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Project title: Prevention of Brain Metastasis of non-Squamous non-Small Cell Lung Cancer by Antiangiogenic Therapy in Mice

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Introduction

Metastasis to the brain is a frequent complication in some tumor entities, including nonsquamous non–small cell lung cancer (nsNSCLC, mainly lung adenocarcinoma), and triple negative and HER2-positive metastatic breast cancer (mBC; ref. 1). Lung cancer is responsible for about 60% of all brain metastases (2). In patients with locally advanced (stage III) nsNSCLC without any residual disease after initial treatment, the incidence of brain metastases is particularly high, ranging from 44% to 63% (3–5). Brain metastases contribute to the bad outcome of these patients, with 5-year survival rates below 20% (6).

If brain metastases occur, treatment options are limited, that include surgery, radiosurgery, and whole-brain radiotherapy. The latter prolongs life by 2 to 5 months, but is associated with unwanted neurotoxic side effects (7). However, relapse rates are high, and median survival after detection of brain metastases is still below one year. Thus, the option to reduce future brain metastases formation from the time of diagnosis on would benefit many cancer patients. Targeted therapeutics hold the promise to achieve that: they are often well tolerated, can be given for prolonged periods of time, and might be more efficient in the early than later steps of the (brain) metastatic cascade (8, 9). However, their potential to prevent metastases has not been addressed in prospective clinical studies so far, and little is known about optimal agents for cancer (sub)types.

The treatment of established brain metastases, but also targeting of early metastatic steps in the brain, are severely hindered by the blood–brain barrier, which might be circumvented by antiangiogenic agents that target the brain endothelial cell (10). This might also be important for the very early stages of brain metastases, when single cancer cells arrive in the brain and the blood–brain barrier is still intact (11, 12). Indeed, using a novel mouse model where single metastasizing cancer cells were tracked by intravital microscopy, we have demonstrated that bevacizumab can prevent an early angiogenic switch that is mandatory for brain outgrowth of nsNSCLC cells (13). In contrast, brain outgrowth of melanoma cells, which grew by cooption of preexisting brain vessels, was not affected by bevacizumab treatment (13).

There has been no clinical data demonstrating metastases prevention by bevacizumab in patients yet. Bevacizumab is safe in the brain metastatic setting, and approved for the treatment of nsNSCLC (14, 15). However, the current clinical benefits achieved by bevacizumab, which is administered primarily to nsNSCLC patients with metastatic disease and high existing tumor burden (16), are modest at best (15, 17). This makes this drug a plausible choice to explore a different mode of action of anti–VEGF-A therapies: their brain metastases preventive potential, which, if present, would benefit patients in earlier disease stages.

Rationale and Aim

To better characterize the effects of bevacizumab on brain metastases prevention, we first retrospectively analyzed three phase III clinical trials about the incidence of brain metastases in the bevacizumab versus control arms in nsNSCLC, and mBC (17–20). We then used mouse models to address questions relevant for clinical studies testing an anti–VEGF-A agent for brain metastases prevention: Is there a differential effect on brain and non-brain metastasis formation? Can the dose of bevacizumab be lowered for preventive application? Will brain metastases prevention result in a survival benefit?

Experimental design

Clinical data

We performed a retrospective analysis to determine the incidence of brain metastases as the first site of recurrence in three randomized phase III trials of bevacizumab (Table 1): AVAiL (nsNSCLC; refs. 17, 20), AVADO (HER2-negative mBC; ref. 19), and AVEREL (HER2-positive mBC; ref. 18). The study designs and patient characteristics are previously described elsewhere (17–20) and summarized in Table 1. Briefly, all studies were multicenter, randomized phase III trials. Patients were randomized to receive either the standard treatment with cisplatin plus gemcitabine for AVAiL, docetaxel for AVADO, and docetaxel plus trastuzumab for AVEREL trials. In the current exploratory analysis, bevacizumab arms were pooled in the two trials that include two different doses of 7.5 mg/kg and 15 mg/kg (AVAiL and AVADO). Histologic classification of the nsNSCLC patients in AVAiL trial revealed 84% adenocarcinoma, 9% large cell carcinoma, 1% mixed carcinoma with predominantly adenocarcinoma component, and 6% other types. Treatment with bevacizumab or placebo was continued until disease progression, unacceptable toxicity, or withdrawal of consent. A total of 1,043, 736, and 424 patients were enrolled in AVAiL, AVADO, and AVEREL trials, respectively. All clinical trials were approved by the local ethical committee.

These three trials were selected because (i) preexisting brain metastases were an exclusion criterion for study entry, and (ii) brain metastases as site of first relapse (event) in control versus bevacizumab groups were recorded in all three trials. According to all study protocols, patients had a baseline brain CT or MRI scan when brain metastases where clinically suspected at study inclusion, and patients were excluded when brain metastases where detected. The onset of brain metastases during follow-up was documented by means of medical chart review: brain metastases were usually detected as a result of the manifestation of neurologic symptoms, followed by a confirmatory CT or MRI scan. Cumulative incidences of brain metastases after 6, 12, 18, and 24 months were evaluated. In addition, any new lesion (not the progression of the existing lesions) outside the brain was noted.

