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Tumor Angiogenesis as a Predictive Marker for Organ Preservation in Patients With Advanced Laryngeal Carcinoma

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Background: **The purpose of this study was to retrospectively investigate tumor angiogenesis as a predictive marker for response to neoadjuvant chemotherapy, organ preservation, and survival in patients with advanced laryngeal carcinoma.** ***Methods:*** **A total of 332 patients with stage III (188 patients) or stage IV (144 patients) squamous cell carcinoma of the larynx were entered in the prospective trial conducted by the Department of Veteran Affairs Laryngeal Cancer Study Group. Of this patient population, 20 pretreatment biopsy specimens were available from the chemotherapy arm for immunohistochemical analysis of Factor VIII expression. Two blinded investigators determined microvessel density in each patient by manual inspection of 10 high-power (400×) fields (HPF).** ***Results:*** **The patients who had a partial response (>50% decrease in tumor volume) or complete response to chemotherapy had a mean value of 20.90 (± 8.09 standard deviation [SD]) blood vessels per HPF. Those who did not respond to chemotherapy and thus required a total laryngectomy had a mean value of 32.99 (± 10.10 SD) vessels per HPF. The difference of the means was statistically significant using a two-tailed *t* test (*P* <.0085). Kaplan-Meier survival curve analysis also revealed that patients with vessel counts above the mean tended to have poorer survival than those below the mean regardless of treatment selection. The most-vascular tumors, those greater than 1 SD above the mean, had a statistically significant difference in survival and laryngeal preservation (*P* = .0345).** ***Conclusions:*** **These results indicate that tumor angiogenesis, as measured by number of vessels per HPF, was associated with decreased responsiveness to chemotherapy and radiation for larynx preservation. The most-vascular tumors also were associated with poorer survival than those with lesser degrees of angiogenesis.** ***Key Words:*** **Angiogenesis, head and neck cancer, organ preservation, predictive markers, Factor VIII.**

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INTRODUCTION

The most significant advance made in the treatment of head and neck carcinoma over the last two decades has been the development of organ preservation protocols for locally advanced cancers of the larynx and hypopharynx. The landmark study, entitled "The Department of Veterans Affairs Cooperatives Studies Program Laryngeal Cancer Study #268," clearly demonstrates that the use of induction chemotherapy followed by definitive radiotherapy can achieve survival rates similar to laryngectomy and postoperative radiation therapy with a high rate of laryngeal preservation.[1] Several subsequent trials have substantiated these results, confirming the important role such treatment paradigms play in the treatment of head and neck malignancies.[2] Major criticisms of the treatment approaches, however, include the long treatment duration, high cost, potential increased morbidity, and an eventual salvage laryngectomy rate of 30% to 40%.[3] Predicting, at the time of diagnosis, which patients would be more likely to respond to organ preservation protocols would greatly decrease the rate of salvage laryngectomy. This would allow for efficient and cost-effective delivery of treatment as well as minimize morbidity. To date, several studies have attempted to predict chemosensitivity as well as ultimate organ preservation, identifying tumor volume, cellular kinetics, p53 expression, PCNA, and apoptotic cell cycle regulators as potential predictive markers.[4] The present study investigates tumor angiogenesis as a marker predictive of chemo-responsiveness, organ preservation, and ultimate survival in the Veterans Affairs Larynx Study.

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MATERIALS AND METHODS

Patient Population

A total of 332 patients with stage III (188 patients) or stage IV (144 patients) squamous cell carcinoma were enrolled in the prospective trial entitled "The Department of Veterans Affairs Cooperative Studies Laryngeal Cancer Study #268."[1] This cooperative study was reviewed and approved by the Central Human Rights Committee at the Hines VA Cooperative Studies Program and the Institutional Review Boards of all participating institutions. Informed consent was obtained from each patient at the time of accrual. Half of the patients enrolled in the study (166 patients) were randomized to receive conventional surgery and postoperative radiation therapy (50–65 Gy). The other half (166 patients) received three cycles of induction chemotherapy (100 mg/m^2 cis-platinum on day 1 and 1000 mg/m^2 5-fluorouracil on days 1–5) and radiation therapy (66–76 Gy). Surgical salvage was performed on those patients who failed to respond after two cycles of chemotherapy, or on those with persistent or recurrent disease at the completion of treatment. This treatment regimen has been previously described in detail.[1] The patients available for the present study were chosen from those randomized to the induction chemotherapy arm of the Veterans Affairs Cooperative Study. All chemotherapy arm patients with sufficient pretreatment biopsy specimens were included in this analysis. Patients with insufficient tissue for analysis were excluded, as were those with incomplete follow-up data.

