The Utility of an *In Vitro* Angiogenesis Score for Prognosis Assessment in Patients with Cervical Cancer

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Abstract. *Background/Aim: Angiogenesis plays a key role in tumour growth and metastasis. Expression of angiogenic factors has been suggested as a marker for tumour malignity, and may help to assess a patient´s individual prognosis. The present study examines the relationship between angiogenic factor expression, an angiogenesis-based histoscore and clinical tumour criteria. Patients and Methods: A total of 81 patients with cervical cancer who underwent follow-up examinations between October 2002, and June 2005, were enrolled, and serum samples were examined for vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), endostatin and VEGF-Receptor1 by means of an ELISA. Based on an endothelial-cell proliferation assay, an angiogenesis score was calculated. Results: Higher endostatin and VEGF expressions indicated advanced disease, and VEGF allowed for a reliable distinction between patients with non-invasive and these with recurrent disease. There were some plausible correlations between the angiogenesis score and clinical criteria and individual angiogenic factors, but the score's discriminating power appears to be limited. Conclusion: The utility of angiogenesis factor testing notwithstanding, the value of an angiogenesis score for the identification of patients with a worse prognosis, and thus a resulting benefit from more aggressive treatment, is arguable.*

Malignancies of the uterine cervix are the second-most frequent cause of death in women worldwide, with well over 250,000 fatal cases annually (1).

The prognosis of uterine cervical cancer in general is favourable, with survival rates over 90% in localised invasive stages (2) and systematic early diagnosis programmes have increased the rate of neoplasms diagnosed in cervical intraepithelial neoplasia (CIN) stages resulting in a higher percentage of patients diagnosed in curable stages (3). However there is a subset of patients who have a significantly poorer prognosis. Despite the identification of a variety of factors for poor prognosis, the exact biological mechanisms that determine the course of progression of this disease are still incompletely understood (4).

The identification of such factors, however, is of pivotal importance for the further improvement of cervical cancer prognosis in a twofold fashion: It might facilitate the stratification of patients in terms of the benefit from a more aggressive general anti-tumour treatment (*i.e.* radio-, chemo- and/or hormone therapy) and thus help to improve the individual risk-benefit assessment for treatment strategies, avoiding undertreatment of high-risk as well as overtreatment of low-risk patients; it could also be a means of developing specifically targeted treatment modalities that interfere with the very mechanisms responsible for tumour progression, which in the long term is a far more promising prospect.

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Angiogenesis has been the subject of substantial scientific attention during the past decade. Not only crucial for tissue development, differentiation, maturation and restoration, it is also centrally involved in tumour growth, invasion, and metastasis (4-6), and there is indeed a growing body of evidence for angiogenesis induction by cervical neoplasms (7, 8).

There is a multitude of factors that are involved in angiogenesis, the individual importance of which is somewhat elusive (9, 10). Factors with a very likely involvement in cervical cancer progression are vascular endothelial growth factor (VEGF) and its receptor VEGF-R1, endothelial 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 cyclo-oxygenase-2 (4, 9, 11-18), and the prognostic value of mediator proteins (p53, c-erbB-2 (12, 18)) and enzymes such as tumour M2 pyruvate kinase (19) has also been investigated. VEGF has received special attention, and gene polymorphisms have recently been implicated as a relevant module in the aetiology of cervical cancer (20). However, attempts to develop a conclusive concept of the importance of individual angiogenesis factors have been futile so far (21).

In order to overcome the aforementioned uncertainty, several biological assays that are based on the assessment of the actual biological effects of angiogenic factors rather than their plasma or serum concentrations have been developed and validated (7, 22). One of these is a histoscore ('angiogenesis score'), first proposed by Brem *et al*. (23) that is employed in the present study.

Beyond prognosis assessment, patient stratification for targeted treatment would be a potential utility of a selective prognostic angiogenic factor since angiogenesis is a validated target for specific therapeutic modalities currently under investigation (5, 6, 24). Therefore, a reliable identification of patients with an angiogenic factor-related poorer prognosis would not only be desirable for risk assessment but also for the implementation of angiogenesis-targeted treatment strategies.