Cell lines and cell culture

The PC14-PE6 line is among the few lung adenocarcinoma cell lines that frequently produces brain metastases in mice, and was generated by intravenous injection of parental PC14 human lung adenocarcinoma cells (21). The PC14-PE6 subline expresses high levels of VEGF-A, which was found to correlate with brain metastasis formation (22). PC14-PE6 cells were obtained from Isaiah Fidler (MD Anderson Cancer Center, Houston, TX) and transduced with a lentiviral pGF1-CMV reporter vector that coexpresses copGFP and firefly luciferase linked by the self-cleaving peptide T2A (System Biosciences), to obtain the PC14-PE6 pGF1 cell line. Flow cytometric isolation of cells by GFP expression was performed on a BD FACSAria (BD Biosciences). The cell line authentication has been performed immediately before initiation of the in vivo experiments using Multiplex human cell line authentication test, which is provided by Multiplexion. To obtain a higher number of brain metastases, a brain-seeking subline of PC14-PE6 pGF1 was generated. Of note, 500,000 PC14-PE6 pGF1 cells were supplemented in 100 mL of PBS and injected into the left cardiac ventricle of NOD/SCID mice. Those mice were followed up weekly with MRI for development of brain metastases. After onset of brain metastases, animals were sacrificed using CO_2, and the brains were removed and harvested immediately. The brains were minced and suspended into 25-mL growth medium, and then transferred to medium sized culture flasks (Nunc). After 2 to 3 weeks, a new cell line formed. These cells were expanded, and reinjected into NOD/SCID mice as explained above. A second round of MRI, harvesting of tumor-bearing brains, and subsequent cell line development in cell culture was done, to obtain the brain-seeking PC14-PE6 pGF1 Br2 cell line. For cell culture, DMEM (PAN Biotech, cat. no: P04-03600) containing 4.5 g/L glucose, sodium pyruvate, 3.7 g/L NaHCO3 without $_L$-glutamine supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, cat. no: 032M3395), 5 mL penicillin/streptomycin (Sigma-Aldrich, cat. no: P4333-10ml), and 5 mL of Glutamax (Gibco, Life Sciences, cat. no: 35050) was used. Cells were kept in a humidified atmosphere of 10% CO_2 at 37_C and passaged every 4 days via trypsinization (Gibco, Life Sciences, cat. no: 25200- 056) when reaching 90% of confluence. To avoid the reduction of GFP-containing cells in the culture, GFP expression was monitored with FACS analysis (BD FACSCanto II flow cytometer, BD Biosciences) and when necessary, FACS sorting of GFP-containing cells was performed.

Mouse metastasis model

All animal work was performed in accordance with the German animal protection law (Approving institution: Regierungspräasidium Karlsruhe). Intra- and extracranial tumor formation was achieved by injecting 5 x 10_5 PC14-PE6 pGF1 maternal and brain-seeking cells in the left cardiac ventricle of 6 to 8 weeks old either NMRI nude mice, or male NOD/SCID mice, respectively (both strains purchased from Charles River Laboratories, mouse weight ranging from 20 to 30 g). For this protocol, cells were prepared according to the routine trypsinization procedure and washed once with PBS (cat. no: 8537, Sigma Life Sciences). Cells were then resuspended in PBS (concentration 5 x 10_5/100 mL), passed through a filter tube (BD-Falcon, BD Biosciences, cat. no: 352235) and injected with a 30-G needle. Animals were anesthetized with xylazine and ketamine [mixture of 0.5 mL from 2% mL bottle (Bayer) and 1.5 mL from 100 mg/mL bottle (Pfizer) in 8 mL of saline, respectively]. Neurologic symptoms were assessed weekly up to the fourth week, afterwards daily.

Intravital imaging and follow-up

Animals were administered 100 to 150 mL of luciferin (30 mg/mL, StayBrite D-Luciferin, cat. no: 7902-1G, Biovision) after 24 hours of injection to take a baseline image using in vivo spectroscopy (IVIS Lumina Imaging system, Caliper Life Sciences). An imaging length of 180

seconds was chosen as optimal.