Immunohistochemical Evaluation of Angiogenesis

Tumor vascularity, or angiogenesis, was detected by the immunohistochemical staining technique first described by Weidner et al.[5] Briefly, 5-μm thick sections were cut through representative invasive cell carcinoma from formalin-fixed and paraffin-embedded tissues. The slides were pretreated with trypsin, and then incubated with a monoclonal antibody raised against Human Factor VIII Related Antigen (Dako, Copenhagen, Denmark). A standard immunoperoxidase method was used for staining (Vectastan ABC Kit; Vector Laboratories, Inc., Burlingame, CA). For each batch of slides, positive and negative controls were performed.

Neovessel Counts

After appropriate immunohistochemical staining, two separate researchers, who were blinded to treatment response, individually counted vessels in the following manner: each slide was scanned under low-power microscopy (100×) to identify areas of high vascularity in the tumor specimen. These regions were then scanned at 400× magnification and individual vessels were counted (Fig. 1). Ten high-power fields per specimen were analyzed, and each individual vessel was counted and recorded.

Statistical Analysis

To examine the similarity of the two patient groups at baseline, all known demographic and disease characteristics having potential prognostic importance were compared. The Fisher's Exact Test and the Student *t* test were used, detecting differences between groups in categorical and continuous measures, respectively. Mean vessel count per specimen was compared between the two patient groups using a Student *t* test. Survival analyses were conducted using the Kaplan-Meier technique; differences between distributions were compared with the log-rank test. Survival was calculated for each patient from the Veterans Affairs Larynx Study randomization date to the date of death; patients remaining alive were censored at the date last known to be alive. The relationship of mean vessel count with overall survival was based on a Cox proportional hazards model. This measure was also stratified using the mean and the mean +1 standard deviation (SD). For all analyses, a two-sided alpha level of 0.05 was considered statistically significant.

RESULTS

Study Population

Twenty patients who were initially randomized to the chemotherapy arm of the Veterans Affairs Laryngeal Cooperative Study had sufficient initial biopsy specimens

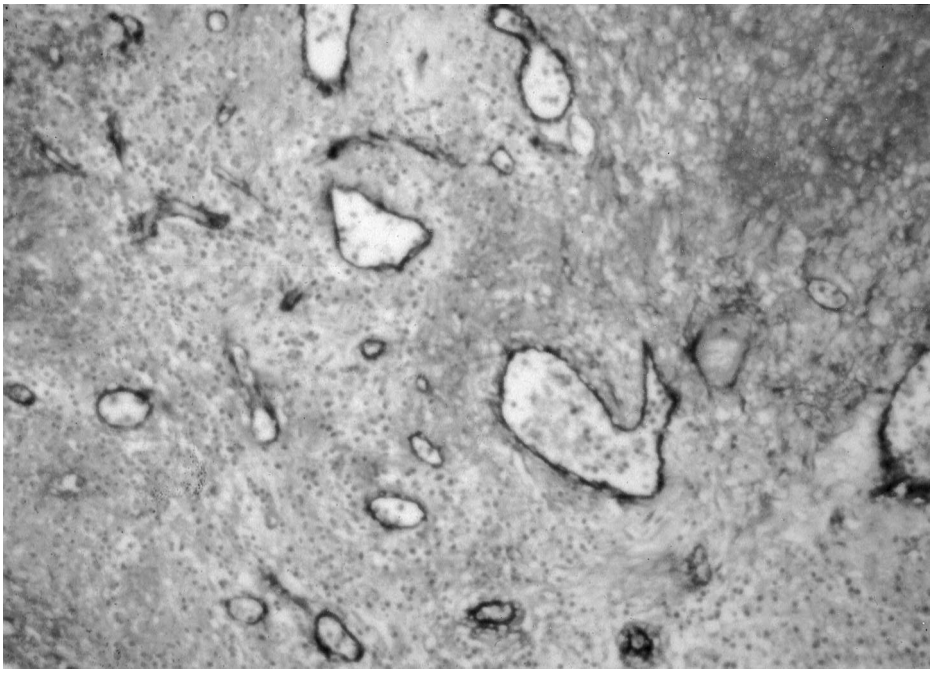

Fig. 1. Photomicrograph illustrating the typical appearance of a Factor VIII-stained specimen in a patient determined to be chemo-resistant. Note the high level of tumor vessel density on this high-power field.

