Prognostic Role of Vascular Endothelial Growth Factor and its Receptor-1 in Patients with Esophageal Cancer

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Abstract. *Background/Aim: To present long-term results regarding the role of vascular endothelial growth factor (VEGF) and its receptor-1 (VEGFR-1) for esophageal cancer. Patients and Methods: In 68 esophageal cancer patients, VEGF, VEGFR-1 plus ten other factors were analyzed for locoregional control (LRC), metastases-free survival (MFS) and survival up to 10 years. Results: On multivariate analysis, improved LRC was associated with hemoglobin during radiotherapy ≥12 g/dl (p=0.001). VEGF-negativity showed a trend for better LRC on univariate analysis. On multivariate analysis, better MFS was associated with hemoglobin ≥12 g/dl (p=0.012), better performance status (p=0.009) and lower tumor stage (p=0.032). On multivariate analysis, improved survival was associated with hemoglobin ≥12 g/dl (p<0.001) and better performance status (p=0.005). Trends for improved survival were observed for VEGF-negativity and VEGFR-1-negativity on univariate analysis. Conclusion: VEGF showed a trend towards worse LRC and survival, VEGFR-1 towards worse survival. Outcomes were associated with hemoglobin, performance status and tumor stage.*

Although novel treatment options are available for patients with locally advanced esophageal cancer, most patients still have very poor outcomes (1-3). A relatively new concept in cancer treatment is the use of more individualized treatment approaches. This idea may be of benefit also for esophageal cancer patients. However, a good knowledge of prognostic factors is mandatory to optimally individualize therapeutic strategies. Pre-clinical prognostic factors, which have been studied more intensively during recent years, include the tumor cell expression of the vascular endothelial growth factor (VEGF) and its receptors (4, 5). These factors have mostly been investigated in patient cohorts with relatively short follow-up times including our own previous study published in 2008 (6). Therefore, the present study has been initiated, which includes a follow-up period of up to 10 years, in order to evaluate a potential long-term impact of the tumor cell expression of VEGF and its receptor 1 (VEGFR-1) on locoregional control (LRC), metastases-free survival (MFS) and survival in patients with stage III cancer of the esophagus.

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Patients and Methods

Patients. The data of 68 patients treated with radio-chemotherapy for stage III esophageal cancer were analyzed in this retrospective study. Twelve patient characteristics were evaluated for locoregional control (LRC), metastases-free survival (MFS) and survival at 3 years, at 5 years and at 10 years after radio-chemotherapy. The twelve investigated characteristics included tumor cell expression of VEGF and VEGFR-1.

Immunohistochemistry. Resected esophagus tissues were fixed in 10% buffered formalin (pH 7.0) (J.T. Baker Inc., Griesheim, Germany), embedded in paraffin and 4-μm-thick serial sections were prepared. Four-micrometer-thick serial sections were de-paraffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was carried out in 0.01 mol/l sodium citrate buffer (pH 6.0) (Sigma-Aldrich Inc., Hamburg, Germany) for 5 min in a steamer-cooker. After the blocking of endogenous peroxidase and of nonspecific binding by incubation with protein block serum (DakoCytomation, Carpinteria, CA, USA), the sections were incubated overnight at 48˚C with anti-human VEGF rabbit polyclonal antibody (clone A-20; 1/450 dilution; Santa Cruz

Biotechnologies Inc., Santa Cruz, CA, USA) and anti-human VEGFR-1 rabbit polyclonal antibody (clone H-225, 1/100 dilution; Santa Cruz Biotechnologies Inc.).

Sections were washed with tris-buffered saline (TBS) containing 0.1% Tween 20 (pH 7.0) (Carl Roth GmbH+Co. KG, Karlsruhe, Germany) and subsequent reaction was performed with the biotin-free horseradish peroxidase enzyme-labelled polymer of Envision+ detection system (DakoCytomation); diaminobenzidine (DAB) complex (Carl Roth GmbH+Co. KG) was used as chromogen. Sections were counterstained with hematoxylin. Negative controls were performed for each tumor section, first by omission of the primary antibody and secondly by incubation with normal rabbit IgG instead of the primary antibody.