The goal of the present study was the development and validation of an individualised method for tumour prognosis prediction. The concentrations of four angiogenic factors (VEGF, bFGF, endostatin and VEGF-R1) and the angiogenesis score, as well as clinical patient, tumour, and outcome criteria, were retrieved from the hospital's database and examined for possible interrelations namely between clinical tumour criteria and the angiogenesis score; between clinical tumour criteria and angiogenic factors; between angiogenic factors and the angiogenesis score; and the dependency of the angiogenesis score on age and menopausal status.

Patients and Methods

Patients. The study participants represent a sample of patients from the ongoing cervical cancer monitoring database of the University Hospital Charité, 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, their serum samples were obtained prior to therapy and stored at –80˚C immediately after collection.

Data acquisition. Information obtained from the database included tumour stage, histology, presence of nodal metastases, lymphatic and venous 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.

All previously collected serum samples were used for an endothelial-cell proliferation assay in November/December, 2005.

Endothelial cell isolation. Endothelial cells (HUVECs) were harvested from fresh human umbilical cords. The human umbilical cords were 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. After careful rinsing, umbilical cords were filled with collagenase type 4 (Invitrogen, Germany) in order to mobilise the cells and then were rinsed with 30 ml PBS. The freshly isolated HUVECs were then 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, the HUVECs were used in the proliferation assay.

Endothelial cell proliferation assay. The endothelial cell proliferation assay was performed according the following protocol: 2,000 HUVECs were plated in a 96-well microtiterplate (Corning Costar, Netherlands) and were cultured for 72 h at 37˚C with 5% CO_2 in RPMI1640 (Invitrogen, Germany) supplemented with 10% foetal calf serum (Biowest, France). The addition of 10% endothelial growth medium 2 (EGM-2) served as positive control. For the determination of the angiogenic activity, EGM-2 was substituted by the human serum from the cervical carcinoma patients (described above). The assay was performed in duplicates. After 72 h the angiogenesis score, *i.e*. the endothelial cell proliferation rate, was determined by intracellular ATP concentration measurement employing the ATP-Lite-M Kit (Perkin-Elmer, Life and Analytical Sciences, Boston, MA, USA). The ATP concentration was measured by chemiluminiscence, and the cell concentration was calculated based on a calibration curve with untreated controls providing the 100% benchmark

Statistical data evaluation. Data were stored in a Microsoft Excel™ spreadsheet and analysed with SPSS™ 14.5 program package (SPSS™ Inc., Chicago, IL). A normal distribution of values was not assumed, and therefore non-parametric methods were employed for analysis. Median and interquartile range (IQR) were used for

Table I. *Baseline characteristics of study patients.*

Criterion	Number	Percentage
Tumour stage		
Non-invasive	15	18.5
CIN I	1	1.2
CIN II	4	4.9
CIN III	10	12.3
Invasive	51	63.0
FIGO I	22	27.2
FIGO II	13	16.0
FIGO III	13	16.0
FIGO IV	3	3.7
Recurrent disease	15	18.5
Tumour histology (only invasive tumours)		
Squamous cell carcinoma	50	75.8
Adenocarcinoma	8	12.1
Adenosquamous carcinoma	5	7.6
Not classifiable	3	4.5
Prognostic criteria		
Nodal metastases	26	32.1
Lymph vessel invasion	22	27.2
Blood vessel invasion	6	7.4
Grading		
G1	2	2.5
G2	34	42.0
G3	26	32.1
None available	19	23.5
Age, years (average)	46.8±13.1	
Menopausal status		
Pre-menopausal	52	64.2
Menopausal	2	2.5
Post-menopausal	27	33.3

