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ORIGINAL RESEARCH ARTICLE

Preclinical Study of a Combination of Erlotinib and Bevacizumab in Early Stages of Unselected Non-Small Cell Lung Cancer Patient-Derived Xenografts

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Abstract
Background The differential outcomes of clinical studies of the targeted therapies for non-small cell lung cancer (NSCLC) indicate that better stratification of patients is required. This could be achieved with the help of patient-derived xenografts (PDX) of epidermal growth factor receptor (EGFR) wild-type patients resistant to erlotinib treatment.
Objective To explore the potential of patient-derived NSCLC xenografts to optimize therapy using 24 well-characterized early-stage NSCLC PDX.
Method Patient tumor tissue was transplanted subcutaneously into nude mice. After engraftment, tumors were expanded and the sensitivity was tested. Gene expression analysis was used to identify differentially expressed genes between erlotinib responder ($n=3$) and non-responder ($n=21$). Tumor tissue was analyzed with TaqMan PCR, immunohistochemistry and ELISA to examine the response of the models.
Results Gene expression analysis revealed vascular endothelial growth factor A (VEGFA) to be up-regulated in erlotinib non-responder. Because of that, the combination of erlotinib with bevacizumab was evaluated in one erlotinib-sensitive and four erlotinib-resistant PDX. Combination treatment was superior to monotherapy, leading to the highest and significant inhibition of tumor growth in all models investigated. A decline of VEGFA protein and an increase of *VEGFA*-mRNA were observed after bevacizumab treatment. Bevacizumab treatment resulted in a distinct decrease of blood vessel number.
Conclusion This study showed that with the help of preclinical PDX models, drug combinations for therapy improvement can be identified on a rational basis. It was observed that a dual blockage of EGFR and VEGFA was more effective than a monotherapy for the treatment of NSCLC in selected PDX models. PDX could be employed to optimize the treatment of cancer patients.

Key Points

Patient-derived xenografts could be used to find optimization for the treatment of cancer patients.

Combined treatment of erlotinib and bevacizumab was superior to monotherapy in non-small cell lung cancer xenografts.

1 Introduction

Lung cancer is one of the leading cancers worldwide, having a high mortality in Europe and the USA with a 5-year survival rate not better than 15 % for all stages combined [1–3]. Non-small cell lung cancer (NSCLC) accounts for more than 75 % of lung cancer cases. Standard treatment does not lead to a significant improvement in outcome during recent years. Targeted therapies that inhibit epidermal growth factor receptor (EGFR) or vascular endothelial growth factor (VEGF) signaling pathways were developed and evaluated in clinical trials [4–6].

Erlotinib is a small-molecule inhibitor of the EGFR tyrosine kinase that is approved by the US Food and Drug Administration and the European Medicines Agency for the treatment of NSCLC [7]. Retrospective and prospective analysis of large clinical trials identified subgroups of patients, e.g. with activating mutations in the EGFR that respond to tyrosine kinase

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inhibitors like erlotinib or gefitinib [8–10]. The identification of patients without mutations who benefit from targeted therapies is still crucial because a significant number of lung cancer patients with EGFR wild-type respond to EGFR tyrosine kinase inhibitors as well [11]. The pathways of VEGF and the EGFR are connected with each other [12] as VEGF is downregulated by EGFR inhibition through hypoxia-inducible factor 1 α (HIF1A)-dependent and -independent mechanisms [13]. Bevacizumab inhibits angiogenesis by binding to VEGFA [14] and is clinically used in combination with chemotherapy for the treatment of non-squamous NSCLC [15].

Because of the tight connection between the EGFR and VEGF signaling pathways, several clinical combination studies with erlotinib and bevacizumab were performed. Targeting both VEGF and EGFR pathways showed synergy effects in preclinical in vivo studies [16]. Resistance to EGFR inhibitors was suggested to be mediated at least partially by targeting VEGF-dependent signaling as an alternative survival pathway [17, 18]. Despite promising preclinical and early clinical evidence [19, 20], a series of clinical trials (BeTa, ATLAS, TASK) failed to demonstrate a clinically relevant benefit from the combination of erlotinib and bevacizumab in molecularly unselected patients [21–24]. Better results were achieved when patients were selected for EGFR mutations [25]. It was shown in a recently published meta-analysis that the addition of bevacizumab to chemotherapy or erlotinib could significantly improve progression-free survival and objective response rate both in first- and second-line treatments of advanced NSCLC. Nevertheless, the question whether bevacizumab plus erlotinib can prolong overall survival will need further validation [26]. These data warrant additional investigations for the optimal setting of a bevacizumab and erlotinib combination regimen.