Treatment. Radiotherapy was delivered with 6-16 MV photons with daily doses of 1.8 or 2.0 Gy, 5 days per week. Initial radiation fields (to 50-50.4 Gy) had superior and inferior margins of 5 cm beyond the primary gross tumor volume. The lateral, anterior and posterior margins were a minimum of 2 cm. Regional lymph nodes were included. For definitive treatment, a boost of 9-10 Gy was delivered to the primary tumor with 2 cm margins and enlarged lymph nodes with a margin ≥1 cm. Two courses of chemotherapy were administered concurrently with radiotherapy. Five-fluorouracil (5-FU) was administered as continuous infusion of 1000 mg/m^2/day for 120 hours (days 1-5 of each course) every four weeks. Cisplatin was administered as intravenous bolus of 75-80 mg/m^2 over 1 hour on day 1 of each course. Surgery for tumors of the upper and middle third was radical *en-bloc* resection of the esophagus and two-field lymphadenectomy. For tumors of the lower third, a trans-hiatal esophagectomy was performed. Esophageal continuity was restored by gastric tube.

Hemoglobin levels during radiotherapy. Because hemoglobin levels were monitored weekly during 5-6.5 weeks of irradiation, 5-6 hemoglobin levels were obtained. Two groups were formed with respect to the majority (3 of 5 or 4 of 6 levels) of hemoglobin levels during radiotherapy, <12 g/dl *versus* ≥12 g/dl. No patient treated for 6 weeks had three hemoglobin levels <12 g/dl and three levels ≥12 g/dl.

Additional potential prognostic factors. In addition to tumor cell expression of VEGF, tumor cell expression of VEGFR-1 and the hemoglobin levels during radiotherapy, the following nine patient characteristics were evaluated: age (≤60 *vs*. ≥61 years), gender, Eastern Cooperative Oncology Group (ECOG) performance score (0-1 *vs*. 2-3), tumor length (≤6 *vs*. >6 cm), tumor histology (squamous cell carcinoma (SCC) *vs*. adenocarcinoma), histologic grading (G1-2 *vs*. G3), tumor stage (T3 *vs*. T4), nodal stage (N0 *vs*. N+) and additional surgery (no *vs*. yes). In those 21 patients who received additional surgery, the impact of the resection margin (R0=no residual tumor, R1/R2=microscopic or macroscopic residual tumor) was investigated. Patient characteristics are summarized in Table I. Patients were followed until death or for a median of 104 months (range: 25-139 months) in those alive at the last follow-up.

Statistical analyses. LRC was defined as no locoregional progression due to endoscopy or endoscopic ultrasound and computed tomography. LRC, MFS and survival were calculated with the Kaplan-Meier method (3) and measured from the last day of radiotherapy. Differences between the Kaplan-Meier curves were evaluated with the log-rank test. Results were considered significant if $p<0.05$. Factors being significant or showing a trend ($p\leq0.09$) on univariate analysis were included in a multivariate analysis, which was done with the Cox proportional hazard model.

Table I. *Patients' characteristics*.

Potential prognostic factor	N patients (%)
VEGF expression	
No	10 (15)
Yes	55 (81)
Unknown	3 (4)
VEGFR-1 expression	
No	13 (19)
Yes	54 (79)
Unknown	1 (1)
Hemoglobin during radiotherapy	
<12 g/dl	31 (46)
≥12 g/dl	37 (54)
Age	
≤60 years	36 (53)
≥61 years	32 (47)
Gender	
Female	9 (13)
Male	59 (87)
ECOG performance score	
0-1	46 (68)
2-3	22 (32)
Tumor length	
≤6 cm	31 (46)
>6 cm	37 (54)
Tumor stage	
T3	35 (51)
T4	33 (49)
Nodal stage	
N0	10 (15)
N+	58 (85)
Histology	
Squamous cell carcinoma	55 (81)
Adenocarcinoma	13 (19)
Histologic grade	
G1-2	37 (54)
G3	31 (46)
Surgery	
No	47 (69)
Yes	21 (31)

VEGF, Vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor receptor-1; ECOG, Eastern Cooperative Oncology Group.

