Gene expression signatures predictive of bevacizumab/erlotinib therapeutic benefit in advanced non-squamous non-small cell lung cancer patients (SAKK 19/05 trial)

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Abstract

Purpose: We aimed to identify gene expression signatures associated with angiogenesis and hypoxia pathways with predictive value for treatment response to bevacizumab/erlotinib (BE) of non-squamous advanced NSCLC patients.

Experimental design: Whole genome gene expression profiling was performed on 42 biopsy samples (from SAKK 19/05 trial) using Affymetrix exon arrays, and associations with the following endpoints: time-to-progression (TTP) under therapy, tumor-shrinkage (TS), and overall survival (OS) were investigated. Next, we performed gene set enrichment analyses using genes associated with the angiogenic process and hypoxia response to evaluate their predictive value for patients’ outcome.

Results: Our analysis revealed that both the angiogenic and hypoxia response signatures were enriched within the genes predictive of BE response, TS and OS. Higher gene expression levels (GELs) of the 10-gene angiogenesis-associated signature and lower levels of the 10-gene hypoxia response signature predicted improved TTP under BE, 7.1 months vs. 2.1 months for low vs. high-risk patients (P = 0.005), and median TTP 6.9 months vs. 2.9 months (P = 0.016), respectively. The hypoxia
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response signature associated with higher TS at 12 weeks and improved OS (17.8 months vs. 9.9 months for low vs. high risk patients, \( P = 0.001 \)).

**Conclusions:** We were able to identify gene expression signatures derived from the angiogenesis and hypoxia response pathways with predictive value for clinical outcome in advanced non-squamous NSCLC patients. This could lead to the identification of clinically relevant biomarkers, which will allow for selecting the subset of patients who benefit from the treatment and predict drug response.

**STATEMENT OF TRANSLATIONAL RELEVANCE**

Clinical outcome of non-small cell lung cancer (NSCLC) could be improved by molecular stratification of patients. Antiangiogenic therapy is approved for treatment of advanced cancers, however because only a subset of patients treated with angiogenesis inhibitors show objective clinical response, there is an increased need for predictive biomarkers. We identified 10-gene signatures associated with angiogenesis and hypoxia-response pathways, which have predictive value for response to combined anti-VEGF/anti-EGFR in non-squamous advanced NSCLC. Subclassification of the patients using these signatures indicated that patients most likely to respond (low-risk) showed higher levels of angiogenesis associated genes mainly involved in maintaining the vascular barrier integrity and lower levels of hypoxia-response genes. Moreover, they showed increased percentile tumor shrinkage and improved overall survival. The inverse trend was observed for the high-risk patients. These findings open the possibility for clinical use of these signatures as predictive biomarkers for identifying patients who would benefit from antiangiogenic therapies.

**Introduction**

Solid tumor cells in their primary goal to avoid senescence and achieve uncontrolled proliferation co-opt neighboring stromal cells to assist them with the expansion of the malignant tissue.\(^1\), \(^2\) These tumor-associated stromal components have been demonstrated to have an important role in tumor growth, disease progression\(^3\) as well as in response and resistance to therapeutic agents.\(^4\), \(^5\) Moreover, tumors are strongly dependent on angiogenesis, the formation of neovessels from the preexisting vasculature, to obtain the nutrients and oxygen essential for their rapid growth.\(^6\) Consequently, antiangiogenic therapeutic strategies were extensively exploited in the last decade in search for more efficient cancer treatments.\(^7\)
Though multiple therapeutic strategies have been developed against vascular endothelial growth factor (VEGF), bevacizumab, a monoclonal antibody targeting VEGFA, is the first approved antiangiogenic drug for clinical use. Bevacizumab is mostly used in combination with standard chemotherapy for treatment of metastatic cancers and as single agent in recurrent glioblastoma. However, survival benefits are observed only in a subset of patients, as a significant number of patients show only modest response presumably because of intrinsic or rapidly acquired resistance to antiangiogenic therapy. One proposed mechanism of resistance to antiangiogenic agents is the onset of hypoxia within the tumor as a result of vessel regression during the course of antiangiogenic therapy. Moreover, effective inhibition of neovascularization using antiangiogenic therapy was shown in some cases to change the phenotype of tumors by increasing their invasion and metastatic potential. Additionally, significant rates of adverse effects were reported for patients receiving anti-VEGF therapy, as well as a mortality rate of 1%, which was a direct consequence of bevacizumab administration. Thus, understanding all cascades of vascular signaling involved in the response to antiangiogenic therapy and subsequent resistance is critical to achieve full potential of this therapeutic approach.

Despite these obvious limitations of antiangiogenic therapy, because a subset of cancer patients treated with angiogenesis inhibitors show objective clinical response, there is an increased need to identify robust predictive biomarkers, which could allow for selecting the subgroup of patients who would benefit from the treatment.

