Biologic Factors Associated with Tumor Oxygenation Are Prognostic in Patients with Stage III Esophageal Cancer: Long-term Results

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Abstract. *Background/Aim: Long-term results of a study investigating potential prognostic factors for treatment outcomes in patients with stage III esophageal cancer are presented. Patients and Methods: In 64 patients, the impact of tumor cell expression of erythropoietin (EPO) and erythropoietin-receptor (EPO-R) and ten additional factors (age, gender, performance status, tumor length, tumor stage (T-stage), nodes (N-stage), histology/grading, hemoglobin levels during radiotherapy, surgery) on survival and loco-regional control was evaluated up to 10 years following radio-chemotherapy. Results: On multivariate analysis, improved survival was associated with low EPO-R expression (p=0.034) and hemoglobin levels during radiotherapy ≥12 g/dl (p=0.026). Low EPO expression was associated with survival on univariate (p=0.010) but not on multivariate analysis (p=0.42). On multivariate analysis, improved loco-regional control was significantly associated with hemoglobin levels during radiotherapy ≥12 g/dl (p<0.001). Conclusion: The long-term results confirm that hemoglobin levels during radiotherapy and tumor cell expression of EPO-R are significant prognostic factors in patients with locally advanced esophageal cancer.*

Patients with stage III esophageal cancer have a very poor survival prognosis with currently available therapies (1). Improvements may be achieved with novel personalized treatment approaches integrating more information regarding prognostic factors and potential treatment targets. Prognostic factors can support the physician when selecting the most appropriate treatment approach for the individual patient. For patients with a locally advanced non-metastatic esophageal cancer, several prognostic factors have already been identified. However, prognostic factors were commonly identified in studies with a relatively short follow-up period. It would be interesting to assess the consistency of prognostic factors in long-term survivors of locally advanced esophageal cancer.

In the present study, we present the long-term results of our study including 64 esophageal cancer patients treated with radio-chemotherapy up to 10 years following treatment (2). The follow-up of our initial study was two years. The current study also focuses in particular on the prognostic impact of factors involved in tumor oxygenation including the tumor cell expression of erythropoietin (EPO) and its receptor (EPO-R), as well as on the hemoglobin levels during radiotherapy.

Patients and Methods

Patients. A total of 64 patients, who received radio-chemotherapy for stage III esophageal cancer, were included in this study. In this cohort of patients, twelve potential prognostic factors were investigated with respect to survival and loco-regional control up to 10 years following treatment. These potential prognostic factors included the tumor cell expression of EPO and EPO-R. Low expression (0-20%) was compared to higher expression (>20%).

Immunohistochemistry. Esophagus tissue samples were fixed in 10% buffered formalin/pH 7.0 (J.T. Baker, 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, Hamburg, Germany) for 5 minutes in a steamer-cooker. Endogenous

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peroxidase was blocked with 0.3% hydrogen peroxidase for 5 min. The sections were incubated with the anti-human EPO rabbit polyclonal antibody (clone H-162; 1/50 dilution; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA) and anti-human EPO-R rabbit polyclonal antibody (clone C-20, 1/450 dilution; Santa Cruz Biotechnologies). Anti-human EPO rabbit polyclonal antibody was raised against a recombinant protein corresponding to amino acids 28 to 189, representing mature EPO of human origin, whereas the anti-human EPO-R antibody was raised against a peptide mapping at the carboxyl terminal of EPO-R of human origin. Sections were then washed with tris-buffered saline (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) containing 0.1% Tween 20 (pH 7.0) and subsequent reaction was performed with the biotin-free horseradish peroxidase enzyme-labelled polymer of Envision+ detection system (DakoCytomation, Carpinteria, CA, USA); diaminobenzidine (DAB) complex (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was used as chromogene. Sections were counterstained with hematoxylin. Negative controls were done for each tumor section, first by omission of the primary antibody and secondly by incubation with the normal rabbit IgG (dilution 1/200, 2 μg/mL concentration, Santa Cruz Biotechnologies) instead of the primary antibody.

Treatment. Radiotherapy was delivered with 6-16 MV photons from a linear accelerator (Siemens Medical Solutions, Concord, CA, USA) with daily doses of 1.8 or 2.0 Gy, 5 days per week. Initial radiotherapy 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. 5-fluorouracil (5-FU) (TEVA GmbH, Ulm, Germany) was administered as continuous infusion of 1,000 mg/m^2/day for 120 h (days 1-5 of each course) every four weeks. Cisplatin (medac GmbH, Wedel, Germany) 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 radiotherapy, 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 EPO and EPO-R and hemoglobin levels during radiotherapy, the following potential prognostic factors were investigated: age (≤60 *vs*. >60 years), gender, Karnofsky Performance Score (KPS) (≤80 *vs*. >80), tumor length (≤6 *vs*. >6 cm), histology (squamous cell carcinoma (SCC) *vs*. adenocarcinoma), histologic grade (G1-2 *vs*. G3), T-stage (T3 *vs*. T4), N-stage (N0 *vs*. N+) and additional surgery (no *vs*. yes). In the 20 patients receiving surgery, the impact of the resection margin (R0=no residual tumor, R1/R2=microscopic or macroscopic residual tumor) was investigated. Patient characteristics are shown in Table I.

