Published OnlineFirst January 14, 2016; DOI: 10.1158/1078-0432.CCR-15-1691

Translational Relevance

The ability to evaluate the effect of a clinical trial drug is central to its success. Although antiangiogenic drugs have demonstrated modest increases in the progression-free survival of breast cancer patients, they do not prolong life expectancy and importantly, lack a formal predictive biomarker. The ability of vascular gene transcripts to provide treatment predictive information in a breast cancer setting remains unexplored. Here, we utilize a set of genes representative of a normal vascular endothelium to identify a subgroup of endocrine-treated breast cancer patients with better and worse long-term distant metastasis-free survival. Moreover, we also note significant increases in signature genes following treatment of metastatic tumors with the angiogenic inhibitor sunitinib, highlighting that evaluation of transcriptional changes in microvascular genes alongside assessment of microvessel density and angiogenic factors in clinical trials of antiangiogenic compounds may be warranted.

performance in subgroups of endocrine and chemotherapy-treated patients.

Materials and Methods

Patients and datasets

Internal datasets. We have previously described both the Uppsala ($N = 253$) and Stockholm ($N = 159$) datasets (17–19) with an extensive overview for both cohorts found here (12). Both microarray studies were approved by the ethics committees at Karolinska Institutet and Karolinska University Hospital (Stockholm, Sweden), respectively and are publically available at NCBI GEO under accession numbers GSE4922 and GSE1456, respectively.

External datasets. Data from the Netherlands Cancer Institute (NKI; $N = 295$; ref. 20), Erasmus Medical Center (Rotterdam; $N = 286$; ref. 21), and the John Radcliffe and Guys hospitals (Oxford and London, United Kingdom; $N = 99$ and 87, respectively; ref. 22) were used, the Oxford and Guys data for further analysis of the findings in relation to endocrine therapy. The NKI dataset is publicly available as a part of the *breastCancerNKI* R package and the Rotterdam and Oxford/Guys datasets are available under accession numbers GSE6532 and GSE5432. Clinical information for 14 patients treated with sunitinib plus docetaxel or docetaxel alone has been previously described (ref. 23; ClinicalTrials.gov identifier NCT00393939) and expression data are publicly available at NCBI GEO under accession number GSE54323. For the comparison of MV score to lymphovascular invasion (LVI), we used previously published data (24), retrievable under the accession number GSE5420.

RNA extraction and array hybridization

Uppsala and Stockholm cohorts. RNA was extracted from homogenized tumor material with RNeasy spin column kits (Qiagen) and quality was assessed with an Agilent 2100 Bioanalyzer. Two to 5 μg of RNA was used to produce biotinylated cRNA. Hybridization to HGU133 A and B microarrays (Affymetrix) and scanning was performed according to Affymetrix protocols. A comprehensive account can be found in Pawitan and colleagues (19).

IHC and MVD

Uppsala cohort. For immunohistochemical analysis, formalin-fixed paraffin-embedded sections (4 μm) were deparaffinized in xylene and rehydrated in graded concentrations of ethanol to TBS. Antigen retrieval by microwave treatment was performed for 20 minutes in Tris-EDTA Buffer (10 mmol/L Tris Base, 1 mmol/L EDTA Solution, 0.05% Tween 20, pH 9.17). CD31 antibody (clone JC70A; DAKO) was diluted 1:50, and staining was carried out in a Tech Mate Autostainer (DAKO). Slides were counterstained with hematoxylin and dehydrated. MVD was determined using previously described methods (25). Briefly, tumor sections were examined at low power to determine the areas containing the greatest numbers of microvessels. Individual microvessels were then counted in these areas at ×400 magnification (three fields per tumor section). Mean counts per high field were then calculated and the resulting value was normalized to yield an MVD score between 0 and 100 (for ease of comparison to microvascular gene expression score) using the rescale command of the "scales" package in R (26).

Microvasculature signature, PAM50 subtypes, and genomic grade

Human homologs for the 57 of 58 previously published mouse microvasculature transcripts (16) were extracted from the HomoloGene database at the National Center for Biotechnology Information (NCBI, Supplementary Table S1). Datasets were RMA normalized and median centered before the expression levels of signature genes were added per tumor, and the resulting sum was scaled to yield a microvasculature signature score (hereafter called MV score) of between 0 and 100 within each dataset using the *rescale* function of the R *genefu* package. The exception to this was the NKI dataset; here, we used the original normalized and median-centered data from the *breastCancerNKI* R package for scaling as described above. Of note, mean centering has been demonstrated as sufficient to remove much of the dataset bias associated with gene expression data from different cohorts, allowing for meaningful prognostic comparisons to be made (27). Fifty-seven of the genes (corresponding to 115 probes) were present on the HGU133 A and B platforms, and could be used for MV score determination in the Uppsala and Stockholm data. In the external datasets, identical methodology was used to determine MV scores and for the NKI, Rotterdam, Oxford, Guys, and metastatic tumor data, 49, 46, 57, 57, and 57 signature genes were present on the respective platforms corresponding to 56, 79, 115, 115, and 115 probes, respectively. In the case of multiple probes mapping to the same gene, an average expression of probes was taken. When data were pooled for combined analysis, the signature score was calculated and scaled in individual datasets before pooling.

PAM50. Molecular subtyping according to the PAM50 signature was performed as outlined in the original publication (7), using the code provided by Parker and colleagues on the UNC Microarray Database website as a data supplement to the original article. Of note, we have previously published our code and the PAM50 subtypes for the Uppsala and Stockholm cohorts (12).

Genomic grade index. Genomic grade index (GGI) was calculated as described in the original publication (6) and we have previously published our code for this signature along with the GGI calls for the Uppsala and Stockholm datasets (12).

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Comparison of MV score to MVD and significance analysis for microarrays and gene ontology analysis

To assess the similarity between the MV and MVD scores, we performed a Pearson correlation comparing (i) both scores directly and (ii) each of the MV score genes to MVD score in 182 patients of the Uppsala cohort. *P* values were adjusted for multiple testing using the FDR method as part of the *p.adjust* command of the R *stats* package. To test for enrichment of biologic themes, the significance analysis of microarrays (SAM; ref. 28; quantitative response) was used to rank genes for association with the MV score (gene expression) and MVD scores (vessel count), respectively. Over-representation of gene ontology categories (GO) reflecting biologic processes was determined with conditional hypergeometric tests using the R *GOstats* package. As many of the MV score genes are annotated as vascular or angiogenesis related, and by necessity will correlate to the signature, the 57 signature genes were removed from the data prior to GO testing. Estimation of false discovery rate control was performed according to Storey and Tibshirani (29) using the R package *qvalue* 2.0.0. All occurring categories in respective gene lists were tested.