In a recent study, aimed at identifying novel biomarkers for response to combined anti-VEGFA/anti-EGFR (epidermal growth factor receptor) therapy in non-squamous NSCLC by exploring gene expression at exon-level, we identified EGFR exon 18 as a predictive marker.
for patients with metastatic non-squamous NSCLC who have received no previous therapy.\textsuperscript{21} The gene expression profiles obtained from this set of microarray data are derived, however, from highly heterogeneous clinical biopsies consisting of both tumor and activated stromal cells. In a previous study, Baty et al. revealed that prediction of survival was independent of tumor cell content present in each NSCLC biopsy.\textsuperscript{22} This suggests a strong predictive contribution from the tumor microenvironment compartments in NSCLC. Additionally, recent studies have demonstrated that tumor microenvironment can provide independent and reliable predictors of clinical outcome.\textsuperscript{23} A key constituent of the tumor microenvironment is the blood vasculature, which undergoes angiogenesis to sustain the high proliferative rate of the tumor, and is the direct target of antiangiogenic therapies. Because there is a high degree of cross-talk between epidermal growth factor receptor EGF(R) and VEGF(R) pathways, they have been identified as potentially synergistic for dual targeting.\textsuperscript{24} EGFR pathway is involved in growth factor–induced angiogenesis, transcriptionally up-regulating VEGF expression.\textsuperscript{25} Additionally, multiple studies demonstrated that hypoxia can trigger the angiogenic switch in solid tumors.\textsuperscript{26} Therefore, we aimed to investigate whether angiogenesis and hypoxia-associated gene expression signatures could predict the combined anti-VEGF/anti-EGFR treatment response in advanced non-squamous therapy naïve NSCLC patients unselected for EGFR mutation status. Our analysis identified 10-gene angiogenesis-associated and hypoxia-response signatures predictive of therapeutic response to bevacizumab/erlotinib (BE) having time-to-progression (TTP) and tumor shrinkage (TS) as endpoints. We also identified for 10-gene signatures with prognostic value. These signatures hold great potential for clinical application allowing for identification of biomarkers, which can identify the patients most likely/ less likely to respond to targeted therapy.
Methods

**Study design.** Our study is based on clinical bronchoscopic biopsies available from 42 patients for which genome-wide gene expression was studied in a microarray platform. These patients (88% adenocarcinoma, 57% female, 31% never smoker) were enrolled in the Swiss Group for Clinical Cancer Research (SAKK) 19/05 phase II trial. Identification of predictive gene-expression signatures from RNA gene expression analysis was a predefined goal of this trial. The detailed clinical information of these patients was published previously. For these patients with stage IIIB or IV (93%) non-squamous NSCLC, BE was used as first-line therapy (independent of the EGFR mutation status) followed by standard platinum-based/gemcitabine chemotherapy (CT) after disease progression, (Figure 1). Time to disease progression and percentage tumor shrinkage at 12 weeks (assessed by CT scans) were defined according to RECIST criteria. To confirm that there was no sample selection bias for the 42 patients included in our study, we performed statistical analysis and found no significant differences between the study group and the patients with no available biopsies. The results are summarized in Supplementary Table 1.

**Gene expression analysis**

Total RNA from 42 bronchoscopic biopsy samples were extracted using miRNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. Affymetrix Human Exon 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) were used for mRNA hybridization. The gene level probesets were preprocessed, quality-checked and normalized using the Robust Multi-array Average (RMA) procedure. Data were expressed as log2 ratio of fluorescence intensities of
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the sample and the reference, for each element of the array. Additional experimental details for this section were previously reported by us. 21

Statistical analysis of gene expression.

Survival and time-to-event analysis were performed by applying univariate Cox proportional hazards regression and principal component analysis (metagene approach) according to a previously described method. 29 We built a binary score (low/high risk) using the median of the metagene scores. The classification accuracy of our algorithm was assessed by leave-one-out cross-validation (LOOCV). Time-to-event and survival results were displayed using Kaplan-Meier curves, and log-rank tests are reported. Hierarchical cluster analysis was carried out using the Euclidean distance together with the complete linkage agglomerative method. The median follow up time was estimated using the reverse Kaplan-Meier method. All statistical tests were performed using the R statistical software version 3.1.0 (http://www.R-project.org). A P value of 0.05 was set as threshold for significance for all study outcomes.

Gene set enrichment analysis (GSEA). We performed enrichment analysis using GSEA v2.1.0 software (http://www.broad.mit.edu/gsea) and GSEA-preranked function. 30 The gene lists were ranked by using as metric –log10(P) resulted from the Cox-regression analysis for all endpoints.

Quantitative real-time PCR analysis. Forty samples of RNA isolated from non-squamous NSCLC biopsies and previously analyzed by microarrays were available for reverse transcription (RT). RT was performed using QuanTitect® Reverse Transcription Kit from
Qiagen starting from 10 ng RNA for each qPCR reaction. The quantity and integrity of RNAs were assessed using an UVS-99 micro-volume spectrophotometer (ACTGene). For this study we investigated the expression levels of nine angiogenesis-associated predictive genes. For RT-qPCR experiments we used predesigned optimized primer sets (annealing temperature 55 °C) for GPR116, EMCN, ITGA9, GNG11, KDR, PECAM1, S1PR1, JAM2 and RHOJ (QuantiTect Primer Assay, Qiagen). For sequences please refer to the Supplementary Table 2.