The differential outcomes of the clinical studies and the lack of understanding of the mechanism of action indicate that better stratification of patients in combining therapies is required. Therefore, a preclinical investigation was initiated with a set of 24 well-characterized patient-derived xenografts (PDX) of NSCLC of early tumor stages [27]. All PDX models had no activating or resistance-mediating mutations in the EGFR. In the course of the characterization of these models, a genome wide gene expression analysis with Affymetrix gene arrays was done that was used as basis for the present study. Frequently, gene expression analysis has been used to identify gene signatures or markers. But numerous (predictive) gene signatures exist that could not qualify as being beneficial [28]. Therefore, a different and simple approach was applied by searching for genes expressing proteins against which approved targeted drugs were available.

It was the aim of this study to explore the potential of patient-derived NSCLC xenografts of early stages for therapy optimization. Xenograft models that were intrinsically resistant to erlotinib were selected in order to improve the response by identifying a combination treatment with the help of gene expression analysis. Further studies were performed to elucidate the mechanisms of response.

2 Methods

2.1 Patient-Derived Xenografts

The establishment and characterization of the PDX was described in detail earlier [27]. In brief, the histological type of the 24 patients was determined according to World Health Organization (WHO) criteria. The collection consisted of 12 (48 %) squamous cell carcinomas, 6 (24 %) adenocarcinomas, and 4 (16 %) carcinomas with a pleiomorphic phenotype. The remaining tumors were diagnosed as large-cell or dedifferentiated carcinomas. The samples originated from female and male patients in equal shares. With one exception, the patients were long-term smokers. The PDX used in this study had no activating or resistance-mediating EGFR mutations; only Lu7860 had a silent Q787Q mutation. LuCa7462, LuCa7466 and LuCa7700 had a Kirsten rat sarcoma (KRAS) mutation.

All mice used in the study were handled in accordance with the Guidelines for the Welfare and Use of Animals in Cancer Research [29] and according to the German Animal Protection Law, approved by the local responsible authorities. Tumor samples were cut into pieces of 3–4 mm and transplanted within 30 minutes subcutaneously into nude mice (in-house breeding). Once tumors became palpable, tumor size was measured twice weekly with a caliper-like instrument. Individual tumor volumes *(V)* were calculated by the formula $V = (\text{length} \times \text{width}^2)/2$ and related to the values at the first day of treatment (relative tumor volume, RTV). Median treated to control (T/C) values of RTV were used for the evaluation of each treatment modality.

After 6 to 19 days, the mean tumor volume reached the indicated starting volume (50–100 mm^3), the mice were randomized to the four treatment arms (six mice per group) and treatment was started. The mice were treated using the following drug dosages and treatment schedules: erlotinib 50 mg/kg, orally, days 1–5 and days 8–12 and/ or bevacizumab 5 mg/kg, intraperitoneally, twice weekly. Control mice were treated with the vehicle alone (saline), orally. At the end of the experiments, tumors were excised, snap frozen and stored at −80 °C for further analyses.

2.2 Gene Expression Analysis

Processing of expression arrays were described in detail in [27]. In brief, from 24 established NSCLC PDX untreated tumor samples ($n = 2$ to 5) were used for hybridization on Affymetrix GeneChip HGU133Plus2.0. In the present paper, GeneSpring GX11.0.2 software was used for data analyses.

The arrays were summarized with the MAS 5.0 summarization algorithm. The detection call was used to filter the probe sets (collection of probes designed to interrogate a given sequence) according to "present", "marginal" or "absent". Statistical testing of the filtered list was done with the Mann–Whitney test. The *p* value was adjusted for multiple comparisons with the Benjamini–Hochberg method to control the false discovery rate. Only probe sets that change more than 1.5-fold between the erlotinib responder ($n = 3$) and non-responder ($n = 21$) and that had a corrected p value < 0.005 were accepted. In the list, only genes were considered when targeted therapies against their proteins existed.

RNA was extracted from $2 \times 2 \times 2$-mm tumor tissue samples which were snap frozen. RNA was isolated with an RNeasy isolation kit (Qiagen) according to the manufacturers' instructions. Total RNA was reversely transcribed using TaqMan reverse transcription reagents [Applied Biosystems (AB)]. TaqMan quantitative real-time polymerase chain reaction (PCR) was performed using cDNA corresponding to 40 ng of RNA per reaction. Gene-specific primers for *VEGFA*, *HIF1A* and *carboanhydrase 9* as well as TaqMan® Fast Universal PCR Mastermix (AB) were used according to the manufacturers instructions, and amplifications were carried out on the StepOnePlus™ real-time PCR system (AB) with 45 cycles. Each sample was done in at least two replicates. Normalized ΔC_T values were obtained by subtracting the house-keeping gene *β-actin* C_T from the gene of interest C_T.

2.3 ELISA Assay

Lysates for enzyme-linked immunosorbent assay (ELISA) were prepared by adding lysis buffer (Cell Signaling Technology) containing protease and phosphatase inhibitors (Sigma-Aldrich) to the tumor tissue. The protein concentration was determined using the BioRad Protein Assay (BioRad Laboratories GmbH). The VEGFA-ELISA assay (PreproTech) was performed according to the manufacturers instructions. The calibration curve was calculated with the four-parameter logistic fit in GraphPad Prism software.