After completion of the imaging, animals were randomized to four types of treatment: (i) control group treated with control IgG (Kiovig, Baxter AG), 25 mg/kg (n.10 for nude mice and n.9 for NodScid mice); (ii) high-dose bevacizumab group treated with bevacizumab (Avastin, Roche) 25 mg/kg (n . 10); (iii) medium dose bevacizumab group treated with bevacizumab 2.5 mg/kg (n . 10); (iv) low-dose bevacizumab group treated with bevacizumab 0.25 mg/kg (n . 10). Bevacizumab inhibits human (tumor-cell) VEGF-A, but not murine (host) VEGF-A; thus, bevacizumab effects obtained in this study can be regarded as minimum effects. According to the previous reports, administration of 25 mg/kg bevacizumab intraperitoneally every 2 days to mice resulted in a plasma concentration of 196.89 mg/mL and 341.3 mg/mL after two and eight injections, respectively (23). This concentration corresponds to 15 mg/kg human dose of bevacizumab, when calculated with the given pharmacokinetic information for humans (24). Although there is no consensus on the standard dose of bevacizumab for the treatment of oncologic patients, most of the clinical trials used a dose ranging between 7.5 and 15 mg/kg (17, 19, 25). On the basis of these data, we defined the 25 mg/kg mouse dose ("high-dose" group) as equivalent to a high but clinical acceptable dose, and selected further subclinical doses: 2.5 mg/kg (a subclinical "medium dose") and 0.25 mg/kg (i.e., a dose two orders of magnitude below the clinical equivalent dose: "low dose"). Treatment was given twice weekly by means of intraperitoneal injection diluted in 200 mL of saline.

Tumor growth has been monitored weekly using IVIS. IVIS images were further processed using Living Image Program (Living Image Version 2.50.1, Xenogen Cooperation). Each metastatic focus was defined as region of interest and the photon flux was quantified. Symptomatic animals, animals with weight loss of 20% and more, and animals with large tumors were immediately sacrificed to prevent suffering. Under general sedation, a left cardiac perfusion was performed. After injecting PBS in the left ventricle, 4% paraformaldehyde (Roti-Histofix, ROTH, cat. no: 22135) was immediately injected and the brains were removed. After a fixation period of 2 hours, the brains were washed with PBS overnight. This was then replaced with 30% of sucrose (cat. no: 84097-1KG, Sigma Life Sciences, diluted in PBS) for further 24 hours. Brain tissue was then frozen with optimal cutting temperature medium (TissueTek, Sakura Finetek) in 80◦C freezer for cutting.

Preparation of slides for histology

Using a cryotome (Cryomicrotome, LeicaCM1950) each brain tissue was cut in 12 µm thick sections with a layer distance of 200 µm. From each layer, two slides were prepared, first for the quantification of the number of metastases, and second for collagen IV staining for the evaluation of brain vessels (26). Slides were applied one drop of Vectashield Mounting medium with DAPI (Vector Laboratories, cat. no: H 1500) and covered with a cover slip. The GFP-containing events were divided into three groups: (i) single cells, up to 3 cells close to each other, (ii) micrometastases, defined as 3 or more cells with a dimension less than 50 µm, and (iii) macrometastases, defined as metastatic formations larger than 50 µm. (Leica DM IRB Microscope, Leica Microsystems).

Immunofluorescence staining of vascular basement membrane

Slides were stained with rabbit anti-collagen IV primary antibody (1/200, Millipore, cat. no: AB756P). Staining was performed as described previously (26). Briefly, slides were air dried under air flow for 10 minutes and washed with ice-cold acetone for further 10 minutes. This was followed by a washing step with PBS for three times each for 5 minutes. Slides were circled with an invisible fat marker (Dako Pen, cat. no: 52002) and a blocking with 10% of donkey serum for 30 minutes was performed. The primary antibody was then applied and the slides were incubated overnight in a light protected chamber on a constant shaker at 4 ◦C. Before applying the secondary antibody (1/400, Alexa Fluor 633, Invitrogen, Life Sciences, cat. no: 21070), slides were washed three times with PBS each for 5 minutes. After an incubation period of 1 hour in the second antibody, slides were again washed as described above and mounted with Vectashield mounting medium and covered with a coverslip. Images were taken by confocal microscopy (Leica TCS SP5 II, Leica Microsystems). For the image processing, FIJI Software (general public license) was used.

Statistical analysis

Statistical significance was calculated using Student t test or Mann–Whitney U test for parametric and nonparametric distribution, respectively. For the differences of metastatic events in the brain of NOD/SCID mice, negative binominal regression test was used. Data were expressed as mean and SD, if not otherwise indicated. Data were considered to be statistically significant when $P < 0.05$. Kaplan–Meier survival curves and log-rank test were used for survival estimation. HR for developing brain metastases depending on the treatment arm was calculated. For calculation of the statistical tests, Microsoft Excel (Microsoft Corporation), SPSS Version 21 (SPSS Inc), and GraphPad Prism 6 were used. GraphPad Prism was further used for the image creation. Statistical analysis of the clinical datasets was performed on individual patient's data; for the AVAiL and AVADO studies, patients receiving both bevacizumab doses (7.5 and 15 mg/kg) were pooled for analysis.

Results, Conclusions and Future Perspectives

Brain as site of first relapse is less frequent in the bevacizumab arm of AVAiL

To explore the potential of bevacizumab to prevent brain metastases in patients, we first analyzed three randomized phase III trials, which investigated the role of bevacizumab in patients with advanced (largely metastasized) solid tumors. In all trials, the incidence of brain and other metastases as site(s) of first relapse was systematically recorded in the databases, and could be analyzed. These datasets allowed unequivocal determination of new occurrence of brain metastases after initiation of study treatment, as brain metastases at study entry were an exclusion criterion; however, it was not expected to find high total incidences of brain metastases, as the study populations were selected against a brain-metastatic pattern. In total, data from 2,203 patients were investigated. Further details of the trials are summarized in Table 1.

