Preradiotherapy Hemoglobin Level but not Microvessel Density Predicts Locoregional Control and Survival in Laryngeal Cancer Treated with Primary Radical Radiotherapy

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ABSTRACT

Purpose: **To evaluate the roles of preradiotherapy hemoglobin level and microvessel density (MVD) as predictive factors for tumor control and survival in patients with laryngeal cancer treated with primary radiotherapy.**

Experimental Design: **Two hundred and fourteen patients with stage I-IV laryngeal cancer were included in the analysis. Patients were treated with once daily fractionated radiotherapy over 6.5 weeks or twice daily fractionated radiotherapy over 4.5 weeks up to total doses of 62 to 68 Gy. Preradiotherapy hemoglobin levels were obtained from patient journals, and pretreatment tumor biopsies were stained with CD34 antibody for the counting of microvessels. The prognostic implication of preradiotherapy hemoglobin level and MVD on tumor control and survival was tested.**

Results: **Five-year locoregional control probability was 88.9% for patients with preradiotherapy hemoglobin levels >137.5 g/L (median) and 64.4% for patients with preradiotherapy hemoglobin levels <137.5 g/L ($P = 0.01$). The corresponding figures for disease-free survival were 87.8 and 62.8% ($P = 0.007$), respectively, and for overall survival 58.1 and 40.3% ($P < 0.001$), respectively.**

In multivariate analysis, tumor stage and preradiotherapy hemoglobin level were significant prognostic factors for locoregional control and disease-free survival, whereas tumor stage, preradiotherapy hemoglobin-level, gender, and age were significant prognostic factors for overall survival. No correlation was found between MVD and tumor control and survival.

Conclusion: **Preradiotherapy hemoglobin level, but not MVD, predicts locoregional control and survival in patients with laryngeal cancer treated with radiotherapy.**

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INTRODUCTION

The two main treatment modalities for laryngeal cancer are radiotherapy and surgery, each with similar results for local disease control and survival. In our institution, as in many others, we usually choose radiotherapy as the initial treatment with surgery saved for salvage because of recurrences. The main rationale for this strategy is that radiotherapy offers a possibility to preserve laryngeal function. For early laryngeal cancer (glottic $T_1T_2N_0M_0$ tumors) treated with radiotherapy in our institution, we have reported a 5-year locoregional control of 85% (1). The corresponding results for advanced laryngeal cancer are 49% for T_3 and 75% for T_4 tumors, respectively, with a larynx preservation rate for the surviving patients of 91% (2).

Given these results, which are comparable with the results from other institutions, advanced-stage tumors are clearly more difficult to cure by radiotherapy, and tumor stage is known to be one of the strongest prognostic factors for the outcome of laryngeal cancer. This may be explained partly by the greater tumor volume and in some cases by tumor invasion of cartilage, both of which have been indicated to be negative prognostic factors for radiotherapy. Other factors found to be of prognostic importance are nodal involvement, gender, impaired vocal cord mobility, and overall treatment time.

However, these prognostic factors are not enough to predict the outcome of radiotherapy for the individual patient. The results above reflect that 15% of patients with small, good-prognosis tumors are not cured by radiotherapy and must undergo salvage laryngectomy and that some of them will even die from their laryngeal cancer. Conversely, a number of patients with advanced-stage, poor-prognosis tumors are cured by radiotherapy and retain good laryngeal function. It is therefore desirable to find more sophisticated biological factors to predict the outcome of radiotherapy for laryngeal cancer.

In this study, we have investigated two such possible factors: preradiotherapy hemoglobin level and microvessel density (MVD), describing the blood oxygen-binding capacity and tumor vascularization of the patient, respectively. Both factors could reflect tumor oxygenation, which is highly important for the response of tumor cells to radiotherapy. Concerning hemoglobin level, reduced tumor oxygenation has thus far only been identified in patients with very low hemoglobin levels Tumor oxygenation can be measured with polarographic techniques, and hypoxic tumors have been shown to have a worse prognosis compared with oxic tumors when treated with radiotherapy (3). Pretreatment hemoglobin level has been reported to be an important prognostic factor for local control and/or survival in various malignancies including cancer of the cervix, lung, prostate, and head and neck (4, 5). Studies done on laryngeal cancer exclusively have reported similar results (6–10). However, in two of these studies the hemoglobin level at the end of treat-

ment, but not before treatment, was found to be a substantial prognostic factor (7, 10).

Tumor vascularization, measured as MVD in histologic specimens, has also been identified as a prognostic factor in various tumors, both in patients treated with surgery or in those treated with radiotherapy. Some studies have found that tumors with a high vascular density have a poorer prognosis than tumors with lower vascularization (11–13), whereas other studies have found the opposite results (14–16).

Many studies investigating the prognostic value of hemoglobin level and tumor vascularization in head and neck cancer have used a heterogeneous study population, with various tumor sites and stages and sometimes with large variations in treatment. The aim of this study was to retrospectively investigate the prognostic significance of preradiotherapy hemoglobin level and MVD on tumor control and survival in 214 patients with laryngeal cancer exclusively treated with radiotherapy in a uniform manner at the Department of Oncology, Sahlgrenska University Hospital, during the period between 1990 and 1998.

MATERIALS AND METHODS

Patients. A total of 214 patients (191 men and 23 women) with primary squamous cell carcinoma of the larynx were diagnosed and treated with curative intent with radiotherapy at the Department of Oncology, Sahlgrenska University Hospital, during the period 1990 to 1998.

Patients were referred to our clinic from a number of hospitals in the southwestern region of Sweden. The diagnostic work-up consisted of clinical examination, evaluation, and biopsy under general anesthesia and a chest X-ray. Patients with stage III and IV tumors also had a computed tomography or magnetic resonance imaging of the larynx and neck as part of their diagnostic work up. Before treatment all patients were examined, the tumors staged, and the treatment discussed at a joint tumor conference including otorhinolaryngologists, oncologists, radiologists, and histopathologists. The medical records of patients were reviewed for standard demographic data, pretreatment classification, and staging of the tumor, preradiotherapy hemoglobin level, mode of radiotherapy (once daily fractionated or accelerated, twice daily fractionated), and outcome of disease. Tumors were classified and staged according to the 1987 version of the Union Internationale Contre le Cancer staging system (17). Ninety-three patients had stage I disease, 45 patients had stage II, 35 patients had stage III, and 41 patients had stage IV disease. Forty-eight patients, all with stage III (18 patients) or IV (30 patients) disease, received 1 to 3 cycles of induction chemotherapy with cisplatin and 5-fluorouracil before radiotherapy.