Results

The LRC rates at 3, 5 and 10 years in the entire cohort were 29%, 21% and 18%, respectively. On univariate analysis (Table II), improved LRC was significantly associated with hemoglobin levels during radiotherapy of ≥12 g/dl ($p<0.001$). VEGF-negativity ($p=0.059$) and T3-stage were of

Table II. *Results of the univariate analysis of locoregional control.*

Potential prognostic factor	At 3 years (%)	At 5 years (%)	At 10 years (%)	*p-Value*
VEGF expression				
No	45	45	30	
Yes	20	12	8	0.059
VEGFR-1 expression				
No	43	43	32	
Yes	21	13	8	0.27
Hemoglobin during RT				
<12 g/dl	0	0	0	
≥12 g/dl	42	33	24	<0.001
Age				
≤60 years	33	22	16	
≥61 years	20	20	13	0.29
Gender				
Female	n.a.	n.a.	n.a.	
Male	28	21	15	0.45
ECOG performance score				
0-1	27	20	20	
2-3	21	21	0	0.88
Tumor length				
≤6 cm	22	22	17	
>6 cm	31	19	12	0.70
Tumor stage				
T3	40	31	22	
T4	7	n.a.	n.a.	0.053
Nodal stage				
N0	10	n.a.	n.a.	
N+	31	24	17	0.36
Histology				
Squamous cell carcinoma	22	22	16	
Adenocarcinoma	46	0	0	0.45
Histologic grade				
G1-2	35	27	19	
G3	8	n.a.	n.a.	0.32
Surgery				
No	22	22	16	
Yes	34	20	14	0.65

n.a., Not available; VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor-receptor 1; RT, radiotherapy, ECOG: Eastern Cooperative Oncology Group.

Table III. *Results of the univariate analysis of metastases-free survival.*

Potential prognostic factor	At 3 years (%)	At 5 years (%)	At 10 years (%)	*p-Value*
VEGF expression				
No	46	46	30	
Yes	33	33	25	0.39
VEGFR-1 expression				
No	48	48	36	
Yes	33	33	25	0.40
Hemoglobin during RT				
<12 g/dl	13	n.a.	n.a.	
≥12 g/dl	47	47	36	0.021
Age				
≤60 years	35	35	28	
≥61 years	37	37	25	0.80
Gender				
Female	n.a.	n.a.	n.a.	
Male	36	36	28	0.62
ECOG performance score				
0-1	46	46	46	
2-3	16	16	0	0.041
Tumor length				
≤6 cm	41	41	30	
>6 cm	33	33	25	0.25
Tumor stage				
T3	49	49	38	
T4	20	n.a.	n.a.	0.018
Nodal stage				
N0	50	n.a.	n.a.	
N+	33	33	25	0.10
Histology				
Squamous cell carcinoma	36	36	26	
Adenocarcinoma	37	37	n.a.	0.77
Histologic grade				
G1-2	40	40	30	
G3	32	n.a.	n.a.	0.44
Surgery				
No	29	29	22	
Yes	50	50	38	0.11

n.a., Not available; VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor-receptor 1; RT, radiotherapy; ECOG, Eastern Cooperative Oncology Group.

borderline significance (p=0.053). VEGFR-1 expression (p=0.12) had no significant impact on LRC. In the multivariate analysis of LRC, hemoglobin levels during radiotherapy remained significant (risk ratio [RR]= 3.09; 95%-confidence interval [CI]= 1.57-6.29; p=0.001), whereas VEGF expression (RR= 1.55; 95%-CI= 0.66-4.23; p=0.33) and T-stage (RR= 1.22; 95%-CI= 0.65-2.34; p=0.53) were not significant. In those 21 patients who received additional surgery, LRC was better after R0-resection than after R1/R2-resection at 3 years (55% *vs*. 0%), at 5 years (33% *vs*. 0%) and at 10 years (22% *vs*. 0%) (p=0.002).

