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ORIGINAL ARTICLE

Predictive value of angiogenic proteins in patients with metastatic melanoma treated with bevacizumab monotherapy

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Abstract

The incidence of malignant melanoma is rising worldwide and survival for metastatic disease is still poor. Recently, new treatment options have become available. Still, predictive biomarkers are needed to optimise treatment for this patient group. In this study, we investigated the predictive value of 60 angiogenic factors in patients with metastatic melanoma treated with the anti-vascular endothelial growth factor A antibody bevacizumab. Thirty-five patients were included in a clinical phase II trial and baseline serum samples were analysed by multiplex protein array. High-serum concentration of Activin A was significantly associated with objective response (OR) to treatment (p = 0.014). Candidate proteins that indicated a borderline association with treatment response were further investigated by immunohistochemistry. Strong expression of Activin A, interleukin-1β, and urokinase-type plasminogen activator receptor in metastases was significantly associated with OR (p = 0.011, p = 0.003, and p = 0.007, respectively), as well as with markers of activated angiogenesis, such as higher number of proliferating vessels and the presence of glomeruloid microvascular proliferations. Our findings indicate that these proteins may be potential predictive markers for treatment with bevacizumab monotherapy.

Keywords: metastatic melanoma; bevacizumab monotherapy; predictive marker; Activin A; uPAR; IL1β; paraffin embedded tissue; serum

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No conflicts of interest were declared.

Introduction

New treatment options have recently become available for patients with metastatic melanoma, and progression-free survival (PFS) [1,2] and overall survival (OS) [3,4] have improved substantially. Combination of different types of immune checkpoint inhibitors is the most promising approach with high-response rates [5,6]. However, limitations such as acquired resistance are recorded for BRAF inhibitors [1], and moderate survival benefits are observed for the CTLA4 antibody ipilimumab [3]. At the same time, incidence rates of malignant melanoma are increasing worldwide among fair-skinned populations, and the 5-year survival rate in metastatic melanoma is poor [7]. Thus, there is a persisting need to identify alternative treatment options for such patients. Validated predictive markers are urgently needed to optimise treatment, avoid side-effects of ineffective treatment, and improve funding of expensive cancer drugs within public healthcare systems.

Angiogenesis is a known hallmark of cancer [8] and is also involved in melanoma progression [9] and metastasis [10,11]. Furthermore, high vascularity in primary melanomas is an adverse prognostic marker [12–14]. Vascular endothelial growth factor A (VEGF-A) is one of the important angiogenesis growth factors and seems also to promote an immunosuppressive pro-tumourigenic environment [15]. Several clinical trials that combine anti-angiogenic treatment and immunotherapy are currently under investigation. Bevacizumab, a monoclonal antibody against VEGF-A, is approved for various solid tumours in combination with chemotherapy. We performed a clinical phase II

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study with bevacizumab monotherapy in patients with metastatic melanoma and observed an objective response (OR) rate of 17%, i.e. patients with complete or partial response, as well as a disease control rate of 31%, i.e. including patients with stable disease for at least 6 months [16]. The addition of bevacizumab to chemotherapy showed promising activity in previous phase II studies for advanced or metastatic melanoma [17–19]. Although biomarker analyses have been performed on blood samples from patients with various tumour types treated with bevacizumab in randomised studies, no predictive markers have yet been established for clinical use [20,21]. In the present study, we investigated the predictive value of 60 angiogenesis-related factors in pre-treatment serum samples. To the best of our knowledge, we are the first to report that the serum concentration of Activin A is associated with OR. For further validation, intra-tumoural expression of candidate proteins was investigated by immunohistochemistry. Strong expression of Activin A, interleukin-1β (IL1β), and uPAR in melanoma metastases was correlated with response to bevacizumab treatment.

Methods

Patients and study design

Thirty-five patients with metastatic melanoma were included in a clinical phase II study at Haukeland University Hospital, Norway, and treated with bevacizumab monotherapy until disease progression or intolerable toxicity (ClinicalTrials.gov: NCT00139360). The study design, patient characteristics, eligibility criteria, and response data were described previously [16]. Treatment response was assessed in accordance to RECIST guidelines. Response data were updated on July 31, 2018. OR was defined as complete or partial response and reported in 7 of 35 patients; 4 additional patients had stable disease. In total, 31% had disease control from this treatment as published previously [16]. Median PFS was 2.1 months (range 0.4–132 months) and median OS was 9.3 months (range 1.1–132 months). The study was approved by the Regional Ethics Committee (processing number: 05/329) and the Norwegian Medicines Agency and conducted according to the ethical principles of the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice. All patients signed informed consent before enrolment.

