In Vitro Vascular Tube Formation Testing as a Tool for Treatment Individualisation in Patients with Cervical Cancer

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Abstract. *Background/Aim: Targeted tumour therapies are promising, but their results in unselected patient populations are modest and tumour growth and metastasis may be promoted rather than suppressed in some cases. The present study investigates the suitability of vascular in vitro tube formation as a tool for the identification of cervical neoplasms that will respond to bevacizumab therapy. Patients and Methods: Fifteen patients with recurrent cervical cancer selected from the ongoing cervical cancer monitoring database of the Charité University Hospital Berlin, Germany, were included. Information obtained from the database included tumour stage, malignancy grade, presence of nodal metastases, lymph vessel invasion, patient age and menopausal status and serum concentrations of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), endostatin and vascular endothelial growth factor receptor 1 (VEGF-R1). Vascular tube formation was assessed with cultured human umbilical vein epithelial cells. Results: Five patients showed a positive, 5 an inverse and 5 no in vitro response to bevacizumab. Tube length showed a marked and significant dependency on bevacizumab response. Besides tube length, VEGF-R1 concentration was the only variable with some correlation to bevacizumab response, with high levels especially for inverse responders. Conclusion: The identification of patients with a likely benefit from targeted therapies is crucial. Tube formation shows substantial potential, but its utility needs to be confirmed in studies on the clinical rather than in vitro response to bevacizumab.*

Uterine cervical cancer is one of the most frequent malignancies in women, causing in excess of 250,000 annual deaths worldwide (1, 2). The establishment of screening programs has led to a decrease in the frequency of advanced stage disease and consequently improved survival (3, 4), but recently, this trend seems to have reached a plateau (5), indicating that the preventive potential of screening is nearly exhausted in most population strata of developed countries.

Overall, the prognosis of patients with locally advanced cervical cancer is favourable (2), but there is a, as yet incompletely defined, cluster of patients in whom the disease takes a markedly more severe course and responds poorly to comprehensive chemotherapeutical regimens (6).

One of the most promising treatment modalities for tumours with poor chemotherapy response is angiogenesis-targeted therapy, most notably with the monoclonal antibody bevacizumab. Introduced as an experimental option for advanced cancer around turn of the millennium (7, 8), bevacizumab has evolved as an established modality for advanced colorectal (9-12), HER2-negative breast (11, 13, 14), non-small cell lung (11, 15), and renal cell cancer (11, 16, 17). The therapeutic potential is in principle undisputed, and since tumour neovascularisation is crucially involved in the development and growth of many different types of cancer, including those of the female genital tract (18), bevacizumab treatment has been evaluated – with promising results – in several further malignancies, including cervical cancer (19, 24).

However, the novel targeted treatment modalities (in addition to bevacizumab, for instance sunitinib, sorafenib and temsirolimus) are all associated with a significant burden

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of cost (25), and more aggressive multimodal treatment regimens impose a cumulative risk of toxicity. Furthermore, there is a more marked variability in treatment response to antiangiogenic treatment than in conventional chemotherapy (26, 27). Therefore, both undertreatment of high-risk and overtreatment of low-risk patients are equally undesirable, and there is a high demand for methods that allow treatment response prediction (28-30).

The recent observation that antiangiogenic therapy can promote rather than diminish tumour progression and spread in some cases (31, 32) makes the identification of patients who will benefit from treatment all the more important.

Since the monoclonal antibody bevacizumab is directed at the vascular endothelial growth factor (VEGF) (23), its expression in tumour tissue is the most obvious target for response diagnostics. However, since tumour angiogenesis is a very complex process with a multitude of interacting factors (*e.g.* VEGF-R1, epithelial growth factor receptor (EGFR), basic fibroblast growth factor (bFGF), thrombospondin-1 (TSP-1), topoisomerase II-α, carbonic anhydrase IX, CD31, CD34, CD45, endoglin (CD105), and cyclooxygenase-2) (6, 33-37) whose specific effects and interactions are currently poorly understood, a simple linear relationship between VEGF expression and bevacizumab response is unlikely and has indeed not been demonstrated yet.

Due to the incomplete understanding of the mechanisms of tumour angiogenesis, an assessment of the actual biological effects is a plausible option. One of those effects that is accessible for measurement is the vascular tube formation on chorioallantoic membranes (CAMs) (38); *in vitro* vascular tube formation has been employed in the assessment of tumour growth and antitumour substance activity (including bevacizumab) in previous studies (39-42).

