A Higher CD105-Assessed Microvessel Density and Worse Prognosis in Elderly Patients With Laryngeal Carcinoma

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Objectives: To ascertain the prognostic role of endoglin (CD105)-assessed microvessel density (MVD) in patients older than 65 years with laryngeal squamous cell carcinoma (LSCC), and whether this MVD differed in the elderly patients from younger adult controls.

Design: Retrospective clinicopathologic investigation.

Setting: Academic tertiary referral center.

Patients: Fifty-seven consecutive elderly patients with LSCC and 19 younger adult controls.

Main Outcome Measure: Image analysis of immunohistochemical reactions.

Results: In LSCCs in elderly patients, N+ stage correlated with a shorter disease-free survival (DFS) (P < .001). A higher CD105-assessed MVD was associated with disease recurrence (P = .006). The DFS was shorter in elderly patients whose CD105 expression was greater than 9.6% than in patients whose CD105 expression was 9.6% or less (P = .001). Among the elderly patients with tumors staged as N0, a higher CD105-assessed MVD correlated with disease recurrence (P = .006) and a shorter DFS (P = .001). CD105-assessed MVD in LSCC occurring in elderly patients did not differ from the situation observed in younger adult controls (P = .74).

Conclusions: In LSCC occurring in elderly patients, CD105-assessed MVD may be a useful N-stage independent, angiogenic prognostic marker for pinpointing: (1) patients at higher risk of disease recurrence; and (2) patients with N0 tumors at higher risk of early recurrence, who may benefit from more aggressive therapy.

Arch Otalaryngol Head Neck Surg. 2011;137(2):175-180
slowly, with less angiogenesis in older than in younger mice. Angiogenesis in human carcinomas occurring in elderly patients is still a controversial topic. The novel aim of the present study was to evaluate the potential prognostic role of CD105-assessed MVD selectively in 57 consecutive elderly patients (>65 years) with laryngeal squamous cell carcinoma (LSCC) all treated with primary surgery. We also aimed to establish whether the CD105-assessed MVD in LSCC occurring in elderly patients differed from the situation observed in a control group of younger adults.

**METHODS**

**PATIENTS**

In the present study, to contain the risk of a significant bias due to the heterogeneity of the series typical of retrospective settings, we chose to investigate only surgical specimens (not biopsy specimens) from LSCCs treated primarily and consecutively by the same surgical team.

Fifty-seven consecutive elderly patients (>65 years) with operable LSCC were considered (52 men and 5 women, mean [SD] age, 72.2 [5.1] years). For comparison, a control group of 19 consecutive younger adult patients was identified (17 men and 2 women, mean [SD] age, 50.5 [3.7] years). Before treatment, all patients underwent upper aerodigestive tract endoscopy, neck ultrasonography (with or without fine-needle aspiration cytology), and microarray analysis with laryngeal biopsy. Head and neck contrast-enhanced computed tomography and/or magnetic resonance imaging, chest radiographs, and liver ultrasonography were also performed.

In this cohort of elderly patients, 15 squamous cell carcinomas (SCCs) were glottic; 33, supraglottic; and 9, transglottic. None of the 17 patients with tumors staged as cT1 (12 elderly and 5 younger) who did not undergo elective dissection developed cervical lymph node metastasis after a mean (SD) follow-up period of 65.9 (21.4) months (range, 34-95 months). No distant metastases (M) were detected at the time of diagnosis. There were no significant clinicopathological differences between the elderly and younger patient groups regarding pT stage ($\chi^2$ test; $P = .86$), N ($\chi^2$ test; $P = .57$), stage ($\chi^2$ test; $P = .65$), or grade ($\chi^2$ test; $P = .99$). The mean duration of follow-up was 54.5 (23.6) months (median duration, 48.0 months) in the elderly patient group and 53.6 (21.5) months (median duration, 51.0 months) in the control group (Mann-Whitney test; $P = .87$). All tissue specimens were fixed in 4% paraformaldehyde and embedded in paraffin wax.