Statistical analysis and software

To test for differences in mean expression of the MV score in relation to clinicopathologic parameters, Student *t* test (for comparing means between two groups) or ANOVA (for comparisons between more than two groups) with *post hoc* Tukey were used as indicated in table legends. Survival analysis was performed using Kaplan–Meier and multivariable proportional hazards (Cox), where only variables with demonstrated prognostic significance in univariable testing were included in the final multivariable model. To ensure consistent survival endpoints across all datasets, distant metastasis-free survival (DMFS) was chosen. Hazard ratio (HR) is reported per 25 increments of MV score and MVD score, to simplify interpretation. ROCs were used to determine best MV score cutoff (with DMFS at 5 years as endpoint) in testing datasets before being applied to validation datasets and correlograms were constructed using the Spearman rank correlation metric. Concordance index (c-index) calculation was performed in R using the "concordance" output from a Cox regression analysis for the GGI and PAM50 signatures alone or in combination with the MV score. All analyses were performed using the R Statistical language (26), with the *GOstats*, *ROCR*, *survival*, and *samr* packages.

Results

The MV score is not correlated to traditional MVD, but does reflect angiogenic/endothelial GO processes

To understand if any similarities exist between genes associated with microvasculature gene expression and traditional MVD score, we stained and scored the tumors of 182 patients from our previously published Uppsala cohort (12,17–19) using the endothelial/MVD marker CD31. Next, we derived a normal MV score using gene expression data from the same tumors by adding the mRNA expression levels for 57 of 58 previously identified (16) endothelial-specific gene transcripts (Supplementary Table S1). Both the MV and MVD scores were then normalized and scaled (for ease of comparison) to yield values between 0 and 100.

A simple correlation analysis did not reveal any clear overall similarity between the MV and MVD scores (Pearson correlation = 0.096, $P = 0.20$, data not shown) and in individual signature gene analysis, only a weak correlation was found between five MV score genes and the MVD score (Supplementary Table S1). Next, we compiled two lists of the top 200 genes most associated with the MV score (having first removed the 57 signature genes) and MVD scores and assessed whether any biologic processes were over-represented in these lists through GO analysis. The top 10 GO terms for both lists are displayed in Table 1, where an enrichment of terms related to angiogenesis and cardiovascular/blood vessel/endothelial development is notable among MV score–correlated genes (Table 1, top list). Conversely, no angiogenic or vascular development terms are found within MVD (CD31) correlated genes, which rather display an enrichment of terms associated with immune response (Table 1, bottom list).

Table 1. GO terms associated with MV score and CD31-correlated genes

Term	GO ID	*P*	*Q* value	Odds ratio	Expected count	Count	Category size
Top 10 gene ontology categories overrepresented in 200 MV score-correlated genes							
Cardiovascular system development	GO:0072358	6.1E−15	3.7E−12	6.7	7.2	34	739
Blood vessel development	GO:0001568	3.0E−13	9.2E−11	7.5	4.6	26	471
Multicellular organismal process	GO:0032501	1.5E−09	3.0E−07	3.4	47.2	78	4836
Endothelium development	GO:0003158	1.7E−08	2.5E−06	16.8	0.7	9	68
Ameboidal cell migration	GO:0001667	2.1E−08	2.5E−06	7.9	2.1	14	216
Epithelium migration	GO:0090132	2.5E−07	2.5E−05	8.7	1.5	11	152
Lymph vessel development	GO:0001945	6.2E−07	5.4E−05	40.9	0.2	5	18
Angiogenesis	GO:0001525	1.2E−06	9.1E−05	7.4	1.7	11	196
Cell adhesion	GO:0007155	1.6E−06	1.1E−04	3.5	8.3	24	849
Positive regulation of locomotion	GO:0040017	1.9E−06	1.1E−04	5.8	2.6	13	267
Top 10 gene ontology categories overrepresented in 200 CD31 correlated genes							
Response to type I interferon	GO:0034340	5.8E−19	2.7E−16	33.9	0.8	17	74
Type I interferon signaling pathway	GO:0060337	5.8E−19	2.7E−16	33.9	0.8	17	74
Interferon-gamma-mediated signaling pathway	GO:0060333	1.0E−13	3.3E−11	26.3	0.7	13	67
Defense response to virus	GO:0051607	9.5E−13	2.3E−10	12.7	1.7	17	168
Antigen processing and presentation of endogenous antigen	GO:0019883	3.0E−12	5.8E−10	180.9	0.1	7	11
Response to other organism	GO:0051707	6.5E−12	1.0E−09	6.2	5.7	27	556
Response to biotic stimulus	GO:0009607	1.4E−11	1.9E−09	6.0	5.9	27	575
Response to interferon-gamma	GO:0034341	1.7E−11	2.0E−09	16.6	1.0	13	98
Positive regulation of immune response	GO:0050778	2.6E−11	2.7E−09	6.8	4.3	23	418
Defense response	GO:0006952	3.2E−11	3.0E−09	5.1	8.7	32	943

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First, these results show that the MV score strongly reflects angiogenic/endothelial processes on a transcriptional level and second, based on the CD31 GO terms, we may be unaware of the extent to which the immune response is involved in blood vessel formation and maintenance.

A low MV score is associated with a Luminal B tumor subtype

With the aim of determining the prognostic and treatment predictive capacity of the MV score, we extended our analysis to three additional gene expression breast cancer datasets (12,19–21). Again, MV scores were normalized and scaled within each dataset and the resulting score was assessed in relation to the clinicopathologic parameters shown in Table 2.

We noted a trend toward lower MV scores in grade 3 tumors across all datasets (vs. grade 1 tumors; Table 2) and similarly, a statistically significant low signature score in the estrogen receptor–positive Luminal B subtype (vs. Luminal A; Table 2, $P \leq 0.001$, 0.008, 0.016, and <0.001, for the Uppsala, Stockholm, NKI, and Rotterdam datasets, respectively). A trend toward a higher MV score was found in tumors of a Normal-like subtype, which reached statistical significance in two of four datasets (vs. Luminal A subtype; Table 2, $P = 0.003$ and 0.047, for the Stockholm and Rotterdam datasets, respectively). Of interest, as information on lymphovascular invasion (LVI) was not available in these cohorts, we analyzed an additional publicly available dataset (ref. 24; $n = 128$) and could not demonstrate a difference in MV score in the absence or presence of LVI (MV score mean ± SEM = 45.71 ± 2.23 and 49.54 ± 2.15, for tumors with and without LVI, respectively. $P = 0.22$, Welch t-test, data not shown).