The present study investigates the suitability of *in vitro* vascular tube formation as a tool for the identification of cervical neoplasms that will respond to bevacizumab therapy. The study attempted to elucidate the correlation between bevacizumab treatment response and vascular tube formation/tube length; between angiogenic factors and vascular tube formation/tube length; and between bevacizumab treatment response and angiogenic factors.

The eventual goal of the study is a valuation of the assay's suitability for the identification of bevacizumab treatment responders.

Patients and Methods

Patients. The study participants represent a sample of patients from the ongoing cervical cancer monitoring database of the Charité University Hospital Berlin, Germany. Data acquisition, storage and processing in this database required written informed consent hence no specific ethical requirements were considered for the present investigation. Patients who underwent diagnostic or follow-up examinations for cervical uterine neoplasms between October 2002 and June 2005 were enrolled into the study. A total of 81 patients were included, and their serum samples were obtained prior to therapy and stored at –80˚C immediately after collection. Out of this sample, 15 patients (18.5%) had recurrent disease, and their sera were employed in vascular tube formation analysis.

Data acquisition. Information obtained from the database included tumour stage, malignancy grade, presence of nodal metastases and lymph vessel invasion, as well as patient age and menopausal status. The sample characteristics regarding the aforementioned criteria are shown in Table I.

The serum concentrations of VEGF, bFGF, endostatin and VEGF-R1 were determined by ELISA (R&D Systems, Minneapolis, MN, USA) as part of the clinical routine, and the respective values were obtained from the database.

Tube formation assay. Endothelial cells (human umbilical vein endothelial cells, HUVECs) were harvested from fresh human umbilical cords and stored in phosphate-buffered saline (PBS; Invitrogen, Germany) with 1% penicillin and streptomycin (Biochrom, Germany) at 4˚C for up to 48 h post partum. Afterwards, the freshly isolated HUVECs were cultured in endothelial cell growth medium 2 (EGM-2; Cambrex, East Rutherford, NJ, USA) in an incubator at 37˚C with 5% CO_2. After three passages, 2,000 HUVECs were plated in a 96-well microtiter plate (Corning Costar, the Netherlands) and were cultured for 72 h at 37˚C with 5% CO_2 in RPMI-1640 (Invitrogen, Germany) supplemented with 10% foetal calf serum (FCS; Biowest, France). The addition of 10% EGM-2 served as positive control (EBM-2 and FCS as negative control). For the vascular tube formation assay, EGM-2 was substituted by sera from the aforementioned 15 patients with recurrent cervical carcinoma. The assay was performed in duplicates.

For preparation, a 48-well plate was coated with Matrigel™ basement membrane matrix (BD Biosciences, San Jose, CA, USA) and stored at 4˚C for 24 h.

Vascular tube formation was assessed microscopically after 20 μl of patient sera and 80 μl EGM-2 with gentamycin had been added to on the 48-well Matrigel™-coated plate, and bevacizumab (2.5mg/ml) was added to one series of specimens. Wells were incubated for 18 h at 37˚C.

The vascular tube length was then assessed with a microscope, and quantitative assessment was performed with dedicated tube formation image analysis software.

Definition of bevacizumab response. Patients were divided in three groups depending on their *in vitro* bevacizumab response according to the following thresholds: Responders: >10% vascular tube formation inhibition after addition of bevacizumab. Non-responders: –10% - +10% change in vascular tube formation after addition of bevacizumab. Inverse responders: >10% increase of vascular tube formation after addition of bevacizumab.

Statistical data evaluation. Data was stored in a Microsoft Excel™ spreadsheet and analyzed with the SPSS™ 15.0 software package (SPSS™ Inc., Chicago, IL). Non-parametric methods were employed for analysis (Kruskal-Wallis test with Scheffé's and Bonferroni-Dunn *post hoc* tests for the differences between non-responders and direct and inverse responders; and Spearman's rank correlation coefficient for linear regression). For all tests, statistical significance was considered when the p-value was <0.05.

Table I. *Baseline characteristics of patients included in this study.*

Criterion	All patients	Response	No response	Inverse response
Tumour histology[n.s.]				
Squamous cell ca.	13	5	4	4
Adenocarcinoma	1		1	
Adenosquamous ca.	1			1
Prognostic criterian.s				
Nodal metastasis	6	2	1	3
Grading				
G 1	-	-	-	-
G 2	9	4	1	4
G 3	5	1	3	1
None available	1	-	1	-
Distant metastasis[n.s.]	10	3	3	4
Age, years (average)[n.s.]	44.9±10.1	44.4±8.7	44.8±11.4	45.4±12.1
Menopausal status[n.s.]				
Pre-menopausal	9	3	3	3
Post-menopausal	6	2	2	2

n.s. Not significant, ca. carcinoma.