available to undergo immunohistochemical analysis. The remaining patients in this arm of the study had pretreatment biopsy specimens that were too small to evaluate by this method. Of the 20 patients who were eligible for this study, 10 were responders to chemotherapy and subsequently retained their larynges. The remaining 10 did not respond to induction chemotherapy and required salvage laryngectomy with postoperative radiation therapy for disease treatment. The former group will be referred to as the responders and the latter as the non-responders to induction chemotherapy. All patients were male, and the mean age at presentation was 62 years (Table I). Fifteen of the 20 patients had stage III disease at the time of presentation, and 5 patients presented with stage IV tumors. Overwhelmingly, T_3 tumors predominated, with 17 T_3 tumors, 1 T_2, and 2 T_4 tumors. Nodal status was determined to be predominantly N_0 (15 patients), with only 2 patients presenting with N_1 neck disease, 2 patients with N_2 disease, and 1 with N_3 disease. The predominant site of the primary tumor was supraglottic larynx (15 of 20). As evident in Table I, there was no statistically significant difference between the groups with regard to stage, T-status, N-stage, race, age, sex, site of the primary, or Karnovsky Performance Scale.

Tumor Angiogenesis Counts

Mean vessel count for all 20 patients was 26.9 vessels per high-power field (HPF), with a median of 25.575. The group of patients who responded to chemotherapy and went on to complete the organ preservation protocol had a mean vessel count of 20.90 ± 8.09 vessels per HPF. The range was 8.75 to 35.90 vessels per HPF. In the group of patients who did not respond to chemotherapy, the mean vessel count was 32.99 ± 10.10 vessels per HPF (range, 21.05–53.10) (Table II). This difference was found to be highly statistically significant ($P = .0085$). Patients with vessel counts above the mean were eight times less likely to respond to chemotherapy (relative risk = 8.0) with a screening sensitivity of 80% (95% confidence interval [CI] 45%, 100%), a specificity of 67% (95% CI 43%, 91%), a positive predictive value of 44% (95% CI 12%, 76%), and a negative predictive value of 91% (95% CI 74%, 100%). Mean tumor vessel counts at the time of presentation did not correlate with nodal stage at presentation ($P = .408$) or disease recurrence ($P = .2105$).

Kaplan-Meier curves evaluating the likelihood of organ preservation based on low versus high vascularity are graphically depicted in Figures 2 and 3. There is a definite trend in both groups toward organ preservation in those patients with the lower vessel counts (Fig. 7). The odds ratio reveals that patients with vessel counts above the mean are 3.5 times more likely to require a laryngectomy than those below the mean. The sensitivity using this value is 60% (95% CI 30%, 90%) with a specificity of 70% (95% CI 42%, 98%), a positive predictive value of 67% (95% CI 36%, 98%), and a negative predictive value of 64% (95% CI 36%, 92%).

Finally, Kaplan-Meier's survival curve analysis revealed that patients in this study whose mean vessel counts were above the mean, compared with those below the mean, tended to display poorer long-term survival, although these differences were not statistically significant ($P = .54$) (Figs. 4 and 6). However, when comparing the most-vascular tumors in this group of patients (those with a mean vessel count 1 SD above the mean) to the rest of the group, a decrease in survival was noted ($P = .0345$) (Fig. 5).

TABLE I.
Results.

	Chemoresponsive	Chemoresistant	*P* Value
Age (yr)	61.69	62.18	.141
Stage III/IV	9/1	6/4	.303
T stage ($T_2/T_3/T_4$)	0/10/0	1/7/4	.171
N status ($N_0/N_1/N_2/N_3$)	7/2/1/0	8/0/1/1	.381
Site (glottic/supraglottic)	3/7	2/8	.606ñ

TABLE II.
Results.

	Vessel Counts (per HPF)	Range	*P* Value
Chemoresponsive	20.90 ± 8.09	8.75–35.90	≤.008
Chemoresistant	32.99 ± 10.10	21.05–53.10	

DISCUSSION

The concept of angiogenesis has been firmly established as a basic feature in tumor growth and metastasis. Although it has long been recognized that solid tumors contain large numbers of highly permeable blood vessels, the critical importance of these vessels in allowing tumor growth remained unappreciated until the early 1970s. At that time, Dr. Judah Folkman in his landmark studies defined the principles governing angiogenesis, and the modern era of research in the field of tumor angiogenesis began. Dr. Folkman hypothesized that new blood vessel formation at the primary tumor site was absolutely required for tumor nodules to grow beyond a diameter of 2 mm.[6,7] This is the size in which diffusion of nutrients and waste products becomes the rate-limiting step to growth. Inhibiting angiogenesis, therefore, would inhibit tumor growth at this 2-mm size. On the other hand, tumors which are able to recruit neovascularity are capable of unlimited growth and metastasis.[6] Folkman and others have demonstrated a strong correlation between tumor growth and vessel concentration in non-head and neck tumors.[8] A measurement of tumor angiogenesis or neovascularity has been suggested as a prognostic marker in malignant melanoma, breast, prostate, ovarian, gastric, and lung carcinomas.[5,9–11] In squamous cell carcinomas of the head and neck, tumor angiogenesis or microvessel density has only recently begun to be investigated. Several studies have investigated the role of tumor angiogenesis in predicting local regional recurrence and survival.[12–19] These studies have investigated multiple sites in the head and neck concurrently, and the results have been mixed, with some revealing a positive and others a nega-