All samples were processed in duplicate for the qPCR with the LightCycler®480 SYBR Green I Master in a 20 µL reaction volume containing 4 µL water, 1 µL PCR primer, 10 µL Master mix and 5 µL cDNA (10 ng). Quantitative real-time PCR experiments were performed on a LightCycler® 480 II instrument using the initial denaturation at 95 °C for 10 minutes followed by 45 cycles: 95 °C for 10 sec, 55 °C for 20 sec, 72 °C for 20 sec. Controls containing no reverse transcriptase were included for each sample. The mRNA expression levels were calculated relative to HPRT1 house-keeping gene and the relative quantification of the gene expression was performed using $2^{-\Delta\Delta C_p}$ method. The correlation between the RT-qPCR gene expression levels (GELs) and microarray GELs was measured by means of Spearman’s correlation coefficient.

**Results**

10-gene angiogenesis-associated and hypoxia-response signatures predict response to BE therapy. We hypothesized that the genes which could associate with the response to BE therapy are genes involved in angiogenic signaling pathways. To investigate this, we performed GSEA using a core gene signature (43 genes) specific for angiogenesis transcriptional program predefined by integrative meta-analysis of the expression profiles of over 1,000 primary human cancers.
GSEA revealed a significant enrichment of the angiogenesis-associated genes within the genes that associate with TTP under BE therapy endpoint (mean rank = 4246, P=0.004, Figure 2A). As VEGF expression is directly activated under hypoxic conditions by the transcription factor hypoxia inducible factor 1 alpha (HIF1α), we decided to additionally investigate hypoxia-response genes by GSEA. For this analysis we used a previously derived common hypoxia metagene (51 genes) across cancer types.33 Within the genes which associate with TTP under BE therapy, we identified by GSEA a significant enrichment of the hypoxia-response genes (mean rank = 4798, P = 0.001, Figure 2B). The top 12-ranked angiogenesis-associated and hypoxia-response genes, which significantly correlate with TTP under BE therapy are given in Figure 2C and 2D.

We then applied unsupervised hierarchical clustering to the top 10-ranked angiogenesis-associated genes to investigate whether there are gene expression patterns correlating with the BE treatment response. The clustering output for the angiogenesis-associated signature is displayed in Figure 3A. We identified three distinct gene clusters: Cluster A1 (low risk, 9 patients, 21 %) was characterized by an increased expression of the angiogenesis-associated signature and associated with improved TTP under BE 7.1 months, 95% confidence interval (95% CI): 4.0 – ∞; Cluster A2 (high risk, 12 patients, 29 %) was characterized by a decreased expression of the angiogenesis-associated gene signature and associated with reduced response to BE treatment with a mean TTP under BE of 2.1 months (95% CI: 1.4 – ∞). The patients within the third cluster A3 (medium risk, 21 patients, 50 %) showed intermediate angiogenesis-associated GELs and a median TTP under BE of 4.1 months (95% CI: 3.1 – 7). The Kaplan-Meier TTP curves are shown in Figure 3C. GELs for the top 10-ranked angiogenesis-associated genes for patients included in each cluster are given in Supplementary Figure 1.
Hierarchical clustering showing the variability of gene expression of hypoxia-response top 10-ranked genes is given in Figure 3B. For this signature, we identified two significant clusters: Cluster **H1** (18 patients, 43%) with lower hypoxia-response GELs and Cluster **H2** (24 patients, 57%) with hypoxia-response higher GELs (Supplementary Figure 2).

Dichotomization of the patients into **low-risk** (Cluster **H1**) and **high-risk** (Cluster **H2**), subgroups based on the gene expression levels of the hypoxia-response signature revealed a marked difference in TTP under BE between the two groups (Figure 3D). We obtained a median TTP for the **high-risk** patients of 2.9 months (95% CI: 1.8 – 4.1) and of 6.9 months (95% CI: 4.0 – 9.7) for the **low-risk** patients.

**Hypoxia-response gene signature predictive of tumor shrinkage after BE treatment in non-squamous NSCLC patients.**

An additional secondary endpoint of interest was tumor shrinkage (TS) measured at 12 weeks after BE treatment, which indicates clinically relevant direct anti-tumor activity. Because of lack of measurements at 12 weeks, 14 patients had to be excluded from this analysis. We analyzed the genes correlating with TS in our patients using GSEA using both the angiogenesis-associated and hypoxia-response gene signatures (Figure 4A and B). Both gene signatures where significantly enriched in the gene set correlating with TS (mean rank = 5104, $P = 0.002$ and mean rank = 7795, $P = 0.038$, respectively). The resulting top 12-ranked genes for both signatures are given in Figure 4C and D. Further, we classified the patients based on the metagene score calculated for the top 10-ranked genes for each gene signature. For the angiogenesis-associated signature, the resulting two patients groups (**low-risk** and **high-risk**) showed a non-significantly different median TS, 13.7 % interquartile range (IRQ): -0.8 – 26.2 vs. 0 %, IQR: -3.2 – 16.4, $P = 0.755$. In contrast, when dichotomization was
performed using the 10-gene hypoxia-response signature, the low-risk patients had a significantly higher median TS than the high-risk patients (16.1%, IRQ: 0 – 26.2 vs. -0.4%, IRQ: -2.5 – 2.6, P = 0.013), indicating a higher potential for assessing treatment response using this signature. A heat map showing the gene expression levels of the top 10-ranked hypoxia response genes predictive of tumor shrinkage is given in Supplementary Figure 3.