Tissue and blood samples

Paraffin embedded tissue from 30 of 35 primary tumours and 35 of 35 metastases was available for investigations. Three of five missing primary melanomas were described earlier [22]; two additional melanomas had to be excluded because of insufficient material. The metastasis diagnosed closest to inclusion in the study was used for immunohistochemical staining. Detailed information about the tissues available from metastases has been reported [22].

Serum samples taken before the start of bevacizumab treatment were available in 28 patients. The samples were taken within 13 days before the first treatment (median 2 days). All samples were processed as reported [22]. The aliquoted samples were stored at –80 °C.

Multiplex angiogenesis array

The Quantibody Human Angiogenesis Array 1000 (RayBiotech Inc., Norcross, GA, USA) was used for analysis of 60 angiogenesis-related proteins (supplementary material, Table S1). This array is based on the sandwich enzyme-linked immunosorbent assay (ELISA)-technology, and each antibody is spotted in quadruplicate. The procedure was performed at room temperature. First, all wells were blocked with sample diluent. Then, the array was incubated with serum samples and the standard dilutions for 2 h. The washing process was followed by incubation with the detection antibody for 2 h. After further washing, Cy3 equivalent was added to each well and incubated for 1 h. Imaging was performed by a microarray scanner (GenePix 4000B, Axon Instruments, San Jose, CA, USA) at different photomultiplier tube gains. Interslide normalisation for the most suitable scan was performed by RayBiotech (Norcross, GA, USA). The concentrations based on linear standard curves were used for further analyses.

Immunohistochemistry

Tissue sections of 4–5 μm were used for immunohistochemical staining with primary antibodies against Activin A (Sigma–Aldrich, St. Louis, Missouri, USA, A1594), IL1β (Santa Cruz Europe, Heidelberg, Germany, sc-7884), and uPAR (American Diagnostics, New York, USA, ADG3937). In brief, sections were deparaffinised in xylene and different alcohol dilutions and rehydrated before antigen retrieval was performed by microwave heating. After blocking with a peroxidase inhibitor, the primary antibody was added. 3-Amino-9-ethylcarbazole was used as chromogen for all staining. Further details are provided in Table 1. Primary antibodies were omitted for negative controls; normal tissue and different cancer types were used as positive controls.

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Table 1. Immunohistochemical staining methods

Primary antibody	Epitope retrieval	Dilution	Incubation	Detection
Activin A, polyclonal goat	MW 6th sense 20 min, pH 8	2.5 μg/ml	60 min, RT	Secondary ab rabbit anti-goat, EnVision-HRP, 30 min RT
IL1β, polyclonal rabbit	MW 6th sense 20 min, pH 6	1:50	120 min, RT	EnVision-HRP, 30 min RT
uPAR, monoclonal mouse	MW 6th sense 20 min, pH 6	1:100	Overnight, 4°C	EnVision-HRP, 30 min RT
VEGF-A sc-152, polyclonal rabbit	MW 6th sense 20 min, pH 9	1:50	60 min, RT	EnVision-HRP, 30 min RT

MW, Microwave; RT, room temperature.

The intra-tumoural expression of VEGF-A, heat shock protein 27 (HSP27), and basic fibroblast growth factor (bFGF) as well as microvessel density [23], the number of proliferating vessels [24] and the presence of glomeruloid microvascular proliferations (GMPs) [25] were presented previously [22] and included here for comparison. Furthermore, histopathological features and clinical parameters that were described earlier were included in the present analyses [16,22].

Evaluation of staining results

The immunohistochemical staining in the cytoplasm of tumour cells was evaluated using a light microscope. The slides were screened at low magnification (×100), staining intensity and area were finally investigated at magnification ×200 and recorded using a semi-quantitative grading. Staining intensity was graded as absent (0), weak (1), moderate (2), or strong (3). The proportion (area) was assessed as 'no positive tumour cells' (0), 'less than 10% positive tumour cells' (1), '10–50% positive tumour cells' (2), or 'more than 50% positive tumour cells' (3). The staining index (SI) is the product of intensity and area with a range from 0 to 9; SI was rated by two observers (CS, OS) blinded for response data.