A low MV score predicts poor outcome in endocrine-treated patients

The prognostic capacity of many first-generation gene expression signatures tends to be limited to ER-positive breast tumors (30). As such, we assessed the MV score in univariable analysis across all, ER-positive, and ER-negative patients in each dataset. No consistent relationship to distant DMFS was found in any of these groupings; however, in all patients, two of four datasets demonstrated a lower HR with increasing MV score (Supplementary Table S2, see "All patients" Uppsala and Stockholm cohorts, HR 0.69; 95% CI, 0.49–0.96 and 0.46; 95% CI, 0.24–0.87, respectively).

To determine whether the type of adjuvant therapy received influenced these results, we subdivided each cohort into patients who did not receive systemic treatment, who received endocrine treatment, or those who received chemotherapy. The MV score did not provide consistent statistically significant information regarding DMFS in untreated, untreated ER-positive, or chemotherapy-treated patients (Fig. 1, see "Untreated," "Untreated (ER positive)," and "Chemotherapy," All). For patients receiving endocrine therapy, a trend toward decreased risk of distant metastasis was observed in two of three datasets (Fig. 1, see "Endocrine

Table 2. Mean MV score in relation to clinicopathologic parameters in four independent datasets

	Uppsala ($N = 253$)			Stockholm ($N = 159$)			NKI ($N = 295$)			Rotterdam ($N = 286$)		
Variable	**Mean MV score**	**±SD**	***P***	**Mean MV score**	**±SD**	***P***	**Mean MV score**	**±SD**	***P***	**Mean MV score**	**±SD**	***P***
ER												
Positive	48.1	16.8		35.8	15.9		58.6	13.4		51.8	17.6	
Negative	43.3	18.0	0.128	33.6	13.7	0.482	**62.5**	**14.5**	**0.040**	55.6	15.1	0.094
PR												
Positive	47.9	17.2		35.1	15.1		—	—		—	—	
Negative	43.5	14.5	0.238	36.1	16.7	0.722	—	—	—	—	—	—
Elston–Ellis grade												
I[a]	53.2	15.7	—	37.0	12.7	—	60.6	14.2	—	58.4	14.6	—
II	47.8	17.6	0.071	39.9	17.8	0.682	60.2	12.3	0.983	58.7	16.6	0.999
III	**39.7**	**13.8**	**<0.001**	30.9	12.8	0.179	58.2	14.6	0.471	51.6	16.3	0.527
Nodal status												
Negative	49.8	16.9		35.7	15.7		59.3	14.3		—	—	
Positive	**43.5**	**16.2**	**0.005**	35.5	15.8	0.952	59.8	13.2	0.752	—	—	—
Tumor size												
≤20 mm	51.7	17.0		37.0	15.0		59.7	14.1		—	—	
>20 mm	**43.1**	**15.8**	**<0.001**	33.6	15.9	0.175	59.4	13.3	0.830	—	—	—
Age												
≤50	41.3	13.5		32.8	14.6		59.6	13.5		51.9	18.6	
>50	**49.2**	**17.4**	**0.002**	36.7	15.8	0.139	58.8	15.6	0.758	53.5	15.6	0.439
Chemotherapy												
Yes	42.2	12.4		31.7	16.5		60.2	12.5		—	—	
No	48.3	17.0	0.087	36.3	15.2	0.146	59.1	14.4	0.495	—	—	—
Endocrine therapy												
Yes	46.0	17.4		36.2	16.7		59.9	13.5		—	—	
No	48.6	16.3	0.252	33.5	11.9	0.331	59.5	13.8	0.839	—	—	—
PAM50												
Luminal A[a]	52.5	14.5	—	36.9	12.0	—	60.3	11.2	—	55.7	16.1	—
Luminal B	**39.7**	**14.2**	**<0.001**	**26.8**	**11.2**	**0.008**	**53.5**	**13.9**	**0.016**	**45.1**	**16.6**	**<0.001**
HER2-enriched	**42.3**	**13.1**	**0.004**	32.0	9.5	0.662	62.2	12.1	0.918	52.3	12.9	0.808
Basal-like	**42.0**	**18.7**	**0.007**	35.4	20.4	0.992	62.2	15.8	0.987	52.6	17.2	0.776
Normal-like	57.9	17.5	0.345	**49.6**	**15.1**	**0.003**	61.4	15.9	0.957	**65.9**	**16.7**	**0.047**

NOTE: P value calculated using Student t test unless otherwise stated.

[a]Reference group, P value based on ANOVA with *post hoc* Tukey analysis. In bold: significant P value of < 0.05.

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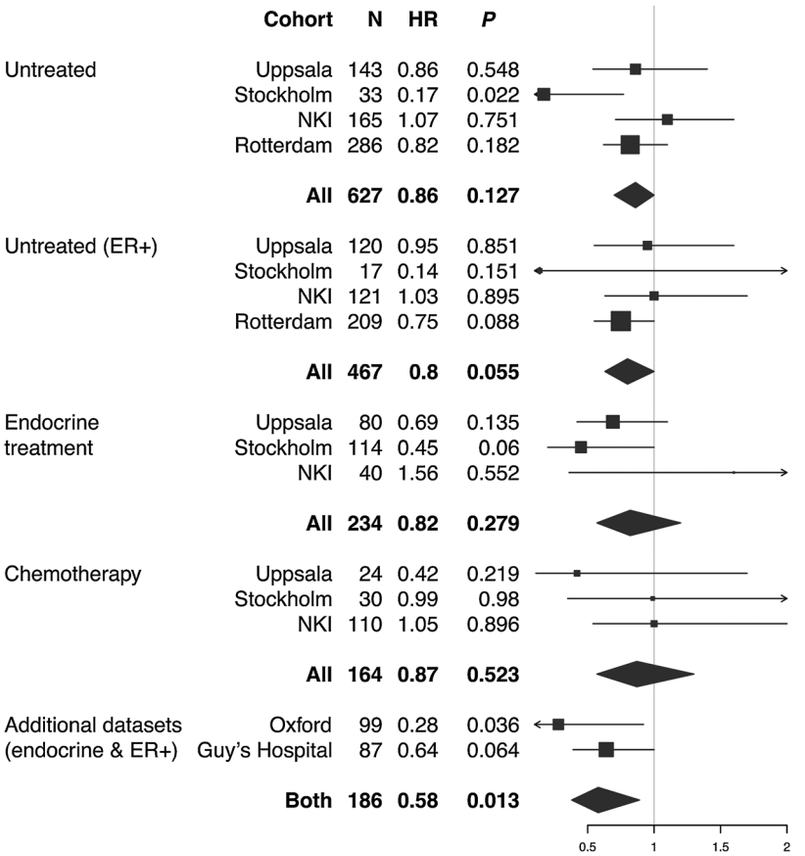

Figure 1.
Distant metastasis-free survival (DMFS), MV score, and systemic breast cancer treatment. HR (Cox proportional hazards regression) are given per 25 increments in MV score expression, for (*N*) patients in the respective stratum.

