Vascular endothelial growth factor (VEGF) -A and -C and VE-cadherin as potential biomarkers in early breast cancer patients

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Abstract

Background: Vascular endothelial growth factor (VEGF) -A and -C act as multifunctional molecules and growth factors, while VE-cadherin (cadherin 5, CDH5) is the endothelial junction protein with differential activities.

Aim: to assess the relationship between intratumoral VEGF -A, -C and CDH5 levels and clinical outcome, in primary, early stage breast cancer patients.

Patients and methods: The study included 69 node-negative (N0) breast cancer patients, all of whom had not received any prior hormonal or chemotherapeutic systemic therapy that would interfere with the course of disease. The median follow-up period was 144 months. Intratumoral mRNA levels of VEGF -A, -C and CDH5 were determined by RT-qPCR. Prognostic performance was evaluated by Cox proportional hazards regression, Kaplan-Meier analysis, as well as by the multivariable approach based on the least absolute shrinkage and selection operator (LASSO) logit regression. Classification of patients into the low and high subgroups was performed using the outcome-oriented cut-off point categorization approach.

Results: Of the measured mRNAs, only CDH5 mRNA (t=-2.17; p=0.04) and VEGF-C mRNA (t=-2.41; p=0.03) showed significant difference between their values in patient subgroups with distant metastasis and those without recurrences. These t-test results were in agreement with the Cox regression by which CDH5 mRNA reached the most pronounced hazard ratio (HR=2.07; p=0.05), followed by VEGF-C mRNA (HR=1.59; p=0.005). HR value above 1.0 indicated that high levels of either CDH5 or VEGF-C mRNAs associated with a higher risk of incurring the distant event. Distant recurrence incidence was 26% for the $CDH5^{high}$ and 3% for the $CDH5^{low}$ subgroup (Kaplan–Meier analysis). Distant recurrence incidence was 23% for the $VEGF\text{-}C^{high}$ and 0% for $VEGF\text{-}C^{low}$ subgroup. The independent prognostic value of VEGF-C mRNA was confirmed by LASSO regression.

Conclusion: Intratumoral VEGF-A levels did not associate with disease outcome in primary, early stage breast cancer patients, whilst raised levels of either CDH5 or VEGF-C prognosticated a high risk of distant metastasis.

Keywords**: **biomarker; breast cancer; VEGF-A, VEGF-C, VE-cadherin.

1. Introduction

Vascular endothelial growth factor (VEGF) family consists of five members of homo- and hetero-dimeric glycoproteins that show distinguishable spectra of functions: VEGF-A/VEGF, VEGF-B, VEGF-C, VEGF-D and placenta growth factor [reviewed by Melincovici et al. 2018]. VEGF-A is a potent stimulator of angiogenesis, and it also plays a vital role as a multifunctional molecule and growth factor [reviewed by Aguilar-Cazares et al. 2019]. It is secreted by inflammatory cells such as activated neutrophils, monocytes/macrophages and activated T cells; as well as by dendritic cells, platelets, endothelial and tumor cells [reviewed by Melincovici et al. 2018 and Lapeyre-Prost et al. 2017]. VEGF-A mediates angiogenesis, vascular permeability and inflammation by combining with high affinity tyrosine kinase receptors VEGFR1 and VEGFR2 expressed mainly on vascular endothelium, but also on tumor cells [reviewed by Zhou et al. 2021 and Melincovici et al. 2018]. VEGF-C is the central factor of lymphangiogenesis [reviewed by Zhou et al. 2021]. It is expressed by tumor cells, macrophages and stromal cells of the tumor microenvironment [Ran et al. 2010]. VEGF-C exerts the major biological effect when bound to high affinity tyrosine kinase receptor VEGFR3 expressed mostly on lymphatic endothelium [reviewed by Zhou et al. 2021 and Melincovici et al. 2018]. VEGF-C also promotes angiogenesis by binding to less affinity VEGFR2, and it regulates inflammation by binding to both VEGFR2 and VEGFR3 [reviewed by Zhou et al. 2021].