Statistical analysis

The final serum concentrations of the multiplex analysis were normalised by log2 and quantile normalised in the R/Bioconductor environment [26]. Dixon test was performed to detect outlier samples. First, unsupervised analysis using Ward hierarchical clustering and heat map diagram was performed. Then supervised analysis was performed for particular proteins. The fold change (FC) between responders and non-responders was calculated based on median concentrations (significance thresholds ≤0.82 and >1.25). Mann–Whitney test (MWT) was computed using Wilcoxon test function in the R/Bioconductor programming environment to detect differences in protein expression between the samples [27]. The boxplots were consistent with the statistical results. Correction for multiple testing was not performed; instead, candidate proteins were validated by immunohistochemistry. Further statistical analyses were performed with SPSS, version 22 (SPSS Inc., Chicago, IL, USA). Quantile normalised data were used for protein concentrations assessed by the multiplex array. MWT was used to identify correlations between interval or ordinal scaled variables and categorical variables. Correlations between two interval scaled variables or an interval and an ordinal scaled variable were calculated by Spearman's rho correlation. Associations between related samples such as protein expression in primary melanomas and metastases were investigated by paired-MWT (pMWT). Kaplan–Meier survival curves were used to investigate PFS; significance differences between the groups were calculated by the log-rank test. The date of inclusion was defined as the start point and the date of confirmed disease progression or July 31, 2018 as the end point. The median was used as cut-off point if not defined differently. The significance threshold for all tests was 0.05. Non-parametric tests were used since not all data were normal distributed. Significant P values are given for all associations assessed by MWT. pMWT is added to the P values when paired analyses were performed. Spearman's rho (r) and the corresponding P value are given for calculations of correlations. Immunohistochemical markers correlated to response were combined in a signature. The signature was calculated by the mean SI of the markers of interest [28,29]. Receiver operating characteristic (ROC) curves [30] were performed for relevant significant single markers and the immunohistochemical signature.

Results

Multiplex array

Analysis of 60 angiogenesis-related factors in serum samples was performed to identify possible predictive markers for treatment with bevacizumab monotherapy in patients with metastatic melanoma. After exclusion of outliers

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Table 2. Candidate proteins that differ between responders and non-responders to bevacizumab monotherapy

Protein	Fold-change	*P* value (Mann-Whitney test)
Activin A	3.29	0.014
LIF	1.71	0.125
AgRP	1.27	0.097
IL1β	0.74	0.178
uPAR	0.82	0.141
VEGF-A	0.69	0.110
IL-12p40	0.80	0.125

Fold-changes are calculated based on median serum concentrations measured by multiplex array. Proteins with fold-change threshold ≤0.82 and >1.25 and corresponding *P* values <0.2 are listed. LIF, Leukaemia inhibitory factor.

based on the Dixon test, 25 of 28 serum samples were included in the final analyses. Unsupervised hierarchical clustering did not show any distinct patterns or global differences between samples (supplementary material, Figure S1). The association between each single protein concentration and OR was calculated. Proteins with a FC threshold ≤0.82 and >1.25 between patients with OR and progressive disease (PD) as well as a *P* value <0.2 were Activin A, AgRP, IL1β, uPAR, VEGF-A, IL-12p40, and LIF (Leukaemia inhibitory factor) (Table 2). Based on our objective to assess predictive markers in patients treated with an anti-VEGF-A antibody, we focused on Activin A, IL1β, uPAR, and VEGF-A in further investigations, since all of these proteins are known to be involved in VEGF-A-related angiogenesis. The FC of Activin A was 3.29 in patients with OR to bevacizumab compared to non-responders (p = 0.014; Figure 1A). High serum concentration was correlated with treatment response. The ROC curve for Activin in serum shows an area under the curve of 0.813 (see supplementary material, Figure S2). Median serum concentrations of IL1β, uPAR, and VEGF-A were lower in patients who had OR to bevacizumab monotherapy (p = 0.178, p = 0.141, and p = 0.110; Figure 1B,D). High concentration of uPAR correlated with high concentration of VEGF-A (r = 0.40, p = 0.047). There was no significant correlation between serum concentrations of Activin A, IL1β, uPAR, or VEGF-A and microvessel density, the number of proliferating vessels, or the presence of GMPs in metastatic tumour tissue. Protein concentrations measured by multiplex array are given in supplementary material, Table S2.

Immunohistochemistry

For further validation, protein expression of Activin A, Il1β, and uPAR was examined in primary tumours and metastases by immunohistochemistry.