Angiogenesis-associated and hypoxia-response gene signatures have prognostic value for non-squamous NSCLC patients. Lastly, we analyzed the prognostic value of both angiogenesis and hypoxia gene signatures by investigating the genes correlating with the overall survival (OS). The median follow-up time was 24.9 months (95% CI, 23.9 – ∞). The results of the GSEA for OS are given in Figure 5A and 5B, and the derived top 12-ranked genes are given in Figure 5C and 5D, respectively.

Both gene signatures where significantly enriched in the gene set correlating with OS (mean rank = 5064, P = 0.031 and mean rank = 4555, P = 0.001, respectively). Using the top 10-ranked genes for both signatures to dichotomize the patients in low-risk (longer OS) and high-risk (shorter OS) revealed marked differences in OS. Figures 5E and 5F show the Kaplan-Meier OS curves for both gene signatures used. Using the angiogenesis-associated gene signature led to a median OS for the high-risk patients of 10.6 months [95% CI, 5.2 – 19.4] and for the low-risk patients of 14.1 months [95% CI, 10.5 – ∞], P = 0.035. The hypoxia-response gene signature showed a higher prognostic value (P = 0.001) resulting in a median OS for the high-risk patients of 9.9 months [95% CI 4.8 – 13.4] and 17.8 months [95% CI 16.6 – ∞] for the low-risk patients. A heat map displaying the gene expression levels of the top
10-ranked hypoxia response genes significantly associated with OS is given in Supplementary Figure 4.

**Validation of microarray gene expression levels (GELs) by RT-qPCR.** RT-qPCR was used to assess the GELs that were established by microarray analysis. RT-qPCR was performed on 40 out of 42 RNA samples and for nine genes, plus three control genes. The Spearman’s rank correlation coefficients (r) were between 0.51–0.71. The correlation was significant for each gene. We obtained P values for these associations of < 0.0008, demonstrating good agreement between the two complementary methods in quantifying the gene expression levels (Figure 6). This outcome indicates a statistically significant correlation between the microarray GELs and GELs assessed by RT-qPCR, which is a more convenient and less expensive technique for routine application in a clinical setting. These results are in agreement with the expected level of correlation considering the fact that there is no designed sequence overlap between the qPCR primers and the microarray probes.

**Discussion**

There are several gene expression signatures identified as prognostic biomarkers for lung cancer, mostly for lung adenocarcinoma. However, gene expression signatures with predictive value for treatment response to antiangiogenic therapy are still lacking. Therefore, the aim of this study was to evaluate the predictive potential of angiogenesis-associated and hypoxia-response gene signatures for the benefit of BE treatment. We performed GSEA for each endpoint, which led to the identification of specific angiogenesis and hypoxia–derived 10-gene signatures. These signatures were then evaluated for their predictive (TTP und TS endpoints) and prognostic value (OS) and tested in an independent
data set. Our findings might shed light on the mechanism of antiangiogenic treatment response and resistance. Moreover, they may lead to the identification of the causal mechanism behind the high proportion of patients treated with angiogenesis inhibitors showing partial response, as demonstrated by increased progression free survival (PFS), however, with no improvement in their OS.

We took a closer look at the angiogenesis-associated genes with predictive value for TTP under BE therapy. The expression of the first ranked gene, adhesion G-protein-coupled receptor 116 (GPR116), was shown to be significantly correlated with tumor progression, recurrence, and poor prognosis in human breast cancer. However, here we identify this gene to play a protective role and its expression to be associated with lower risk of disease recurrence in NSCLC. The biological role of GPR116 in angiogenesis and endothelial proliferation remains to be assessed, however this protein is highly expressed in normal human lung tissue and it was recently demonstrated to regulate lung surfactant homeostasis. The second ranked gene, endomucin (EMCN), is an endothelial sialomucin and an endothelial-specific marker, involved in cell-cell and cell-extracellular matrix interactions. Integrin α9 (ITGA9), which forms a heterodimeric receptor with activated β1 integrin, has been demonstrated to bind directly to VEGFA, and to contribute to angiogenesis. α9 β1 integrin ligand, tenascin-C, enhances secretion of S1P (sphingosine 1-phosphate) in endothelial cells. In turn, S1P promotes endothelial cell barrier integrity, acting as an anti-permeability agent and modulating vessel integrity through its cognate receptor S1PR1. S1PR1 inhibits VEGFR2 signaling and suppresses endothelial hypersprouting via stabilization of junctional VE-cadherin, which leads to enhanced cell-cell adhesion. PECAM-1 and JAM2 are adhesive proteins that accumulate in adherens junctions and maintain the restrictiveness of the endothelial barrier. RhoJ, an endothelial-enriched Rho
GTPase, regulates angiogenesis and vessel integrity.\textsuperscript{41} GNG11 is a member of the γ subunit family of heteromeric G-protein, which regulates cellular senescence in response to environmental stimuli.\textsuperscript{42} To obtain the ranking of the discriminating power for each gene, we performed optimized between-group classification (OBC)\textsuperscript{43} sensitivity analysis for this signature. OBC suggests that for angiogenesis-associated signature \textit{S1PR1}, \textit{GPR116}, and \textit{PECAM1} have the highest discriminating power for TTP under BE (Supplementary Figure 5a).