Vascular endothelial cadherin (VE-cadherin), also known as cadherin 5 (CDH5), plays a crucial role in endothelial adherens junction assembly and maintenance, thus controlling the integrity and permeability of vessels [reviewed by Veyssière et al. 2022]. CDH5 forms distinct complexes with different partners on the same cell, and these complexes might transfer different types of signals, depending on the functional conditions [reviewed by Giannotta et al. 2013]. CDH5 association with one or another mediator is reversible and temporally and spatially regulated [reviewed by Giannotta et al. 2013]. For example, its interactions with the growth factor receptors VEGFR2, fibroblast growth factor receptor 1 (FGFR1) and transforming growth factor beta – receptor (TGFβ-R) complex, module their downstream signaling and promote cancer progression [reviewed by Giannotta et al. 2013, Labelle et al. 2008]. Permeability-increasing agents such as VEGF-A and thrombin, act by inducing CDH5 internalization into clathrin-coated vesicles, thereby reducing the amount of CDH5 at adherens junctions and disrupting the endothelial cell barrier [reviewed by Giannotta et al. 2013]. In response to VEGF-A stimulation, tyrosine kinase domain of VEGFR2 is activated

and downstream Src kinase pathway is initiated, resulting in the phosphorylatation of serine residue in the cytoplasmic tail of CDH5; the subsequent binding of β-arrestin to serine-phosphorylated CDH5 promotes its internalization [reviewed by Giannotta et al. 2013, Gavard and Gutkind, 2006].

Breast cancer is one of the most frequent malignancies in women worldwide, and metastasis is the leading cause of death. A recent review study by Aguilar-Cazares et al. [2019] proposed a concept that the process of angiogenesis may be occurring at a very early stage of tumor development to favor the arrival of immune cells, and not necessarily at the point of hypoxia-induced angiogenic switch. In that context, the aim of this study was to assess the relationship between intratumoral VEGF -A, -C and CDH5 mRNA levels and clinical outcome, as well as their association with clinicopathological parameters, in primary, early stage breast cancer patients.

2. Material and Methods

2.1 Patients

This retrospective study included 69 early stage (T1/2N0) breast cancer patients who underwent surgical resection at the Institute of Oncology and Radiology of Serbia. The report was written according to REMARK recommendations for tumor marker prognostic studies [Altman et al. 2012]. Histological specimens were examined and classified according to the criteria of the American Joint Committee on Cancer / Union International Contre le Cancer (AJCC/UICC) for TNM stage, histological type and grade. Patient data were received in an anonymized form without indirect identifiers that could enable re-identification (Safe-Harbour methodology of the 2012 Health Insurance Portability and Accountability Act).

This non-interventional, retrospective, study was approved by the Institutional Ethics committee of the Institute of Oncology and Radiology of Serbia and conforms to The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18th July 1964) and its later amendments.

This group of T1/2 and N0 breast cancer patients had not received any prior hormonal or chemotherapeutic systemic therapy that would interfere with the course of disease. We assembled this specific patient group from a period of over 25 years when low recurrence-risk breast cancer patients were not prescribed systemic therapy at our hospital. This was in line with recommendations valid at that time for the lower-risk T1/2 and N0 patients [Rosner and Lane, 1990]. In this patient group, 75% (52/69) of patients had positive estrogen receptor

(ER) status and 35% (24/69) had positive progesterone receptor (PR) status. Twenty two percent (15/69) of patients had human epidermal growth factor receptor 2 (HER2) gene amplified. For steroid hormone receptor (ER, PR) and HER2 determination, please refer to our previous report [Milovanović et al. 2022].

To study whether the angiogenic factors VEGF -A, -C and CDH5 gave prognostic information in early stage breast cancer patients, we evaluated whether intratumoral mRNA levels of VEGF -A, -C and CDH5 were associated with the, retrospectively recorded, occurrence of local or distant recurrences. Local recurrence refers to the development of locoregional changes, whilst distant recurrence refers to metastasis in another organ such as the bone, lung, liver and brain. During the follow-up, 23% (16/69) of patients developed recurrence: 9% (6/69) developed local recurrences whilst 14% (10/69) developed distant metastasis. The median follow-up period was 144 months.