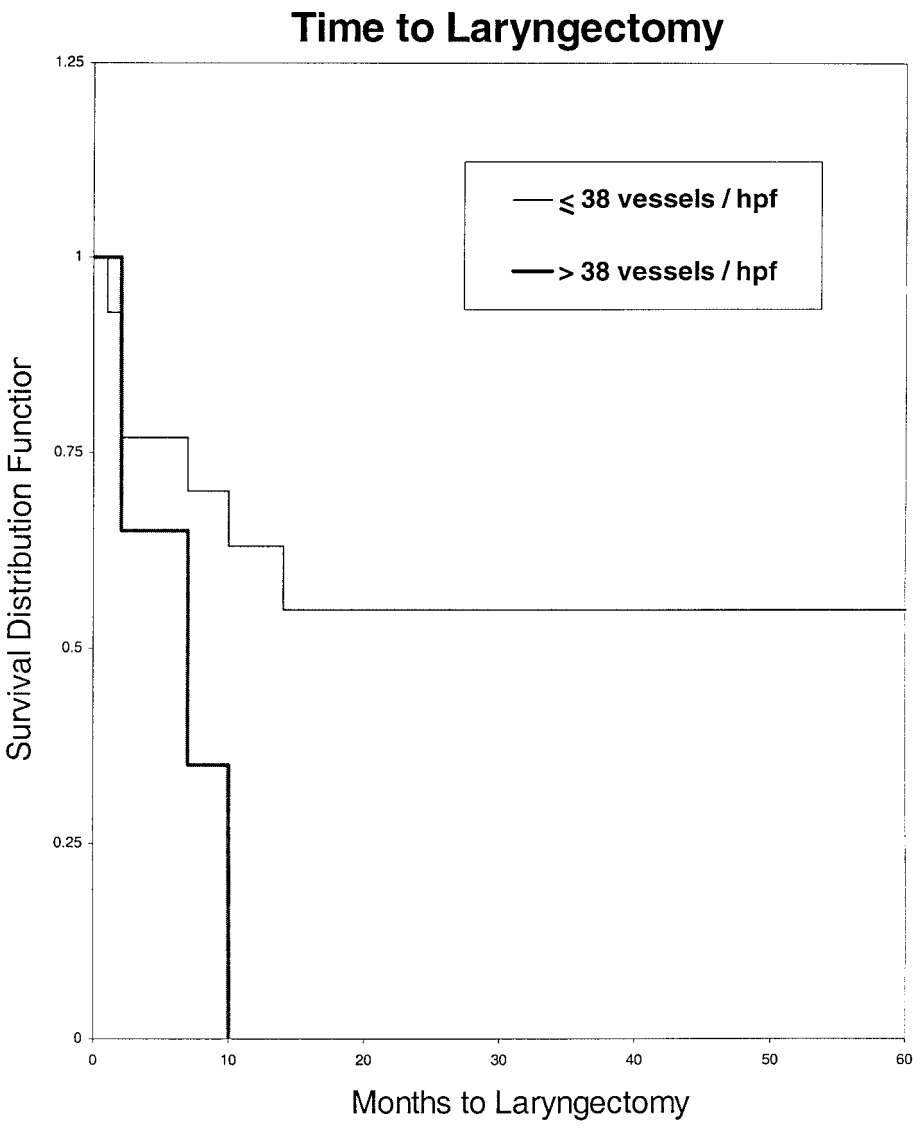

Fig. 2. Kaplan-Meier plot evaluating the likelihood of organ preservation for patients whose vessel counts were 1 standard deviation above the mean (38 vessels per high-power field, n = 3) versus those that were below this value (P = .09).

tive impact on overall prognosis. The only other study in the literature investigating laryngeal cancer exclusively was conducted by Murray et al.[20] They concluded that tumor angiogenesis is a significant predictor of local regional recurrence in N_0 (node-negative) patients. They further postulated that angiogenesis could be used as an independent prognostic indicator to determine which clinically N_0 patients are at higher risk for metastasis and could benefit from adjuvant chemotherapy.[20]