treatment," Uppsala and Stockholm, HR 0.69; 95% CI, 0.42–1.12 and 0.45; 95% CI, 0.20–1.03, respectively); however, this trend did not reach overall statistical significance (Fig. 1, see "Endocrine treatment," All, HR 0.82; 95% CI, 0.57–1.18). As the HR for the NKI dataset in endocrine-treated patients (Fig. 1, HR 1.56; 95% CI, 0.36–6.6) was in the opposite direction to that of the Uppsala and Stockholm datasets, and as 50% of the endocrine patients in the NKI datasets also received chemotherapy, we further examined the MV score in an independent dataset of 186 patients collected at the John Radcliffe and Guy's Hospitals (Oxford/Guys dataset; ref. 22). All patients had ER-positive tumors and received adjuvant tamoxifen monotherapy. Here, a higher MV score was associated with a reduced risk of DMFS in endocrine-treated patients (Fig. 1, see "Additional datasets," Both, HR 0.58; 95% CI, 0.38–0.89), consistent with the trend found in Uppsala and Stockholm endocrine-treated patients. This statistical significance

Table 3. Multivariable analysis of prognostic markers in the pooled endocrine-treated patients of the Uppsala ($N = 80$), Stockholm ($N = 114$), Oxford ($N = 99$), and Guys ($N = 87$) cohorts, $N = 380$ in total

	Histologic grade ($N = 324$[a])		Genomic grade ($N = 366$[a])	
	HR (95% CI)	***P***	**HR (95% CI)**	***P***
Age[b]	1.67 (0.76–3.70)	0.200	1.43 (0.69–2.99)	0.340
Size[c]	**2.30 (1.39–3.81)**	**0.001**	**2.09 (1.33–3.30)**	**0.002**
Nodal Status[d]	1.30 (0.81–2.07)	0.270	1.44 (0.94–2.22)	0.090
Histologic grade[e]				
G1	ref (—)	—	ref (—)	—
G2	**4.11 (1.63–10.34)**	**0.003**	—	—
G3	**3.38 (1.26–9.08)**	**0.016**	—	—
Genomic grade[f]	—		**1.62 (1.29–2.04)**	**<0.001**
MV score (continuous)	**0.70 (0.50–0.97)**	**0.030**	0.89 (0.65–1.21)	0.450

NOTE: Patient numbers in each group (total $N = 380$) Distant metastasis-free survival, HR per 25 increments in MV score.
Abbreviation: ref, reference category.
[a]Reduced number of patients, missing cases shown below.
[b]Age $\leq$ 50 years ($N = 51$) vs. Age > 50 years ($N = 329$).
[c]Size $\leq$ 20 mm ($N = 177$) vs. size > 20 mm ($N = 201$), missing ($N = 2$).
[d]Nodal status, negative ($N = 177$) vs. positive ($N = 191$), missing ($N = 12$).
[e]Histologic grade, G1 ($N = 71$) vs. G_2 ($N = 179$) vs. G3 ($N = 86$), missing ($N = 44$).
[f]Genomic grade, GG1 ($N = 224$) vs. GG3 ($N = 156$).

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remained in multivariable analysis combining the Uppsala, Stockholm, Oxford, and Guys data, when considering standard prognostic markers (Table 3, $n = 380$, see "Histologic Grade," MV score HR 0.70; 95% CI, 0.50–0.97). Taken together, these data suggest an endocrine therapy predictive capacity for the MV score.

A low MV score predicts poor outcome in patients with endocrine-treated, low genomic grade breast tumors

Although numerous gene expression signatures exist for endocrine-treated patients (5,9,31), their prognostic capacity relies on proliferation-related genes (12,32). To address a potential relationship to proliferation for the MV score, we classified the Uppsala, Stockholm, and Oxford/Guys datasets according to the GGI, a strongly proliferation-related gene signature (12,22). When histopathologic grade was replaced by genomic grade in multivariable analysis, the prognostic ability of the MV score was lost (Table 3, see "Genomic Grade," MV score HR 0.89; 95% CI, 0.65–1.21). Similarly, neither MV score nor the PAM50 subtypes were prognostic in a multivariable analysis containing both variables (Supplementary Table S3, MV score HR 0.84; 95% CI, 0.58–1.21). To further examine the reason for this loss of prognostic power, we first identified a cutoff (of 39) for the MV score in our training datasets (Uppsala and Stockholm cohorts) that would perform best for prediction of DMFS in endocrine-treated patients (Supplementary Fig. S1A, iv). Second, we used this cutoff to produce Kaplan–Meier curves for both our training and validation datasets (Oxford and Guys cohorts), split according to genomic grade (GG1 or GG3). This analysis showed that the strength of the MV score resides in the lowly proliferative genomic grade 1 group of tumors (Fig. 2A, GG1, training group, MV score high/low $n =$ 66/48; Fig. 2B, GG1 validation group, MV score high/low $n = 84/$ 26; and Fig. 2C, GG1 both groups, MV score high/low $n = 150/74$; $P = 0.31$, 0.012, and 0.007, respectively. Compare to Fig. 2D–F, GG3, training, validation, and both groups together, respectively.). In this subgroup, as in the full set of endocrine-treated tumors, the MV score had independent prognostic capacity over standard prognosticators (Supplementary Table S4, HR 0.47; 95% CI, 0.26–0.84). It is pertinent to highlight here that although the separation of GG1 curves does not reach formal statistical significance in the training dataset (Fig. 2A), there is a crossing of curves after approximately 4 years, potentially rendering the log-rank test underpowered. Moreover, this cutoff displays strong prognostic capacity in other treatment subgroups of the training dataset (Supplementary Fig. S1B, i–iii). Next, we hypothesized that a combination of both signatures (MV and GGI) would provide more prognostic information than either one alone. To test this,