2.2 Sample size calculation

The prospective sample size calculation was based on a pilot experiment with 30 patients. The calculation parameters for intratumoral VEGF -A, -C and CDH5 obtained from the pilot experiment were: target power of 0.8, effect size by hazard ratio (HR) of 7, significance level of 0.05, variability of 0.58 – 0.69 and the event rate of 12%. We calculated the variability for each feature (expressed in standard deviation, SD) as a distance between average values of the patient subgroups with and without the actual recurrence.

The required numbers were 65 patients with six events. The actual patient number was 69, with ten distant events and six local events. The average SD distance given between the subgroups with and without recurrence was 0.64 for distant events and 0.43 for local events. The event rate was 9% for local and 14% for distant events. The effect size for distant events for CDH5 mRNA was 2.07 and 1.59 for VEGF-C. This resulted in the actual power of 0.88 for prognostication of distant events. Calculations were performed by the two-sided stpower cox test *(Stata/MP 17 software, StataCorp, College Station, TX, USA)*.

2.3 Real-Time PCR Assay

Samples of breast tumour tissue with an approximate volume of 2 mm^3 were homogenized on ice in the presence of ceramic microbeads for 60 seconds with an MP Fast Prep 24 homogenizer in 600 µL guanidinium thiocyanate solution (RLT buffer, *Qiagen Inc., Santa Clarita, CA*) supplemented with 0.1 M 2-mercaptoethanol. The homogenate was

further processed by centrifugation for 2 min at 12 000 x g in a QIAshredder homogenizer *(Qiagen)*.

Total RNA was then isolated with the *RNeasy mini kit (Qiagen)*. The integrity of the isolated RNA was examined by the *Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA)*. Only RNA samples with integrity number (RIN) > 7 were subsequently reverse transcribed with the *High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific,Waltham, MA)* by use of hexanucleotide random primers.

Quantitative PCR was performed with the Taqman Universal PCR Master Mix, *No AmpErase UNG kit* containing AmpliTaq Gold DNA polymerase *(Thermo Fisher Scientific)*. The following TaqMan assays were used: Hs00901465_m1 for CDH5, Hs00900055_m1 for VEGF-A, Hs01099203_m1 for VEGF-C. All assays contained the probe that spanned exons. Transcripts were amplified for 40 cycles for 15 s at 95 $^{\circ}$C, and 60 s at 60 $^{\circ}$C by a 7900TH TaqMan robot *(Thermo Fisher Scientific)*. 18S rRNA was used as a normalization control for mRNA input. Only samples with a Ct < 15 for 18S rRNA were considered for further analysis.

2.4 Prognostic performance evaluation

Kaplan-Meier analysis was done for the period from tumor resection until the occurrence of local or distant event *(IBM SPSS Statistics for Windows version 28, IBM Corp. Chicago, IL)*. Univariate Cox proportional hazards regression test was performed for comparison of the prognosticated and actual (local or distant) events, and this test was based on continuous numerical values for each variable. The advantage of prognostic evaluation based on continuous data values was the absence of a bias introduced by data categorization. The hazard ratio (HR) designates the effect size by Cox regression, corresponding to recurrence rates in high- and low-risk groups of patients *(IBM SPSS v28)*. Each feature satisfied the proportional hazards assumption based on the Schoenfeld residuals by phtest *(Stata/MP)*.

2.5 Feature selection

The feature selection utilized the multivariable approach based on the least absolute shrinkage and selection operator (LASSO) logit regression. LASSO is a supervised machine learning algorithm that identifies the features with non-redundant and internally cross-validated association with the outcome [Tibshirani, 1996]. We performed LASSO calculations by use of continuous feature values and the binary disease outcome. LASSO was also used to

generate the prognostic signature based on the selected features and their calculated coefficients, according to the formula (1):

(1) Coefficient value = feature 1 * coefficient 1 + feature 2 * coefficient 2

2.6 Validation

The p-values and confidence intervals (95%CI) of the calculated HRs were corrected for bias by the bootstrap internal validation *(IBM SPSS v28)*. Bootstrap resample validation tests the model stability by adjusting the confidence intervals and calculating p-values. The bootstrap based on “resampling with replacement” produces new "surrogate" data sets with the same number of cases as the original data set. This is performed by a random selection of observations from the original sample until the same number of observations is achieved, followed by calculation of the 95%CI and p-value. The “resampling with replacement” bootstrap used here did not remove the selected observations from the pool during resampling. Therefore, some measurements may be selected multiple times while certain observations may not appear in a resample. By creating 10,000 different resamples, bootstrapping offers prognostic performance with increased stability [Efron, 1982].