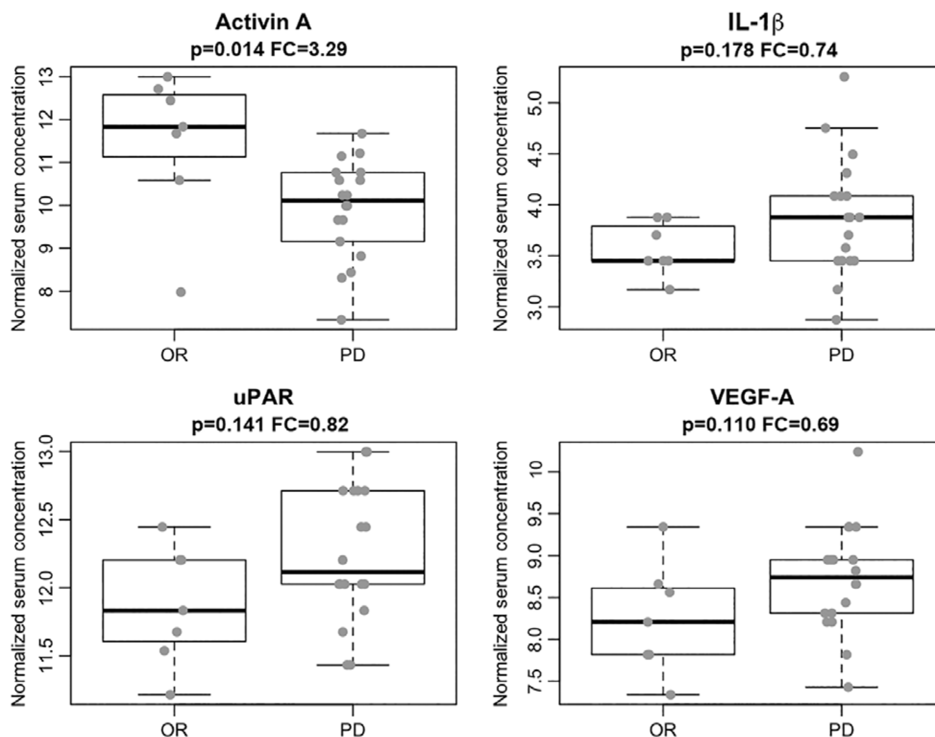

Figure 1. Associations between serum concentration of candidate proteins measured by multiplex array and objective response (OR) to bevacizumab monotherapy in patients with metastatic melanoma. Mann-Whitney test was used to calculate the difference in concentration between patients with OR and progressive disease (PD). FC, fold change.