In the AVAiL trial of patients with nsNSCLC, the rate of brain metastases as first site of recurrence was significantly lower in the bevacizumab arm when compared with the control chemotherapy arm (2.6% vs. 5.8%; P = 0.01; Fig. 1A), with a lower risk of brain metastases development over time (HR = 0.36, P = 0.001; Fig. 1B). This effect of bevacizumab appeared to be most prominent during the time most patients received the drug (Fig. 1B). Moreover, in nsNSCLC patients developing brain lesions, the median time to brain metastases was shorter in the control arm (4.5 vs. 7.8 months with bevacizumab, P < 0.01, log-rank test). Brain metastases as first site of recurrence were not significantly different in the two breast cancer bevacizumab trials (Fig. 1A and Table 2); when a meta-analysis of these two mBC trial was performed, an HR of 0. 69 (CI, 0.46–1.03) was calculated, indicating that an effect of bevacizumab on the occurrence of brain metastases was, if present at all, smaller in mBC than in nsNSCLC.

Table 1. Summary of bevacizumab trials and populations analyzed

	AVAiL	AVADO	AVEREL
Trial design	Double-blind, placebo-controlled, randomized phase III	Double-blind, placebo-controlled, randomized phase III	Open-label, randomized phase III
Tumor type	Stage IIIB, IV, or recurrent nsNSCLC	HER2-negative mBC	HER2-positive mBC
Chemotherapy backbone	Cisplatin 80 mg/m^2 + gemcitabine, 1,250 mg/m^2, q3w (up to 6 cycles)	Docetaxel 100 mg/m^2, q3w (up to 9 cycles)	Docetaxel 100 mg/m^2, q3w (at least 6 cycles) + trastuzumab 8≥6 mg/kg q3w
Bevacizumab dose, mg/m^2 q3w	7.5 or 15	7.5 or 15	15
Number of patients	1,043	736	424
Control group	347	241	208
Bevacizumab group(s)	696	495	216

Abbreviation: q3w, 3 weeks interval.

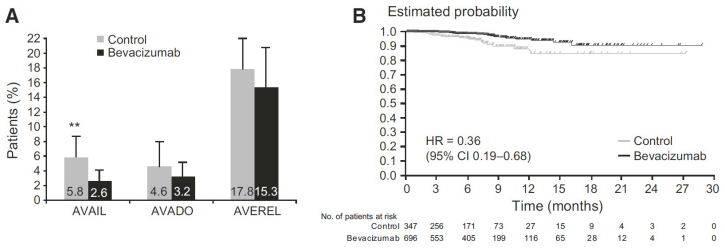

Figure 1.
Reduced incidence of brain metastases as site of first relapse in bevacizumab-treated patients with advanced nsNSCLC, but not metastatic breast cancer (mBC). A, incidence of brain metastases by trial and treatment arm (AVAiL: stage IIIb/IV nsNSCLC; AVADO: HER2-negative mBC; AVEREL: HER2-positive mBC); **, $P = 0.01$. Vertical lines represent 95% confidence interval (95% CI). B, time to new brain lesion in the control and bevacizumab arms of the AVAiL trial. The difference between control and treatment group was statistically significant ($P = 0.01$,log-rank test).

Table 2. Summary and detailed information about brain metastases formation in the three phase III bevacizumab trials included in this study

	AVAiL		AVADO		AVEREL	
	Co ($n = 347$)	Bev ($n = 696$)	Co ($n = 241$)	Bev ($n = 495$)	Co ($n = 208$)	Bev ($n = 216$)
Patients with brain lesions, *n* (%)	20 (5.8)	18 (2.6)	11 (4.6)	16 (3.2)	37 (17.8)	33 (15.3)
Time from randomization to brain metastases, HR (95% CI)	0.36 (0.19–0.68)		0.6 (0.28–1.3)		0.73 (0.46–1.17)	
Log-rank test	$P = 0.001$		$P = 0.19$		$P = 0.19$	
1-year brain lesion-free rate, %	88 (82–94)	95 (92–98)	92 (87–97)	96 (93–98)	86 (81–92)	91 (86–95)
6-month brain lesion free rate, %	95 (92–98)	98 (98–99)	97 (95–99)	98 (97–99)	96 (93–99)	98 (96–99)
Median time from randomization to brain metastases, months (range)	4.5 (0.3–12.1)	7.8 (1.1–16.2)	6.2 (1.3–11.9)	7.4 (2.0–15.9)	10.6 (2.3–34.9)	13.0 (2.1–33.0)

NOTE: "Brain metastases" means those recorded at first relapse.
Abbreviations: Co, control; Bev, bevacizumab.