Split-sample cross-validation was used as a validation tool for selecting an optimal penalty coefficient λ within the LASSO regression analysis in *Stata/MP 17*. The advantages of this study include an internal validation performed by bootstrap and cross-validation, which suggested that the model is generalizable. It is important that the measured features are robust. Another advantage is therefore the performed LASSO selection of features that are most robust to cross-validation. A full model with all predictors generally has the lowest bias and maximal in-sample predictive performance but suffers from overfitting, meaning that the model is likely to provide poor performance if applied to new data. Therefore, LASSO was used to reduce the model complexity by removing predictors, while also minimizing overfitting through cross-validation.

3. Results

Statistical analysis was performed based on the distant or local events as endpoints. Table 1 indicates statistical analysis of the prognostic performance of clinicopathological and measured mRNA parameters by Cox regression. Of the available clinicopathological parameters in this patient group, the age, tumor size and ER significantly associated with the increased risk of distant or local events (Table 1). Only the age showed prognostic

significance for both local and distant events. Table 1 also shows the prognostic evaluation of the measured intratumoral mRNA levels of VEGF -A, -C and CDH5. None of these mRNA levels provided significant prognostic value when calculated against the local events endpoint (Table 1). Considering all the clinical and measured mRNA parameters, CDH5 mRNA reached the most pronounced HR (HR=2.07 and p=0.05), followed by VEGF-C mRNA (HR=1.59 and p=0.005). HR value above 1.0 indicated that high levels of either CDH5 or VEGF-C mRNAs associated with a higher risk of incurring the distant event.

The average ± standard deviation (SD) dCt CDH5 mRNA level was 17.4 ± 1.3 for the patients with distant metastases, 15.6 ± 1.4 for those with local recurrences and 16.4 ± 1.9. for patients without any events. The average dCt VEGF-A mRNA level for the patients with distant metastases was 13.7 ± 1.5, with local recurrences 12.8 ± 1.3 and without any events 13.4 ± 1.4. The average dCt VEGF-C mRNA level for the patients with distant metastases was 19.4 ± 1.4, with local recurrences 17.6 ± 0.9 and without any events 18.3 ± 1.7. Besides the Cox regression, the prognostic value of the measured features was also evaluated using the independent samples. Thereby, of the measured mRNAs, only CDH5 mRNA (t=-2.17; p=0.04) and VEGF-C mRNA (t=-2.41; p=0.03) showed statistically significant difference between their values in patient subgroups with distant metastasis and those without any events (not shown). These t-test results were thus in agreement with the Cox regression analysis (Table 1). The point-biserial correlation analysis (Table 2) revealed the statistically significant positive correlations between the following parameters: age and ER, age and CDH5 levels, tumor size and VEGF-A, tumor size and VEGF-C, VEGF-A and VEGF-C.

Logit cross-validation LASSO was utilized as a multivariate machine learning approach to select the features that are prognostically least redundant and most robust. This multivariate analysis included all available clinicopathological parameters and measured mRNAs. Similarly to the univariate analysis, LASSO also considered the distant or local recurrences as endpoints (Table 3). VEGF-C mRNA was thereby highlighted as the least redundant and most robust to the cross-validation performed during the LASSO procedure. The independent prognostic value of VEGF-C mRNA was confirmed by LASSO regression. It is of note that LASSO feature selection could select features only for the distant metastasis as the outcome (Table 3), indicating the weak robustness of the measured features in prognostication of local recurrences.