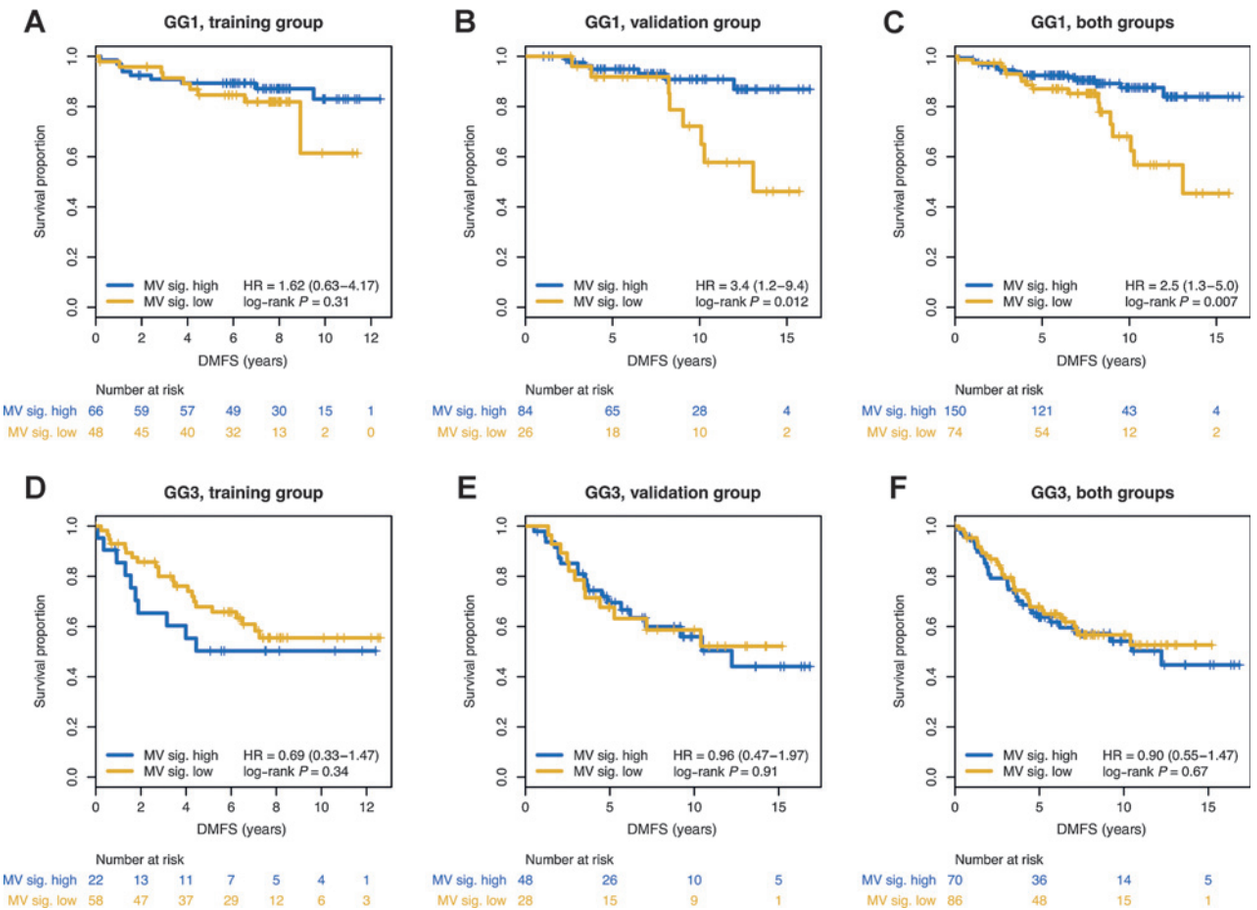

Figure 2.
Kaplan–Meier survival curves for distant metastasis-free survival (DMFS) in endocrine-treated patients ($N = 380$). Patients from the Uppsala and Stockholm data sets (A and D); the Guy's and Oxford data sets (B and E); all datasets (C and F). Patients stratified by genomic grade (low genomic grade, GG1; A–C and high genomic grade, GG3; D–F). *P* values given for a log-rank test of microvascular signature score $\geq$39 (MV score high, blue curves) versus <39 (MV score low, yellow curves).

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we calculated the c-index for the GGI, MV, and PAM50 signatures alone and in combination in all patients of our original four datasets. In line with our hypothesis, we found that, in general, the addition of the MV score to the PAM50 and GGI gene signatures may provide more prognostic information all datasets than either signature alone (Supplementary Fig. S2, compare green vs. blue bars—PAM50 vs. PAM50 + MV score and red vs. yellow bars—GGI vs. GGI + MV score, in all datasets).

To further explore the relevancy of our signature in a clinical setting, we calculated the change in MV score in a previously published cohort of 14 metastatic breast tumors (7 from the control arm and 7 from the treated arm) before and after treatment (14 days) with the angiogenic inhibitor sunitinib (23). These samples were taken as part of a substudy from a recent phase III clinical trial comparing the efficacy of sunitinib and docetaxel versus docetaxel alone in an advanced breast cancer setting (33). A heatmap displaying the intrapatient change in the expression of the MV score genes before and after treatment in both clinical trial arms is shown in Fig. 3A, where in general, a greater change in signature genes was found in the combination arm (Fig. 3A, red bar). Concomitantly, a significant increase in MV score was noted after treatment in the combination arm (Fig. 3B, right, $P = 0.031$ vs. baseline); however, low patient numbers prevent further analysis regarding survival. For the sake of completeness, we also show a table of the change in MV score (14 days, baseline) and RECIST response for all patients, no clear trend is observable (Supplementary Table S5). These results are in line with the concept of vascular normalization following treatment with an angiogenic inhibitor (34,35), but notably, are the first demonstration of this principle on a transcriptional level in human breast tumor samples.

Discussion

In this study, we found that the abundance of normal microvascular transcripts was reproducibly related to both the Luminal B breast cancer subtype and the clinical endpoint DMFS. In 993 primary breast carcinomas, a simple summary signature was expressed at lower levels in Luminal B tumors and in endocrine-treated patients, high expression of the MV score displayed a trend toward a more favorable outcome in two of three datasets and a similar finding was noted in a set of 186 patients subjected to tamoxifen monotherapy. Multivariate and subgroup analysis revealed that this association was only present in a subgroup of tumors characterized by low genomic grade. In addition, a significant increase in signature score was found in seven metastatic breast tumors after 14 days treatment with sunitinib + docetaxel, an increase that was not present in tumors treated with docetaxel alone.

Although others have also produced microvascular gene expression signatures in a breast cancer setting, these signatures have generally been designed to capture the transcriptional differences between microdissected normal versus tumor microvasculature. In short, these signatures likely represent a tumor endothelium that is increasingly thought of as angiogenically active and chronically inflamed (36). This is in contrast to our signature score that is derived from a physiologically normal microvasculature and as such is highly expressed in low-risk tumors. These differences are further emphasized when comparing the overlap of our module genes with other published angiogenesis signatures: number of genes from our module present in Bhati and colleagues signature, 1/48; in Masiero and colleagues signature 13/43; Pepin and colleagues 0/494; and Mannelqvist and colleagues 0/18 (data not shown; refs. 37–40). Of note, two recently published endothelial metagenes do display a greater degree of overlap with our signature, those being "signature 4" and "signature 5" from Winslow and colleagues (ref. 41; overlap = 4/9 and 2/3, respectively). Interestingly, these metagenes were derived through correlation analysis to a core set of genes enriched in tumor stromal compartments. Related to this, we also characterized the MV score in the context of other published signatures and gene expression modules (42) and found that that the MV score (Supplementary Fig. S3A–S3D, MV_SIG, red arrows) is strongly inversely correlated to the AURKA proliferation–related gene module when considering all patients (Supplementary Fig. S3A–S3D, AURKA, blue arrows). Of note, although we also see moderate correlations to the GGI, PLAU, and Stoma1 modules in all patients, these become weakly correlated or not statistically significant in the endocrine-treated, low GG subgroup (data not shown). A second link between our signature and proliferation was in evidence when we examined the MV score within the PAM50 molecular subgroups. Here, the MV score was consistently lower in the Luminal B tumors of all four tested datasets relative to Luminal A tumors. It has previously been demonstrated that the one of the main factors distinguishing these two tumors groups is level of proliferation with higher levels found in Luminal B tumors (43). However, given that our signature retains prognostic significance in the lowly proliferative GG1 tumor subgroup (Supplementary Table S4), it is reasonable to state that the prognostic capacity of signature extends beyond that of a simple proliferative marker.