Finally, in an exploratory analysis of first sites of relapse other than brain, no significant differences between the treatment arms could be observed in the AVAiL trial (data not shown).

Bevacizumab does not prevent metastases outside the brain in a preclinical model

To further investigate the potential metastases-preventive effects of anti–VEGF-A therapies, we established an animal model of hematogenous nsNSCLC (lung adenocarcinoma) metastasis. After a follow-up period of 36 days, first mice from the control group became moribund. The average load of non-brain (extracranial) metastases as

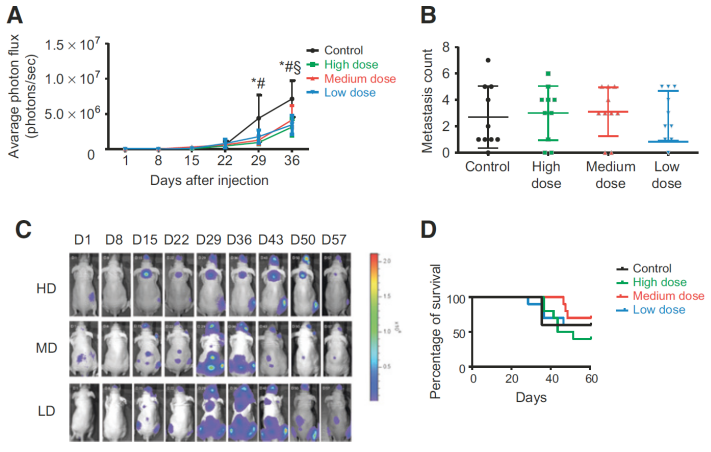

Figure 2.
Bevacizumab does not reduce the incidence of metastases outside the brain, and does not improve survival in a mouse model of systemic nsNSCLC metastasis. A, growth of extracranial metastases over 36 days in the control group (25 mg/kg control antibody), and the three bevacizumab groups: high dose (equivalent to human clinical dose, 25 mg/kg), medium dose (subclinical, 2.5 mg/kg), and low dose (subclinical, 0.25 mg/kg). *In vivo* imaging using IVIS was performed every week. Data shown are mean ± SD. *, $P < 0.05$ between control and high-dose group; #, $P < 0.05$ between control and medium dose; §, $P < 0.05$ between control and low dose. B, number of extracranial metastases per animal at day 36: the time when first animals in the control group became moribund. No statistically significant difference was detected between the groups. C, weekly IVIS images of 3 representative mice in which shrinkage of large extracranial metastases was observed during continued bevacizumab administration. IVIS color bar with increasing photon count from blue to red has been shown on the right side. A secondary remission was not observed in any of the mice treated with the control antibody. Of note, histologic analyses confirmed that none of the metastases detected by IVIS in the cranial region where actually located in the brain parenchyma. D, Kaplan–Meier survival curves during the study period of 60 days ($n = 10$ mice per group). Differences between the control and the three bevacizumab groups were not statistically significant (log-rank test, $P > 0.05$).

measured by the photon flux in IVIS was significantly lower in the high-dose and medium-dose bevacizumab groups on day 29, and for all groups on day 36 (Fig. 2A). In general, measurements of size and incidence of extracranial metastases by IVIS were verified by standard histology; here, no brain metastases could be detected using this particular animal model (data not shown). To clarify whether the reduced signal from extracranial macrometastases was due to a preventive effect, we counted their number on day 36. Interestingly, no relevant differences were found between the groups (Fig. 2B), arguing against a preventive effect of bevacizumab on the incidence of extracranial metastases in this model. Further analyses revealed that during continued bevacizumab treatment, metastases stopped to grow in some animals and continuously reduced their size over time (Fig. 2C). Two, four, and three animals from the high-dose, medium-dose, and low-dose bevacizumab groups, respectively, showed this phenomenon, while this was not observed in the control group. When the growth kinetics of all metastases in all four groups was analyzed, a growth-suppressive effect on established metastases was confirmed. All in all, a therapeutic effect on established macrometastases can explain why the total tumor load was reduced in the bevacizumab groups, while no prevention of the occurrence of extracranial metastases could be detected. Importantly, the lack of prevention of extracranial metastases was associated with a lack of survival differences between any of the treatment groups and the control group (Fig. 2D), demonstrating that the limited therapeutic effects on extracranial macrometastases did not relevantly change the clinical course of the disease.

Bevacizumab prevents brain metastases formation and prolongs survival in a mouse model of nsNSCLC brain metastasis

Because we did not detect a successful metastatic outgrowth in the brain using parental PC14-PE6 lung adenocarcinoma cells in nude mice, we established a brain-seeking subline (PC14-PE6 pGF1 BR2) in NOD/SCID mice. This allowed us to investigate the effects of a subclinical ("medium") dose of bevacizumab on brain metastases formation. In this model, a total of 112 brain metastatic events (single cells, micrometastases, and macrometastases) were observed in the 8 control animals available for analysis, but only two brain metastatic events in the 10 bevacizumab-treated animals ($P < 0.001$; Table 3). Importantly, survival was now prolonged in the bevacizumab group when compared with the control group (Fig. 3A).