The most important finding in our study is that patients with a high level of tumor angiogenesis are less likely to benefit from organ-sparing protocols than those with lower levels of angiogenesis. We clearly illustrated that there was a statistically significant difference between the vessel counts of tumors that responded to chemotherapy and those tumors that did not. In addition, we further showed by Kaplan-Meier analysis that there was a strong trend toward the need for laryngectomy in patients with high vessel counts regardless of their chemosensitivity status. These findings seem to be counterintuitive at first glance. One would expect improved response to chemotherapy and increased rates of organ preservation in those patients with a high degree of vascularity resulting from the more effective delivery of the cytotoxic agent. The opposite, however, appears to be true, and rationale for this has been alluded to by Folkman[6] and Gorski et al.[21] It is well established that all solid tumors are dual-component systems consisting of tumor cells and a vascular infrastructure. Cytotoxic therapy (i.e., chemotherapy or radiation therapy) affects predominantly the tumor component of this system, leaving the vascular infrastructure in place. Any residual tumor, therefore, would have abundant nourishing vascularity to support rapid growth and tumor repopulation. Further supporting this and our findings in this study, Huang et al.[22] combined cytotoxic therapy with the antiangiogenic agent C225, and showed markedly improved response rates compared with the cytotoxic therapy alone. Therefore, the lack of chemoresponsiveness in the most-vascular tumors, as seen in this study, has been theorized previously and been corrob-

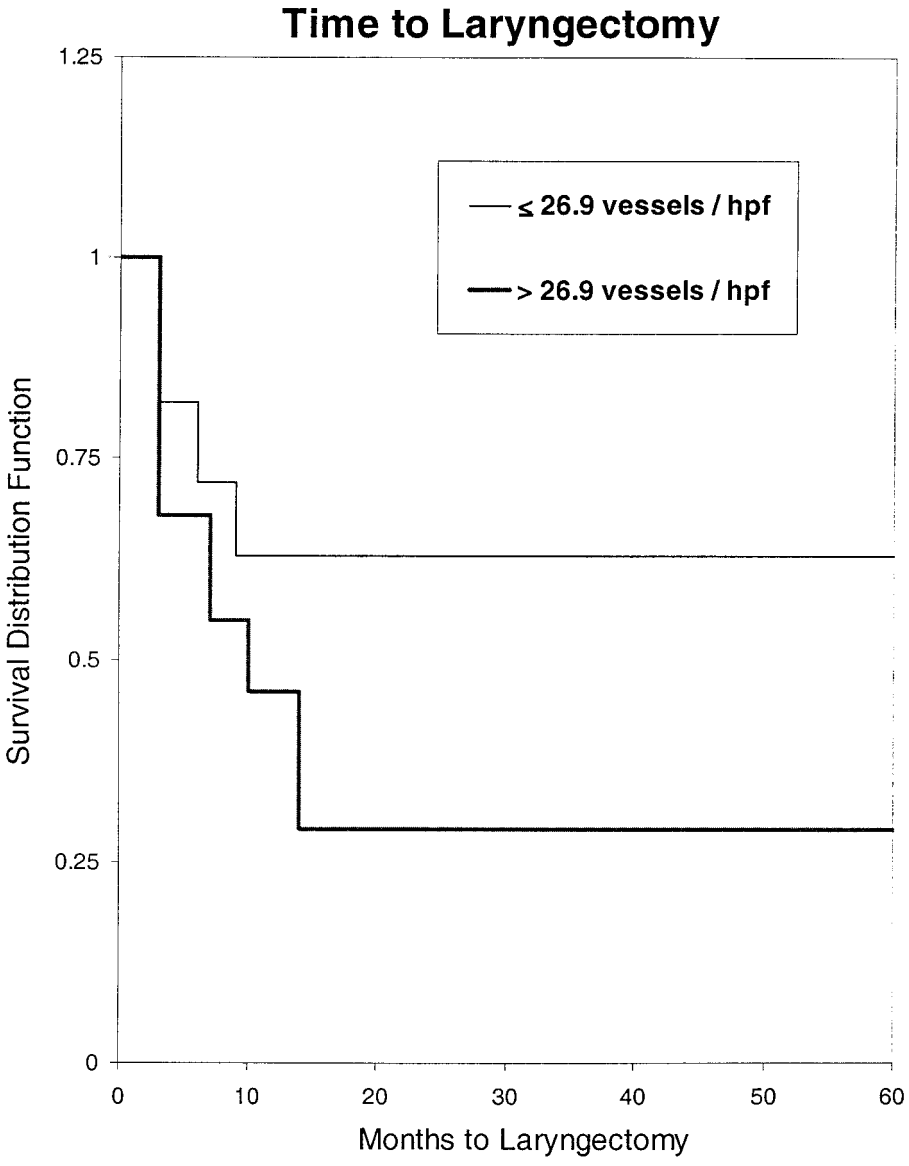

Fig. 3. Kaplan-Meier plot evaluating the rate of organ preservation for those patients above and below the mean (26.9 vessels per high-power field) ($P = .21$).

orated with at least one clinical study in head and neck patients.