MFS rates at 3, 5 and 10 years were 36%, 36% and 28%, respectively. On univariate analysis (Table III), MFS was significantly better regarding hemoglobin levels during radiotherapy of ≥12 g/dl (p=0.021), an ECOG performance score of 0-1 (p=0.041) and T3-stage (p=0.018). Tumor cell expressions of VEGF (p=0.39) and VEGFR-1 (p=0.040) had no significant impact on MFS. In the multivariate analysis of MFS, hemoglobin levels (RR= 2.48; 95%-CI= 1.23-5.05; p=0.012), ECOG performance score (RR= 2.51; CI= 1.27-4.90; p=0.009) and T-stage (RR= 2.06; 95%-CI= 1.06-4.10; p=0.032) achieved significance. In those 21 patients who received additional surgery, MFS rates after R0-resection at 3, at 5 and at 10 years were 71%, 71% and 53%, respectively, and not available for R1/R2-resection (p=0.010).

Survival rates at 3, 5 and 10 years were 19%, 17% and 11%, respectively. On univariate analysis (Table IV), improved survival was significantly associated with hemoglobin levels during radiotherapy of ≥12 g/dl ($p<0.001$), male gender ($p=0.007$) and T3-stage ($p=0.008$). A trend was seen for VEGF-negativity ($p=0.07$), VEGFR-1-negativity ($p=0.08$), ECOG performance score of 0-1 ($p=0.09$) and additional surgery ($p=0.07$). In the multivariate analysis of survival (Table V), hemoglobin levels ($p<0.001$) and the ECOG performance score ($p=0.005$) were significant, while T-stage showed a trend ($p=0.08$). In the 21 patients receiving additional surgery, survival was better ($p<0.001$) after R0-resection than after R1/R2-resection at 3 years (62% *vs*. 0%), at 5 years (62% *vs*. 0%) and at 10 years (33% *vs*. 0%).

Table IV. *Results of the univariate analysis of survival.*

Potential prognostic factor	At 3 years (%)	At 5 years (%)	At 10 years (%)	*p-Value*
VEGF expression				
No	40	40	27	
Yes	14	12	6	0.07
VEGFR-1 expression				
No	38	38	29	
Yes	14	12	6	0.08
Hemoglobin during RT				
<12 g/dl	3	0	0	
≥12 g/dl	32	32	21	<0.001
Age				
≤60 years	19	19	12	
≥61 years	18	14	9	0.48
Gender				
Female	0	0	0	
Male	22	20	13	0.007
ECOG performance score				
0-1	24	21	18	
2-3	9	9	0	0.09
Tumor length				
≤6 cm	22	17	13	
>6 cm	16	16	9	0.28
Tumor stage				
T3	31	31	20	
T4	6	0	0	0.008
Nodal stage				
N0	10	n.a.	n.a.	
N+	21	18	12	0.77
Histology				
Squamous cell carcinoma	18	16	11	
Adenocarcinoma	23	23	n.a.	0.61
Histologic grade				
G1-2	27	27	18	
G3	9	0	0	0.11
Surgery				
No	13	10	8	
Yes	33	33	17	0.07

n.a., Not available; VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor-receptor 1; RT, radiotherapy; ECOG, Eastern Cooperative Oncology Group.

Discussion

Prognostic factors are important instruments to provide an optimal individualized treatment for cancer patients. Most patients with locally advanced cancer of the esophagus have poor prognoses. However, long-term survivors do exist. It is important to identify such patients prior to the start of treatment, since the patient's prognosis likely has a major impact on the treatment approach to be selected for him or her. For example, since the risk of radiotherapy and chemotherapy late sequelae increases with time, the possibility of such potential adverse effects must be particularly emphasized to patients with a long expected survival.

In the present study, patients who had received radio-chemotherapy for locally advanced cancer of the esophagus were followed-up to 10 years. According to the multivariate analyses of our study, LRC was associated with the hemoglobin levels during radiotherapy, MFS with the hemoglobin levels during radiotherapy, the ECOG performance score, and the tumor stage, and survival with the hemoglobin levels during radiotherapy and the ECOG performance score. These findings are different from those of our preceding study with a shorter follow-up period (6). In the multivariate analyses of the preceding study, only the hemoglobin levels during radiotherapy achieved significance for LRC and survival. This emphasizes the importance of reporting long-term results. Results regarding MFS were not presented in our study published in 2008 (6).