Kaplan-Meier estimator plots illustrate the prognostic association of the intratumoral CDH5 and VEGF-C mRNAs, which showed the best association with the disease outcome (Figure 1A and 1B). Classification of patients into the low and high subgroups was performed

using the categorization approach based on the outcome-oriented cut-off point. The cut-off point for intratumoral CDH5 mRNA was at 16.8 dCt and 18.0 dCt for VEGF-C mRNA. P-values were calculated by the univariate Cox regression test. A wider separation between upper and lower curves indicates better prognostic performance. Distant recurrence incidence was 26% for the CDH5^{high} and 3% for the CDH5^{low} subgroup (Figure 1A). Distant recurrence incidence was 23% for the VEGF-C^{high} and 0% for VEGF-C^{low} subgroup (Figure 1B). High levels of either CDH5 or VEGF-C mRNAs at time of surgery thereby prognosticated higher risk of distant recurrence.

4. Discussion

Tumor cell-derived factors impact cancer progression by modulating endothelial cell activation at the (pre-)metastatic niche, which affects tumor cell dissemination as well as the outgrowth of seeded metastatic cells [reviewed by Preuss et al. 2023]. Among them, VEGF family participates in (pre-)metastatic niche formation and re-growth of metastatic tumor cells in distal organs via VEGF-induced primitive vasculature formation [reviewed by Yang & Cao, 2022]. On the other hand, cadherins play an important role in tissue homeostasis, as they are responsible for cell-cell adhesion during embryogenesis, tissue morphogenesis, differentiation and carcinogenesis [reviewed by Kaszak et al. 2020]. Especially, epithelial to mesenchymal transition (EMT) is a mechanism by which epithelial cadherin (E-cadherin) expression is lost during tumor progression [reviewed by Gloushankova et al. 2018]. In that context, we assessed the relationship between intratumoral VEGF -A, -C and CDH5 levels and clinical outcome, in primary, early stage breast cancer patients.

In general, studies have revealed that VEGF-A positive tumors are biologically more aggressive and are associated with a poor outcome in breast cancer patients. A study by Ghosh et al. [2008] analyzed tumor-specific expression of VEGF-A on a large cohort (n=642) of primary breast cancer tissue microarray, and found high VEGF-A levels significantly associated with worse outcome. A large study by Liu et al. [2011] examined VEGF-A expression in 1,788 primary invasive breast cancers using immunostaining of tissue microarray sections. In this study, VEGF-A expression was not significantly associated to worse survival when all cases were considered together, but in 262 women untreated systemically, VEGF-A expression was significantly associated with breast cancer-specific mortality [Liu et al. 2011]. A study by Ali et al. [2011] examined the expression pattern of VEGF-A in serum and tissues of 120 untreated breast cancer patients. Serum and tissue VEGF-A were positively correlated and strongly associated with grade III, large tumor size,

positive lymph node, negative hormone receptor (HR) status, positive HER2 status and poor survival [Ali et al. 2011]. A recent study by Shera et al. [2019] also found tissue VEGF-A expression correlated well with the grade and stages of breast tumor. On the contrary, a meta-analysis of 37 studies (n=5,001 patients) found no association of VEGF-A immunohistochemical (IHC) expression with clinicopathological parameters, including age, tumor size, grade and receptor status [Su et al. 2016]. In our study, intratumoral VEGF-A levels positively correlated with tumor size, but not with other clinicopathological parameters (Table 2). Recent review study analyzed recurrence-free (RFS) and overall survival (OS) rates of breast cancer patients stratified by VEGF-A mRNA expression [reviewed by Al Kawas et al. 2022]. In this study, all breast cancer subtypes benefit from low VEGF-A mRNA expression as a prognostic biomarker [reviewed by Al Kawas et al. 2022]. Two other prognostic studies, one on metastatic and the other on triple-negative breast cancer patients (n=253 and n=303, respectively) found elevated levels of serum VEGF-A significantly associated with the unfavourable outcome [Banys-Paluchowski et al. 2018, Wang et al. 2019]. Although breast cancer studies have indicated the prognostic significance of VEGF-A, we found no significant association between intratumoral VEGF-A levels and clinical outcome. This could be explaind by the multifunctional nature of VEGF-A molecule. For example, the recruitment of inflammatory cells promoted by VEGF-A enhance the local production of plethora of cytokines, chemokines and other factors [reviewed by Zhou et al. 2021 and De Palma et al. 2017, Todorović-Raković et al. 2017]. Evidently, higher intratumoral VEGF-A expression does not ensure inflammatory response that favors worse patient survival, because complex regulatory mechanisms will form a comprehensive impact [reviewed by Zhou et al. 2021].