The other important finding in this study included the fact that the patients with the highest degree of microvessel density, regardless of chemo-responsiveness or laryngectomy status, appeared to have a poorer overall survival. Recently, numerous studies have shown a statistically significant relationship between increased intratumoral microvessel density and decreased overall survival. This has been shown for patients with breast carcinoma, non-small-cell lung cancer, prostate carcinoma, gastric carcinoma, and nasopharyngeal carcinoma, to name a few.[5,10,14] Again, our study is in agreement with the studies in the literature in other malignancies, as well as in selected head and neck tumors.

Clearly, our results indicate that advanced laryngeal tumors with a high degree of vascularity tend to be less responsive to chemotherapy, be more likely to require laryngectomy, and have decreased overall survival. However, to provide more clinically useful information, we analyzed this small group of patients to identify a single cut-off value, which could be used to optimally separate patients into those who would benefit from organ preservation versus those who would require laryngectomy. When using the mean (26.9 vessels per HPF) as a cut-off value, the patients above this value were 3.5 times more likely to require laryngectomy and eight times less likely to respond to chemotherapy. It is important to note, however, that despite significant overlap of the vessel counts between the various groups (Figs. 4 and 5), the ability to predict which patients will not respond to chemotherapy (negative predictor value) was 91%. Despite our small numbers, these trends are very compelling, and it is possible that with a larger study population a more clinically useful cut-off value can be identified.

There are several presumed weaknesses in this study that need to be formally addressed. First and foremost is the fact that the sample size is somewhat limited. As

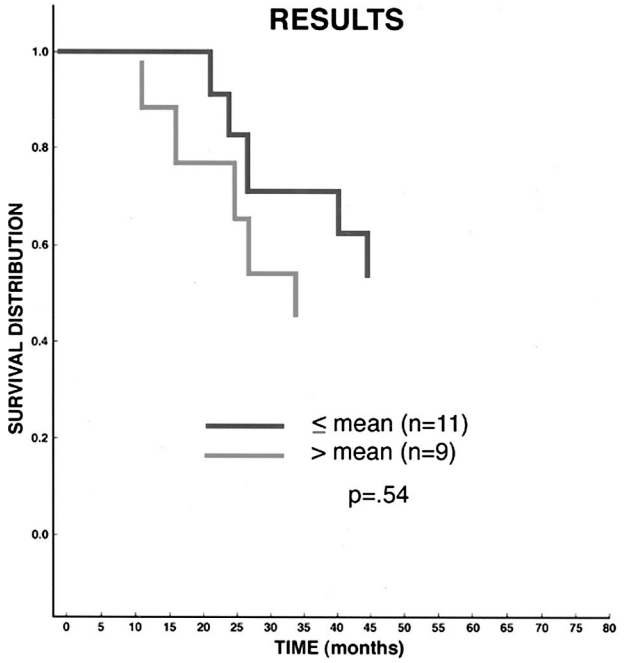

Fig. 4. Kaplan-Meier survival curves suggesting shorter long-term survival in patients with intratumoral vessel counts that were above the mean. This, however, did not reach statistical significance.

described in the *Methods* section, of the 166 patients in the chemotherapy arm, only 20 patients had sufficient pretreatment specimen available for immunohistochemical