Table I. *Patient characteristics*.

Potential prognostic factor	N patients (%)
Age	
≤60 years	33 (52)
>60 years	31 (48)
Gender	
Female	8 (13)
Male	56 (88)
Karnofsky Performance Score	
≤80	35 (55)
>80	29 (45)
Tumor length	
≤6 cm	31 (48)
>6 cm	33 (52)
T-Stage	
T3	34 (53)
T4	30 (47)
N-stage	
N0	10 (16)
N1	54 (84)
Histology	
SCC	51 (80)
Adenocarcinoma	13 (20)
Histologic grade	
G1-2	36 (56)
G3	28 (44)
Surgery	
No	44 (69)
Yes	20 (31)
Hemoglobin levels during radiotherapy	
<12 g/dl	27 (42)
≥12 g/dl	37 (58)
EPO-R-expression	
≤20%	29 (45)
>20%	31 (48)
EPO-expression	
≤20%	26 (41)
>20%	35 (55)

Statistical analyses. Loco-regional control was defined as absence of loco-regional progression based on findings of endoscopy, endoscopic ultrasound, or computed tomography. Survival and loco-regional control 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 $p<0.05$. Those factors being significant or showing a trend ($p\leq0.08$) in the univariate analysis were additionally included in a multivariate analysis, performed with the Cox proportional hazard model.

Results

Patients were followed-up until death or for a median of 84 months (range=25-139 months) in survivors. In the entire cohort, the survival rates at 3 years, 5 years and 10 years following radio-chemotherapy were 21%, 19% and 14%,

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

Potential prognostic factor	At 3 years (%)	At 5 years (%)	At 10 years (%)	*p*-Value
Age				
≤60 years	24	24	19	
>60 years	19	14	9	0.28
Gender				
Female	0	0	0	
Male	24	22	17	0.009
Karnofsky Performance Score				
≤80	8	8	0	
>80	38	33	28	0.004
Tumor length				
≤6 cm	28	23	17	
>6 cm	15	15	11	0.18
T-Stage				
T3	35	35	26	
T4	7	0	0	0.008
N-stage				
N0	10	n.a.	n.a.	
N1	24	21	16	0.98
Histology				
SCC	21	19	15	
Adenocarcinoma	23	23	n.a.	0.71
Histological grade				
G1-2	28	28	21	
G3	13	n.a.	n.a.	0.24
Surgery				
No	14	11	8	
Yes	39	39	29	0.026
Hemoglobin levels during radiotherapy				
<12 g/dl	4	0	0	
≥12 g/dl	34	34	26	<0.001
EPO-R-expression				
≤20%	31	31	21	
>20%	15	10	10	0.08
EPO-expression				
≤20%	35	35	29	
>20%	9	4	0	0.010

n.a.=Not available.

Table III. *Results of the univariate analysis of loco-regional control.*

Potential prognostic factor	At 3 years (%)	At 5 years (%)	At 10 years (%)	*p*-Value
Age				
≤60 years	37	22	22	
>60 years	20	20	13	0.27
Gender				
Female	30	23	19	
Male	29	21	18	0.26
Karnofsky Performance Score				
≤80	19	19	0	
>80	39	28	28	0.08
Tumor length				
≤6 cm	30	30	22	
>6 cm	28	14	14	0.32
T-Stage				
T3	48	36	30	
T4	6	n.a.	n.a.	0.006
N-stage				
N0	10	n.a.	n.a.	
N+	34	25	21	0.35
Histology				
SCC	24	24	20	
Adenocarcinoma	46	0	0	0.50
Histological grade				
G1-2	36	27	22	
G3	15	n.a.	n.a.	0.37
Surgery				
No	22	22	17	
Yes	39	19	19	0.56
Hemoglobin levels during radiotherapy				
<12 g/dl	0	0	0	
≥12 g/dl	46	34	29	<0.001
EPO-R-expression				
≤20%	38	26	19	
>20%	16	16	16	0.24
EPO-expression				
≤20%	37	31	25	
>20%	10	0	0	0.18

n.a.=Not available.