The mean and median ages were 66.5 and 68.7 years, respectively (range 21.8–90.7).

Table 1 shows some patient and tumor characteristics.

Information on preradiotherapy hemoglobin level was available for 209 of the patients. Evaluable archival pathologic specimens from pretreatment diagnostic biopsies were available for 170 of the patients. Histologic sections from the pathologic specimens were reviewed by one observer (B. M.) to confirm the diagnosis of laryngeal squamous cell carcinoma. Informed consent was obtained from patients still alive at the time of analysis, and the Research Ethics Committee at the University of Göteborg approved the study.

Table 1 Patient and tumor characteristics

Characteristics	No. of patients (%)
Male gender	191 (89)
Female gender	23 (11)
Median age (years)	68.7
Range	(21.8–90.7)
Tumor site	
Supraglottic	29 (13.5)
Glottic	162 (76)
Subglottic	8 (3.5)
Transglottic	15 (7)
Differentiation	
WDSCC	41 (19)
MDSCC	105 (49)
PDSCC	47 (22)
SCC	21 (10)
T-classification	
T_1	94 (44)
T_2	50 (23)
T_3	29 (14)
T_4	41 (19)
Nodal involvement	
N_0	194 (91)
N_+	20 (9)
UICC stage	
I	93 (43.5)
II	45 (21)
III	35 (16.5)
IV	41 (19)
Fractionation	
Once daily	101 (47)
Twice daily	113 (53)
Induction chemotherapy	
Yes	48 (22)
No	166 (78)

Abbreviations: WDSCC, well differentiated squamous cell carcinoma, MDSCC, moderately differentiated squamous cell carcinoma, PDSCC, poorly differentiated squamous cell carcinoma, SCC, squamous cell carcinoma with degree of differentiation not specified; UICC, Union Internationale Contre le Cancer (International Union Against Cancer).

Chemotherapy. Forty-eight patients with stage III (18 patients) or IV (30 patients) disease received induction chemotherapy with 1 to 3 cycles of cisplatin and 5-fluorouracil. This treatment procedure has been described in detail in an earlier report from our institution (2).

Radiotherapy. All patients were immobilized in a plastic mask and treated on linear accelerators with 4 to 6 mega voltage (MV) photon beams. A conformal three-dimensional radiotherapy technique, mainly with multi-leaf collimator, was used for all patients. The planning target volume consisted of gross tumor volume with a margin of 2 cm, but at least the whole larynx, and positive neck nodes. For stage III and IV tumors, bilateral neck nodes including the supraclavicular nodes were included in the adjuvant target volume. Most patients were treated by two lateral fields often with a slight anterior tilt of 10° to 20°. The fields were also often noncoplanar with a couch rotation of 5° to 10°. The reason that we used a combination of anteriorly directed gantry angles and couch rotation was to avoid the shoulders of the patient. To obtain a homogeneous

dose distribution in the whole planning target volume, low-weighted anterior and posterior fields were added in some cases.

One hundred and one patients (86 T_1, 5 T_2, 2 T_3, and 8 T_4) were treated with radiotherapy, receiving one daily fraction up to a total dose of 62 to 66 Gy over 6.5 weeks. One hundred and thirteen patients (8 T_1, 45 T_2, 27 T_3, and 33 T_4) were treated with accelerated radiotherapy (twice daily fractionated) over 4.5 weeks. Five of these patients were treated with two daily fractions with a concomitant boost technique up to 68 Gy. The other 108 patients were treated with a split-course, hyperfractionated accelerated radiotherapy regimen, receiving two daily fractions of 1.7 Gy up to a total dose of 61.2 to 68.0 Gy. This regimen has been described in more detail in previous reports from our institution (1–2).

A computer-based three-dimensional radiotherapy planning program was used to calculate the target dose, and the dose was prescribed at the iso-center in the middle of the planning target volume. The 95% iso-dose of the prescribed dose surrounded the planning target volume.

Follow-up. All patients had their first follow-up examination 6 to 8 weeks after completion of radiotherapy. They were then routinely evaluated under general anesthesia at 3 to 4 months after completion of radiotherapy, and if there was any suspicion of local recurrence, a biopsy was taken. Patients were then followed-up with a clinical examination every 3 months for the first 2 years, every 4 months during the 3rd year, and biannually during the 4th and 5th year. After 5 years, follow-up was scheduled once a year. Most patients were followed-up at our clinic for the entire study period. However, some patients, especially elderly ones with long travel distances, were followed-up at our clinic during the 1st and 2nd year and thereafter at the department of otorhinolaryngology at their local hospital. Patients who developed a local recurrence and underwent salvage laryngectomy mostly continued their follow-up at the department of otorhinolaryngology in our hospital after their laryngectomy. The mean and median follow-up times were 34.7 and 28.5 months, respectively (range 0.9–118.2 months). Follow-up time was calculated from the last day of treatment to the date of the last follow-up examination or death.

Preradiotherapy Hemoglobin Level. The preradiotherapy hemoglobin values were retrieved from the patient's record and were available for 209 patients. Hemoglobin levels were measured as grams per liter (g/L). The reference values for hemoglobin in our hospital are 132 to 166 g/L for men and 116 to 149 g/L for women.

Immunohistochemistry. Evaluable archival pathologic specimens were available for 170 patients. Four μm sections of formalin-fixed, paraffin-embedded tumor tissue from biopsies taken at diagnosis were tested for the presence of immunohistochemically detectable microvessels by staining with a monoclonal CD34 antibody (CD34 class II QBEend 10, dilution 1:50, DAKO, Carpinteria, CA). A dextran-secondary antibody-peroxidase complex kit (Dako EnVision) was used, applying capillary gap staining technique with the automated TechMate 500 immunostaining system.