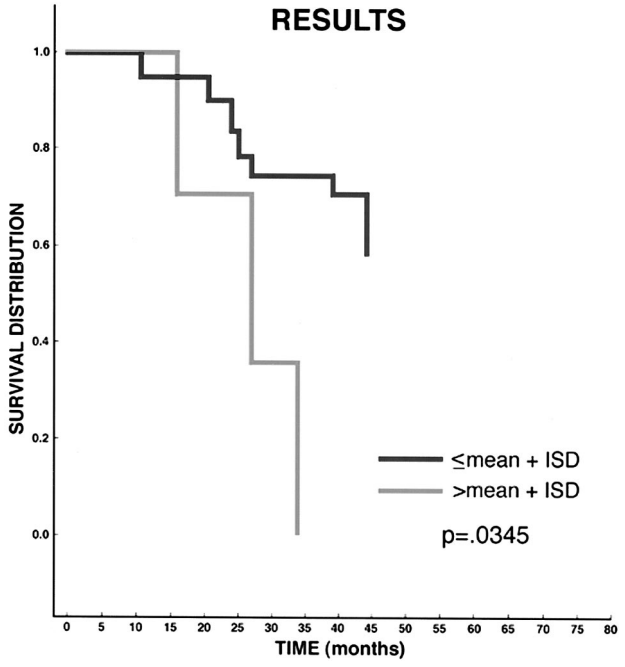

Fig. 5. Kaplan-Meier survival curve clearly demonstrating a poorer survival in patients with the most-vascular tumors (n = 3). The tumors represented by the red line had vessel counts greater than the mean plus 1 SD, whereas the blue line represents those tumors whose values were less than or equal to the mean + 1 SD. This was statistically significant at a value of $P = .0345$.

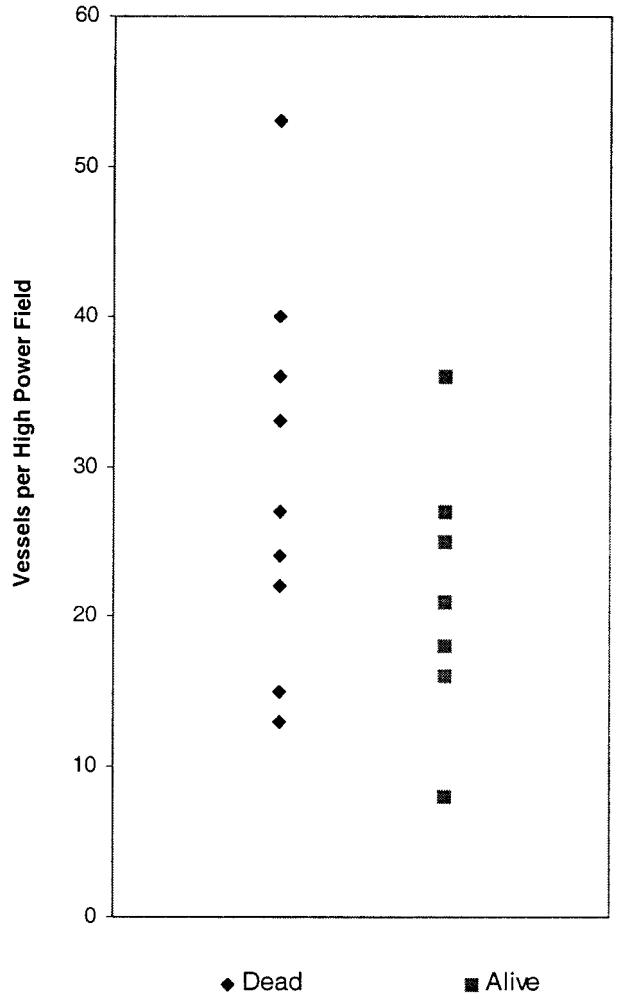

Fig. 6. Scatter plots revealing graphically the raw data of tumor vessel counts in pretreatment biopsy specimens of patients who died versus those who survived their advanced laryngeal carcinomas.

analysis. This occurred because the Veterans Affairs Laryngeal Cooperative Study tissue bank has been studied extensively by numerous investigators. The 20 samples available for this study were chosen, therefore, simply based on tissue availability. No other selection bias or criteria were used to include samples in the study. Surprisingly, however, the number of specimens is not as limited as one might think. There were only 30 patients who failed induction chemotherapy; therefore, the 10 patients analyzed in this study represent a full one third of all available specimen. To address this shortcoming of our study, a tissue-conserving microarray is being constructed on the remaining pretreatment biopsies in the Veterans Affairs Larynx Study. This will allow for confirmation of our results, as well as further testing on this valuable database. Therefore, despite the limited availability of specimen, we think our results are compelling and important enough to warrant reporting and further investigation.

The second factor that may be considered a limitation of this study is our choice of Factor VIII antibody as an

immunohistochemical marker of tumor angiogenesis. Although the literature is replete with reports of Factor VIII microvessel density staining, some think CD31 antibody staining gives a more-accurate assessment of tumor vascularity.[20] Factor VIII antibody is thought to stain both vasculature and lymphatics, whereas CD31 is thought to be more specific to the vasculature alone. Of the limited experience in the study of angiogenesis in head and neck tumors,[12–19] there has been no consensus as to the antibody of choice; and, indeed, both markers have been used with equal frequency. We chose Factor VIII immunohistochemical staining because of our previous favorable experience with this antibody, and we do not think it has altered the outcome of this study.