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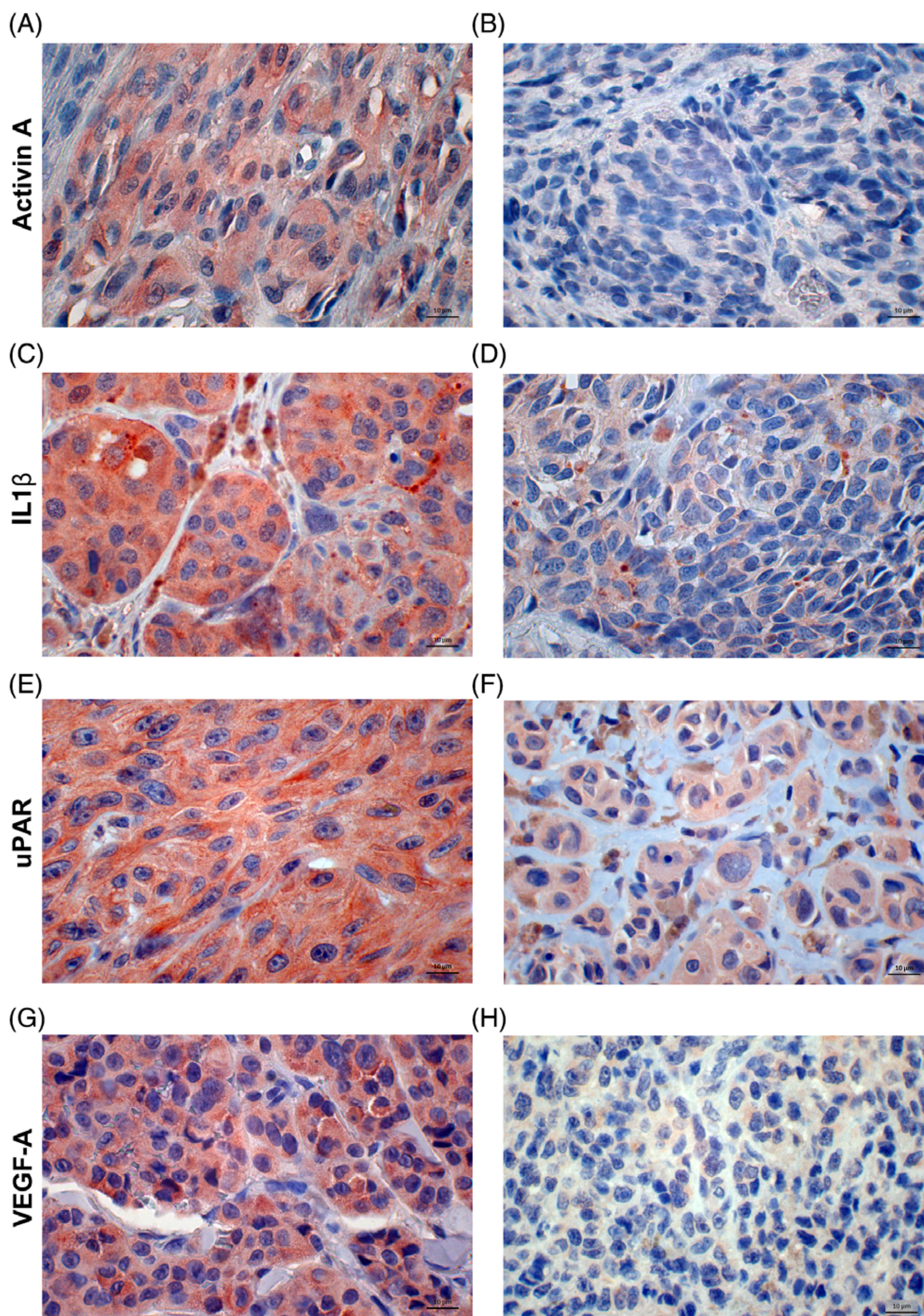

Figure 2. Immunohistochemical staining of candidate proteins in tumour cells. (A) Intermediate expression of Activin A. (B) Negative staining for Activin A. (C) Strong and (D) weak expression of IL1β. (E) Strong and (F) weak expression of uPAR. (G) Strong and (H) weak expression of VEGF-A.

Positive cytoplasmic staining for Activin A was observed in 25 of 29 primary tumours and 22 of 32 metastases (Figure 2A,B). The median SI was 2 in primary melanomas and metastases, and median SI was significantly higher in metastases from patients with OR to bevacizumab monotherapy compared with patients with PD (SI 3 versus SI 2, p = 0.011; Figure 3A). The ROC curve shows an area under the curve of 0.809 and a cut off value of 2.5 (sensitivity: 0.857, 1 − specificity: 0.280) (supplementary material, Figure S2). Activin A expression in metastases correlated with a higher number of proliferating

