optimal cutpoint while correcting for the use of optimal p-value statistics using the Miller-Siegmund p-value correction [19]. Briefly, when making multiple comparisons to find the minimum p-value using the log rank test, the false positive rate (i.e. the percentage of times a marker that has no true prognostic value will be found to have a $p < 0.05$) can approach 40%. This statistical adjustment generates a minimum p-value corrected to yield a true false-positive rate of 5%. Our calculations were performed using an epsilon of 0.10. Survival calculations were subsequently performed by Kaplan-Meier analysis with log-rank for determining statistical significance. Hazard ratios were assessed by the univariate and multivariate Cox Proportional Hazards models with the use of the likelihood ratio test for determining statistical significance. Residuals were examined for influential observations, linearity of variables, and proportional hazards. All survival analyses were performed at 20-year cutoffs. Spearman's Rho correlations were determined for AQUA score correlations between two-fold redundant arrays. Correlations between biomarkers (VEGF, VEGFR-1, VEGFR-2, NP-1) were made with Pearson's correlation coefficient. These analyses were performed using Statview software (version 5.0.1; SAS Institute Inc., Cary, NC) and R (GNU, Boston, MA).

Results

Patient characteristics

There were 642 patients with primary breast carcinomas who met the inclusion criteria for our patient cohort. Although full treatment information was unavailable for this cohort, most patients were treated with a combination of surgical excision +/- local irradiation and/or hormonal therapy. Approximately 10% of patients received some form of chemotherapy. Although not explicitly stated, it appears that both chemotherapy and radiation were delivered postoperatively in the majority of cases. Demographic and clinicopathologic information is summarized in Table 1. Nuclear grade and ER/PR/HER2 characteristics were all evaluated on this cohort of patients using standard criteria.

Immunostaining patterns

VEGF, VEGFR-1, VEGFR-2, and NP-1 all showed a predominantly membrane/cytoplasmic distribution in the tumor (Figure 1). Raw AQUA scores were then normalized to each other and a regression plot was constructed with Spearman's Rho calculation (Figure 2). This showed relatively good correlation between the redundant arrays (Spearman Rho values of 0.660.420,

0.537, and 0.559 for VEGF, VEGFR-1, VEGFR-2, and NP-1 respectively). Thus normalized scores were averaged between the two-fold redundant arrays and natural log transformed for subsequent analyses. The X-tile program was then used to generate optimal cutpoints for VEGF, VEGFR-1, VEGFR-2, and NP-1 (80.5, 39.2, 64.3, 24.5 respectively) (Figure 2). Patients with expression less than or greater than these cutpoints were subsequently classified as low or high expressors respectively (Table 1).

Validation of TMA cohort/univariate survival analysis

Univariate analysis showed that established prognostic markers including tumor size, nodal status, ER/PR levels, and nuclear grade were all significantly associated with outcome (Table 2). In addition, high VEGF, VEGFR-1, VEGFR-2, and NP-1 expression were also significantly associated with poor survival.

As shown in figure 3, Kaplan-Meier analysis showed that high expression of all four markers was also associated with worse survival compared with low expression (median survival 87 months versus >240 months for VEGF, $P_{cor} = 0.0020$ after Miller Siegmund correction; 107 months versus >240 months for VEGFR-1, $P_{cor} = 0.0160$ after Miller Siegmund correction; 118 months versus >240 months for VEGFR-2, $P_{cor} = 0.0320$ after Miller Siegmund correction; 154 months versus >240 months for NP-1, $P_{cor} = 0.0170$ after Miller Siegmund correction).

Multivariate analysis

Using the Cox Proportional Hazards model with the likelihood ratio test for determining statistical significance, multivariate analysis showed that in addition to HER2 status, tumor size, and nodal status, VEGF and NP-1 retained statistical significance. Interactions between variables were investigated; however, none were significant. Linearity in VEGF and NP-1 was verified. Proportional hazards for the final model was assessed by adding a time-varying coefficient for each variable (keeping the other coefficients constant) and testing its significance as well as the tests and graphs of the scaled Schoenfeld residuals. There was an indication of non-proportional hazards due to a decrease in the effect of nodal status over time. To account for this, the multivariate model was stratified on nodal status allowing separate baseline hazard functions for nodal status = negative and nodal status = positive. VEGFR-1 and VEGFR-2 did not attain statistical significance in this analysis (Table 2).

Biomarker correlations

Among the markers tested, correlation was greatest between VEGF and NP-1 (Table 3). Correlation between VEGF and its other receptors are also noted in Table 3.