respectively. In the univiariate analysis, survival was significantly associated with tumor cell expression of EPO ≤20% (p=0.010), male gender (p=0.009), KPS >80 (p=0.004), T3-stage (p=0.008), surgery (p=0.026) and hemoglobin levels during radiotherapy ≥12 g/dl (p<0.001). Low expression of EPO-R showed a trend towards better overall survival (p=0.08). The results of the univariate analysis are given in Table II. On multivariate analysis, survival was significantly associated with expression of EPO-R (risk ratio [RR]: 1.94; 95%-confidence interval [CI]: 1.05-3.61; p=0.034) and hemoglobin levels during radiotherapy (RR: 2.25; 95%-CI: 1.10-4.61; p=0.026). KPS showed a trend (RR: 1.86; 95%-CI: 0.96-3.70; p=0.067). Tumor cell expression of EPO (RR: 1.34; 95%-CI: 0.66-2.74; p=0.42), gender (RR: 1.79; 95%-CI: 0.69-4.15; p=0.22), T-stage (RR: 1.62; 95%-CI: 0.77-3.50; p=0.20) and surgery (RR: 1.54; 95%-CI: 0.68-3.57; p=0.30) were not significant in the multivariate analysis.

In the entire cohort, the loco-regional control rates at 3, 5 and 10 years following radio-chemotherapy were 29%, 21% and 18%, respectively. On univariate analysis, better loco-regional control was significantly associated with T3-stage (p=0.006) and hemoglobin levels ≥12 g/dl during radiotherapy (p<0.001). Tumor cell expression of EPO

(p=0.18) and EPO-R (p=0.24) had no significant impact on loco-regional control. The results of the univariate analysis of loco-regional control are summarized in Table III. In multivariate analysis of loco-regional control, hemoglobin levels during radiotherapy maintained significance (RR: 3.44; 95%-CI: 1.75-6.94; p<0.001), whereas T-stage was not significant (RR: 1.64; 95%-CI: 0.84-3.28; p=0.15).

Discussion

The present study presents the long-term results up to 10 years following treatment of a study that investigated the potential prognostic impact of twelve potential prognostic factors with respect to survival and loco-regional control in patients receiving radio-chemotherapy for stage III esophageal cancer (2). The twelve potential prognostic factors included the tumor cell expression of EPO and its receptor EPO-R. In our previous report with a follow-up period of 2 years, high EPO expression (p=0.049) was negatively associated with survival in the univariate analysis and high EPO-R expression showed a trend towards worse survival (p=0.09) (2). On multivariate analysis, EPO expression did not achieve significance (p=0.86). In the present analysis with a follow-up period of up to 10 years, the results of the univariate analysis of survival have been confirmed. The p-values were p=0.010 for EPO expression and p=0.08 for EPO-R expression. In the multivariate analysis of the current study, EPO-R expression achieved significance, which is different from the previous study. EPO expression was not significant in the multivariate analysis of survival, similar to the preceding study. However, it was negatively associated with survival in the univariate analysis. This finding is consistent with the scarce data from the literature. Acs *et al*. suggested increased EPO expression to be associated with a worse prognosis in a retrospective study of 107 patients with uterine cancer (4). Another retrospective study of Seibold *et al*. that investigated 144 head-and-neck cancer patients has suggested that both EPO expression (RR: 4.77; p=0.003) and EPO-R expression (RR: 2.36; p=0.010) of tumor cells are independent prognostic factors negatively-associated with survival (5). In addition, Vukelic *et al*. presented the hypothesis that less differentiated laryngeal carcinomas will have a higher level of endogenous expression of EPO and EPO-R (6). Therefore, one can expect less favorable outcomes for tumors with EPO- and EPO-R-expression.

In addition to low tumor cell expression of EPO-R, higher hemoglobin levels during radiotherapy (majority ≥12 g/dl) were associated with improved survival in multivariate analysis. This finding has already been observed in the preceding study with a follow-up of 2 years and another retrospective study (2, 7). It can be explained by the fact that the effect of radiotherapy depends on tumor oxygenation, which is poor in case of anemia (8). Tumor hypoxia leads to a decreased production of radiation inducing cytotoxic-free radicals resulting in both decreased radiation-induced DNA damage and less tumor cell kill.

In the multivariate analyses of the present long-term study, a better performance status (Karnofsky performance score >80) showed a trend towards improved survival (p=0.067). In the multivariate analysis of the preceding study, the association between performance status and survival had even been significant (RR: 1.88; p=0.045). The prognostic value of the performance status has also been previously described by other authors (9-11).

In conclusion, the present long-term results mostly confirm the findings of the preceding study. Improved survival was associated with low (≤20%) tumor cell expression of EPO and EPO-R, and with the majority of hemoglobin levels during radiotherapy being ≥12 g/dl. A Karnofsky performance score of >80 showed a trend towards better survival. Loco-regional control was positively-associated with hemoglobin levels during radiotherapy of ≥12 g/dl. These biological factors associated with oxygenation are prognostic factors that may help physicians to better personalize the treatment of patients with stage III esophageal cancer.

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