Table II. *Angiogenesis score (%) of study patients.*

Parameter		Median	IQR	Range	*p*-Value
Entire sample		53.3	27.7	10.4-110.9	n/a
Tumour stage					0.017
Non-invasive		37.3	14.0	16.5-96.8	0.045†
Invasive		58.4	30.9	15.0-110.9	
Recurrent disease		52.2	28.8	10.4-86.3	n.s.‡
CIN	I	30.3	-	-	n.s.‡
	II	29.3	16.1	16.5-37.3	
	III	42.5	28.9	30.7-96.8	
FIGO	I	57.4	23.6	17.0-110.9	
	II	57.4	33.0	18.9-88.4	
	III	64.0	25.7	29.9-82.5	
	IV	30.1	24.3	15.0-47.4	
Recurrent disease		52.2	28.8	10.4-86.3	
Grading				0.012	
G1	39.5	33.8	22.6-56.4	n.s.‡	
G2	57.4	28.2	19.7-110.9		
G3	58.5	32.3	17.0-87.8		
None available		37.3	14.8	10.4-96.8	
Menopausal status				n.s.‡	
Pre-menopausal		55.5	30.6	15.0-110.9	n.s.‡
Menopausal		46.5	58.9	17.0-76.9	
Post-menopausal		51.4	21.2	10.3-88.4	

†*Pos-hoc* test, non-invasive *vs*. invasive; ‡all (other) pairwise comparisons.

description of concentrations. The changes in adhesion molecule concentrations before and after treatment were assessed with the Wilcoxon test for paired differences, and differences between subgroups of the sample were analysed with the Mann-Whitney *U*-test (for two subgroups) or the Kruskal-Wallis test (more than two subgroups, with Scheffé's post hoc test). The χ^2 test was used for the comparison of frequency distributions, and Spearman's rank correlation coefficient for linear regression. For all tests, statistical significance was considered when the *p*-value was <0.05.

Results

Angiogenesis score and clinical characteristics. The results of the angiogenesis score evaluation are displayed in Table II. The median score of the entire sample was 53.3% (IQR 27.7, range 10.4-110.9%). There was a significant dependency on tumour stage (χ^2 test $p<0.05$), and the angiogenesis score increased continually from cervical intraepithelial neoplasia (CIN) I to FIGO III. Patients in stage FIGO IV (only three) and with recurrent disease had a lower value. None of the paired differences of the angiogenesis score between stages, however, were significant in *post-hoc* testing. Tumour cell differentiation and tumour grading according to Bloom and Richardson showed a significant correlation with the angiogenesis score. There was no relationship between menopausal status and the angiogenesis score, which renders the angiogenesis score some potential as a promising independent prognosis criterion. All in all, the angiogenesis score showed some potential for risk stratification.

Angiogenesis score and angiogenic factors. The angiogenesis score showed a significant correlation with only one of the angiogenic factors, namely endostatin (Pearson's R –0.227). This correlation was, however, inverse. Endostatin also clearly increased with tumour stage, but showed no correlation with tumour grading (Table III). The formally significant correlations of endostatin and VEGF-R1 with the menopausal status should be ignored because of the non-continuous behaviour of the values.

The serum VEGF level was the only angiogenic factor with a strong and plausible correlation with tumour prognosis. It showed a marked, relevant and statistically significant correlation with tumour stage and grading, and its correlation with menopausal status, albeit not significant, was continuous and plausible (Table III).

Applying the manufacturer-recommended cut-off point of 115 pg/ml plasma, VEGF also provided an excellent stratification of stages: whereas no patient with non-invasive disease had elevated concentrations of VEGF in serum, all but two patients with recurrent disease did (Figure 1).

Table III. *Angiogenic factors and their correlation with clinical criteria and the angiogenesis score.*