Sections were deparaffinized and rehydrated and then placed in a TRIS/EDTA buffer (pH 9) and treated in a microwave oven to the boiling point for at least 10 minutes. Next they were cooled and washed in PBS before immunostaining. All tissues were then exposed to primary antibody for 25 minutes, blocking of endogenous peroxidase by phosphate buffer and nitrogen-peroxide (15 mmol/L $NaNO_3$) for 7.5 minutes, dextran-secondary antibody-horseradish-peroxidase complex for 25 minutes, substrate buffer containing hydrogen peroxidase, diaminobenzidine and chromogen for 15 minutes, and hematoxylin as counterstain for 4 minutes. The incubations were done at room temperature. Between incubations, the sections were washed with buffer. Sections of human liver were used as positive controls. Antibody was replaced by buffer in negative controls.

Counting of MVD. After appropriate immunostaining, MVD was counted as follows: for each case, one researcher (B. M.) scanned the slide under low-power microscopy (×100) to identify areas representative of vascularized tumor tissue. These regions were then scanned at ×250 magnification and thereafter photographed with a digital camera (Leica DC 100, Leica, Heerbrugg, Switzerland) for analysis on a computer screen. Two separate observers (H. H. and B. M.), who were blinded to treatment response, analyzed the photographed microscopic fields (each field corresponding to an area of 1 mm^2) by counting the number of microvessels per field. In 150 cases two microscopic fields could be analyzed, whereas in 20 cases only one microscopic field could be analyzed because of a limited amount of tumor tissue. For each case, MVD was finally expressed as the mean number of vessels for the four (or two) observations per millimeter squared.

Statistics. We did statistical analyses using the SAS System for Windows, Release 8.02 CTS Level 02M0, Windows version 5.0.2195 (1999–2001, SAS Institute Inc., Cary, NC).

Mean, SD, median, and range were calculated for descriptive purposes. For comparison between groups, the Mann-Whitney test was used for continuous variables.

Correlations were analyzed with the Spearman nonparametric correlation coefficient. Survival estimates were calculated from the 1st day of treatment by the Kaplan-Meier method and formally tested with the log-rank test. Overall survival includes deaths because of any cause. Disease-free survival includes deaths because of laryngeal cancer. Overall survival estimates were calculated from the first day of treatment to the date of the last follow-up examination or death. Disease-free survival estimates were calculated from the 1st day of treatment to the day of recurrence or death.

Stepwise Cox proportional hazard regression model was used for multivariate survival analysis.

Reliability was expressed as the mean for the difference between measurements, SD for the differences between measurements, limits of agreement, intraindividual SD (IISD; ref. 18), and coefficient of variation. Mean differences and SD for difference were calculated by subtracting values between the two observers. Limits of agreement were defined by $mean_{diff} \pm 1.96 \cdot SD_{diff}$, which is the confidence interval for the difference. IISD is defined as the SD within measures. The difference between the two observers, measurements for the same subject is expected (with 95% accuracy) to be $< \sqrt{2} \cdot 1{,}96 \cdot IISD$. Coefficient of variation is defined as $IISD/\chi_{total} \cdot 100$ (expressed in %). Systematic differences between observers were tested with the Wilcoxon-signed rank test.

Table 2 Reasons for death by tumor stage

	Tumor stage				
	I (n = 93)	II (n = 45)	III (n = 35)	IV (n = 41)	Total (n = 214)
Local recurrence	4	3	7	6	20
Loco-regional recurrence	1	1	4	3	9
Distant metastases	0	0	1	2	3
Treatment related	0	1	1	1	3
New malignancy	9	4	2	3	18
Intercurrent disease (other than malignancy)	10	13	7	8	38
Total	24	22	22	23	91

All tests were two-tailed and conducted at 5% significance level.

RESULTS

Tumor Control and Survival Results. Local tumor control probabilities at 5 years were 86.3% (SE = 0.09) for patients with stage I disease, 92.2% (SE = 0.04) for stage II, 64.7% (SE = 0.11) for stage III, and 73.3% (SE = 0.11) for patients with stage IV disease. The corresponding figures for locoregional control probabilities at 5 years were 83.4% (SE = 0.09), 87.4% (SE = 0.06), 52.3% (SE = 0.11), and 63.7% (SE = 0.11) for patients with stage I to IV disease respectively.

Disease-free survival probabilities at 5 years were 83.4% (SE = 0.09) for stage I disease, 87.4% (SE = 0.06) for stage II, 50.3% (SE = 0.11) for stage III, and 58.3% (SE = 0.11) for patients with stage IV disease. The corresponding figures for overall survival probabilities at 5 years were 65.2% (SE = 0.07), 44.7% (SE = 0.10), 26.8% (SE = 0.09) and 24.4% (SE = 0.01) for patients with stage I to IV disease, respectively. There was a statistically significant difference between patients with stage I/II disease compared with patients with stage III/IV disease for all of the above parameters analyzed (local control, $P < 0.001$; locoregional control, $P < 0.001$; disease-free survival, $P < 0.001$; overall survival, $P < 0.001$).

A total of 32 patients (15%) died of their laryngeal cancer: 20 from a local recurrence, 9 from regional recurrence, and 3 from distant metastasis. Three patients died from treatment-related complications, of which two had advanced disease. Fifty-six (26%) patients died from intercurrent diseases. Eighteen of these deaths were caused by a second malignancy, whereas cardiovascular diseases and pulmonary infections were the cause of most of the remaining deaths. Reasons for death by subgroup are listed in Table 2.

Preradiotherapy Hemoglobin. The median preradiotherapy hemoglobin value for the 209 evaluable patients was 137.5 g/L (range 81.0–176.0 g/L). Sixty-two of 186 men (33%) and 5 of 23 women (22%) had preradiotherapy hemoglobin levels <132 and <116, respectively, and were hence suffering from anemia according to the reference values of the hospital laboratory. Preradiotherapy hemoglobin values were higher in patients with stage I and II disease compared with stage III and IV disease with median values of 143 g/L for stage I, 143 g/L for stage II, 126 g/L for stage III, and 120 g/L for stage IV. Local control, locoregional control, disease-free survival, and overall survival probabilities at 5 years for patients with preradiotherapy hemoglobin values below or above the median value (<137.5 or >137.5 g/L) are shown in Table 3 and in Fig. 1*A–D*. The 5-year results for locoregional control, disease-free survival, and overall survival were significantly better for patients with preradiotherapy hemoglobin values >137.5 g/L compared with those with values <137.5 g/L (locoregional control, $P = 0.01$; disease-free survival, $P = 0.007$; overall survival, $P < 0.001$). There was also a trend toward better local control for patients with preradiotherapy hemoglobin values >137.5 g/L, but the difference did not reach statistical significance ($P = 0.087$).