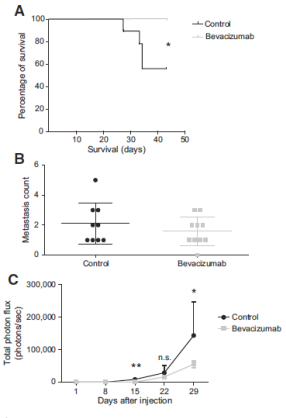

Figure 3.
Bevacizumab prolongs survival in a mouse model of nsNSCLC brain metastasis. A, Kaplan–Meier survival curves from control group and bevacizumab group (2.5 mg/kg, twice weekly; $n = 8$ and $n = 10$, respectively). Differences between the groups were statistically significant (log-rank test; *, $P = 0.02$). B, number of metastatic foci outside the brain among two study groups. The difference was not statistically significant. C, average photon count (photons/sec) of metastases outside the brain among control and treatment groups with bevacizumab within 29 days of tumor cell injection. *In vivo* imaging using IVIS was performed every week. Data shown in mean ± SD. Differences at day 15 and 29 were statistically significant (Mann–Whitney *U* test; *, $P = 0.02$; **, $P = 0.006$; n.s., not significant).

We next wanted to rule out that this difference in survival was partially caused by additional effects of bevacizumab on extracranial metastases in this model. Therefore, we analyzed the number of extracranial metastases (Fig. 3B), and the total metastases load (Fig. 3C) in the bevacizumab versus control group using IVIS. When compared with extracranial metastases in the model of systemic nsNSCLC metastasis, both models showed no bevacizumab effects on total metastases incidence (Figs. 2B and 3B), and a similar, modest effect on total metastases load (Fig. 2A, medium dose; 3C). Taken together, these data support a lack of preventive activity of bevacizumab administration on extracranial metastases formation, and also confirm that bevacizumab activity on the extracranial disease did not change in the brain-seeking mouse model.

Table 3. Brain metastatic events (single cells, micrometastases, and macrometastases, *n*) analyzed by histology of frozen brain tissue in mice treated with control antibody versus bevacizumab (2.5 mg/kg)

		Tumor type			
Mouse group	Mouse ID	Cells	Micromets	Macromets	Total events
Control	1	0	0	0	0
	2	0	0	0	0
	3	7	22	7	36
	4	0	0	0	0
	5	0	0	0	0
	6	59	6	4	69
	7	4	1	0	5
	8	0	2	0	2
Bevacizumab	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	5	0	1	0	1
	6	0	0	0	0
	7	0	0	0	0
	8	0	0	1	1
	9	0	0	0	0
	10	0	0	0	0
Significance		$P < 0.001$	$P < 0.001$	$P = 0.09$	$P < 0.001$

Effects of bevacizumab on blood vessels of brain metastases

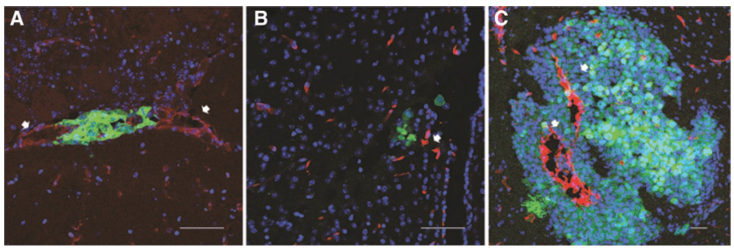

Figure 4.
Vessel wall structure of brain metastases in control versus bevacizumab-treated animals. Investigation of blood vessel wall morphology by collagen IV staining of brain tissues from representative mice in the control (A) and bevacizumab (B and C) groups. GFP-expressing tumor cells are green; collagen IV basement membrane staining is red. Nuclear staining with DAPI is shown in blue. Abnormal basement membrane typical for angiogenic blood vessels is evident in the control group (A), whereas the vasculature in the one micrometastasis found in bevacizumab-treated mice had a normal-appearing morphology (B; magnification ×40). The one macrometastasis found in the bevacizumab group had a vascular wall similar to the control group (magnification ×20; C). Scale bars, 50 μm for all images. Arrows, vascular structures.

Next, we investigated the morphology of the vasculature in brain metastases. In control animals, a thickened and abnormal vascular wall identified by collagen IV staining (26) was observed where metastatic tumor cells coopted the perivascular niche, which was regularly found in micro- and macrometastases (Fig. 4A). In contrast, cerebral microvessels in vicinity to the single micrometastasis in the bevacizumab group showed a more normal vascular wall (Fig. 4B), which is consistent with the prevention of an early angiogenic switch by VEGF-A inhibition (13). However, in the single macrometastasis that developed in one animal of the bevacizumab group, blood vessel wall morphology was also pathologic (Fig. 4C), which indicates an angiogenic escape mechanism during VEGF-A inhibition in this single animal.