Parameter (Median, IQR)	Plasma VEGF (pg/ml)	Serum bFGF (pg/ml)	Serum endostatin (pg/ml)	Serum VEGF-R1 (ng/ml)
Entire sample	89.7, 127.2	<3, 7.3	111.0, 39.7	75.4, 27.7
Tumour stage	***	n.s.	***	n.s.
Non-invasive	32.1, 11.9	<3, 4.5	96.6, 19.8	87.8, 19.5
Invasive	89.7, 107.9	<3, 6.0	108.0, 32.5	79.3, 25.1
Recurrent disease	161.0, 142.5	7.0, 10.8	151.0, 73.3	66.5, 23.5
Grading	***	n.s.	n.s.	n.s.
G 1	34.0, 1.5	7.5, 5.0	87.2, 8.0	76.5, 23.5
G 2	120.0, 122.1	<3, 7.0	112.5, 51.3	74.5, 26.0
G 3	118.5, 115.4	<3, 9.0	121.0, 51.0	71.7, 30.2
Non available	35.6, 16.6	<3, 6.8	105.0, 32.8	87.8, 16.2
Menopausal status	n.s.	n.s.	*	*
Pre-menopausal	82.5, 99.6	<3, 7.3	103.5, 33.2	85.1, 29.0
Menopausal	110.4, 151.2	<3, 6.5	81.8, 19.7	53.7, 30.9
Post-menopausal	120.0, 138.5	<3, 7.5	123.0, 43.0	73.6, 25.4
Angiogenesis score (R)	n.s. 0.107	n.s. 0.142	–0.227*	n.s. 0.049

n.s. Not significant, *p<0.05, ***p<0.0001.

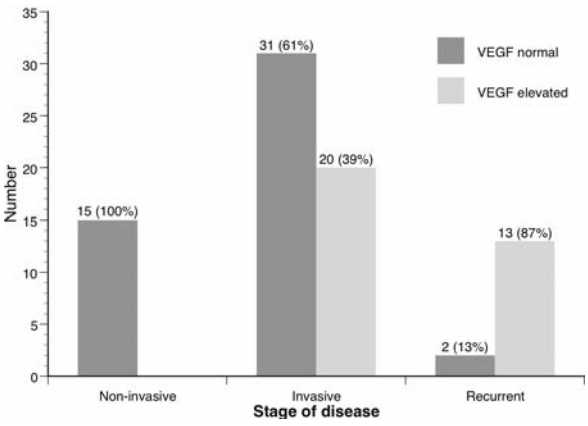

Figure 1. *Normal/elevated VEGF plasma concentrations depending on tumour stage in patients with cervical carcinoma.*

Discussion

The present study does not confirm the value of the angiogenesis score as a selective prognostic marker in patients with cervical cancer. While there was indeed a certain association between lower angiogenesis scores and better prognosis, the variation of individual values between groups did not convincingly outweigh that within groups, resulting in a lack of statistical significance, as well as of diagnostic discriminatory power.

The angiogenesis score shows no promising potential as a maker for tumour progression and its prevention by targeted modalities. The prognostic significance of a high angiogenesis score thus seems to be very limited. This may at first glance be in contrast to published material; however, studies that assert a prognostic value do so only when the angiogenesis score is combined with other criteria (see *e.g.* 7). Taken in isolation, the results of Zaghloul *et al.* (7) concerning the angiogenesis score are perfectly in keeping with those of the present study, except for a higher baseline value in our patients (~53% *vs.* 40%).

On the other hand, the clear and plausible relationship between VEGF concentrations and tumour stage, as well as grading, corroborates the key role of angiogenesis in cervical cancer spread that has been demonstrated in numerous studies (4, 8, 11, 12, 14, 16, 25-28). According to the results of the present study, the normal range of ≤115 pg/ml plasma concentration for VEGF may be suitable as a cut-off point for stratification, and, in particular, for the identification of patients developing recurrent disease during post-treatment monitoring, but this needs to be confirmed by further studies.

Consequently, the development of a tool to identify the subset of patients with a particularly poor prognosis who will benefit from more aggressive general and anti-angiogenic treatment modalities is in principle possible, but the angiogenesis score examined in the present study most likely does not provide a sufficient basis for this. It is, however, unlikely that a single circulating marker will turn out to be the 'magic wand' for an accurate prognosis prediction. More probably, a diagnostic index will have to be developed that includes a number of variables, and according to the present study, plasma VEGF concentrations will be a likely candidate for this, especially since VEGF binding and subsequent inactivation is the key mechanism of bevacizumab action (6).

However, meticulous analysis of the presently available circulating angiogenic factors performed by our group (21, 29) has failed to identify a promising approach, and therefore a closer biological model of angiogenesis-induced tumour progression such as tube formation (*e.g.* 22) may be more suitable.

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