2.4 Microvessel Density

Frozen tissue was sliced with a cryotome (Leica CM1900), fixed with 4 % paraformaldehyde and blocked with 20 % goat serum. The sections were incubated with anti-mouse CD31 antibody (Becton Dickinson, Clone MEC 13.3) for one hour, followed by the secondary horseradish peroxidase (HRP)-conjugated anti-rat antibody (Southern Biotech, cat. no. 3050–05). The staining was visualized with the 3-amino-9-ethylcarbazole (AEC) system (Vector Laboratories) and counterstained with hematoxylin. Three mice per group, randomly chosen, were stained and counted for the number of vessels in at least two representative fields per sample.

2.5 Statistics

All statistical analyses were performed using GraphPad Prism version 5.02 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com. Analysis of variance (ANOVA) was applied if more than two groups were compared. In vivo data were expressed as mean ± standard error. $P < 0.05$ was considered statistically significant. All statistical tests were two-sided.

3 Results

3.1 Gene Expression Analysis Revealed Higher VEGFA Expression Levels in Erlotinib Non-Responders

According to their erlotinib response, the xenografts were divided into responders ($n = 3$) and non-responders ($n = 21$) using a cut-off level of T/C = 30 %. Around 3100 probe sets, representing around 2200 fully annotated genes were found to be differentially expressed. In order to search for therapy optimization, we considered only genes expressing proteins against which targeted drugs are available. Furthermore, the ideal candidate should be higher expressed in the non-responders. A detailed analysis among the differentially expressed genes revealed *VEGFA*, *SRC* and *PARP1* that were higher expressed in the non-responders. Four probe sets of *VEGFA* were identified with a two-fold difference in expression levels between responder and non-responder and a corrected P value <0.002. So the hypothesis was derived that combination with a VEGFA inhibitor, like bevacizumab, could improve the response rate. The other therapy options like dasatinib against *SRC* and olaparib against *PARP* will be investigated in separate studies.

3.2 Combined Inhibition of EGFR and VEGFA In vivo

Out of the available panel of NSCLC PDX, one erlotinib-sensitive model (7466) and four resistant models (7126, 7462, 7700 and 7860) were selected for the present study. Erlotinib monotherapy did not significantly reduce the tumor volume in all models but a moderate inhibition was observed in the sensitive model, although this PDX had an EGFR wild-type. The monotherapy with bevacizumab delayed tumor growth in three out of five models significantly ($P < 0.05$). In the sensitive model, the extent of the bevacizumab response was comparable to erlotinib. The combination treatment of erlotinib and bevacizumab was superior to the monotherapy, leading to the highest and significant ($P < 0.05$) inhibition of tumor growth in all models investigated (Fig. 1).

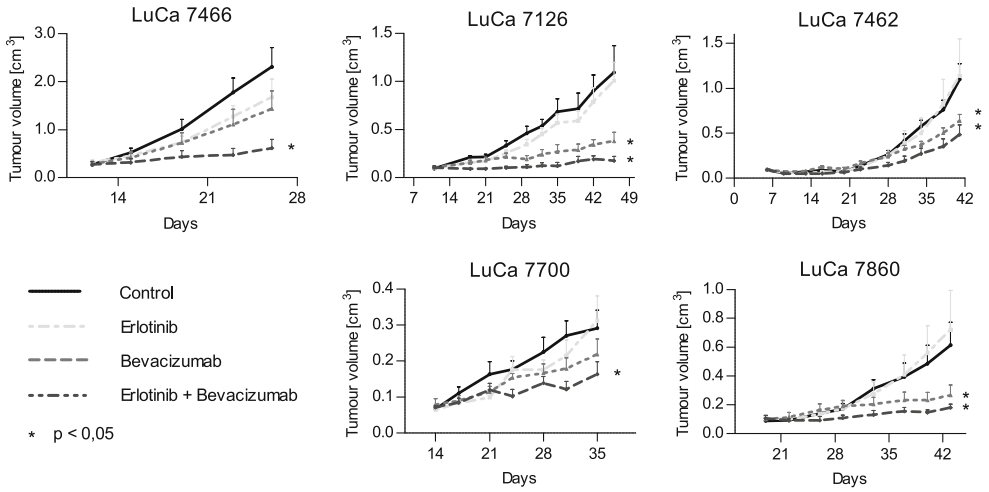

Fig. 1 Sensitivity of five NSCLC xenografts. Tumor growth curves of five selected models after monotherapy with erlotinib (*light grey*), bevacizumab (*grey*) or the combination (*dark grey*). The model 7466 was sensitive in former studies, whereas the models 7126, 7462, 7700 and 7860 did not respond to erlotinib. Mean tumor volume ($n = 6$) with standard error of the mean was shown. The x-axis shows days after tumor transplantation. * statistically significant $P < 0.05$ compared to control group

3.3 Analysis of VEGFA Expression in Tumor Tissue

The target molecule of bevacizumab, human VEGFA, was analyzed in order to elucidate its mechanism of action and to correlate with treatment and response (Fig. 2). Erlotinib treatment had no significant influence on the protein concentration in all models tested (Fig. 2a). A decreased protein expression could be observed after bevacizumab monotherapy in two out of five models. After combination with erlotinib, a significant decline of VEGFA expression was seen in the erlotinib-sensitive and two erlotinib-resistant models.