Several studies have identified CDH5 as a novel biomarker of metastatic breast cancer, but its prognostic value in early stage breast cancer patients is still undefined. A study by Fry et al. [2016] showed that serum CDH5 levels were significantly elevated in patients who had developed a distant metastasis (n=52) compared to those who had remained recurrence-free (n=60). Another study on 141 hormone-resistant metastatic breast cancer patients, found elevated levels of serum CDH5 significantly associated with a shorter progression-free (PFS) and overall survival (OS) [Rochefort et al. 2017]. Only one study found elevated CDH5 in primary breast tumor samples (n=114) associated with a poor survival of patients [Martin et al. 2005]. In our study, intratumoral CDH5 levels associated significantly with the increased risk of distant metastasis (Table 1, Figure 1A). That could be in accordance with its role during cancer progression related to epithelial to mesenchymal transition (EMT). Within the motility/invasion phase of the metastatic cascade, a critical mechanism that allows tumor

cells to acquire the necessary skills is the EMT [reviewed by Aguilar-Cazares et al. 2019]. However, the EMT allows tumor cells to develop vasculogenic mimicry, the condition in which tumor cells mimic endothelial cells to form extracellular matrix-rich tubular channels [Liu et al. 2013]. As part of the EMT, CDH5 is expressed in tumor cells favoring both vasculogenic mimicry and metastasis [reviewed by Aguilar-Cazares et al. 2019]. Moreover, the differential activities of the endothelial junction protein CDH5 reflect the versatile behavior of endothelial cells between vascular quiescence and angiogenesis [reviewed by Wallez et al. 2006]. CDH5 function and signaling are deeply modified in proliferating cells, and this conversion is accompanied by CDH5 phosphorylation and enhanced transcription of its gene [reviewed by Wallez et al. 2006].

The prognostic value of VEGF-C has been evaluated in many breast cancer studies and the results are contradictory. In a study by Bando et al. [2006], VEGF-C levels were measured in the tumor lysate supernatant by ELISA in 193 primary breast cancer patients. In this study, high VEGF-C levels significantly associated with low grade tumors, smaller tumor size, a favourable disease-free and overall survival [Bando et al. 2006]. By contrast, two studies confirmed the negative prognostic value of VEGF-C based on its mRNA levels in breast tumor tissue [Linardou et al. 2012, Lin et al. 2015]. High VEGF-C mRNA levels were associated with worse grade, more lymph node metastasis, a shorter progression-free and overall survival in breast cancer patients [Lin et al. 2015]. Several meta-analyses strongly supported the negative prognostic value of VEGF-C in breast cancer patients and reported higher tumoral VEGF-C expression significantly associated with poorer relapse-free and overall survival [Zhang et al. 2016, Liang & Li 2014, Wang et al. 2012]. In contrast, two meta-analyses reported no significant association between VEGF-C expression and disease-free or overall survival in breast cancer patients [Wang et al. 2016, Gao et al. 2014]. Moreover, a recent study by Maañón et al. [2018] found higher serum VEGF-C levels associated with better relapse-free survival in 174 node-negative breast cancer patients. In this study, serum VEGF-C levels did not correlate with any clinical or pathological variables, but the five-year RFS rate was higher in patients with VEGF-C levels above the median than in patients with lower levels [Maañón et al. 2018]. A study by Gisterek et al. [2010] also reported that higher serum VEGF-C levels predicted better overall survival in 349 breast cancer patients, but a study by Al-Mowallad et al. [2007] found no association between plasma VEGF-C levels and overall survival in 122 breast cancer patients. A meta-analysis of 37 studies (n=5,001 patients) showed that VEGF-C IHC expression was not associated with any clinicopathological parameter except HER2 (VEGF-C overexpression correlated with HER-2 positivity) [Su et al.