**IMMUNOHISTOCHEMICAL ANALYSIS FOR CD105**

Five-micron sections were obtained in all cases for immunohistochemical evaluation and CD105 reactivity was evaluated in each sample. The sections were rehydrated, preincubated with protein block (Novocastra Laboratories Ltd, Newcastle-upon-Tyne, England) for 5 minutes and stained with mouse monoclonal antibody CD105 (clone SN6h, diluted 1:10 [DAKO, Glostrup, Denmark]). Postprimary block (Novolink Polymer Detection System, Novocastra Laboratories Ltd) was then applied to the specimens for 20 minutes, then they were washed with phosphate-buffered saline (PBS) (pH, 7.0) for 3 minutes and incubated with Novolink Polymer for 20 minutes. The color was developed using 3,3′-diaminobenzidine (DAKO) for 4 minutes. The sections were counterstained with Meyer hematoxylin. An angiosarcoma sample was used as a positive control for CD105, while for the negative control the antibody was substituted with PBS.

Cytoplasmic CD105 staining was determined in endothelial cells from the LSCCs. The sections were scanned at 100 × original magnification to select the 3 areas with the greatest degree of vascularization (hot spots), free of necrosis or hemorrhage (confirmed by one of us [S.B.]) within the less well-differentiated areas of the tumor.

**IMAGE ANALYSIS**

All measurements on hot spots were performed at 400 × original magnification with a CYRES workstation image analysis (IA) system (Zeiss, Jena, Germany) consisting of a conventional microscope (Axiostar; Zeiss) connected to a 3-CCD (charge-coupled device) color video camera (KY-F35BE; JVC, Yokohama, Japan). The microscopic field was 0.785 mm² in size. The images to analyze were captured with a frame grabber (Kontron, Eching, Germany). The frame grabber and the IA software, operating online with the camera, were hosted in a personal computer. During all measurement sessions, the lighting was kept constant, and any stray light effect was reduced by using the Koehler illumination setting. The online segmenta-
tion and measurement routine enabled the artifacts to be rejected and all the areas selected to be checked after obtaining the measurements. A spatial calibration was also performed with a stage micrometer: the value obtained was 0.25 micron/pixel, in agreement with the Shannon-Nyquist sampling theory. All objects measured were within the range of 60 to 117 on the gray scale.

The proportion of the fields considered as being occupied by CD105-assessed microvessels (the area fraction) was determined for each specimen.

### STATISTICAL ANALYSIS

The following statistical tests were performed: the χ² test, Fisher exact test, the Mann-Whitney test, and the Kruskal-Wallis test for trend. The receiver operating curve (ROC) approach was used to determine the analytically best-fitting cutoff for the CD105-assessed MVD in terms of the survival analysis. The Kaplan-Meier survival function and the log-rank test were used, respectively, to display and evaluate the different disease-free survival (DFS) rates for patients stratified according to the variables selected. Considering the cohort of elderly patients, in the multivariate analysis, Cox proportional hazard regression determined significant predictors of DFS.

P < .05 was considered significant. The STATA 8 (Stata Corp, College Station, Texas) statistical package was used for all evaluations.

### RESULTS

#### CLINICAL OUTCOME AND FOLLOW-UP IN ELDERLY PATIENTS WITH LSCC

Thirty-five of the 57 elderly patients with LSCC experienced no recurrence of their malignant tumor after treatment (Table 1). Twenty-two patients developed locoregional recurrences after a mean (SD) period of 15.6 (11.9) months. The mean duration of follow-up was 54.9 (24.9) months in patients without recurrent disease and 53.9 (22.0) months in cases that recurred (Mann-Whitney test; P = .87). Between the 2 subcohorts of elderly patients with vs without locoregional carcinoma recurrences, the χ² test found significant differences in the distribution for lymph node status (cN0 or pN0 vs N+) (P = .02) and stage (P = .009), but not for pT (P = .12) or tumor grade (P = .14). The log-rank test showed a significant difference in DFS (in months) when patients were stratified by N (P < .001) and stage (P < .001), but not for pT (P = .34) or grade (log-rank test; P = .16).

The recurrence rate was higher in patients given postoperative RT (Fisher exact test; P = .01), and these patients’ DFS was also significantly shorter (log-rank test; P = .003).

#### CD105-ASSESSED MVD IN LSCC OCCURRING IN ELDERLY PATIENTS COMPARED WITH YOUNGER ADULT PATIENTS

CD105 intensively stained the vessels within the tumor tissue from both elderly and younger patients. There was no CD105 expression in the LSCC cells or the tumor’s stromal components. Rare vessels in normal, healthy laryngeal mucosa adjacent to the carcinoma also reacted to CD105 in both groups.