Results

Vascular tube formation. Figure 1 shows the endothelial cell organization in a positive control sample (Figure 1a, vascular tube length=17,709 units), a negative control sample (Figure 1b, vascular tube length=28,236 units) and after incubation with patient serum (Figure 1c, vascular tube length=33,837 units).

The vascular tube length of samples was between 19,711 and 45,364 (mean 32,075±8,435, median 31,944) units, *i.e.*, comparably well defined with a near-normal distribution. The addition of bevacizumab left the mean tube length practically unaltered, except for a certain narrowing of the range (15,530-37,679, mean 30,564±5,731, median 32,108; Figure 2).

Bevacizumab treatment response and vascular tube formation. There was a marked difference in vascular tube length depending on the *in vitro* response to bevacizumab. The average tube length (before bevacizumab addition) in responders (38,995±9,559) was about 14,000 units (36%) higher than in patients with an inverse response (24,889±4,911), the non-responders falling practically in the middle between the two groups (32,341±3,007). The total difference marginally failed to be statistically significant (p=0.0539, Kruskal-Wallis test), but the individual difference between positive and inverse responders was significant both in the Scheffé and Bonferroni-Dunn *post hoc* tests (Figure 3).

Angiogenic factors and vascular tube formation. The correlation between vascular tube formation/tube length and individual angiogenic factors was barely appreciable, with the notable exception of VEGF-R1, which displayed a marked inverse correlation that was of borderline significance, mainly owing to the small sample size (Table II, Figure 4). This correlation was substantially weakened after the addition of bevacizumab to the assay (Table II).

Bevacizumab treatment response and angiogenic factors. VEGF and VEGF-R1 showed no significant association with bevacizumab treatment response, and VEGF-R1 concentrations in responders and non-responders were very similar. Inverse responders had a notably higher serum concentration of VEGF- R1. There was a relatively clear tendency for a higher serum VEGF concentration in responders as compared to non-responders; however, the potential utility of this finding is substantially marred by the fact that values for inverse responders (*i.e.* those in whom bevacizumab promotes rather than inhibits tube formation) lie between those for responders and those for non-responders (Table III).

Discussion

The potential of antiangiogenic modalities in cervical cancer treatment is undeniable (23), but the success of currently available treatment methods is relatively modest, and the mechanisms of resistance are poorly understood (27). Ultimately, all tumours become resistant to antiangiogenic treatment (43), and therefore the sensitivity of a given malignancy appears to be a state rather than a trait.

The recent literature suggests that the feasibility of patient stratification for targeted tumour therapies may not be as straightforward as had been hoped (44-45). Signal transduction in the malignant cell is highly complex, and molecules involved in cell invasion, metastasis, apoptosis, cell-cycle control, and tumour-related angiogenesis do not have a single function but are implicated in several processes with diverse consequences, involving both tumourtype- and patient-related pathways (27).

The detection of the 'window of opportunity' for antiangiogenic treatment has been elusive so far. Since serum concentrations of angiogenic factors provide no reliable method for the selection of antiangiogenic drug-sensitive malignancies, a more closely modelled diagnostic approach was employed in the present study.

The vascular tube formation (tube length) indeed showed a promising correlation with the *in vitro* response to bevacizumab, and the virtual absence of correlations with soluble angiogenic factors – with the notable exception of VEGF-R1 – corroborates the limited explanatory power of simpler diagnostic methods.

The findings of our study suggest two different tumour entities: Those that do, and those that do not respond to bevacizumab, respectively. The former group further