The above analyses were carried out separately for patients receiving once daily fractionated radiotherapy and for patients receiving accelerated radiotherapy. For patients receiving once daily fractionated radiotherapy, the 5-year results for locoregional control, disease-free survival, and overall survival were significantly better for the patients with preradiotherapy hemoglobin values >137.5 g/L compared with those with values <137.5 g/L (locoregional control, $P = 0.015$; disease-free survival, $P = 0.015$; overall survival, $P = 0.024$). However, for patients receiving accelerated radiotherapy, there was no statistically significant difference between patients with preradiotherapy hemoglobin values >137.5 g/L compared with those with values <137.5 g/L for any of the above parameters, even if there was a trend toward better outcome for patients with preradiotherapy hemoglobin values >137.5 g/L (Table 4). Again, the difference in local control did not reach statistical

Table 3 5-year control and survival probabilities for patients with pre-radiotherapy hemoglobin values below or above the median value (<137.5 g/L or >137.5 g/L)

5-year control/survival probabilities	Preradiotherapy hemoglobin <137.5 g/L	Preradiotherapy hemoglobin >137.5 g/L	P value
Local control	74.5% (SE = 0.09)	91.8% (SE = 0.03)	0.087
Loco-regional control	64.3% (SE = 0.09)	88.9% (SE = 0.04)	0.010
Disease free survival	62.8% (SE = 0.08)	87.8% (SE = 0.04)	0.007
Overall survival	40.3% (SE = 0.06)	58.1% (SE = 0.06)	<0.001

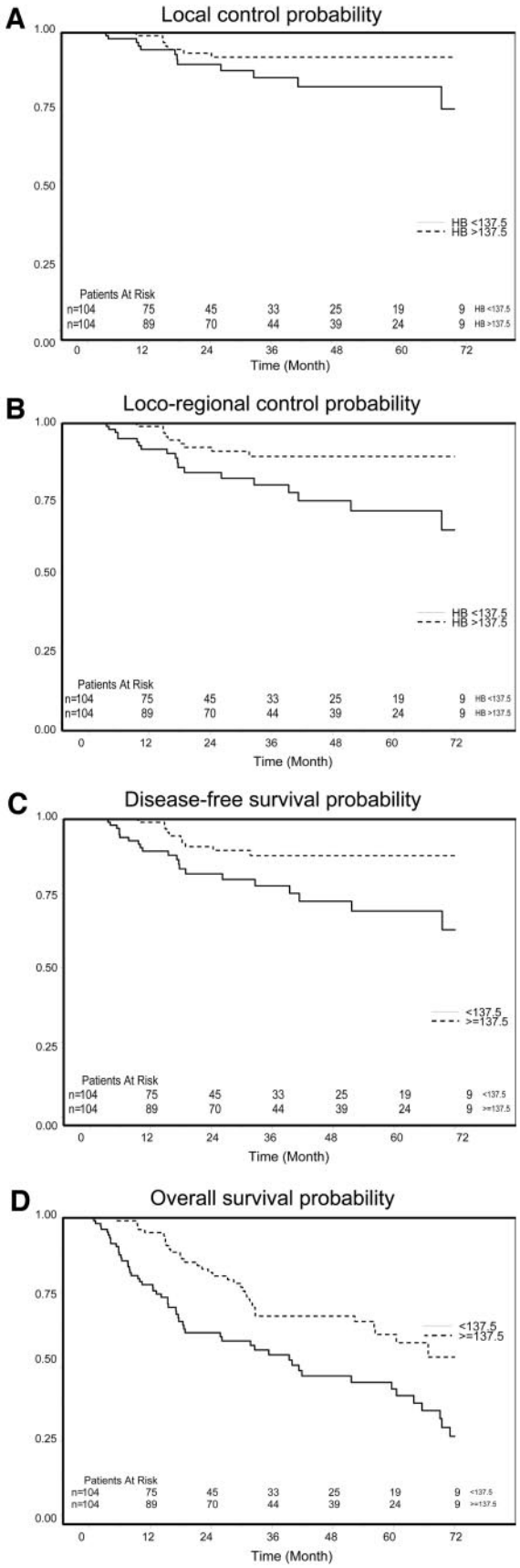

Fig. 1 *A*. Local control probability for patients with preradiotherapy hemoglobin levels >137.5 g/L (*dotted line*) or <137.5 g/L (*solid line*). *B*, locoregional control probability for patients with preradiotherapy hemoglobin levels >137.5 g/L (*dotted line*) or <137.5 g/L (*solid line*). *C*, disease-free survival probability for patients with preradiotherapy hemoglobin levels >137.5 g/L (*dotted line*) or <137.5 g/L (*solid line*). *D*, overall survival probability for patients with preradiotherapy hemoglobin levels >137.5 g/L (*dotted line*) or <137.5 g/L (*solid line*).

significance, neither for patients receiving once daily fractionated radiotherapy nor for patients receiving accelerated radiotherapy.

Microvessel Density. Microvessel density (MVD) ranged from 5 to 70 microvessels per field (1 mm^2). The median MVD was 19 vessels per field (mean = 21 vessels per field). There was no difference in median MVD between low-stage and advanced-stage tumors (stage I, 20 vessels per field; stage II, 18 vessels per field; stage III, 19 vessels per field; stage IV, 20 vessels per field). Figure 2*A* and *B* show microvessels (CD34 staining) in a pretreatment biopsy from a patient with a $T_3N_0M_0$ glottic cancer. The variation in microvessel density in different areas within the same tumor is illustrated here.

There was high agreement in microvessel counts between the two observers, even regarding the higher counts, where the two observers differed the most. In >95% of all observations, the difference in microvessel count between the two observers was not >2.88 vessels (mean difference between measurements = −0.11, SD for the difference between measurements = 1.47, limits of agreement = −0.11, intra-individual SD = 1.04, coefficient of variation = 4.15%; ref. 18). When testing for systematic differences between the two observers, no statistically significant difference was found ($P = 0.39$).