The hypoxia-response genes with predictive value for TTP under BE were mostly genes involved in the glycolytic and other metabolic pathways\textsuperscript{44}: PFKP (phosphofructokinase, platelet), LDHA (lactate dehydrogenase A), GPI (glucose-6-phosphate isomerase), ALDOA (aldolase A), ACOT7 (acyl-CoA thioesterase 7), PGK1 (phosphoglycerate kinase), SLC25A32 (solute carrier family 25, mitochondrial folate carrier, member 32) and SLC2A1 (solute carrier family 2, glucose transporter, member 1). The first-ranked hypoxia-response gene DDIT4 (DNA-damage-inducible transcript 4 protein, also known as REDD1) has been shown to be implicated in inhibition of the mTORC1 (mammalian target of rapamycin kinase) signaling pathway, which is relevant in tumor suppression;\textsuperscript{45} MIF (macrophage migration inhibitory factor) has been revealed to act within the tumor microenvironment to stimulate angiogenesis and promote immune evasion;\textsuperscript{46} ADM (adrenomedullin) is involved in promoting tumor progression by sustaining proliferation and angiogenesis.\textsuperscript{47} For this signature, OBC indicated that DDIT4 and MIF have the highest discriminatory power (Supplementary Figure 5b). Importantly, the hypoxia-response signature shows both high predictive and prognostic value and could potentially guide future clinical decisions.

Our gene expression analyses suggest that the low-risk patients (most likely to respond to antiangiogenic combined BE therapy and have better outcome) have a tumor
microenvironment that sustains controlled angiogenesis, developing blood vessels with increased levels of integrity and reduced permeability. The controlled angiogenesis is associated with lower risk of hypoxia within the tumors (lower levels of hypoxia response genes). On the other hand, the high-risk patients (less likely to respond to BE treatment and have worse outcome) have a tumor microenvironment that sustains aberrant angiogenesis with reduced vascular stability and increased vascular permeability. This phenotype is further associated with increased tumor hypoxia and an earlier onset of disease progression. Moreover, we observed that the 10-gene hypoxia-response signature has a higher prognostic power than the angiogenesis-associated signature. This most likely indicates that CT has a relatively high contribution to OS, which is expected taking into account that hypoxic tumors are less responsive to CT than normoxic tumors.

Additionally, we performed LOOCV from the original dataset in order to test the robustness of the gene signatures associated with TTP under BE and OS endpoints. The perturbations resulting from LOOCV had an insignificant impact on our findings demonstrating the robustness of the discriminatory power of our gene signatures (Supplementary Table 3). Unfortunately, we could not investigate the performance of our gene expression signatures using an identical independent data set, as an additional gene expression data set from pretreatment biopsies of treatment-naïve NSCLC patients receiving the same therapeutic scheme is not available for validation. However, we tested our 10-gene signatures correlating with TTP under BE using a data set comprising GELs of biopsies from a pretreated NSCLC population (BATTLE-1 study, trial registration ID: NCT00409968, raw data GEO series accession number: GSE33072). We analyzed the association between our 10-gene expression signatures and PFS for two treatment arms: erlotinib (25 patients, all EGFR-WT) and sorafenib (31 patients, all EGFR-WT; the patients with squamous cell or adenosquamous
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carcinoma were excluded). This analysis revealed that our 10 gene angiogenesis-associated signature had no predictive value for either erlotinib or sorafenib response as second line therapy. Likewise, the 10-gene hypoxia-response signature had no predictive value for erlotinib response. However, for the second line sorafenib-treated patient group (majority erlotinib resistant), we found that the 10-gene hypoxia-response signature discriminated between low-risk (responders) and high-risk patients (non-responders): PFS 3.65 months (95%CI: 1.87 – 8.74) and 2.61 months (95%CI: 1.81 – 3.61), respectively (P = 0.0186, Supplementary Figure 6). Sorafenib, a multikinase inhibitor, achieves antiangiogenic effects by blocking VEGFR and PDGFR. In addition, several studies suggest that sorafenib exerts a negative regulatory effect on angiogenesis by suppressing expression of VEGF via inhibition of HIF-1α accumulation and activation.⁵⁰ Although caution in extrapolating data from one clinical trial to the other is required, our findings suggest that the hypoxia-response signature may be a predictive biomarker of anti-VEGF(R) treatment response (such as bevacizumab and sorafenib) and has no predictive power for erlotinib activity. The lack of predictive value of the 10-gene angiogenesis in the context of second line sorafenib treatment could indicate that the expression of the angiogenesis-associated genes significantly changes from treatment naïve tumors to CT resistant tumors, whereas hypoxia-response GELs are affected to a lesser extent. Importantly, only 18 patients in sorafenib-treated group had biopsies originating from the lung; the gene expression signatures of tumor-associated vascular endothelial cells originating from other organs could vary greatly. Nonetheless, our findings suggest that the 10-gene hypoxia-response has a high predictive value of treatment response even in the context of second line antiangiogenic therapy and a great potential for future clinical use.
The positive correlation between pretreatment angiogenesis-associated and hypoxia-response GELs from tumor biopsies and clinical outcomes following BE treatment derived from our analyses supports further evaluation of these candidate gene signatures as potential biomarkers for the selection of the patient subpopulation most likely to obtain benefit from antiangiogenic therapy. There are, however, several limitations that accompany our study. Our study comprises a relatively low number of patients, and control groups (no treatment, and bevacizumab-only and erlotinib-only treatment) are absent. Further validation with larger number of patients and adequate control arms is needed. Nevertheless, we found highly statistically significant differences in the hypoxia-response GELs of responders vs. non-responders to antiangiogenic therapy in both our data set and an additional independent data set. This is very promising and suggests that the identified signatures may be clinically useful for further stratifying non-squamous NSCLC patients and allow for personalized treatment to avoid unnecessary costs and patient exposure to toxicity.