Four out of five PDX models showed an up-regulation (lower delta C_T values) of the *VEGFA* mRNA after bevacizumab treatment that was significant ($P < 0.05$) in the sensitive model 7466 and in three resistant models (Fig. 2b).

3.4 Influence on Blood Vessels

The potential influence of the different treatment schemes on tumor angiogenesis was assessed by immunohistological staining of the murine endothelial protein CD31. The microvessel number was slightly decreased after erlotinib treatment in four out of five models. Bevacizumab led to a distinct decrease of blood vessel number in all models. This effect was strengthened in the combination group, leading to a (significant) decline of this parameter in all models. It was further observed that the morphology of the blood vessels changed in the treated samples compared to the control group (see Fig. 3).

3.5 No Impact on Hypoxia Marker Genes in Response to Bevacizumab

After observing a different regulation of *VEGFA* mRNA, we further investigated if this was due to hypoxia caused by the treatment. *HIF1A* and *carboanhydrase 9* were analyzed on mRNA level. Neither *HIF1A* nor *carboanhydrase 9* mRNA were found to be differentially expressed in the control or treated tumor samples (data not shown).

4 Discussion

Our study was initiated to show that PDX can be used to propose an optimization of treatment for cancer patients. We found a combinatorial treatment to overcome erlotinib resistance. During recent years, intense research has been performed to improve the prognosis of NSCLC after chemotherapeutic treatment. Targeted agents were broadly tested in clinical studies or entered the clinics but have shown only limited efficacy as monotherapy. One explanation could be the use of cell lines for the generation of preclinical data. The in vitro system is relatively easy to handle, allows molecular manipulations, functional analyses and a high degree of standardization. Numerous cell line-derived xenografts were established to perform investigations under in vivo settings. But the individuality of the patient tumor is missing as cell lines with high passage numbers show an increased dedifferentiation [30]. Further, they only possess limited histological and molecular congruence with the primary tumor [31]. PDX can be seen as a link between the bench and the clinic as they combine features from both systems. The individual characteristics like response rate of the patient are maintained [27, 30] and sufficient tumor material for analysis is available. The use of PDX as a preclinical tool is a step towards the realization of an individualized medicine.

The PDX models in this set were subdivided into intrinsically sensitive and resistant xenografts based on erlotinib response. It should be noted, this approach is challenging due to