To assess the predictive value of MVD on local control, locoregional control, disease-free survival, and overall survival, patients were divided into a low and a high group with the median as the cutoff-value. No correlation was found between MVD and any of the four parameters.

When testing the correlation between MVD and preradiotherapy hemoglobin levels, no correlation was found (correlation coefficient = 0.003, $P = 0.96$).

Multivariate Analysis for Predictive Factors. After univariate analysis, the effects of preradiotherapy hemoglobin (continuous data), gender (male *versus* female), tumor stage (stage I/II *versus* III/IV), and age (continuous variable) were tested for 209 patients in multivariate analysis with a stepwise Cox proportional hazard regression model. Microvessel count (CD34) did not meet the 0.05 significance level criteria and was therefore not included in the analysis.

Tumor stage ($P < 0.001$) was the only variable that significantly influenced local control, whereas tumor stage ($P = 0.012$) and preradiotherapy hemoglobin level ($P = 0.019$) both significantly influenced locoregional control. Tumor stage ($P = 0.04$) and preradiotherapy hemoglobin level ($P = 0.002$) also significantly influenced disease-free survival, whereas overall survival was significantly influenced by all four variables (tumor stage, $P = 0.003$; preradiotherapy hemoglobin, $P = 0.003$; gender, $P = 0.001$; age, $P = 0.003$). Results of the multivariate analysis are shown in more detail in Table 5.

Table 4 5-year control and survival probabilities for patients with once daily fractionated or twice daily fractionated radiotherapy and preradiotherapy hemoglobin values below or above the median value (<137.5 g/L or >137.5 g/L)

5-year control/ survival probabilities	Once daily fractionated hemoglobin			Twice daily fractionated hemoglobin		
	<137.5 g/L	>137.5 g/L	*P* value	<137.5 g/L	>137.5 g/L	*P* value
Local control	85.8% (SE = 0.09)	95.7% (SE = 0.03)	0.1	79.1% (SE = 0.07)	86.9% (SE = 0.05)	0.493
Locoregional control	73.0% (SE = 0.13)	95.7% (SE = 0.02)	0.015	68.1% (SE = 0.09)	80.1% (SE = 0.07)	0.259
Disease-free survival	72.9% (SE = 0.13)	95.6% (SE = 0.03)	0.015	65.7% (SE = 0.08)	77.7% (SE = 0.07)	0.218
Overall survival	50.7% (SE = 0.11)	67.3% (SE = 0.09)	0.024	34.4% (SE = 0.07)	47.3% (SE = 0.09)	0.078

DISCUSSION

Oxygen plays an important role in the radiation response of tumors, with hypoxic tumors being more radioresistant than well-oxygenated ones. It has long been known that hypoxia exists in tumors. The growth of solid tumors requires growth of new blood vessels, a process named angiogenesis. These new blood vessels are often primitive and dysfunctional and may be inadequate for meeting the needs of the growing tumor. Therefore, nutrient-deprived and oxygen-deprived regions develop within the tumor. However, the hypoxic cells existing in these regions may still be viable. It has been shown that most experimental solid tumors contain hypoxic cells, and the proportion of hypoxic cells to nonhypoxic cells (hypoxic fraction) has been estimated to vary between 1 and 50%. Several trials have attempted to improve tumor oxygen status and hence the outcome of radiotherapy for head and neck cancer by using different hypoxic cell sensitizers. Both the Danish Head and Neck Cancer Study 5-85 protocol, using the hypoxic radiosensitizer nimorazole (19), and the phase II ARCON study by Kaanders *et al.* (20) combining accelerated radiotherapy with carbogen (95% O_2 and 5% CO_2) and nicotinamide, showed improved locoregional control results for patients treated with the sensitizing regimes. In the ARCON study, this was particularly true for laryngeal cancer.

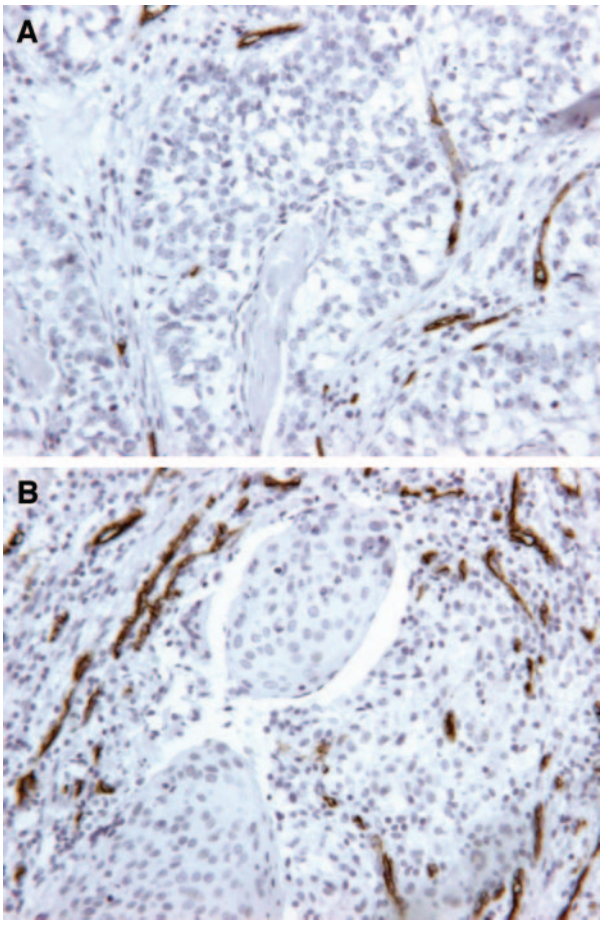

Fig. 2 *A*, CD 34 staining in a pretreatment biopsy specimen from a patient with $T_3N_0M_0$ glottic cancer showing an area of tumor tissue with a relatively low MVD (13 vessels/m^2). *B*, CD 34 staining in a pretreatment biopsy specimen from the same patient with $T_3N_0M_0$ glottic cancer showing an area of tumor tissue with higher MVD (46 vessels/m^2), illustrating MVD variation within the same tumor.