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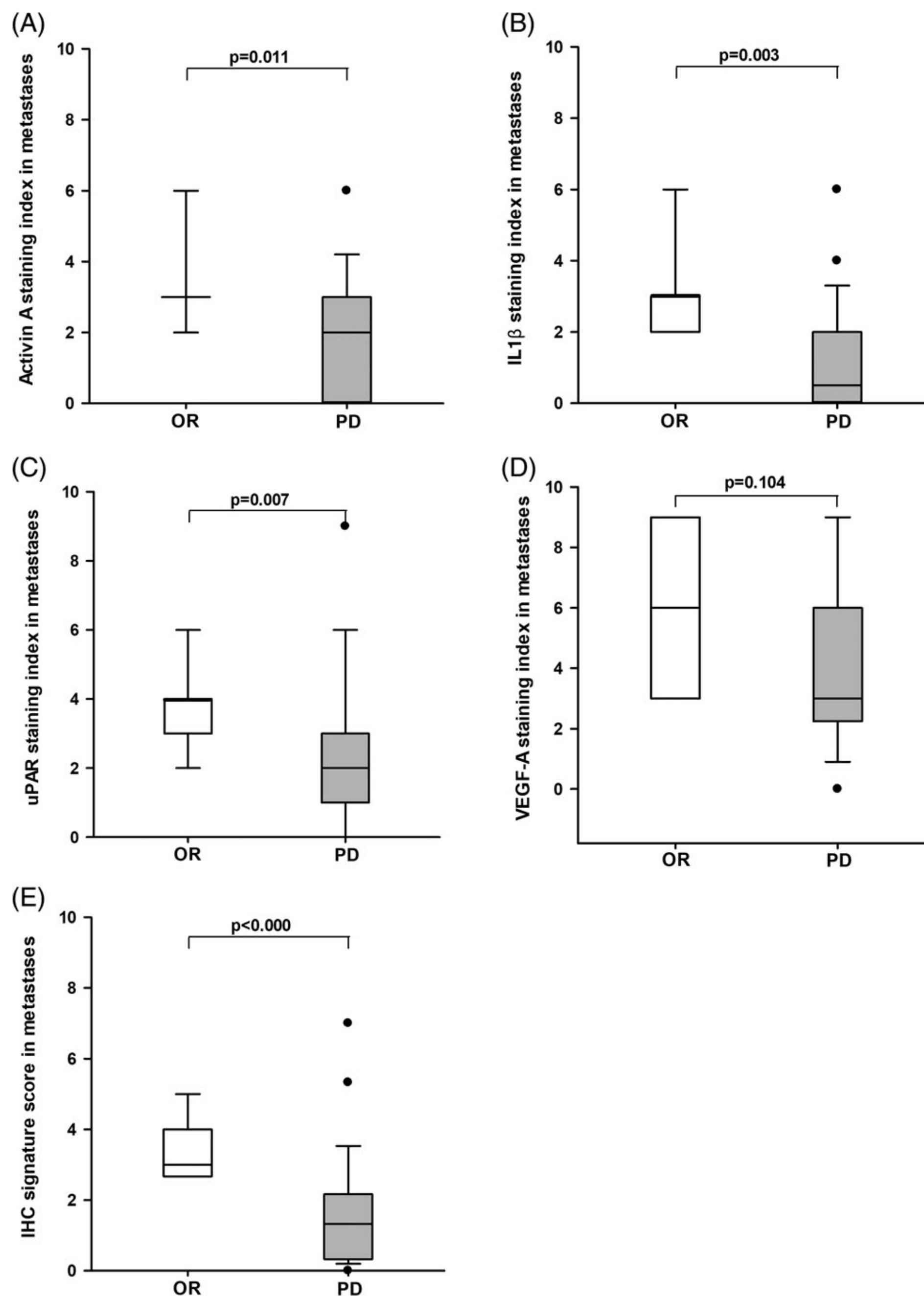

Figure 3. Association between intra-tumoural expression of candidate proteins and objective response (OR) to bevacizumab monotherapy in patients with metastatic melanoma. Mann-Whitney test was used to calculate the difference in staining indices between patients with OR and progressive disease (PD). A, activin A; B, IL1β; C, uPAR; D, VEGF-A; E, immunohistochemistry (IHC) signature score.

microvessels ($r = 0.44$, $p = 0.012$) and the presence of GMPs in metastases ($p = 0.001$). Additionally, strong expression of Activin A in metastases correlated with strong cytoplasmic expression of HSP27 ($r = 0.36$, $p = 0.043$), VEGF-A ($r = 0.49$, $p = 0.005$), and bFGF ($r = 0.59$, $p < 0.001$). Immunohistochemical expression of Activin A in metastases did not correlate significantly with Activin serum concentration measured by multiplex array.

IL1β was expressed in the cytoplasm of 28 of 30 primary tumours and in 20 of 33 metastases (Figure 2C, D). The median SI was significantly higher in primary melanomas compared to metastases in which staining was absent in 40% of the cases (SI 3 versus SI

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2, $p = 0.032$; pMWT). IL1β expression in metastases was significantly stronger in patients with OR to bevacizumab monotherapy (SI 3 versus 0.5, $p = 0.003$; Figure 3B). The ROC curve shows an area under the curve of 0.854 and a cut-off value of 2.5 (sensitivity: 0.714, 1 − specificity: 0.154) (supplementary material, Figure S2). A higher number of proliferating vessels and the presence of GMPs was seen in metastases with strong expression of IL1β ($r = 0.43$, $p = 0.015$, respectively; $p = 0.034$). Strong IL1β expression in metastases correlated with strong expression of VEGF-A, bFGF, and Activin A ($r = 0.42$, $p = 0.016$; $r = 0.62$, $p < 0.001$; and $r = 0.77$, $p < 0.0005$). IL1β concentration measured by the multiplex array did not correlate significantly with IL1β expression in metastases.