In the univariate analyses of the current long-term study, tumor cell expression of VEGF showed a strong trend towards worse LRC ($p=0.059$) and a trend towards worse survival ($p=0.07$). VEGFR-1 expression showed a trend towards worse survival ($p=0.08$). In contrast to these findings, our previous study suggested a negative association with the patients' prognosis only for the expression of VEGF. Since the negative associations between tumor cell expression of VEGF and VEGFR-1 could not be confirmed in the corresponding multivariate analyses, one should be quite cautious when using these factors for treatment decisions.

On the other hand, two recent meta-analyses of retrospective studies suggested a negative impact of VEGF overexpression on the survival of esophageal cancer patients with a hazard ratio of 1.80 (95%-CI= 1.51-2.14) and a risk ratio of 1.26 (95%-CI= 1.16-1.37), respectively (4, 5). Further retrospective studies that were published following the meta-analysis supported these findings. Kozlowski *et al*. suggested that pre-treatment VEGF levels reflect lymph

Table V. *Multivariate analysis of survival*.

	Risk ratio	95%-Confidence interval	*p*-Value
VEGF expression	1.41	0.49-4.37	0.53
VEGFR-1 expression	1.34	0.57-3.63	0.53
Hemoglobin during RT	2.94	1.58-5.59	<0.001
Gender	1.96	0.86-4.03	0.10
ECOG performance score	2.41	1.30-4.43	0.005
Tumor stage	1.64	0.94-2.90	0.08
Surgery	1.31	0.63-2.79	0.47

VEGF, Vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor-receptor 1; RT, radiotherapy; ECOG, Eastern Cooperative Oncology Group.

node metastases and advanced stage disease in patients with cancer of the esophagus (8). Similar findings were observed by Sun *et al*. who reported 3-year results of 82 patients receiving surgery for esophageal cancer (9), and by Tanaka *et al*. who presented a series of 106 patients undergoing radical esophagectomy (10). In the latter study, VEGF expression levels were higher in N+ patients and associated with worse survival. In the retrospective study of Liu et al. of 73 patients with SCC of the esophagus, median survival times were 10.4 months for patients with a VEGF-positive tumor and 28.5 months for those with a VEGF-negative tumor (p=0.003) (11).

An important finding of our present study was the negative impact of anemia (hemoglobin levels <12 g/dl) during radiotherapy on treatment outcomes. This result was found in our preceding study from 2008 with a shorter follow-up as well as in another study published in 2006 (6, 12). Anemia results in an impairment of tumor oxygenation (13). Adequate oxygenation of the tumor tissue is important for a maximum effect of irradiation. The tumor killing effect of radiotherapy is dependent on the presence of oxygen as it is primarily due to the production of radiation-induced cytotoxic free oxygen-radicals. The oxygen radicals lead to radiation-induced DNA damage which kills tumor cells.

In addition, treatment outcomes were significantly associated with performance status and tumor stage in the present long-term study. These prognostic factors were previously identified by other authors (1, 3, 14). For example, in a study of 154 patients treated with radiotherapy or radio-chemotherapy for esophageal cancer, advanced tumor stage was significantly associated with survival in both univariate and multivariate analyses (1). In a patterns-of-care study of 400 patients, both performance status (Karnofsky performance score 90-100 *vs*. 60-80; RR= 0.61; 95%-CI= 0.46-0.86; p=0.004) and tumor stage (stage I/II *vs*. stage III; RR= 0.66; 95%-CI= 0.47-0.93; p=0.017) were significantly associated with survival in the multivariate analysis (3).

In conclusion, in this study presenting long-term results up to 10 years following radio-chemotherapy, better treatment outcomes were significantly associated with hemoglobin levels during radiotherapy of ≥12 g/dl, an ECOG performance score of 0-1 and T3-stage. Tumor cell expression of VEGF showed a trend towards worse LRC and survival, tumor cell expression of VEGFR-1 a trend towards worse survival. These findings can help the physician when designing individualized-therapy for patients with stage III cancer of the esophagus.

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