Tissue oxygenation is dependent on a number of factors: the O_2 content of arterial blood, cardiac output, and a functioning microvessel network. The O_2 content of arterial blood in turn depends on the hemoglobin concentration, reflecting the capacity of the blood to transport oxygen. The binding of oxygen to hemoglobin is again dependent on the oxygen partial pressure, with the O_2 content and saturation of blood increasing progressively with increasing pO_2. The O_2 binding to hemoglobin depends not only on O_2 tension but also on the partial pressure of CO_2 and the pH of the blood. The HbO_2 dissociation curve is shifted to the right by a rise in pCO_2 or a decrease in pH. This shift in the oxy-hemoglobin dissociation aids in the delivery of oxygen to tissues, including tumor tissues with an acidic environment.

Carbon monoxide (CO), produced, for instance, by cigarette smoking also influences the O_2-binding capacity of hemoglobin. CO has >200 times the affinity for hemoglobin than does O_2. Because CO competes with O_2 for the binding sites on the hemoglobin molecule, the amount of HbO_2 will consequently be reduced. The combination of CO with hemoglobin also affects O_2 binding and causes the O_2 dissociation curve to shift to the left, causing a deficit in O_2 uptake that is greater than might be expected from just the decrease in carrying capacity of

Table 5 Results from stepwise Cox proportional hazard regression analysis

	Regression coefficient	Standard error	Hazard ratio	95% hazard ratio confidence interval	*P* value
Local control					
Tumor stage (I/II vs. III/IV)	1.7	0.495	5.475	(2.075–14.448)	<0.001
Pre-radiotherapy Hb					
Gender(male /female)					
Age					
Loco-regional control					
Tumor stage (I/II vs. III/IV)	1.248	0.500	3.485	(1.306–9.298)	0.012
Preradiotherapy Hb	−0.031	0.013	0.969	(0.944–0.995)	0.019
Gender (male /female)					
Age					
Disease-free survival					
Tumor stage (I/II vs. III/IV)	1.508	0.479	4.518	(1.765–11.567)	0.002
Preradiotherapy Hb	−0.025	0.012	0.975	(0.952–0.999)	0.004
Gender (male /female)					
Age					
Overall survival					
Tumor stage (I/II vs. III/IV)	0.773	0.262	2.166	(1.297–3.615)	0.003
Preradiotherapy Hb	−0.022	0.007	0.978	(0.964–0.992)	0.003
Gender (male/female)	−1.553	0.473	0.212	(0.084–0.535)	0.001
Age	0.035	0.012	1.036	(1.012–1.060)	0.003

hemoglobin for O_2. The result is that less O_2 can be transported to the tissues. The release of O_2 from HbO_2 to the tissues is regulated by the specific compound 2,3-diphosphoglycerate, present in high concentrations in red blood cells. This compound has a high affinity for hemoglobin and binds preferentially to deoxygenated hemoglobin. This reduces the affinity of hemoglobin for O_2, and the HbO_2 dissociation curve is shifted to the right, making more O_2 available to the tissues. The synthesis of 2,3-diphosphoglycerate increases in hypoxic conditions, such as anemia.

Given the background for tissue oxygenation above, one can hypothesize that tumor hypoxia can be caused by inadequate blood supply caused by dysfunctional microvessels or even a scarce distribution of microvessels, a low hemoglobin concentration, a low blood oxygen tension (*e.g.*, in patients with respiratory diseases), an acidic microenvironment, or an increased level of HbCO (cigarette smoking). In our study we examined two of these factors, preradiotherapy hemoglobin concentration and MVD, to investigate their influence on the outcome of patients with laryngeal cancer treated with radical radiotherapy. We found a strong correlation between preradiotherapy hemoglobin concentration and both locoregional tumor control and survival. Preradiotherapy hemoglobin was revealed to be a significant prognostic factor for locoregional control, disease-free survival, and overall survival in multivariate analysis. Studies investigating tumor oxygenation with polarographic measurements have found a strong correlation between tumor hypoxia and low hemoglobin concentrations (21–22). A physiologic explanation for this relationship could be that given the hypoxic and acidic environment known to exist in tumors, the oxy-hemoglobin curve should be shifted to the right, releasing more oxygen to the tumor tissues. However, with a low hemoglobin level, the amount of oxygen available for delivery to the tumor tissues may be insufficient to make the tumor oxic, leaving at least part of the tumor hypoxic. In head and neck cancer, where the majority of patients are smokers, the situation is likely to be worsened because of the increased level of CO causing decreased O_2 uptake and transport (23). Retrospective analyses yield evidence for the prognostic importance of hemoglobin concentration for the outcome of radiotherapy in head and neck cancer, and this has been confirmed in prospective studies (24–25). Retrospective analyses on laryngeal cancer exclusively have also showed better local control and survival for patients with normal or high hemoglobin values (6–10, 26–27), supporting our results. In contrast to our study, most studies on laryngeal cancer have investigated only early glottic (T_1/T_2) tumors treated with conventional fractionation (6–26). One retrospective study reported on patients with advanced laryngeal cancer (T_3-T_4), but this study also included patients with surgery as part of their primary treatment (27).

A particularly interesting finding was the difference in influence of preradiotherapy hemoglobin level for patients treated with once daily fractionated radiotherapy and patients treated with accelerated radiotherapy. Patients treated with once daily fractionated radiotherapy had significantly better locoregional control, disease-free survival and overall survival if their preradiotherapy hemoglobin value was >137.5 g/L. For patients treated with accelerated radiotherapy, however, this difference was not statistically significant. Half of the patients in this study, mainly with stage II to IV tumors, were treated with accelerated, twice daily fractionation. In a previous study, we found similarly positive 5-year local control results for T_1 and T_2 glottic tumors when treating the T_2 tumors with accelerated fractionation (1), indicating that a shortening of the overall treatment time is of importance even in small laryngeal tumors. An explanation for the above finding that hemoglobin concentration seems to be of more importance when using conventional fractionation compared with accelerated fractionation could be that the accelerated fractionation, reducing the repopulation of tumor cells between fractions, to some degree compensates for the low hemoglobin concentration causing hypoxia and hence more radioresistant tumor cells.