Table 1 summarizes the mean CD105-assessed MVD in different clinicopathological subgroups of our sample of LSCCs in elderly patients. Statistical analysis failed to identify any significant relationship between CD105-assessed MVD and pT (Kruskal-Wallis test for trend; P = .34), N (Mann-Whitney test; P = .69), stage (Kruskal-Wallis test for trend; P = .76), or grade (Kruskal-Wallis test for trend; P = .85). In the cohort of elderly patients, the Mann-Whitney test ruled out any significant difference between the mean (SD) CD105-assessed MVD found in supraglottic (6.5% [5.0%]) vs glottic SCCs (7.1% [7.1%]) (P = .72). The mean CD105-assessed MVD was 11.0% (8.5%) and 5.2% (4.8%) in the LSCC tissue from elderly patients with and without locoregional recurrences, respectively. Fisher exact test indicated a significant association between a higher CD105-assessed MVD and recurrent disease (P = .01). When the N0 cases were analyzed selectively, moreover, Fisher exact test disclosed a significant direct correlation between a higher CD105-assessed MVD and recurrent disease (P = .006).

The ROC approach was used to find the analytically best-fitting cutoff for the CD105-assessed MVD, which was calculated to be 9.6% (area under the curve, 0.74; 95% confidence interval [CI], 0.61-0.88; positive likelihood ratio, 4.13). A CD105-assessed MVD greater than 9.6% was found in 17 of 57 elderly patients (Figure, A and B) and in 5 of 19 younger ones. Statistical analysis showed that DFS was significantly shorter in elderly patients with a CD105 expression greater than 9.6% than in patients whose CD105-assessed MVD was 9.6% or less (log-rank test; P = .001) (Figure, C). In the subcohort of N0 cases, DFS was again significantly shorter in elderly patients with a CD105-assessed MVD greater than 9.6% than in those whose CD105-assessed MVD was 9.6% or less (log-rank test; P = .001) (Figure, D).

The CD105-assessed MVD in the LSCCs from the elderly and the younger patient groups were 7.5% (7.0%) and 6.3% (4.8%), respectively. The Mann-Whitney test ruled out any significant difference between the mean CD105-assessed MVD found in the elderly vs the younger patients (P = .74). When the subcohorts of N0 and N+ cases were considered, statistical analysis revealed no significant differences between the CD105-assessed MVD in the elderly vs younger patients (Mann-Whitney test; P = .67 and P = .78, respectively) in either case.

#### ELDERLY PATIENTS WITH LSCC: MULTIVARIATE ANALYSIS

Multivariate DFS estimates were based on the Cox proportional hazards model and assumed no interactions between significant variables in the final model. Considering the cohort of elderly patients with LSCC, CD105-assessed MVD status (MVD > 9.6% or MVD ≤ 9.6%), age, pT stage, and N stage were entered into this multivariate model to determine their relation with DFS. On analysis synthesized in Table 2, N stage (relative risk 3.23; 95% CI, 1.16-8.97; P = .02) and CD105-assessed MVD status (relative risk, 3.68; 95% CI, 1.49-9.06;
COMMENT

The literature abounds with conflicting reports on the influence of a patient’s age on survival after contracting cancer and other parameters relating to having a malignant tumor. Several studies investigated LSCC in particular, and some of them concluded that the prognosis for LSCC is better for younger patients, while others came to the opposite conclusion.

In the present study, we used a computer-based IA system to measure CD105-assessed MVD for the accuracy, precision, and reproducibility achievable with immunostained slide analysis. The relationships between CD105 expression and selected clinicopathological and prognostic features in LSCC were first investigated by our group. Data analysis showed a significantly higher locoregional carcinoma recurrence rate and a shorter DFS in patients with a higher CD105-assessed MVD. In 2006, we first investigated the relationship between CD105-assessed MVD and nuclear MASPIN (a tumor-suppressing serpin and potent angiogenesis inhibitor) in 35 consecutive LSCCs, finding mean nuclear MASPIN expression higher in patients without recurrent LSCC than in cases with LSCC recurrences, and statistical analysis confirmed that the mean CD105-assessed MVD was significantly higher in patients whose LSCC recurred. These preliminary results pointed to the crucial role of the subcellular nuclear localization of MASPIN in LSCC angiogenesis (ie, CD105-assessed MVD was significantly lower in LSCCs with MASPIN nuclear staining than in those with mainly cytoplasmic localization).
toplastic staining). Over the past 2 years, the Montenegro Clinical Centre in Podgorica has investigated CD105 expression specifically in supraglottic\textsuperscript{14} and glottic\textsuperscript{15} SCCs.