Twenty-eight of the 30 primary tumours and 28 of 34 metastases showed cytoplasmic staining for uPAR (Figure 2E,F). uPAR expression in primary melanomas was significantly stronger than in metastases (median SI 3.5 versus 2, $p = 0.017$; pMWT). Metastases in patients with OR to bevacizumab showed significantly stronger expression of uPAR than those of non-responders (SI 4 versus 2, $p = 0.007$, Figure 3C). The ROC curve shows an area under the curve of 0.823 and a cut-off value of 2.5 (sensitivity: 0.857, 1 − specificity: 0.259) (supplementary material, Figure S2). Strong expression of uPAR in metastases was associated with higher microvessel density and a higher number of proliferating vessels in metastases ($r = 0.41$, $p = 0.018$; $r = 0.38$, $p = 0.027$). Additionally, the SI for uPAR was higher in metastases with GMPs (SI 5 versus SI 2, $p = 0.021$). Strong uPAR expression correlated with strong HSP27, Activin A, and IL1β staining in metastases ($r = 0.52$, $p = 0.002$; $r = 0.65$, $p < 0.001$; and $r = 0.62$, $p < 0.001$ respectively). The expression of uPAR, VEGF, and bFGF in metastases was not correlated. uPAR expression in metastases did not correlate with serum concentration of uPAR measured by the multiplex array.

Cytoplasmic staining of VEGF-A (Figure 2G,H) in metastases was not associated with response to bevacizumab, although it did correlate with higher microvessel density [22]. VEGF-A concentration in serum samples was not associated with immunohistochemical expression of VEGF-A in metastases [22].

The staining indices of Activin-A, IL1β, and uPAR were combined in a signature (IHC signature). Patients with a high IHC signature score had significantly better response to treatment with bevacizumab (median score 3.0 versus 1.3, $p < 0.000$, MWT; Figure 3E). The ROC curve shows an area under the curve of 0.920 and a cut-off value of 2.5 (sensitivity: 1.0; 1 − specificity: 0.08) (supplementary material, Figure S2).

Discussion

Anti-angiogenic treatment with bevacizumab has been an established treatment option for various tumours since its approval in 2004. Its role in treatment of metastatic melanoma is still under investigation. Angiogenesis is a complex process regulated by the interplay of many different growth factors, cytokines, and interactions with the microenvironment [31] which makes it challenging to identify single proteins as predictive factors. No attempt to establish validated predictive biomarkers for treatment with bevacizumab has been successful to this day [20,21]. Biomarker studies have mostly been performed on blood or tissue samples from patients receiving bevacizumab in combination with chemotherapy versus chemotherapy alone. Conversely, our material is from a clinical trial with bevacizumab monotherapy which was performed to investigate response rates and potential predictive markers of response in patients with metastatic melanoma. Overall, 7 of 35 patients (20%) had complete or partial response [16]. Based on the promising results in a patient group with otherwise poor prognosis, we investigated potential predictive factors in tissues and serum samples.

Strong uPAR expression in metastases at baseline was associated with OR to bevacizumab monotherapy. In addition, we observed a significant correlation between strong expression of uPAR in metastases and a higher number of microvessels, proliferating vessels, and GMPs supporting the relevance of uPAR for tumour-associated angiogenesis. uPAR is expressed to a higher extent in various kinds of tumour compared to normal tissues [32]. In melanoma, uPAR expression is stronger in more advanced primary lesions and metastatic melanoma compared to thin melanomas or nevi [33]. uPAR is an important player in extracellular matrix degradation, cell motility, invasion, proliferation, and cell survival, and it is involved in multiple intra-cellular signalling pathways [34]. Furthermore, VEGF-A-induced re-localisation of uPAR to the leading edge of migrating endothelial cells promotes angiogenesis [35] in addition to its proteolytic function. Our findings indicate that patients with strong uPAR expression in metastases and activated angiogenesis may benefit from treatment with bevacizumab. Loss of uPAR expression in metastases may characterise clones of metastases which do not depend on angiogenesis to the same extent [36]. In our view, uPAR is a promising predictive marker for treatment with bevacizumab in melanoma patients.