In the present study we found that preradiotherapy hemoglobin concentration was a substantial prognostic factor not only

for locoregional tumor control and disease-free survival but also for overall survival. Although this has been found in other studies (5), the underlying mechanisms are unknown. Overall survival reflects deaths because of any cause and not only deaths because of laryngeal cancer. In the present study 56 of 214 patients (26%) died of intercurrent diseases not related to their laryngeal cancer. The most common causes of death were new primary cancers and cardiovascular and pulmonary diseases. Cigarette smoking is the most likely common risk factor. However, one can speculate that a low hemoglobin concentration leading to tissue hypoxia by itself could play a direct role in the development or progression of these intercurrent diseases or by indirect mechanisms such as the formation of free radicals and the modification of hypoxia-inducible genes (*e.g.*, vascular endothelial growth factor, hypoxia-inducible factor-1α, p53, etc.).

In our study, we found no correlation between MVD and tumor control or survival. Previous studies have shown conflicting results regarding the correlation between MVD and response to radiotherapy in head and neck cancer. Zätterström *et al.* (14) found low MVD to be a negative prognostic factor for survival in a retrospective study of 48 patients with various head and neck malignancies receiving preoperative radiotherapy. However, in a subsequent report from the same group, studying 39 patients with oral cancer receiving preoperative radiotherapy, the opposite result was found, with a worse clinical outcome for patients with high MVD scores (13). Kamijo *et al.* (15–16) found better local control for tumors with high MVD values in two retrospective studies on patients with T_1 and T_2 laryngeal cancer. One realistic criticism of these studies is that the number of patients was quite small (31 and 55 patients, respectively). Furthermore, the total radiation doses varied greatly (52–70 Gy), and the lower doses must be considered suboptimal for curative radiotherapy of laryngeal cancer. Studies of MVD in more advanced laryngeal cancer have mostly investigated surgically treated patients. Murray *et al.* (28) found a correlation between high MVD values and higher risk of nodal recurrence in a series of 51 surgically treated patients, whereas Kupisz *et al.* (29) reported a correlation between high MVD and lower survival in a series of 60 patients. Pignataro *et al.* (30) found no correlation between MVD and survival in a retrospective study of 122 patients treated with primary surgery. Considering tumor hypoxia, a high MVD should be beneficial because of the increased amount of vessels for oxygen transport to tissues, making the tumor cells less hypoxic and hence more radiosensitive. From another perspective, a high MVD could increase the risk of hematogenous tumor spread and hence increase the risk of death from metastatic disease. In laryngeal cancer, the latter aspect is a much smaller problem than in many other types of cancer. In this retrospective study of 214 patients, only 3 patients (all with stage III or IV disease) died of metastatic disease, reflecting that laryngeal cancer is a locoregional disease.

A possible explanation for the lack of correlation between MVD and tumor outcome found in our study is that MVD yields only the number of microvessels within a defined area of tumor tissue and no information about the function of these vessels. A tumor with a high MVD could be just as hypoxic or even more hypoxic than a tumor with a low MVD, because despite many vessels, these vessels could be defective or in other ways unsuitable for oxygen transport. We also found that the MVD could vary greatly (range 5–70 vessels per field) not only between tumors, but also between different areas within the same tumor (Fig. 2). Therefore, even in small laryngeal tumors with a high MVD, there could be areas with very few microvessels possibly containing more radioresistant hypoxic cells. It is also possible that the capacity of hemoglobin to bind and transport oxygen is of greater importance for tumor oxygenation than MVD in laryngeal cancer. If that is the case, then a high or low hemoglobin value will affect tumor oxygenation and hence radio response more than variations in MVD.

The data supporting the prognostic importance of hemoglobin concentration for the outcome of radiotherapy in head and neck cancer have lead to trials investigating the effects of manipulating the hemoglobin concentration. Increasing the hemoglobin level by transfusion has not resulted in any substantial benefit (19). The use of erythropoietin has given rise to new opportunities for increasing the hemoglobin level. In contrast to transfusions, erythropoietin is capable of producing a graduate increase in hemoglobin concentration resulting in a graduated increase in and a durable oxygen supply over time. A retrospective study by Glaser *et al.* (31) on patients with oral and oro-pharyngeal cancer treated with chemoradiation and surgery showed improved locoregional control and overall survival for anemic patients receiving recombinant erythropoietin. However, in a recent randomized study by Henke *et al.* (32) of 351 patients undergoing radiotherapy for advanced head and neck cancer, treatment with epoetin-β corrected anemia but did not improve locoregional control or survival. On the contrary, the treatment outcome in the group that received erythropoietin was reported to be inferior to that of the control group. This study has been criticized for its selection of patients and questioned for its treatment standards. The study population was heterogeneous, including patients who received radiotherapy both primarily and postoperatively as well as patients receiving radiotherapy for recurrent tumors. The outcome in the trial was poor by international standards, with low locoregional control rates, and the number of protocol violations was high. The results of this study should therefore be interpreted with caution. Our study of a relatively large group of patients with laryngeal cancer treated in a uniform manner with radiotherapy supports previous reports of the importance of hemoglobin concentration for the outcome of radiotherapy. Increasing the hemoglobin concentration in head and neck cancer patients treated with radiotherapy is a desirable task, and despite the discouraging results in the study by Henke *et al.*, the potential benefit of erythropoietin as an adjuvant in cancer treatment should be further explored. We recommend using new, better-designed, randomized trials with a homogenous study population and treatment.

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REFERENCES

1. Haugen H, Johansson K-A, Mercke C. Hyperfractionated-accelerated or conventionally fractionated radiotherapy for early glottic cancer. Int J Radiat Oncol Biol Phys 2002;52:109–19.

2. Haugen H, Johansson K-A, Ejnell H, Edström S, Mercke C. Accelerated radiotherapy for advanced laryngeal cancer. Acta Oncologica 2004.

3. Hockel M, Knoop C, Schlenger K, et al. Intratumoral pO_2 predicts survival in advanced cancer of the uterine cervix. Radiother Oncol 1993;26:45–50.

4. Brizel DM, Dodge RK, Clough RW, Dewhirst MW. Oxygenation of head and neck cancer: changes during radiotherapy and impact on treatment outcome. Radiother Oncol 1999;53:113–7.