Given the vast morphologic differences between the endothelium lining, the cardiovascular/lymphatic systems, and tumor endothelium, disparities in the quantity and type of genes expressed are to be expected. The tumor endothelium is characterized by atypical cell morphology, blood flow that can range from chaotic to nonexistent (44), and intracellular gaps that leak fluids and blood into the surrounding tissue (45). Taken together, these hallmarks of endothelial dysfunction not only influence gene expression patterns but also make pathologic assessments of microvascular density challenging. Indeed, spotty CD31 staining has been highlighted in the tumor endothelium *in vivo*, owing to a lack of expression in some cells and an absence of cells entirely in some areas of the vessel wall (46). This is likely one of the reasons as to why we found no overt similarity when comparing the MV score to microscopic assessment of MVD with CD31 staining. Staining issues notwithstanding, the value of MVD assessment as a prognostic marker has, on the whole, been called into question. In a systematic review of MVD and outcome, Uzzan and colleagues reported risk ratios in the range of 1.5 to 2, concluding that MVD has significant but weak prognostic capacity in breast cancer, and that standardization of MVD assessment is needed (47). Similar weak/negative findings were recently reported by Cheng and colleagues in a recent renal cell carcinoma meta-analysis of MVD (48).

This study had some limitations, the foremost of those being that this is a retrospective study performed in multiple patient cohorts (rather than a single, large, homogeneously-treated cohort) and that the patient numbers in the metastatic cohort are low ($N = 14$), as expected from a feasibility study. Furthermore, not all analyses were prespecified; our initial aim was to characterize the MV score in terms of its relationship to traditional MVD and to standard breast cancer clinicopathologic parameters

Published OnlineFirst January 14, 2016; DOI: 10.1158/1078-0432.CCR-15-1691

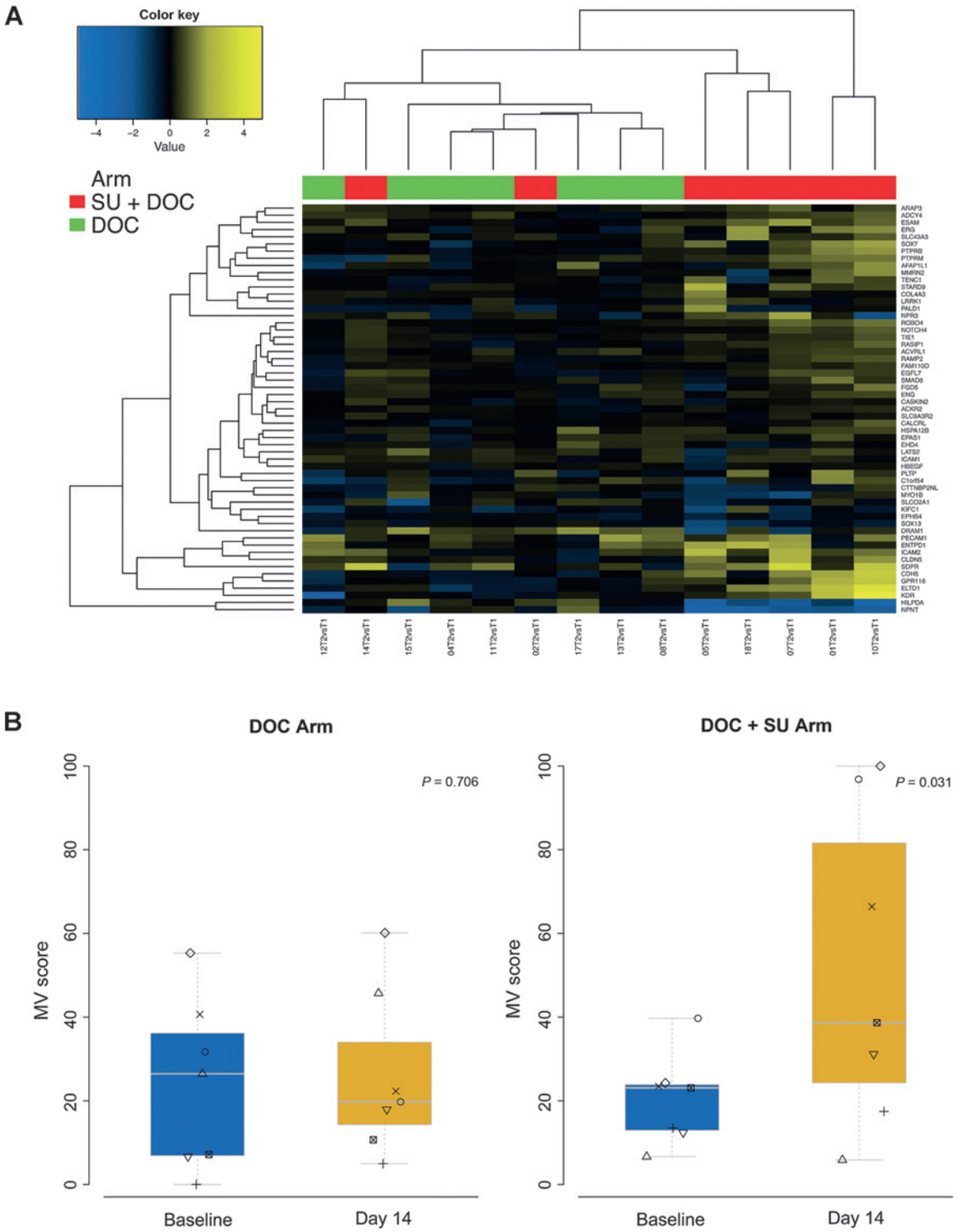

Figure 3.
Changes in MV signature score gene expression after 14 days treatment with antiangiogenic therapy ($N = 14$). The change in MV signature score gene expression before and after 14 days treatment with sunitinib plus docetaxel or docetaxel alone was determined in the metastatic breast tumors of 14 patients. A, heatmap showing the intra-patient changes in MV signature score gene expression. Columns represent the difference in signature gene expression for individual patients before and after treatment and rows represent signature genes. Red and green bars highlight different treatment arms; sunitinib plus docetaxel or docetaxel alone, respectively. B, boxplots showing MV score at baseline (before treatment) and after 14 days treatment, split by clinical trial treatment arm. Left, docetaxel alone arm ($n = 7$), $P = 0.706$ versus baseline. Right, sunitinib and docetaxel arm ($n = 7$), $P = 0.031$ versus baseline. Markers represent individual patients matched across baseline and day 14 boxplots within each treatment arm (e.g., the circle in the baseline boxplot of the docetaxel (DOC) arm is the same patient as the circle in the day 14 boxplot of the DOC arm). P values are based on Student t test.

in the context of treatment subgroups and DMFS. The subgroup analysis splitting patients into GG1 and GG3 and the examination of change in signature score before and after treatment in the metastatic cohort were exploratory in nature.