Conclusions

We identified 10-gene angiogenesis and hypoxia signatures, which can predict the subgroup of patients with higher likelihood of responding to angiogenic therapy. These patients had higher GELs of the genes mainly involved in maintaining the vascular barrier integrity and lower levels of hypoxia-response genes. Moreover, these patients showed improved OS and 75% of them experienced a high tumor shrinkage level (between 16 - 76% TS) at 12 weeks after the beginning of treatment. Although there is a very important implication to patient selection for antiangiogenic therapy, the results of this study are preliminary and need to be further validated.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Franzini, M. H. Brutsche

Development of methodology: A. Franzini, M. H. Brutsche

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Franzini, I. I. Macovei, C. Droege, D. Betticher, F. Zappa

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Franzini, F. Baty, I. I. Macovei, O. Dürr, D. Klingbiel, M. H. Brutsche

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Supplementary Information

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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References

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IIIB/IV) followed by platinum-based chemotherapy (CT) at disease progression: a multicenter phase II trial (SAKK 19/05). Lung Cancer 2012;78:239-44.

Figure legends:

Figure 1: Treatment course for patients with no prior therapy included in the SAKK 19/05 phase II trial, study endpoints and microenvironment compartments analyzed by GSEA; TTP – time to progression, TS – tumor shrinkage, OS – overall survival.

Figure 2: Enrichment plot from GSEA shows statistically significant enrichment of the angiogenesis-associated genes (P=0.004) (A) and in the hypoxia-response genes (P=0.001) (B) in the gene set predictive of TTP under BE. Top 12-ranked angiogenesis-associated genes (C) and hypoxia-response genes (D) derived from GSEA.

Figure 3: Hierarchical clustering of top 10-ranked angiogenesis-associated genes (A) and hypoxia-response genes (B) significantly associated with TTP under BE showing gene expression variation among patients. The heat maps represent relative intensity values of gene expression levels. Kaplan–Meier TTP under BE curves of the three patients groups (low-risk, medium-risk and high-risk) defined by the 10-gene angiogenesis-associated signature, P = 0.013 for Cluster A1 vs. Cluster A2, (C) and two patient groups (low-risk and high-risk) defined by the 10-gene hypoxia-response signature, P = 0.016 (D). The P values of these associations were determined by log-rank test.

Figure 4: Angiogenesis and hypoxia-associated gene signatures associate with tumor shrinkage in NSCLC. Enrichment plot from GSEA shows statistically significant enrichment of the angiogenesis-associated genes (P=0.002) (A) and in the hypoxia-response genes (P=0.038) (B) in the gene signature correlating with TS. Top 12-ranked angiogenesis-associated genes (C) and hypoxia-response (D) derived from GSEA, which correlate with TS. Correlation between the two patients groups defined by the 10-gene angiogenesis-associated signature (E) and hypoxia-response signature (F) (high-risk and low-risk; n = 28) and tumor shrinkage at 12 weeks after BE treatment (box plots). The boxes represent the median ± interquartile range (IQR). Whiskers delimit the highest and lowest non-outlier data points (defined as greater/less than 1.5 × IQR).

Figure 5: Angiogenesis and hypoxia-associated gene signatures predicting OS in NSCLC. Enrichment plot from GSEA shows statistically significant enrichment of the angiogenesis-associated genes (P = 0.031), (A) and hypoxia-response genes (P = 0.001) (B) in the gene signature predictive of OS. Top 12-ranked angiogenesis-associated genes (C) and top 12-
ranked hypoxia-response genes (D) derived from GSEA, which significantly correlate with OS. Kaplan–Meier OS curves of the two patients groups (low-risk and high-risk) defined by the 10-gene angiogenesis-associated signature (P = 0.035) (E) and 10-gene hypoxia signature (P = 0.001) (F). The P values of the associations were determined by log-rank test.