5. Caro JJ, Salas M, Ward A, Gloss G. Anemia as an independent prognostic factor for survival in patients with cancer. Cancer (Phila) 2001;91:2214–21.

6. Warde P, O'Sullivan MB, Bristow RG, et al. T1/T2 glottic cancer managed by external beam radiotherapy: the influence of pretreatment hemoglobin on local control. Int J Radiat Oncol Biol Phys 1998;41: 347–53.

7. Tarnawski R, Skladowski K, Maciejewski B. Prognostic value of hemoglobin concentration in radiotherapy for cancer of supraglottic larynx. Int J Radiat Oncol Biol Phys 1997;38:1007–11.

8. Fein DA, Lee WR, Hanlon AL, et al. Pretreatment hemoglobin level influences local control and survival of T1–T2 squamous cell carcinomas of the glottic larynx. J Clin Oncol 1995;13:2077–83.

9. van Acht MJJ, Hermans J, Boks DES, Leer JWH. The prognostic value of hemoglobin and a decrease in hemoglobin during radiotherapy in laryngeal cancer. Radiother Oncol 1992;23:229–35.

10. Skladowski K, Tarnawski R, Maciejewski B, Wygoda A, Slosarek K. Clinical radiobiology of glottic T1 squamous cell carcinoma. Int J Radiother Oncol Biol Phys 1999;43:101–6.

11. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis–correlation in invasive breast cancer. N Engl J Med 1991;324:1–8.

12. Weidner N, Caroll PR, Flax J, Blumenfield W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol 1993;143:401–9.

13. Brun E, Zätterström U, Kjellén E, et al. Prognostic value of histopathological response to radiotherapy and microvessel density in oral squamous cell carcinomas. Acta Oncol 2001;40:491–6.

14. Zätterström UK, Brun E, Willén R, Kjellén E, Wennerberg J. Tumor angiogenesis and prognosis in squamous cell carcinoma of the head and neck. Head and Neck 1995;17:312–8.

15. Kamijo T, Yokose T, Hasebe T, et al. Potential role of microvessel density in predicting radiosensitivity of T1 and T2 stage laryngeal squamous cell carcinoma treated with radiotherapy. Clin Cancer Res 2000;6:3159–65.

16. Kamijo T, Yokose T, Hasebe T, et al. Image analysis of microvessel surface area predicts radiosensitivity in early-stage laryngeal cancer treated with radiotherapy. Clin Cancer Res 2001;7:2809–14.

17. Union Internationale Contre le Cancer. UICC TNM atlas, illustrated guide to the TNM/pTNM classification of malignant tumors, 3rd edition. Berlin, Heidelberg, New York, London, Paris, Tokyo, Hong Kong, Barcelona, Budapest: Springer Verlag, 1990. p. 32–43.

18. Bland JM, Altman DG. Measurement error. British Med J 1996; 313:744–53.

19. Overgaard J, Sand Hansen H, Overgaard M, et al. Randomized double-blind phase III study of nimorazole as a hypoxic radiosensitizer of primary radiotherapy in supraglottic larynx and pharynx carcinoma. Results of the Danish Head and Neck Cancer Study (DAHANCA) protocol 5–85. Radiother Oncol 1998;46:135–46.

20. Kaanders JH, Pop LA, Marres HA, et al. ARCON: experience in 215 patients with advanced head and neck cancer. Int J Radiat Oncol Biol Phys 2002;52:769–78.

21. Becker A, Stadler P, Lavey RS, et al. Severe anemia is associated with poor tumor oxygenation in head and neck carcinomas. Int J Radiat Oncol Biol Phys 2000;46:459–66.

22. Clavo B, Pérez JL, López L, et al. Influence of haemoglobin concentration and peripheral muscle pO_2 on tumor oxygenation in advanced head and neck tumours. Radiat Oncol 2003;66:71–4.

23. Overgaard J, Horsman MR. Modification of hypoxia-induced radioresistance in tumors by the use of oxygen and sensitizers. Semin Radiat Oncol 1996;6:10–21.

24. Lee WR, Berkey B, Marcial V, et al. Anemia is associated with decreased survival and increased locoregional failure in patients with locally advanced head and neck carcinoma: a secondary analysis of RTOG 85–87. Int J Radiat Oncol Biol Phys 1999;42:1069–75.

25. Overgaard J, Horsman MR, Andersen AP, et al. Misonidazole combined with split-course radiotherapy in the treatment of invasive carcinoma of the larynx and pharynx: report from the DAHANC 2 study. Int J Radiat Oncol Biol Phys 1989;16:1065–8.

26. Cho EI, Sasaki CT, Haffty BC. Prognostic significance of pretreatment hemoglobin for local control and survival in T1–T2N0 larynx cancer treated with external beam radiotherapy. Int J Radiat Oncol Biol Phys 2004;58:1135–40.

27. Nguyen-Tan PF, Le QT, Quivey JM, et al. Treatment results and prognostic factors of advanced T3–4 laryngeal carcinoma: the University of California, San Francisco (UCSF) and Stanford University Hospital (SUH) experience. Int J Radiat Oncol Biol Phys 2001;50:1172–80.

28. Murray JD, Carlson GW, McLaughlin K, et al. Tumor angiogenesis as a prognostic factor in laryngeal cancer Am J Surg 1997;174:523–6.

29. Kupisz K, Chibowski D, Klatka J, Klonowski S. Tumor angiogenesis in patients with laryngeal cancer. Eur Arch Otorhinolaryngol 1999; 256:303–5.

30. Pignataro L, Carboni N, Midolo V, et al. Clinical relevance of microvessel density in laryngeal squamous cell carcinomas. Int J Cancer 2001;92:666–70.

31. Glaser C, Millesi W, Kornek GV, et al. Impact of hemoglobin level and the use of recombinant erythropoetin on efficacy of preoperative chemoradiation therapy for squamous cell carcinoma of the oral cavity and oropharynx. Int J Radiat Oncol Biol Phys 2001;50:705–15.

32. Henke M, Laszig R, Rube C, et al. Erythropoietin to treat head and neck cancer patients with anemia undergoing radiotherapy: randomized, double-blind, placebo-controlled trial. Lancet 2003;362:1255–60.

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