Discussion

We used our automated imaging system using molecular co-localization techniques to assess the prognostic significance of VEGF, its two principal tyrosine kinase receptors, and NP-1 on our breast cancer tissue microarray. Univariate analysis showed that high levels of all four markers were all robust predictors of clinical outcome. Multivariate analysis also showed that VEGF, and NP-1 both remained significant independent of other standard breast cancer prognostic variables.

A limitation of our study, as is often the case in any retrospective analysis, was the lack of complete treatment information on our cohort of patients. Clearly, surgical, radiotherapy, and systemic treatments varied over the period of time on our array. However, the vast majority (88%) of the patients were diagnosed and treated between 1960–1980. In that time, systemic chemotherapies were not often utilized, and when used, often only for more advanced node

positive disease. There was no significant difference in our results looking at only node positive or node negative subsets (data not shown), suggesting, that these results may be treatment independent. The vast majority of patients received only surgery and/or radiation and in this era, there was fairly minimal use of tamoxifen.

In breast cancer, a number of studies have looked at the prognostic significance of VEGF expression in the tumor. Using cytosol-based methods, several studies have shown that VEGF is expressed in the majority of cancers to some degree and that expression was in general correlated with worse survival and associated with treatment resistance [20,21]. Although immunohistochemistry-based studies seem less consistent, a number of studies have also found similar relationships between VEGF expression and outcome [20,22]. Immunohistochemistry is certainly susceptible to a variety of inconsistencies including antibody specificity and sensitivity and variabilities surrounding staining conditions. However, our current study uses a frequently used and validated antibody in one of the largest cohort of patients in TMA format with a quantitative method of measuring *in situ* protein expression and have found similar associations with clinical outcome. These data suggest that this relationship is indeed a biologically relevant phenomenon. There are only limited number of studies looking at tumoral or vascular expression of and clinical consequences of altered VEGFR expression in breast cancer [4,23,24]. However, some have suggested autocrine and paracrine loops based on studies looking at immunohistochemical staining patterns of VEGF, VEGFR-1, and VEGFR-2 in serial sections showing similar staining patterns within tumors and within tumors and surrounding stroma/endothelium [25].

NP-1 also has been shown to be expressed on breast tumor vasculature and tumor cells and has been shown to be possibly associated with tumor growth and progression in a small sample size of malignant and premalignant breast samples [26]. Other *in vitro* work has also suggested functional importance to its expression in breast cancer. In a NP-1 positive, VEGFR-2 negative breast cancer cell line, VEGF-165 (which binds to NP-1) prevented apoptosis and the levels of NP-1 (either overexpression or downregulation by an inhibitory peptide) directly correlated with cell survival [27,28]. In addition, NP-1 is also thought to play a role in mediating breast cancer migration and metastasis. The class 3 semaphorin SEMA3F bind to neuropilins and can inhibit cell spreading whereas VEGF has the opposite effect suggesting that these ligands may compete for binding [29] and in another study, SEMA3A/VEGF ratios correlated with chemotactic rate of breast cancer cells [10]. These studies provide preclinical evidence that NP-1 may be expressed on both tumor vessels as well as on the tumor cells and may play a role in promoting VEGF-induced proangiogenic/pro-tumor growth and semaphorin-induced antiangiogenic/anti-tumor growth properties.

Despite the clinical interest in anti-angiogenic targeted drug therapies in cancers, there are currently no reliable surrogate markers for efficacy or toxicity for these treatments. As of yet, standard measures of VEGF in tissue (e.g. qualitative assessment of VEGF expression in tumors), and in serum and other body fluids have not consistently been correlated with disease state or with responses to anti-VEGF-based therapies such as bevacizumab [20,30]. Many potential factors are possible for these findings including the fact that there may be sequestered but biologically active pools of VEGF. Irrespective, it may be ever more important to investigate VEGF receptor occupancy and functional consequence of receptor activation as a more representative test in addition to other potentially useful surrogate measures of angiogenesis that utilize proteomic analysis, expression profiling, levels of circulating ECs and endothelial precursor cells, and vascular imaging-based techniques. Furthermore, our study is the first to look at NP-1 expression and clinical significance in a large scale fashion and validates further studies to develop NP-1 as a therapeutic target.

Financial support/Acknowledgements

G.G. Chung received support from the DOD Breast Cancer Idea Award, W81XWH-04-1-0277, Anna and Argall Hull YCC Translational Research Award, and the Susan G. Komen Breast Cancer Foundation BCTR0504220. A.M. Molinaro received support from NCI-K22CA123146.