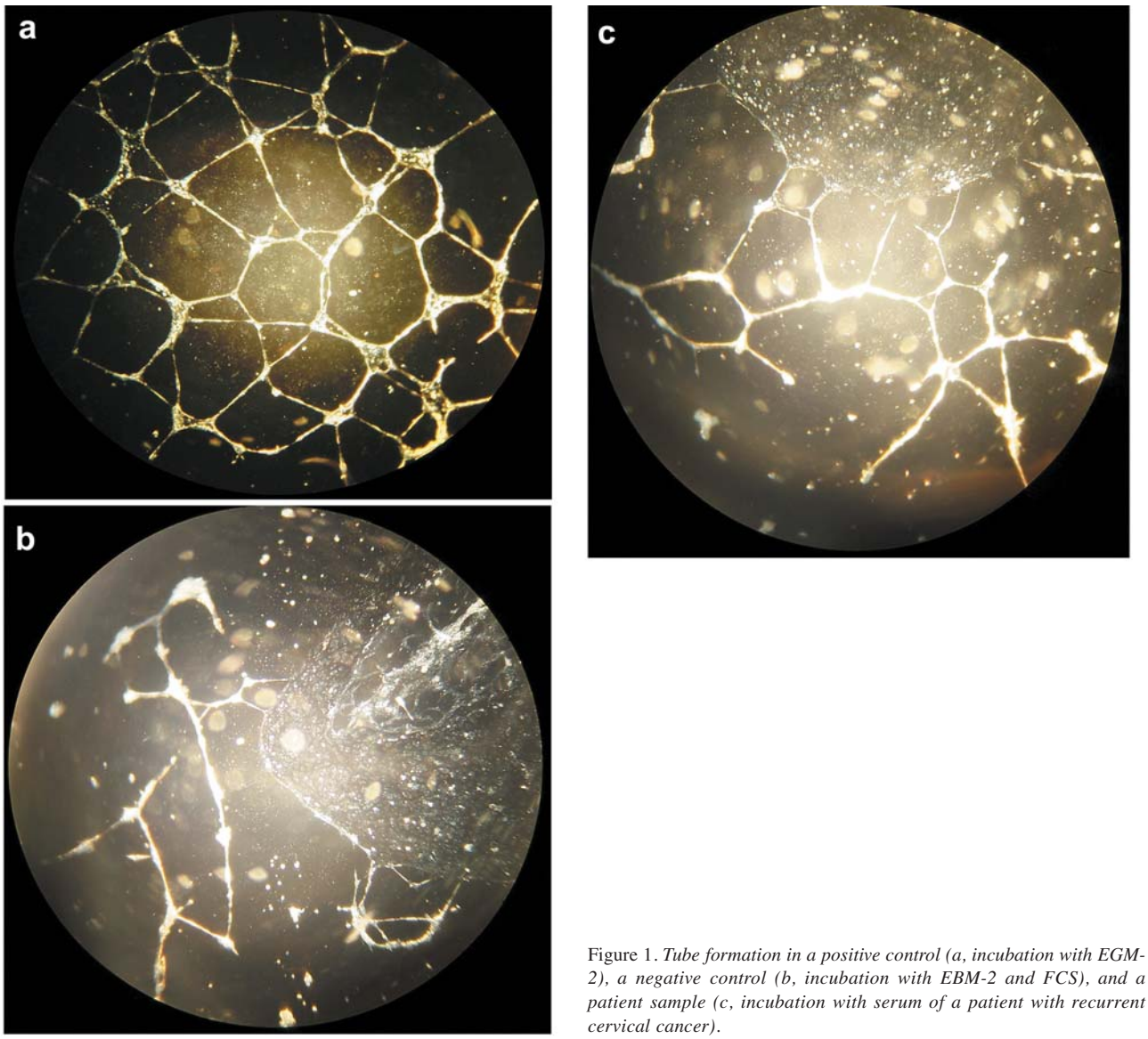

Figure 1. *Tube formation in a positive control (a, incubation with EGM-2), a negative control (b, incubation with EBM-2 and FCS), and a patient sample (c, incubation with serum of a patient with recurrent cervical cancer).*