In summary, we report a MV score representative of a normal endothelium that reproducibly describes differential expression between Luminal A and B molecular subtypes, and identifies a subgroup of endocrine-treated patients with worse outcome. Moreover, we show the first evidence of normalization of tumor vasculature on a transcriptional level in response to an angiogenic inhibitor in human metastatic breast cancer samples. In light of these findings, evaluation of transcriptional changes in microvascular genes alongside assessment of MVD and angiogenic factors in clinical trials of antiangiogenic compounds appears warranted.

Disclosure of Potential Conflicts of Interest

J. Bergh reports receiving research funding, through Karolinska University Hospital or Karolinska Institutet, from Astrazeneca, Amgen, Bayer, Merck, Pfizer, Roche, and Sanofi-Aventis. T. Foukakis reports receiving a commercial research grant, Karolinska Hospital, from Roche. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Wennmalm, T. Foukakis, G. Landberg, J. Bergh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.P. Tobin, K. Wennmalm, L. Lindstrom, T. Foukakis, L. He, G. Genove, A. Ostman, G. Landberg, C. Betsholtz
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Acknowledgments

The authors thank Dr. John Lövrot, Dr. Johanna Klinge, and Julie Lorent, for essential technical assistance and discussion.

Grant Support

This work was supported by grants from the Swedish Cancer Society (to J. Bergh, C. Betsholtz, G. Genové), the Stockholm Cancer Society (to J. Bergh), BRECT (to J. Bergh), the King Gustav V jubilee fund (to J. Bergh), the ALF Foundation (to J. Bergh), the Linné Foundation (to J. Bergh), the Swedish Research Council [to J. Bergh, C. Betsholtz, LSL (grant nos.: 524-2014-4483 and 521-2014-2057)], the Swedish Research Council for Health, Working life and Welfare, FORTE [LSL (grant no.: 2014-1962)], the Märit & Hans Rausing Foundation (to J. Bergh), the Knut and Alice Wallenberg's Foundation (to J. Bergh, C. Betsholtz), and AstraZeneca (to J. Bergh).

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Received July 16, 2015; revised December 23, 2015; accepted January 2, 2016; published OnlineFirst January 14, 2016.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012: Globocan 2012. Int J Cancer 2015;136:E359–86.
2. Perez EA, Romond EH, Suman VJ, Jeong J-H, Davidson NE, Geyer CE, et al. Four-year follow-up of trastuzumab plus adjuvant chemotherapy for operable human epidermal growth factor receptor 2-positive breast cancer: joint analysis of data from NCCTG N9831 and NSABP B-31. J Clin Oncol 2011;29:3366–73.
3. Slamon D, Eiermann W, Robert N, Pienkowski T, Martin M, Press M, et al. Adjuvant trastuzumab in HER2-positive breast cancer. N Engl J Med 2011;365:1273–83.
4. Van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AAM, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415:530–6.
5. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med 2004;351:2817–26.
6. Sotiriou C, Wirapati P, Loi S, Harris A, Fox S, Smeds J, et al. Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. J Natl Cancer Inst 2006;98:262–72.
7. Parker JS, Mullins M, Cheang MCU, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol 2009;27:1160–7.
8. Paik S, Tang G, Shak S, Kim C, Baker J, Kim W, et al. Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. J Clin Oncol 2006;24:3726–34.
9. Albain KS, Barlow WE, Shak S, Hortobagyi GN, Livingston RB, Yeh I-T, et al. Prognostic and predictive value of the 21-gene recurrence score assay in postmenopausal women with node-positive, oestrogen-receptor-positive breast cancer on chemotherapy: a retrospective analysis of a randomised trial. Lancet Oncol 2010;11:55–65.
10. Prat A, Lluch A, Albanell J, Barry WT, Fan C, Chacón JI, et al. Predicting response and survival in chemotherapy-treated triple-negative breast cancer. Br J Cancer 2014;111:1532–41.
11. Sotiriou C, Pusztai L. Gene-expression signatures in breast cancer. N Engl J Med 2009;360:790–800.
12. Tobin NP, Lindström LS, Carlson JW, Bjöhle J, Bergh J, Wennmalm K. Multi-level gene expression signatures, but not binary, outperform Ki67 for the long term prognostication of breast cancer patients. Mol Oncol 2014;8:741–52.
13. Crown JP, Diéras V, Staroslawska E, Yardley DA, Bachelot T, Davidson N, et al. Phase III trial of sunitinib in combination with capecitabine versus capecitabine monotherapy for the treatment of patients with pretreated metastatic breast cancer. J Clin Oncol 2013;31:2870–8.
14. Gligorov J, Doval D, Bines J, Alba E, Cortes P, Pierga J-Y, et al. Maintenance capecitabine and bevacizumab versus bevacizumab alone after initial first-line bevacizumab and docetaxel for patients with HER2-negative metastatic breast cancer (IMELDA): a randomised, open-label, phase 3 trial. Lancet Oncol 2014;15:1351–60.
15. Von Minckwitz G, Puglisi F, Cortes J, Vrdoljak E, Marschner N, Zielinski C, et al. Bevacizumab plus chemotherapy versus chemotherapy alone as second-line treatment for patients with HER2-negative locally recurrent or metastatic breast cancer after first-line treatment with bevacizumab plus chemotherapy (TANIA): an open-label, randomised phase 3 trial. Lancet Oncol 2014;15:1269–78.
16. Wallgard E, Larsson E, He L, Hellström M, Armulik A, Nisancioglu MH, et al. Identification of a core set of 58 gene transcripts with broad and specific expression in the microvasculature. Arterioscler Thromb Vasc Biol 2008;28:1469–76.
17. Bergh J, Norberg T, Sjögren S, Lindgren A, Holmberg L. Complete sequencing of the p53 gene provides prognostic information in breast cancer patients, particularly in relation to adjuvant systemic therapy and radiotherapy. Nat Med 1995;1:1029–34.
18. Miller LD, Smeds J, George J, Vega VB, Vergara L, Ploner A, et al. An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. Proc Natl Acad Sci U S A 2005;102:13550–5.
19. Pawitan Y, Bjöhle J, Amler L, Borg A-L, Egyhazi S, Hall P, et al. Gene expression profiling spares early breast cancer patients from adjuvant therapy: derived and validated in two population-based cohorts. Breast Cancer Res 2005;7:R953.
20. Van de Vijver MJ, He YD, van 't Veer LJ, Dai H, Hart AAM, Voskuil DW, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002;347:1999–2009.