CONCLUSION

A high level of tumor angiogenesis in patients with advanced laryngeal carcinoma was found to be associated with a poor response to chemotherapy, a higher rate of laryngectomy, and a poorer overall survival. Tumor microvessel density should be considered a potentially important prognostic factor in determining which patients are most likely to benefit from laryngeal-sparing protocols. The findings in this study also suggest that there may be a role for the use of anti-angiogenic agents in such protocols in an effort to improve response rates and prevent rapid tumor repopulation following therapy.

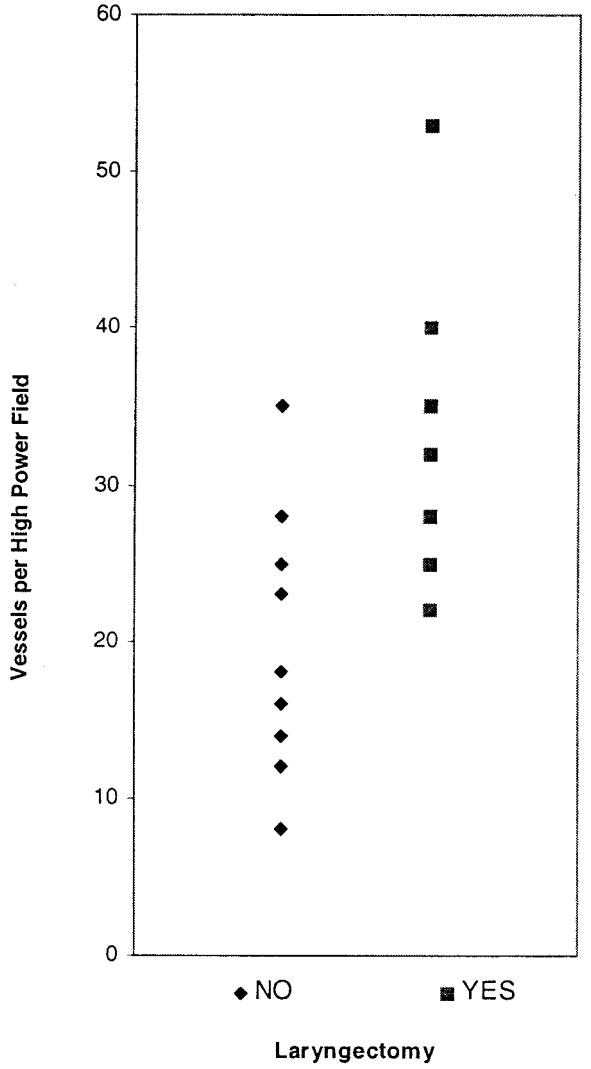

Fig. 7. Raw data shown in a scatter plot format revealing the pretreatment tumor vessel counts between patients who ultimately underwent laryngectomy versus those who did not require laryngectomy.

Acknowledgments

Participants of the Department of Veterans Affairs Laryngeal Cancer Study Group include the following: Study Chairman, Gregory T. Wolf, MD (Ann Arbor VAMC, Ann Arbor, MI); Study Co-chairman, Waun Ki Hong, MD (M.D. Anderson Hospital, Houston, TX); Biostatistician, Susan G. Fisher, PhD (Hines VA CSP Coordinating Center, Hines, IL); Susan Urba, MD (Ann Arbor VAMC, Ann Arbor, MI); James W. Endicott, MD (Tampa VAMC, Tampa, FL); Lanny Close, MD (Dallas VAMC, Dallas, TX); Samuel R. Fisher, MD (Durham VAMC, Durham, NC); Robert J. Toohill, MD (Wood VAMC, Milwaukee, WI); Daniel Karp, MD (Boston VAMC, Boston, MA); Donald M. Miller, MD, PhD (Birmingham VAMC, Birmingham, AL); Nae K. Cheung, MD (East Orange VAMC, East Orange, NJ); Arthur Weaver, MD (Allen Park VAMC, Allen Park, MI); Allen D. Hillel, MD (Seattle VAMC, Seattle, WA); Monica Spaulding, MD (Buffalo VAMC, Buffalo, NY); Barbara K. Chang, MD (Augusta VAMC, Augusta, GA); Brian Dougherty, MD (Miami VAMC, Miami, FL); Ronald DeConti, MD (Albany VAMC, Albany, NY); Harinder Garewal, MD (Tucson VAMC, Tucson, AZ); and Carol Fye, MS (Research Pharmacy Coordinating Center, Albuquerque, NM).

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