2016]. However, it is important to note that the positive biomarker expression was defined according to different cut-off values in various studies. In our study, intratumoral VEGF-C levels associated significantly with a larger tumor size as well as with the increased risk of distant metastasis (Tables 1 and 2, Figure 1B). Multivariate analysis confirmed the independent prognostic value of VEGF-C (Table 3). According to literature, VEGF-C expression in breast cancer is associated with lymphatic vessel proliferation, lymphatic cell migration, tumor cell dissemination via lyphmatic and blood vessels, and distant metastasis formation [Ran et al. 2010]. A recent study revealed a novel mechanism by which cancer cell-derived VEGF-C remodels lymphovascular microenvironment by upregulating CXC chemokine production in lymphatic endothelial cells to promote cancer invasion [Chen et al. 2019]. In addition, these chemokines also stimulated the expression of serum amyloid A1 in cancer cells, enhancing their lymphatic invasion by increasing CDH5 phosphorylation, junction disruption and permeability of lymphatic endothelium [Chen et al. 2019].

The advantages of this study include an internal validation performed by bootstrap and cross-validation, which suggested that the model is generalizable.

In conclusion, our results indicate prognostic significance of CDH5 and VEGF-C in a specific, adjuvantly untreated, node-negative, patient group with a long term follow-up. In our study, intratumoral VEGF-A levels did not associate with disease outcome but raised levels of either CDH5 or VEGF-C prognosticated a high risk of distant metastasis. Multivariate analysis confirmed the independent prognostic value of VEGF-C. Summarized, our results suggest that CDH5 and VEGF-C have prognostic potential as biomarkers for the identification of breast cancer patients at high risk of recurrence.

Highlights

- Intratumoral VEGF-A levels did not associate with disease outcome
- Raised levels of CDH5 and VEGF-C associated with a high risk of distant metastasis
- Raised intratumoral levels of VEGF -A and -C correlated with a larger tumor size
- Raised VEGF-C levels associate independently with unfavorable disease outcome

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Declaration of competing interest

The authors declare no conflict of interest.

Authors' contributions

All authors have made substantial contributions to: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) and final approval of the version to be submitted.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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6. Tables

Table 1. Prognostic performance of clinicopathological and angiogenic parameters[a]

Variable	HR[a] 95% CI[b] P-value*	
	Distant metastasis	**Local recurrence**
Age	1.15 1.01 – 1.22 **0.04***	1.03 0.86 – 1.00 **0.05***
Tumour size	1.08 1.02 – 1.14 0.30	1.01 0.93 – 1.07 **0.03***
ER	1.01 0.99 – 1.0 **0.05***	1.01 0.92 − 0.99 0.29
PR	1.02 0.36 – 1.02 0.29	1.03 0.45 – 1.00 0.54
Tumour grade	1.87 0.12 – 2.34 0.23	1.08 0.88 − 48.1 0.27
HER2	1.67 0.03-10.4 0.48	1.23 0.02-9.80 0.82
CDH5 mRNA	2.07 1.00 – 2.21 **0.05***	1.15 0.32 – 1.23 0.12
VEGF-A mRNA	1.65 0.54 – 1.95 0.92	0.22 0.22 – 1.23 0.17
VEGF-C mRNA	1.59 1.01 – 2.38 **0.005***	1.19 0.28 – 1.07 0.17

[a] Univariate Cox proportional hazards regression test, based on continuous prognosticator data.
[b] Confidence intervals (95% CI) and P-values were corrected by bootstrap.
* $P \leq 0.05$
Abbreviations: ER, estrogen receptor; PR, progesterone receptor; CDH5, Cadherin 5; VEGF, Vascular endothelial growth factor.

Table 2. Correlations between intratumoural CDH5 and VEGF mRNA levels and the major clinicopathological parameters[a]

Predictor	*Age*	*pT*	*ER*	*PR*	*Grade*	*HER2*	*CDH5*	*VEGF-A*
pT	0.03							
ER	**0.31***	0.13						
PR	0.11	0.10	0.23					
Grade	0.09	0.05	0.09	-0.02				
HER2	0.05	-0.05	0.02	-0.04	0.16			
CDH5	**0.32***	0.009	0.25	0.14	0.14	0.21		
VEGF-A	-0.02	**0.46***	-0.02	0.04	-0.03	0.01	-0.07	
VEGF-C	-0.03	**0.43***	0.11	0.04	-0.13	-0.11	-0.14	**0.65***

[a] Continuous numerical values were used for calculation of point-biserial coefficients except for tumour grade and HER2 score which are inherently categorical.