In the present cohort of LSCCs in elderly patients, N+ stage correlated strongly with a poor prognosis, in terms of disease recurrence rate and DFS. Statistical analysis identified a significant association between a higher CD105-assessed MVD and disease recurrence. In this series, the DFS was also significantly shorter in elderly patients with a CD105 expression greater than 9.6% than in cases whose CD105-assessed MVD was 9.6% or less. It is worth noting that data analysis showed that a higher CD105-assessed MVD correlated significantly with carcinoma recurrence and shorter DFS in the N0 subcohort of elderly patients, too. The multivariate analysis showed that CD105-assessed MVD greater than 9.6% had a prognostic significance with relation to DFS in elderly patients with LSCC. In particular, it was noted that, in the elderly patients’ group, the relative risk of poor DFS with CD105-assessed MVD greater than 9.6% was 3.68. Moreover, multivariate model evidence confirmed the hypothesis that CD105-assessed MVD was an N-stage independent indicator of a poor prognosis for LSCC in elderly patients.

Very recently, after assessing MVD using VE cadherin in a mouse model of head and neck SCC, Bojovic and Crowe\textsuperscript{16} concluded that tumors in elderly mice were poorly vascularized and necrotic, and produced significantly fewer lymph node metastases. When observational clinical studies were considered, there were reports of fewer blood vessels in breast cancers and lung adenocarcinomas occurring in elderly patients than in younger patients.\textsuperscript{17-19} Conversely, the present study on CD105-assessed MVD in 57 consecutive cases of LSCC occurring in elderly patients revealed no significant differences vis-à-vis a clinicopathologically homogeneous control group of younger adults. Chandrachud et al\textsuperscript{2} also reported findings to indicate that vascularity, as measured by the density or volume of vessels stained with panendothelial markers in 88 resected non–small-cell lung cancers, was unrelated to the patient’s age.

**CONCLUSIONS**

The results of the present investigation bear out the theory that angiogenesis continues to play a crucial part in laryngeal carcinoma prognosis in elderly patients too. In LSCCs occurring in elderly patients, CD105-assessed MVD can be considered an angiogenic prognostic marker, N-stage independent, and potentially useful for identifying: (1) patients at higher risk of disease recurrence after treatment and (2) patients with tumors staged as N0 at higher risk of early locoregional recurrence, who may benefit from more aggressive therapy.

Convincing experimental findings have suggested that anti-CD105 monoclonal antibodies could become strongly localized in the endothelium of the tumor-associated vasculature and that they are efficient in inhibiting tumor angiogenesis, growth, and metastasis in animal models, thereby pointing to CD105 as a suitable vascular target for an antibody-based therapeutic approach to cancer.\textsuperscript{6} Shiozaki et al\textsuperscript{20} developed a human/mouse chimerical antibody to CD105 and generated encouraging pharmacokinetic and toxicity data in nonhuman primates. Regarding active vaccination, it has been found in a murine tumor model that immunization with swine CD105 induced specific anti-CD105 antibodies that inhibited angiogenesis and enhanced apoptosis in subsequently induced tumors.\textsuperscript{21,22}

Carefully designed clinical trials involving patients likely to respond to CD105 inhibition may be able to demonstrate the efficacy of treatment combinations associating angiogenesis-targeted and conventional therapies for LSCC in elderly patients, too.

**Submitted for Publication:** June 7, 2010; final revision received August 11, 2010; accepted September 14, 2010.

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**Financial Disclosure:** None reported.

**Funding/Support:** This study was supported in part by grant 60A07–4404/09 to Dr Marioni from the University of Padova.

**Additional Information:** Dr Ralli is the past president of the Italian Association of Otorhinolaryngology and Geriatrics.

**Additional Contributions:** Frances Coburn revised the final English version of this article.

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