Published OnlineFirst January 14, 2016; DOI: 10.1158/1078-0432.CCR-15-1691

21. Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, Yang F, et al. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet 2005;365:671–9.
22. Loi S, Haibe-Kains B, Desmedt C, Lallemand F, Tutt AM, Gillet C, et al. Definition of clinically distinct molecular subtypes in estrogen receptor-positive breast carcinomas through genomic grade. J Clin Oncol 2007; 25:1239–46.
23. Foukakis T, Lövrot J, Sandqvist P, Xie H, Lindström LS, Giorgetti C, et al. Gene expression profiling of sequential metastatic biopsies for biomarker discovery in breast cancer. Mol Oncol 2015;9:1384–91.
24. Lu X, Lu X, Wang ZC, Iglehart JD, Zhang X, Richardson AL. Predicting features of breast cancer with gene expression patterns. Breast Cancer Res Treat 2008;108:191–201.
25. Bottini A, Berruti A, Bersiga A, Brizzi MP, Allevi G, Bolsi G, et al. Changes in microvessel density as assessed by CD34 antibodies after primary chemotherapy in human breast cancer. Clin Cancer Res 2002;8: 1816–21.
26. R Core Team (2015). R: A language and environment for statistical computing, Vienna, Austria. URL https://www.R-project.org/.
27. Sims AH, Smethurst GJ, Hey Y, Okoniewski MJ, Pepper SD, Howell A, et al. The removal of multiplicative, systematic bias allows integration of breast cancer gene expression datasets – improving meta-analysis and prediction of prognosis. BMC Med Genomics 2008;1:42.
28. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci 2001; 98:5116–21.
29. Storey JD, Tibshirani R. Statistical significance for genomewide studies. Proc Natl Acad Sci 2003;100:9440–5.
30. Győrffy B, Hatzis C, Sanft T, Hofstatter E, Aktas B, Pusztai L. Multigene prognostic tests in breast cancer: past, present, future. Breast Cancer Res 2015;17:11.
31. Desmedt C, Giobbie-Hurder A, Neven P, Paridaens R, Christiaens M-R, Smeets A, et al. The Gene expression Grade Index: a potential predictor of relapse for endocrine-treated breast cancer patients in the BIG 1–98 trial. BMC Med Genomics 2009;2:40.
32. Paik S. Is gene array testing to be considered routine now?Breast Edinb Scotl 2011;20 Suppl3:S87–S91.
33. Bergh J, Bondarenko IM, Lichinitser MR, Liljegren A, Greil R, Voytko NL, et al. First-line treatment of advanced breast cancer with sunitinib in combination with docetaxel versus docetaxel alone: results of a prospective, randomized phase III study. J Clin Oncol 2012;30:921–9.
34. Goel S, Wong AH-K, Jain RK. Vascular normalization as a therapeutic strategy for malignant and nonmalignant disease. Cold Spring Harb Perspect Med 2012;3:a006468. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3282493/.
35. Sennino B, M. McDonald D. Controlling escape from angiogenesis inhibitors. Nat Rev Cancer 2012;12:699–709.
36. Dudley AC. Tumor endothelial cells. Cold Spring Harb Perspect Med 2012;2:a006536.
37. Bhati R, Patterson C, Livasy CA, Fan C, Ketelsen D, Hu Z, et al. Molecular characterization of human breast tumor vascular cells. Am J Pathol 2008;172:1381–90.
38. Masiero M, Simões FC, Han HD, Snell C, Peterkin T, Bridges E, et al. A core human primary tumor angiogenesis signature identifies the endothelial orphan receptor ELTD1 as a key regulator of angiogenesis. Cancer Cell 2013;24:229–41.
39. Pepin F, Bertos N, Laferrière J, Sadekova S, Souleimanova M, Zhao H, et al. Gene-expression profiling of microdissected breast cancer microvasculature identifies distinct tumor vascular subtypes. Breast Cancer Res 2012;14: R120.
40. Mannelqvist M, Wik E, Stefansson IM, Akslen LA. An 18-gene signature for vascular invasion is associated with aggressive features and reduced survival in breast cancer. PLoS ONE 2014;9:e98787.
41. Winslow S, Leandersson K, Edsjö A, Larsson C. Prognostic stromal gene signatures in breast cancer. Breast Cancer Res 2015;17:23.
42. Ignatiadis M, Singhal SK, Desmedt C, Haibe-Kains B, Criscitiello C, Andre F, et al. Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. J Clin Oncol 2012;30: 1996–2004.
43. Cheang MCU, Chia SK, Voduc D, Gao D, Leung S, Snider J, et al. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst 2009;101:736–50.
44. Padera TP, Stoll BR, Tooredman JB, Capen D, di Tomaso E, Jain RK. Pathology: cancer cells compress intratumour vessels. Nature 2004;427: 695.
45. Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, et al. Openings between defective endothelial cells explain tumor vessel leakiness. Am J Pathol 2000;156:1363–80.
46. Di Tomaso E, Capen D, Haskell A, Hart J, Logie JJ, Jain RK, et al. Mosaic tumor vessels: cellular basis and ultrastructure of focal regions lacking endothelial cell markers. Cancer Res 2005;65:5740–9.
47. Uzzan B, Nicolas P, Cucherat M Perret G-Y. Microvessel density as a prognostic factor in women with breast cancer: a systematic review of the literature and meta-analysis. Cancer Res 2004;64:2941–55.
48. Cheng S-H, Liu J-M, Liu Q-Y, Luo D-Y, Liao B-H, Li H, et al. Prognostic role of microvessel density in patients with renal cell carcinoma: a meta-analysis. Int J Clin Exp Pathol 2014;7:5855–63.

Published OnlineFirst January 14, 2016; DOI: 10.1158/1078-0432.CCR-15-1691

Clinical Cancer Research

An Endothelial Gene Signature Score Predicts Poor Outcome in Patients with Endocrine-Treated, Low Genomic Grade Breast Tumors

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Clin Cancer Res 2016;22:2417-2426. Published OnlineFirst January 14, 2016.