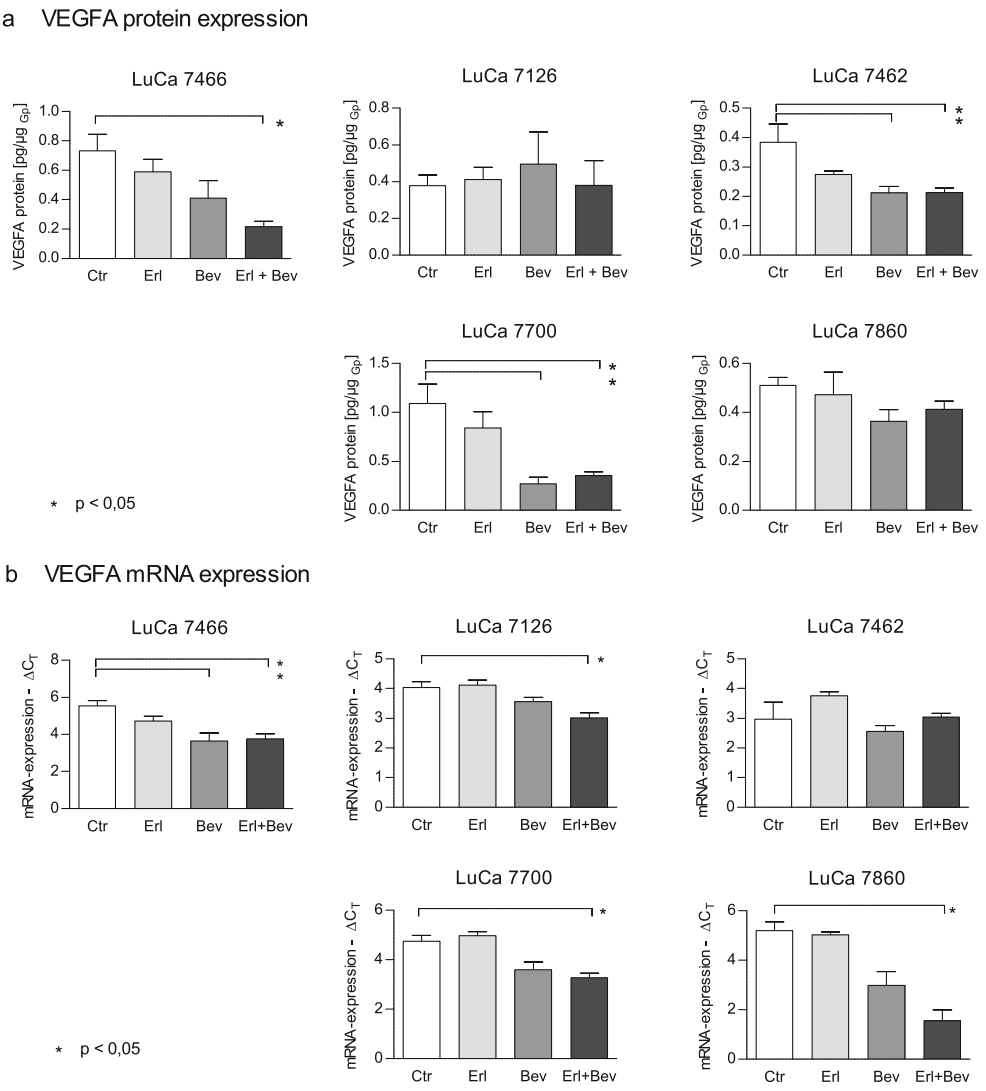

Fig. 2 VEGFA mRNA and protein expression after treatment in NSCLC xenograft models. **a** A decrease of VEGFA protein expression was determined after bevacizumab and slightly after erlotinib treatment. **b** An increase of mRNA levels of *VEGFA* was observed after bevacizumab monotherapy and after combination therapy. Mean expression ($n = 4$ to 6) with standard error of the mean was shown. Ctr: control, Erl: erlotinib, Bev: bevacizumab, * statistically significant $P < 0.05$

the laborious generation of xenograft models and the resulting limited number of models included. The gene expression analysis revealed that *VEGFA*was differentially expressed among others. *VEGFA* was selected as bevacizumab is approved for the treatment of non-squamous NSCLC. Although EGFR wild type, one model responded to erlotinib. Like known from other publications the KRAS mutated PDX were erlotinib resistant [32]. The combination of erlotinib and bevacizumab led in all five NSCLC PDX to a significant tumor regression compared to controls and led to better response rates compared to the monotherapies. That effect was seen both in the erlotinib-sensitive and erlotinib-resistant PDX models. Published (preclinical) data confirmed an additive anti-tumor effect after treatment with erlotinib and bevacizumab in NSCLC [33] and xenografts of other cancer types [34, 35] and may be explained by increasing concentrations of erlotinib in the tumor during treatment [33].

VEGFA mRNA and protein were analyzed as molecular markers for the targeted mechanism of action. Bevacizumab binds to VEGFA and neutralizes the growth factor by forming an immune complex which will be degraded [36, 37]. That leads to a decrease in the VEGFA protein which could be observed in all treated models and is consistent with results of previous studies [33, 38]. In parts, the decrease of the protein could also be explained by interference of the antibody bevacizumab with the ELISA assay. A slight decline of VEGFA was found in the erlotinib-treated group and the protein decreased further after bevacizumab treatment in all models. The level of reduction was not clearly associated with the response to bevacizumab. Only VEGFA expressed by the human PDX tissue was detected because bevacizumab binds exclusively to the human VEGFA and not to the murine protein, and VEGFA expression was analyzed with a human-specific ELISA assay and primers.

As reported in the literature, cetuximab therapy reduces pro-angiogenic factors like VEGF in preclinical models of lung cancer [39]. Similar observations were described for tyrosine kinase inhibitors [40]. A compensatory increase in the