IL1β is produced by tumour cells, macrophages, and myeloid derived cells; it is absent in homeostatic

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conditions and plays an important role in immune response and tumour-mediated angiogenesis [37]. Here, we found that strong IL1β expression in metastases correlated with OR to treatment with bevacizumab monotherapy. In addition, strong IL1β expression in metastases correlated with a higher number of proliferating vessels and strong cytoplasmic expression of VEGF-A and bFGF in metastatic lesions. These observations are in line with findings indicating interactions between IL1β, VEGF-A, bFGF, and endothelial cells in angiogenesis [37,38]. IL1β produced by tumour cells recruits myeloid cells and stimulates them to secrete pro-angiogenic proteins; in addition, IL1β activates expression of VEGF-A, and other angiogenic cytokines in endothelial cells and influences migration and tube formation [37]. Carmi *et al* reported crosstalk between VEGF-A and IL1β during angiogenesis in a model of B16 melanoma cells [39]. In IL1β KO mice, limited angiogenesis was seen and the number of VEGF-producing cells was reduced. These findings demonstrate an importance of IL1β for induction of VEGF-A dependent angiogenesis. In line with this, we observed an association between expression of IL1β and pro-angiogenic proteins as well as vessel density, and response to bevacizumab.

High concentration of Activin A in serum samples correlated strongly with OR to bevacizumab monotherapy. In line with this, Bai *et al* found a significant correlation between high Activin A concentration in plasma samples and better PFS in patients with colorectal cancer treated with chemotherapy and bevacizumab [40]. Furthermore, we found an association between strong expression of Activin A in metastases and OR. Activin A expression also correlated with the presence of the pro-angiogenic markers VEGF-A, bFGF, and HSP27 as well as a higher number of proliferating vessels in metastases. Others reported that Activin A induced VEGF-A and bFGF expression *in vitro* and observed increased tubulogenesis when Activin A and one of these angiogenic proteins were added simultaneously [41,42]. Activin A stimulated VEGF-A expression in various tumour cell lines [43], corneal epithelial cells, and when using an *in vivo* model of corneal neovascularisation [44].

VEGF-A is one of the key pro-angiogenic proteins in growing tumours [45,46]. However, the level of expression in metastases did not predict response to treatment with the anti-VEGF antibody bevacizumab [22]. Increased serum concentration of VEGF-A is reported to predict reduced survival in patients with melanoma [47], colorectal cancer [48], and ovarian cancer, indicating prognostic value [49]. The role of VEGF-A as a predictive marker for response to bevacizumab has been examined in previous studies in different cancer types [20,21]. The complexity of angiogenesis and the heterogenous effects of VEGF inhibition on tumours make investigation of predictive biomarkers challenging. There is still a lack of such markers as a decision-making tool for anti-VEGF treatment [20,50].

Serum concentrations and tissue expression of uPAR, IL1β, Activin A, and VEGF-A were not correlated in our material. These findings are in line with other reports on a discrepancy between serum and tissue concentrations for uPAR [51]. Woods *et al* measured low concentrations of IL1β in supernatants from squamous cancer cell lines that expressed intra-tumoural IL1β [52]. Also, in ovarian cancer, VEGF-A expression did not correlate with serum concentration as recently published [49]. Others reported an inverse correlation between local and systemic levels of VEGF-A during angiogenesis in wound healing. Differences in intra- and extra-tumoural protein expression may be explained by complex tumour–stroma interactions and reflect the challenge to identify robust biomarkers. In addition, the soluble form of proteins is investigated in serum samples whereas cellular surface receptors or intra-cellular proteins are detected by immunohistochemistry. As discussed by Komatsu *et al* [49], intra- and inter-tumoural heterogeneity may partly explain the lack of association between the protein expression in one single biopsy and the serum concentration.

Conclusion

We identified uPAR, IL1β, and Activin A as potential predictive markers for objective response to treatment with bevacizumab monotherapy in metastatic melanoma. In particular, a high score in the IHC signature including expression of Activin A, IL1β, and uPAR in metastases correlated significantly with treatment response. In addition, the serum concentration of Activin showed promising results. The role of these proteins as predictors in anti-VEGF-A treatment should be further investigated. Our results shed light on the complex interplay between VEGF-A, as the target of bevacizumab, on the one side and other proteins involved in angiogenesis and inflammation on the other.

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Author contributions statement

CS carried out experiments. CS, LAA, TS, and OS analysed data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Unsupervised hierarchical clustering of the multiplex array data

Figure S2. Receiver operating characteristic curve analysis including area under the curve

Table S1. Distribution of angiogenic factors included in the multiplex array

Table S2. Protein concentrations measured by multiplex array

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