** P < 0.01 (2-tailed)

* P < 0.05 (2-tailed)

Abbreviations: pT, tumour size; ER, estrogen receptor; PR, progesterone receptor; CDH5, Cadherin 5; VEGF, Vascular endothelial growth factor.

Table 3. Feature selection[a]

Feature	Lambda	Coefficient	95%CI	CV mean deviance	p-value
Distant metastasis					
Age	0.071	0.006	–	0.1315	–
VEGF-C mRNA	0.078	0.042	–	0.1314	–
Selected lambda[b]	0.130	–	–	0.1303	–
Index	–	10854	8.1 – 14495592	–	<0.001
Local relapse					
–	–	–	–	–	–

[a] Variable selection performed by use of an adaptive LASSO regression.
[b] Lambda selected by cross-validation in final adaptive step.
P ≤ 0.05 was used as the significance threshold.
Abbreviations: LASSO, least absolute shrinkage and selection operator.

7. Figures

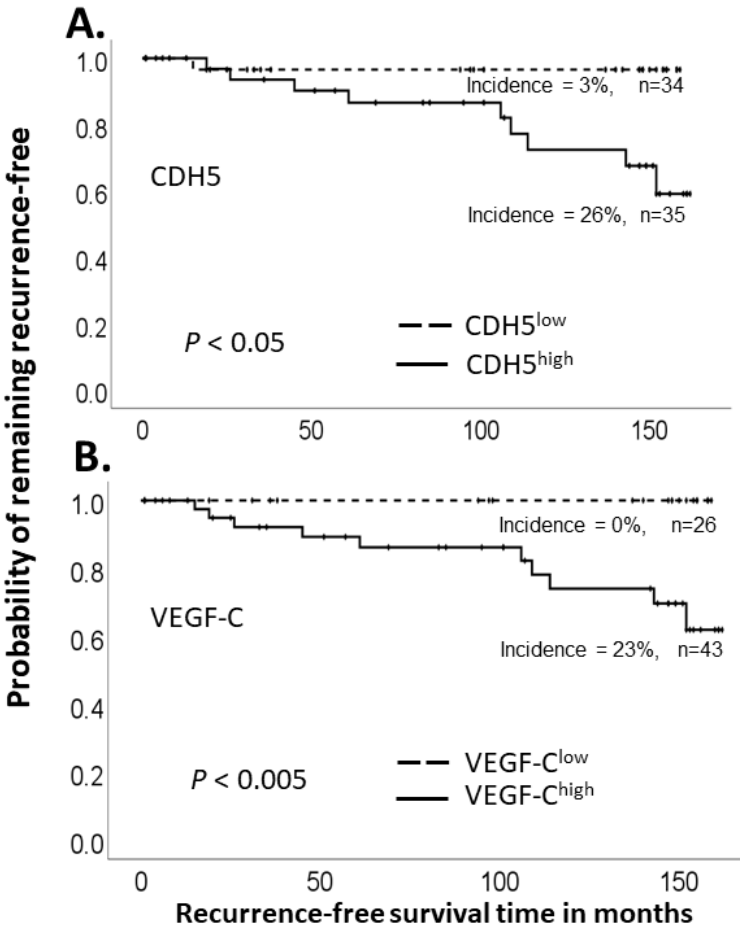

Figure 1. Kaplan-Meier analysis of the intratumoral CDH5 and VEGF-C mRNA levels in prognosis of distant events. (A) Prognostic performance of CDH5 mRNA. (B) Prognostic performance of VEGF-C mRNA. Classification of patients was performed using the categorization approach based on the outcome-oriented cut-off point. The cut-off point for intratumoral CDH5 mRNA was at 16.8 dCt and 18.0 dCt for VEGF-C mRNA. P-values were calculated by the univariate Cox regression test. A wider separation between upper and lower curves indicates better prognostic performance.

Abbreviations: CDH5, Cadherin 5; VEGF-C, Vascular endothelial growth factor C.