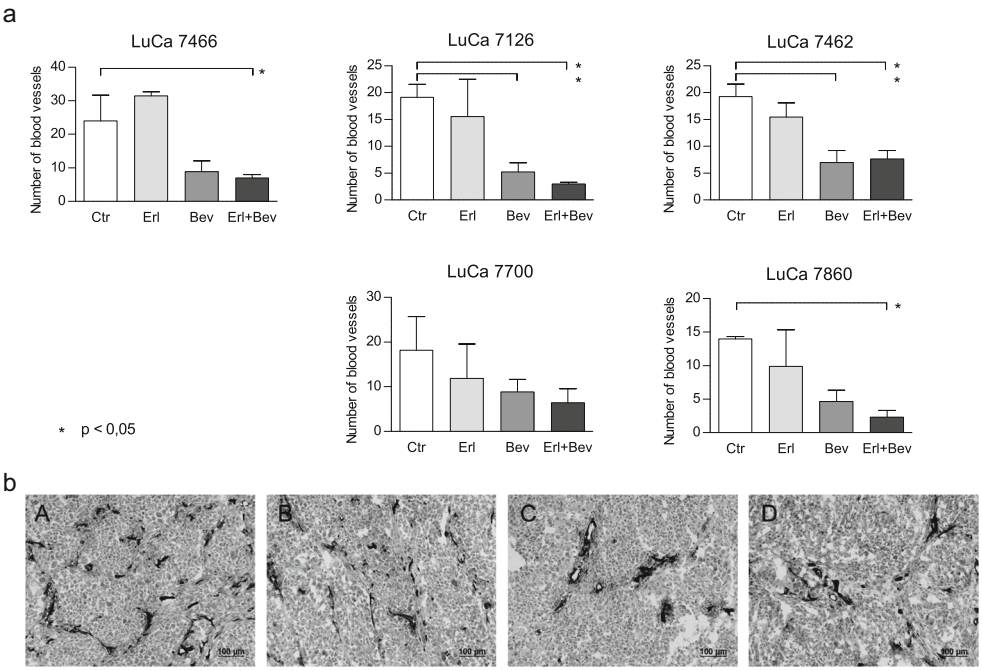

Fig. 3 Determination of blood vessel density in NSCLC xenografts after anti-EGFR and anti-angiogenic treatment. **a** The number of blood vessels decreased in all models after the treatment with bevacizumab (Bev). **b** One representative example for CD31 staining. The number of blood vessels decreased from the control group (*A*) to the erlotinib group (*B*) and further in the bevacizumab (*C*) monotherapy group. The lowest number of blood vessels was observed in the combination group (*D*). Ctr: control, Erl: erlotinib, Bev: bevacizumab, * statistically significant $P < 0.05$

VEGFA mRNA level was described in tumor cells [41] and a higher mRNA level was also found in our experiments under bevacizumab monotherapy as well as in the combination group. These results indicate that a dual blockage of signaling pathways has the potential for improved NSCLC treatment.

A decrease of VEGFA protein is suggested to inhibit angiogenesis. Therefore, CD31 was used as a marker for endothelial cells in order to visualize the vasculature. Almost all models showed a reduced number of blood vessels under bevacizumab and erlotinib treatment. In other preclinical xenografts, anti-EGFR treatment with cetuximab or tyrosine kinase inhibitors was also associated with a decrease of VEGF and a reduced number of microvessels and metastases [40, 42]. In our study, it seemed that bevacizumab had the major impact on the reduction of blood vessels, but a further decrease was observed in the combination groups. Thus, it can be hypothesized that EGFR and VEGFA blockage have an additive effect on blood vessel density. According to the literature and our own observations, treatment with anti-angiogenic drugs not only prevents neo-angiogenesis but also normalizes the morphology of the vasculature [43, 44] as a probable precondition for increased drug delivery in preclinical studies [45, 46]. But there is a window of normalization that differs from patient to patient [47, 48] as well as some differences of VEGF kinetics and distribution in mice versus human [49] which might explain the differences of preclinical and clinical data.

A tight connection between VEGF induction and hypoxia exists because VEGF is down-regulated by EGFR inhibition through HIF1A-dependent and -independent mechanisms [13]. Treatment with drugs could cause hypoxic conditions in the tumor itself. The mRNA expression of *HIF1A* and *carboanhydrase 9*, which was formerly shown to be up-regulated by HIF1A [50], was analyzed. No regulation of *HIF1A* or *carboanhydrase 9* on the RNA level was seen in the present study. Hence, it could be concluded that the change of VEGFA expression was not indirectly induced via hypoxia but could be due to another unidentified mechanism like an EGFR blockade.

This study showed that with the help of preclinical PDX models representing tumors of individual patients, drug combinations for therapy improvement can be identified on a rational basis. It was observed that a dual blockage of EGFR and VEGFA was more effective than monotherapy for the treatment of NSCLC in the unselected PDX models used here. The relative VEGFA expression in tumors could be used for a preliminary stratification of patients for an additional anti-VEGF treatment but it needs further validation in larger cohorts. The present response rates coincide with the recently published meta-analysis, but, on the other hand, differ compared to some clinical trials [21–23, 26]. Schedule and dosing of such combination therapies require substantial considerations. Further investigations to determine an optimized treatment schedule should be done. In the frame of such a study, the mechanism of action could be analyzed and validated in the tumor samples by different methods. In the future, investigations exploring the mechanism of erlotinib resistance could be done within this molecularly selected PDX panel. That should help to better understand preclinical data and translate them to the clinic to increase efficacy. In that way, high drug costs and redundant patient treatments could be avoided.

5 Conclusions

PDX are preclinical models that should be used to find rational optimization strategies for the treatment of cancer patients. It could be shown that a dual blockage of EGFR and VEGFA was more effective than a monotherapy for the treatment of unselected NSCLC.

Compliance with Ethical Standards

Funding None.