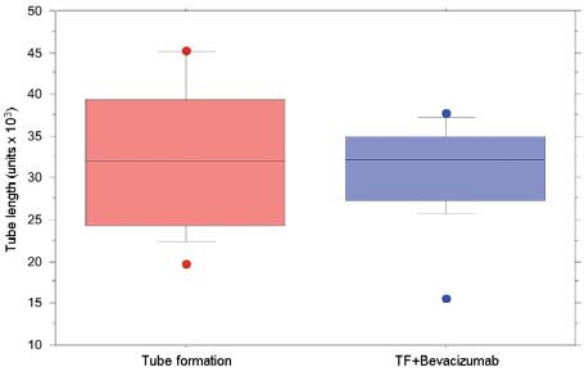

Figure 2. *Box plot of vascular tube length with and without bevacizumab.*

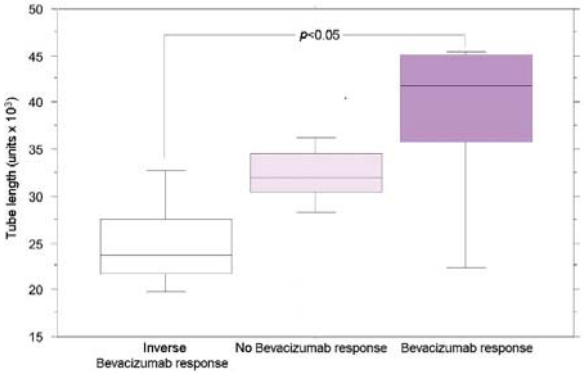

Figure 3. *Tube length according to bevacizumab treatment response.*

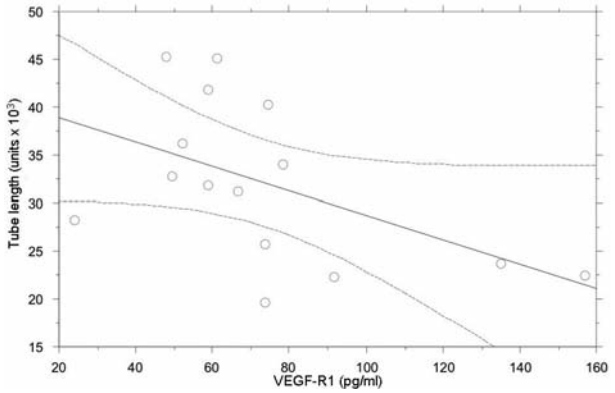

Figure 4. *Correlation between tube length and VEGF-R1.*

Table II. *Correlation of vascular tube formation/tube length and individual angiogenic factors.*

Angiogenic factor	Spearman's R	*p*-Value
VEGF (pg/ml, plasma)		
Alone	0.155	0.580
With bevacizumab	0.298	0.280
Endoglin (ng/ml, serum)		
Alone	0.023	0.936
With bevacizumab	0.002	0.995
Endostatin (ng/ml, serum)		
Alone	0.294	0.287
With bevacizumab	0.374	0.169
Angiogenin (ng/ml, serum)		
Alone	0.120	0.670
With bevacizumab	0.231	0.408
VEGF-R1 (pg/ml, serum)		
Alone	–0.510	0.052
With bevacizumab	–0.342	0.212

differentiates into tumours with the desired response and those with an inverse response, and these two groups show a marked degree of similarity in clinical terms. In the present study, the only parameter with a reasonable discriminating power in terms of bevacizumab response was serum VEGF-R1. It should be emphasized that the present study is based on *in vitro* rather than clinical bevacizumab response; the utility of serum VEGF-R1 concentrations for the prediction of clinical response is controversial (46, 47).

The vascular tube formation assay appears to have a unique ability to predict bevacizumab response *in vitro*. Consequently, it would be very interesting to verify (or falsify) its predictive value for the clinical response of tumours to bevacizumab treatment. Whereas the assay in its present form is probably too time consuming for clinical routine application, it might lead the way to more economical methods if its clinical explanatory power can be proven.

The clinical application of antiangiogenic treatment needs an evidence-based reappraisal, and the distinction of tumours with desired response on the one hand and with inverse response on the other is crucial. Apparently, the response of a tumour to targeted treatment modalities is not a constant biological property but varies with time and under treatment (31, 43, 48). Eventually, the desired effects of antiangiogenic treatment wane and an inverse effect occurs; this clinical observation is in agreement with our *in vitro* finding of an inverse response to bevacizumab.

The window of opportunity for targeted treatment methods needs to be carefully defined, and the current evidence suggests that a closely modelled approach may be required. The vascular tube formation assay shows substantial potential in this regard, but its utility needs to be confirmed in clinical studies.

Table III. *VEGF and VEGF-R1 concentration according to bevacizumab treatment response.*

Angiogenic factor	Mean±std.dev.	Median, range	*p*-Value
VEGF (pg/ml, plasma)			0.379
Response	291.6±172.1	279, 125-529	
No response	145.8±119.6	146, 120-168	
Inverse response	195.2±198.9	178, 104-341	
VEGF-R1 (pg/ml, serum)			0.344
Response	66.8±16.9	61.2, 47.7-91.7	
No response	55.9±20.4	58.9, 23.9-78.2	
Inverse response	97.7±45.9	73.6, 49.2-157.0	

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