Discussion

Most cancer patients do not die of the primary tumor, but of existing and developing metastases. In locally advanced (but not yet metastasized) nsNSCLC, but also triple-negative and HER2-positive breast cancer and melanoma, there is a particularly high risk to develop brain metastases. Although these patients receive intensive local treatment and also chemotherapy, there is no drug with proven efficacy to reduce the incidence of future brain metastases. Here, we characterize the potential of anti–VEGF-A therapeutics with respect to metastasis prevention, both in preclinical models and by analyzing data from clinical trials. We find preventive activity of the anti–VEGF-A antibody bevacizumab in nsNSCLC limited to the brain as site of metastatic spread.

By retrospective analysis of three clinical phase III bevacizumab trials, we identified that bevacizumab might prevent or delay the formation of brain metastases in nsNSCLC, but we could not detect a signal of similar strength for brain metastases prevention in mBC, and for nsNSCLC metastasis outside the brain. The HR of 0.36 found for brain metastases reduction in the bevacizumab arms in nsNSCLC in the current study had little overall benefit for the study population analyzed (17), but that might change for nsNCLC patients at high risk to develop brain metastases: in locally advanced (particularly stage IIIA) nsNSCLC, brain metastases occur in 40% to 50% of patients within 2 years after diagnosis (3–5), many of them as site of first relapse. Similarly, a recent retrospective analysis of noncontrolled data of a smaller number of advanced NSCLC patients (n = 159) implicated less brain metastases and a better outcome when bevacizumab was part of the treatment regimen (27).

We did not see a clear signal for brain metastasis prevention in breast cancer in the two breast cancer trials analyzed (AVADO and AVEREL). Thus, a tumor-type–specific preventive activity of bevacizumab (probably not including breast) is one important finding reported of our study. In the BEATRICE trial (phase III triple-negative breast cancer; ref. 28), reduction in brain metastasis was just a trend (11% vs. 7%) in the bevacizumab arm, confirming our results where we see a similar small trend, but without reaching statistical significance. The apparent failure of bevacizumab to relevantly prevent brain metastases in breast cancer is most likely due to differential growth patterns, with early vascular cooption and only late occurrence of angiogenesis seen during the breast cancer brain metastatic cascade in mouse models (Yunxiang Liao and colleagues, unpublished data), and angiogenesis being one crucial step of the early brain metastatic cascade in nsNSCLC (13). In the studies analyzed, nsNSCLC and mBC patients received different chemotherapeutic drugs in addition to bevacizumab. Although we cannot exclude that this fact might also have some influence on the incidence of brain metastases, it appears not very likely, because the chemotherapeutics used cannot cross the intact blood–brain barrier and act on micrometastatic brain lesions, while bevacizumab exerts its activity by inhibiting the endothelial cell, which does not require to

cross the blood–brain barrier.

Next, the clinical data were confirmed and further characterized in mouse nsNSCLC metastasis models investigating different doses of bevacizumab. Outside the brain, bevacizumab had some growth-inhibitory, partially even regressive effects on established macrometastases, but did not prevent their occurrence, which resulted in a failure to relevantly alter the course of the extracranial disease, which is in accordance with previous reports (29). A specific brain metastases preventive effect was present when using brain-seeking lung adenocarcinoma cells with a subclinical bevacizumab dose, which translated into a survival benefit in these mice. These differential effects of VEGF-A inhibition on the metastatic outgrowth in the brain versus other sites might result from a higher level of angiogenesis in patients' brain metastases, when compared with metastases of other anatomical sites, and a particular strong angiogenic reaction observed in brain metastases from lung adenocarcinoma (nsNSCLC) patients (13, 30, 31). Together, these data support the concept that antiangiogenic treatments can effectively inhibit metastasis formation by interfering with early steps of organ colonization (32), and add to this concept that organ-specific and tumor-type–specific differences must be taken into account.

In general, although formation of distant metastases over the course of the disease is a central problem for cancer patients, there is an urgent need for better preventive strategies (33). A successful prevention approach has been introduced for bone metastasis in prostate cancer patients (34). In case of small-cell lung cancer, prophylactic cranial radiotherapy (pWBRT) of patients resulted in a prolongation of brain metastases-free and overall survival (35), whereas a clear survival benefit was not seen in NSCLC (36). The relevant neurotoxicity of WBRT (7), and the inclusion of squamous NSCLC with far lower risks to develop brain metastases (3, 4) might explain this failure.

Finally, some experimental limitations should be noticed: (i) as the brain-seeking subline was established in NOD/SCID mice, we performed brain metastasis studies in this mouse strain, although we used nude mice to study the incidence of non-brain metastases; (ii) with the general paucity of lung cancer cell lines forming brain metastases in a meaningful number of mice, we restricted our analysis to one lung adenocarcinoma cell line, and rather investigated different bevacizumab doses in these animals; (iii) bevacizumab was used as a monotherapy in our animal experiments, but in combination with chemotherapy in the clinical study.