Conflict of Interest J. Rolff, M. Becker, J. Hoffmann and I. Fichtner are employees of EPO Berlin-Buch GmbH. J. Merk declares no conflic of interest. All animals used were handled according to the Guidelines for the Welfare and Use of Animals in Cancer Research and according to the German Animal Protection Law, approved by the local responsible authorities.

References

1. American Cancer Society Global Cancer Facts & Figures 2012. http://www.cancer.org/research/cancerfactsstatistics/global. 2012. Accessed Apr 2015.
2. Ferlay J, Parkin DM. Steliarova-Foucher E Estimates of cancer incidence and mortality in Europe in 2008. Eur J Cancer. 2010;46:765–81.
3. Jemal A, Bray F, Center MM et al. Global cancer statistics. CA Cancer J Clin. 2011;61:69–90.
4. Pakkala S, Ramalingam SS. Combined inhibition of vascular endothelial growth factor and epidermal growth factor signaling in non-small-cell lung cancer therapy. Clin Lung Cancer. 2009;10(1):S17–23.
5. Pal SK, Figlin RA, Reckamp K. Targeted therapies for nonsmall cell lung cancer: an evolving landscape. Mol Cancer Ther. 2010;9:1931–44.
6. Belani CP, Goss G, Blumenschein Jr G. Recent clinical developments and rationale for combining targeted agents in nonsmall cell lung cancer (NSCLC). Cancer Treat Rev. 2012;38:173–84.
7. Shepherd FA, Rodrigues PJ, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med. 2005;353:123–32.
8. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science. 2004;304:1497–500.
9. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from Bnever smokers^ and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A. 2004;101:13306–11.
10. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. 2004;350:2129–39.
11. Laurie SA, Goss GD. Role of epidermal growth factor receptor inhibitors in epidermal growth factor receptor wild-type nonsmall-cell lung cancer. J Clin Oncol. 2013;31:1061–9.
12. Byers LA, Heymach JV. Dual targeting of the vascular endothelial growth factor and epidermal growth factor receptor pathways: rationale and clinical applications for non-small-cell lung cancer. Clin Lung Cancer. 2007;8 Suppl 2:S79–85.
13. Pore N, Jiang Z, Gupta A, et al. EGFR tyrosine kinase inhibitors decrease VEGF expression by both hypoxia-inducible factor (HIF)-1-independent and HIF-1-dependent mechanisms. Cancer Res. 2006;66:3197–204.
14. Brekken RA, Overholser JP, Stastny VA, et al. Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. Cancer Res. 2000;60:5117–24.
15. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med. 2006;355:2542–50.
16. Tonra JR, Deevi DS, Corcoran E, et al. Synergistic antitumor effects of combined epidermal growth factor receptor and vascular endothelial growth factor receptor-2 targeted therapy. Clin Cancer Res. 2006;12:2197–207.
17. Lichtenberger BM, Tan PK, Niederleithner H, et al. Autocrine VEGF signaling synergizes with EGFR in tumor cells to promote epithelial cancer development. Cell. 2010;140:268–79.
18. Larsen AK, Ouaret D, El OK, et al. Targeting EGFR and VEGF(R) pathway cross-talk in tumor survival and angiogenesis. Pharmacol Ther. 2011;131:80–90.
19. Herbst RS, Johnson DH, Mininberg E, et al. Phase I/II trial evaluating the anti-vascular endothelial growth factor monoclonal antibody bevacizumab in combination with the HER-1/epidermal growth factor receptor tyrosine kinase inhibitor erlotinib for patients with recurrent non-small-cell lung cancer. J Clin Oncol. 2005;23:2544–55.
20. Tortora G, Ciardiello F, Gasparini G. Combined targeting of EGFR-dependent and VEGF-dependent pathways: rationale, preclinical studies and clinical applications. Nat Clin Pract Oncol. 2008;5:521–30.
21. Hainsworth J, Herbst RA. phase III, multicenter, placebocontrolled, doubleblind, randomized clinical trial to evaluate the efficacy of bevacizumab (Avastin) in combination with erlotinib (Tarceva) compared with erlotinib alone for treatment of advanced non-small cell lung cancer after failure of standard first-line chemotherapy. J Thorac Oncol. 2008;3(4):302.
22. Miller VA, O'Connor P, Soh C, et al. A randomized, doubleblind, placebo controlled, phase IIIb trial (ATLAS) comparing bevacizumab (B) therapy with or without erlotinib (E) after completion of chemotherapy with B for first-line treatment of locally advanced, recurrent, or metastatic NSCLC. J Clin Oncol. 2009;27:799.
23. Ciuleanu T, Tsai CM, Tsao CJ, et al. A phase II study of erlotinib in combination with bevacizumab versus chemotherapy plus bevacizumab in the first-line treatment of advanced nonsquamous non-small cell lung cancer. Lung Cancer. 2013;82:276–81.
24. Johnson BE, Kabbinavar F, Fehrenbacher L, et al. ATLAS: randomized, double-blind, placebo-controlled, phase IIIB trial comparing bevacizumab therapy with or without erlotinib, after completion of chemotherapy, with bevacizumab for first-line treatment of advanced non-small-cell lung cancer. J Clin Oncol. 2013;31:3926–34.
25. Seto T, Kato T, Nishio M, et al. Erlotinib alone or with bevacizumab as first-line therapy in patients with advanced nonsquamous non-small-cell lung cancer harbouring EGFR mutations (JO25567): an open-label, randomised, multicentre, phase 2 study. Lancet Oncol. 2014;15:1236–44.
26. Sun L, Ma JT, Zhang SL, et al. Efficacy and safety of chemotherapy or tyrosine kinase inhibitors combined with bevacizumab versus chemotherapy or tyrosine kinase inhibitors alone in the treatment of non-small cell lung cancer: a systematic review and meta-analysis. Med Oncol. 2015;32:473.
27. Fichtner I, Rolff J, Soong R, et al. Establishment of patientderived non-small cell lung cancer xenografts as models for the identification of predictive biomarkers. Clin Cancer Res. 2008;14:6456–68.
28. Kuner R. Lung Cancer Gene Signatures and Clinical Perspectives. Microarrays. 2013;2:318–39.