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Figure 1. VEGF, VEGFR-1, VEGFR-2, and NP-1 expression

Representative AQUA images of breast cancer TMA histospots demonstrating high levels of VEGF (A), VEGFR-1 (B), VEGFR-2 (C), and NP-1 (D) expression. Cytokeratin panel shows a raw image of the membranous pattern seen for the cytokeratin stain and inset shows the binary gating used to create a tumor mask. VEGF in tumor (for example) panel shows the quantitative amounts of VEGF co-localized to the tumor compartment (red). The other two panels show DAPI and marker (e.g. raw VEGF staining) with blue and red pseudocolors respectively.

Figure 2. Correlations between redundant tissue arrays and frequency histogram

The left column shows regression plots for raw AQUA scores on the two-fold redundant TMAs for VEGF, VEGFR-1, VEGFR-2, and NP-1 with Spearman Rho calculations. On the right are the respective frequency histograms for the normalized/averaged scores.

Figure 3. Kaplan-Meier survival analysis

KM curves showing association of VEGF (A), VEGFR-1 (B), VEGFR-2 (C), and NP-1 (D) with survival.

Table 1

TMA Patient cohort characteristics (n=642)

		N (%)	Median (Range)
Follow-up (years)			8.9 (0.19–41)
Age (years):	< 50	170 (26)	58 (24–88)
	≥50	465 (73)	
	Not specified	7 (1)	
Histology:	Infiltrating duct	520 (81)	
	Infiltrating lobular	14 (2)	
	Carcinoma NOS	83 (13)	
	Other	25 (4)	
Tumor Size (cm):	<2	212 (33)	2.5 (0.13–14.5)
	≤2 < 5	279 (44)	
	≥5	99 (15)	
	Not specified	52 (8)	
Nodal status:	Positive	317 (49)	
	Negative	320 (51)	
Nuclear grade:	1	112 (17)	
	2	310 (49)	
	3	169 (26)	
	Not Specified	51 (8)	
ER:	Positive	320 (50)	
	Negative	287 (45)	
	Not available	35 (5)	
PR:	Positive	298 (47)	
	Negative	290 (46)	
	Not available	49 (7)	
HER2:	0	368 (58)	
	1	119 (19)	
	2	42 (6)	
	3	67 (10)	
	Not specified	46 (7)	
AQUA VEGF:	High	143 (22)	
	Low	402 (63)	
	Unevaluable	97 (15)	
AQUA VEGFR-1:	High	187 (29)	
	Low	359 (56)	
	Unevaluable	96 (15)	
AQUA VEGFR-2:	High	237 (37)	
	Low	315 (49)	
	Unevaluable	90 (14)	
AQUA NP-1:	High	396 (62)	

		N (%)	Median (Range)
	Low	122 (19)	
	Unevaluable	124 (19)	

Table 2

Univariate and multivariate analyses of 20 year survival by Cox regression

Marker	Univariate P-value	Hazard Ratio (95% CI)	Multivariate P-value	Hazard Ratio (95% CI)
ER Positive	<0.01	0.72 (0.56–.92)		
PR Positive	0.01	0.73 (0.57–.94)		
HER2 positive	0.19	1.23 (0.90–1.67)	0.04	1.42 (1.01–1.99)
Tumor Size > 2 (cm)	<0.01	2.01 (1.55–2.60)	<0.01	1.59 (1.20–2.10)
Nodal Status Positive	<0.01	2.38 (1.85–3.05)		<u>Strata</u>
Nuclear Grade High	<0.01	1.52 (1.170–1.97)		
AQUA VEGF	<0.01	1.83 (1.41–2.37)	0.01	1.50 (1.11–2.03)
AQUA VEGFR-1	<0.01	1.40 (1.09–1.80)		
AQUA VEGFR-2	<0.01	1.38 (1.08–1.76)		
AQUA NP-1	<0.01	1.77 (1.26–2.49)	0.02	1.49 (1.05–2.10)

Table 3Correlations between continuous biomarkers[†]

Marker	VEGF	VEGFR-1	VEGFR-2	NP-1
VEGF	1.00 (1.00–1.00)	0.33 (0.25–0.40)	0.29 (0.21–0.36)	0.41 (0.33–0.48)
VEGFR-1		1.00 (1.00–1.00)	0.38 (0.31–0.45)	0.04 (–0.05–0.13)
VEGFR-2			1.00 (1.00–1.00)	0.22 (0.13–0.30)
NP-1				1.00 (1.00–1.00)

[†] Pearson's Correlation

Coefficient (95% CI)