In conclusion, we show that anti–VEGF-A treatment has the potential to effectively inhibit brain metastases formation in nsNSCLC patients, with low doses necessary to achieve this preventive effect in animal models. The results of our study imply that those patients that are macroscopically tumor-free, but at high risk to develop future brain metastases, and die from it, might benefit most from antiangiogenic agents. This calls for a controlled clinical trial in stage III nsNSCLC patients with no detectable disease after standard radiochemotherapy, which are at particularly high risk to develop brain metastases in the future. Anti–VEGF-A agents, preferably in low doses, could be tested regarding their brain metastases preventive potential in these patients. Although potential benefits must be balanced against cost aspects and toxicities, and better stratification factors are warranted to better identify patients at high risk for brain metastases development, a demonstration of effective brain metastases prevention by a non-neurotoxic treatment would make a relevant difference in oncology.

List of Publications and Presentations Resulting from the Translational Research Project "insert name of project"

- Ilhan-Mutlu A, Osswald M, Liao Y, Gömmel M, Reck M, Miles D, Mariani P, Gianni L, Lutiger B, Nendel V, Srock S, Perez-Moreno P, Thorsen F, von Baumgarten L, Preusser M, Wick W, Winkler F (2016). *Bevacizumab prevents brain metastases formation in lung adenocarcinoma* Mol Cancer Ther. 15(4):702-10

- Ilhan-Mutlu, A; Prevention of brain metastasis by inhibiton of angiogenesis?, WFNO Magazine 06/2016

- Oral Talk, CCC-Welcome Meeting, Vienna, Austria 10/2014

- Oral Talk, Austrian Society of Oncology Yearly Meeting, Salzburg, Austria, 04/2015

- Oral Talk and Poster Presentations: 5th Turkish Society of Oncology Meeting, Antalya, Turkey, 03/2014

- Progress report in Neuro-Oncology Laboratory, German Cancer Research Center, Heidelberg, Germany, 05/2014

- Oral talk: 2nd Young Neuro-Oncology Investigator Meeting, Berlin, Germany, 01/2014

- Progress report in Neuro-Oncology Laboratory, German Cancer Research Center, 01/2014

- Progress reports of the Frank Winkler Lab, which takes place weekly

List of Publications and Presentations resulting from other projects during the fellowship period (if applicable)

- One publication, where I am the second author is currently under submission (last actualization September 2018)

Selection of Courses and Workshops Attended During the Fellowship

- FELASA B Course (Federation for Laboratory Animal Science Association), 10/2013, DKFZ, Heidelberg

- Central Nervous System Malignancies – Serono International Symposia, 10/2013, Vienna, Austria

- Carrier Development Days, Heidelberg, 05/2014, Heidelberg, Germany

- Several other seminars and lectures, which takes place in DKFZ routinely

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Personal Feedback

I appreciate the support very much from ESMO, which enabled me to conduct a very exciting work in one of the most renowned oncological research centers on the world, namely German Cancer Research Center. I not only learned new techniques and methodologies, but also knew excellent people there, with whom I still have personal and scientific contact. The knowledge, which is gathered during this scientific stay has been basis for some further studies and maybe even for a clinical trial. The support from ESMO is very valuable and I highly encourage younger colleagues to plan an abroad scientific stay and apply for the ESMO Grant

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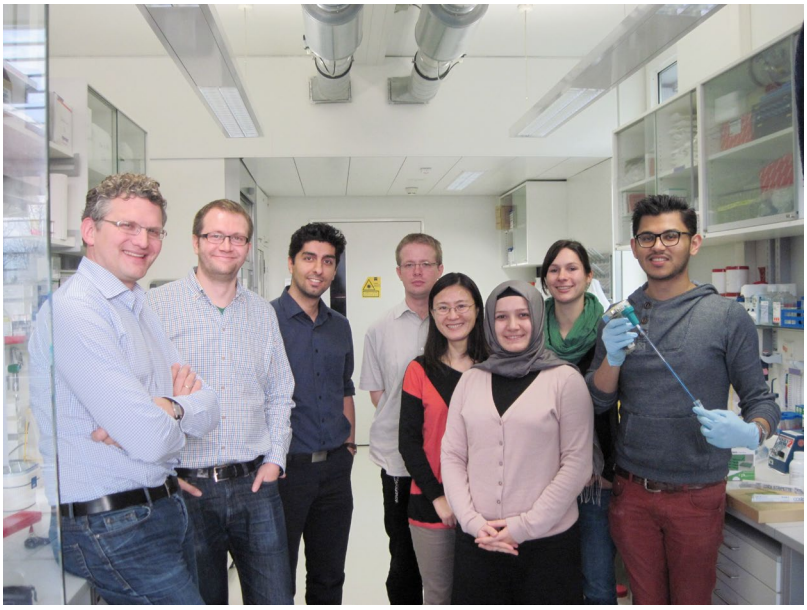
My goodbye party at the Neurooncology Labaratory DKFZ, September 2014

Working Group Professor Winkler, April 2014



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