29. Workman P, Aboagye EO, Balkwill F, et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer. 2010;102:1555–77.
30. Fiebig HH. Burger AM Human tumor xenografts and explants. In: Teicher, B.A, editors. Animal models in cancer research. Humana Press; 2002. pp. 113–137.
31. Daniel VC, Marchionni L, Hierman JS, et al. A primary xenograft model of small-cell lung cancer reveals irreversible changes in gene expression imposed by culture in vitro. Cancer Res. 2009;69:3364–73.
32. Pao W, Wang TY, Riely GJ, et al. KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. PLoS Med. 2005;2:e17.
33. Li H, Takayama K, Wang S, et al. Addition of bevacizumab enhances antitumor activity of erlotinib against non-small cell lung cancer xenografts depending on VEGF expression. Cancer Chemother Pharmacol. 2014;74:1297–305.
34. Jung YD, Mansfield PF, Akagi M, et al. Effects of combination anti-vascular endothelial growth factor receptor and antiepidermal growth factor receptor therapies on the growth of gastric cancer in a nude mouse model. Eur J Cancer. 2002;38:1133–40.
35. Naumov GN, Nilsson MB, Cascone T, et al. Combined vascular endothelial growth factor receptor and epidermal growth factor receptor (EGFR) blockade inhibits tumor growth in xenograft models of EGFR inhibitor resistance. Clin Cancer Res. 2009;15:3484–94.
36. KimKJ LB, Houck K, et al. The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies. Growth Factors. 1992;7:53–64.
37. Presta LG, Chen H, O'Connor SJ, et al. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res. 1997;57:4593–9.
38. Stefanini MO, Wu FT, Mac GF, et al. Increase of plasma VEGF after intravenous administration of bevacizumab is predicted by a pharmacokinetic model. Cancer Res. 2010;70:9886–94.
39. Yu YF, Chen ZW, Li ZM, et al. The effects of cetuximab alone and in combination with endostatin on vascular endothelial growth factor and interleukin-8 expression in human lung adenocarcinoma cells. Curr Ther Res Clin Exp. 2009;70:116–28.
40. Ellis LM. Epidermal growth factor receptor in tumor angiogenesis. Hematol Oncol Clin North Am. 2004;18:1007–21.
41. Swinson DE, O'Byrne KJ. Interactions between hypoxia and epidermal growth factor receptor in non-small-cell lung cancer. Clin Lung Cancer. 2006;7:250–6.
42. Perrotte P, Matsumoto T, Inoue K, et al. Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. Clin Cancer Res. 1999;5:257–65.
43. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science. 2005;307:58–62.
44. Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat Rev Cancer. 2008;8:579–91.
45. Tong RT, Boucher Y, Kozin SV, et al. Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer Res. 2004;64:3731–6.
46. Wildiers H, Guetens G, De BG, et al. Effect of antivascular endothelial growth factor treatment on the intratumoral uptake of CPT-11. Br J Cancer. 2003;88:1979–86.
47. Batchelor TT, Sorensen AG, di Tomaso E, et al. AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. Cancer Cell. 2007;11:83–95.
48. Winkler F, Kozin SV, Tong RT, et al. Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. Cancer Cell. 2004;6:553–63.
49. Yen P, Finley SD, Engel-Stefanini MO, et al. A twocompartment model of VEGF distribution in the mouse. PLoS One. 2011;6:e27514.
50. Wykoff CC, Beasley NJ, Watson PH, et al. Hypoxiainducible expression of tumor-associated carbonic anhydrases. Cancer Res. 2000;60:7075–83.