**Figure 6:** Comparison between microarray and RT-qPCR GELs. Spearman's correlations were performed between relative gene expression levels (GELs) determined by RT-qPCR (x-axis) and RNA microarray (y-axis) for nine angiogenesis-associated genes for 40 out of the 42 patients. The mRNA levels for each gene of interest were determined by RT qPCR and correlated with microarray expression scores determined after data processing, both calculated relative to HPRT1 gene. r, Spearman’s rank correlation coefficient.
Biopsies from patients with stage IIIB or IV non-squamous NSCLC with no prior therapy.

Endpoints: TS, TTP under BE, OS

Compartments: Angiogenesis, Hypoxia

Bevacizumab/Erlotinib (BE) therapy for n × 3 weeks until progression.

Standard chemotherapy (CT) for 6 × 6 weeks or until progression.

12 weeks
Figure 2

Angiogenesis

Enrichment score (ES)

0 5000 10000 15000

Rank in Ordered Dataset

Ranked list metric

Hypoxia

Enrichment score (ES)

0 5000 10000 15000

Rank in Ordered Dataset

Ranked list metric

Table 1: Gene symbols, rank in gene list, rank metric score, and enrichment score (ES)

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Rank in gene list</th>
<th>Rank metric score</th>
<th>Enrichment score (ES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPR116</td>
<td>403</td>
<td>1.924</td>
<td>0.027</td>
</tr>
<tr>
<td>EMCN</td>
<td>611</td>
<td>1.700</td>
<td>0.060</td>
</tr>
<tr>
<td>ITGA9</td>
<td>648</td>
<td>1.667</td>
<td>0.103</td>
</tr>
<tr>
<td>LDB2</td>
<td>707</td>
<td>1.629</td>
<td>0.143</td>
</tr>
<tr>
<td>MEF2C</td>
<td>805</td>
<td>1.561</td>
<td>0.179</td>
</tr>
<tr>
<td>GNG11</td>
<td>829</td>
<td>1.546</td>
<td>0.219</td>
</tr>
<tr>
<td>CALCR1</td>
<td>865</td>
<td>1.522</td>
<td>0.257</td>
</tr>
<tr>
<td>KDR</td>
<td>987</td>
<td>1.455</td>
<td>0.289</td>
</tr>
<tr>
<td>PECAM1</td>
<td>1019</td>
<td>1.435</td>
<td>0.326</td>
</tr>
<tr>
<td>S1PR1</td>
<td>1117</td>
<td>1.391</td>
<td>0.357</td>
</tr>
<tr>
<td>JAM2</td>
<td>1288</td>
<td>1.313</td>
<td>0.382</td>
</tr>
<tr>
<td>RH0J</td>
<td>1364</td>
<td>1.289</td>
<td>0.412</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Rank in gene list</th>
<th>Rank metric score</th>
<th>Enrichment score (ES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDIT4</td>
<td>18</td>
<td>3.539</td>
<td>0.082</td>
</tr>
<tr>
<td>MIF</td>
<td>33</td>
<td>3.276</td>
<td>0.157</td>
</tr>
<tr>
<td>PFKP</td>
<td>53</td>
<td>2.991</td>
<td>0.226</td>
</tr>
<tr>
<td>ADM</td>
<td>69</td>
<td>2.835</td>
<td>0.291</td>
</tr>
<tr>
<td>LDHA</td>
<td>132</td>
<td>2.513</td>
<td>0.346</td>
</tr>
<tr>
<td>GPI</td>
<td>137</td>
<td>2.494</td>
<td>0.404</td>
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<tr>
<td>ALDOA</td>
<td>238</td>
<td>2.211</td>
<td>0.450</td>
</tr>
<tr>
<td>ACOT7</td>
<td>373</td>
<td>1.975</td>
<td>0.488</td>
</tr>
<tr>
<td>SLC25A32</td>
<td>976</td>
<td>1.460</td>
<td>0.487</td>
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<tr>
<td>PGK1</td>
<td>1110</td>
<td>1.394</td>
<td>0.512</td>
</tr>
<tr>
<td>TUBA1B</td>
<td>1238</td>
<td>1.331</td>
<td>0.535</td>
</tr>
<tr>
<td>SLC2A1</td>
<td>1420</td>
<td>1.268</td>
<td>0.554</td>
</tr>
</tbody>
</table>
Figure 4

A. Angiogenesis

B. Hypoxia

C. Table for Gene Symbols and Scores

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Rank in gene list</th>
<th>Rank metric score</th>
<th>Enrichment score (ES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDB2</td>
<td>622</td>
<td>1.818</td>
<td>0.010</td>
</tr>
<tr>
<td>VWF</td>
<td>664</td>
<td>1.785</td>
<td>0.053</td>
</tr>
<tr>
<td>MYCT1</td>
<td>669</td>
<td>1.784</td>
<td>0.098</td>
</tr>
<tr>
<td>TEK</td>
<td>700</td>
<td>1.763</td>
<td>0.141</td>
</tr>
<tr>
<td>EMCN</td>
<td>734</td>
<td>1.745</td>
<td>0.183</td>
</tr>
<tr>
<td>CDH5</td>
<td>749</td>
<td>1.735</td>
<td>0.227</td>
</tr>
<tr>
<td>TIE1</td>
<td>788</td>
<td>1.716</td>
<td>0.268</td>
</tr>
<tr>
<td>JAM2</td>
<td>1072</td>
<td>1.559</td>
<td>0.291</td>
</tr>
<tr>
<td>ZNF423</td>
<td>1139</td>
<td>1.533</td>
<td>0.326</td>
</tr>
<tr>
<td>ROBO4</td>
<td>1197</td>
<td>1.506</td>
<td>0.361</td>
</tr>
<tr>
<td>ADCY4</td>
<td>1398</td>
<td>1.425</td>
<td>0.386</td>
</tr>
<tr>
<td>PTPRB</td>
<td>1690</td>
<td>1.328</td>
<td>0.402</td>
</tr>
</tbody>
</table>

D. Table for Gene Symbols and Scores

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Rank in gene list</th>
<th>Rank metric score</th>
<th>Enrichment score (ES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRPL15</td>
<td>61</td>
<td>2.960</td>
<td>0.090</td>
</tr>
<tr>
<td>PSMA7</td>
<td>82</td>
<td>2.836</td>
<td>0.179</td>
</tr>
<tr>
<td>ANKRD37</td>
<td>170</td>
<td>2.465</td>
<td>0.252</td>
</tr>
<tr>
<td>MIF</td>
<td>228</td>
<td>2.285</td>
<td>0.321</td>
</tr>
<tr>
<td>LDHA</td>
<td>473</td>
<td>1.951</td>
<td>0.368</td>
</tr>
<tr>
<td>GPI</td>
<td>605</td>
<td>1.829</td>
<td>0.418</td>
</tr>
<tr>
<td>TUBA1B</td>
<td>1212</td>
<td>1.501</td>
<td>0.430</td>
</tr>
<tr>
<td>YKT6</td>
<td>1522</td>
<td>1.382</td>
<td>0.456</td>
</tr>
<tr>
<td>PFKP</td>
<td>2118</td>
<td>1.206</td>
<td>0.459</td>
</tr>
<tr>
<td>ACOT7</td>
<td>3154</td>
<td>0.991</td>
<td>0.429</td>
</tr>
<tr>
<td>ALDOA</td>
<td>3247</td>
<td>0.975</td>
<td>0.455</td>
</tr>
<tr>
<td>PGK1</td>
<td>3600</td>
<td>0.922</td>
<td>0.463</td>
</tr>
</tbody>
</table>

E. Box plots for Patients at risk (metagene score)

F. Box plots for Patients at risk (metagene score)
Figure 5

A. Angiogenesis

B. Hypoxia

C. Gene symbol | Rank in gene list | Rank metric score | Enrichment score (ES)
--- | --- | --- | ---
GPR116 | 504 | 1.798 | 0.030
MYCT1 | 811 | 1.563 | 0.064
ITGA9 | 1228 | 1.346 | 0.084
RHOJ | 1369 | 1.297 | 0.118
KDR | 2137 | 1.059 | 0.108
CDH5 | 2260 | 1.031 | 0.135
NGN1 | 2354 | 1.009 | 0.163
CALCRL | 2379 | 1.004 | 0.195
LDB2 | 2485 | 0.987 | 0.222
ESAM | 2601 | 0.962 | 0.247
JAM2 | 3091 | 0.879 | 0.247
ROBO4 | 3220 | 0.859 | 0.268

D. Gene symbol | Rank in gene list | Rank metric score | Enrichment score (ES)
--- | --- | --- | ---
MIF | 100 | 2.699 | 0.061
ALDOA | 265 | 2.177 | 0.105
MRPL15 | 290 | 2.124 | 0.156
LDHA | 333 | 2.059 | 0.204
ADM | 387 | 1.957 | 0.249
GPI | 388 | 1.955 | 0.297
DDT4 | 442 | 1.880 | 0.341
ACOT7 | 466 | 1.844 | 0.385
SHCBP1 | 757 | 1.598 | 0.407
PFKP | 931 | 1.489 | 0.434
PSMA7 | 977 | 1.460 | 0.467
NDRG1 | 1307 | 1.320 | 0.480

E. Cumulative survival (%)

F. Cumulative survival (%)
Figure 6

GPR116

EMCN

ITGA9

GNG11

KDR

PECAM1

S1PR1

JAM2

RHOJ

*GELs, RT-qPCR/HPRT1*

*GEL, microarray/HPRT1*

$r = 0.6912$

$P < 0.0001$

$r = 0.7032$

$P < 0.0001$

$r = 0.7131$

$P < 0.0001$

$r = 0.7030$

$P < 0.0001$

$r = 0.6534$

$P < 0.0001$

$r = 0.5102$

$P = 0.0008$

$r = 0.7030$

$P < 0.0001$

$r = 0.6588$

$P < 0.0001$
Gene expression signatures predictive of bevacizumab/erlotinib therapeutic benefit in advanced non-squamous non-small cell lung cancer patients (SAKK 19/05 trial)

Anca Franzini, Florent Baty, Ina I Macovei, et al.

Clin Cancer Res  Published OnlineFirst April 28, 2015.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-14-3135

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2015/04/29/1078-0432.CCR-14-3135.DC1

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