Laryngeal Carcinoma Recurrence Rate and Disease-free Interval are Related to CD105 Expression but not to Vascular Endothelial Growth Factor 2 (Flk-1/Kdr) Expression

GINO MARIONI[1], LUCIANO GIACOMELLI[2], EMILIANO D'ALESSANDRO[2], CLAUDIA STAFFIERI[1], VINCENZA GUZZARDO[2], ALBERTO STAFFIERI[1] and STELLA BLANDAMURA[2]

[1]Department of Medical and Surgical Specialties, Section of Otolaryngology, and [2]Department of Medical/Diagnostic Sciences and Special Therapies, Section of Anatomic Pathology, University of Padova, Padova, Italy

Abstract. *Background: Tumour angiogenesis is the result of an inbalance between anti- and pro-angiogenic factors. CD105 (endoglin) is a component of the receptor complex of transforming growth factor (TGF-β1). Vascular endothelial growth factor receptor 2 (VEGFR2 or Flk-1/KDR) belongs to the high-affinity VEGF receptors. The aim of the study was to investigate the expression, cellular localization and role of CD105 and VEGFR2 in laryngeal carcinoma. Patients and Methods: Sections of 62 laryngeal carcinomas were stained with CD105 and VEGFR2/Flk-1/KDR antibodies. Results: A significant association between CD105 expression and locoregional recurrence was found (p=0.009). Interestingly, in N0 patients CD105 expression was significantly associated with locoregional recurrence of the carcinoma (p=0.03). The log-rank test showed a significant difference in the disease-free interval in patients stratified according to CD105 expression (p=0.02). Statistical analysis showed no significant associations between vessel endothelial cell or laryngeal carcinoma cell VEGFR2 expressions and recurrence of disease or disease-free intervals. Conclusion: CD105 expression but not VEGFR2 expression correlated with carcinoma recurrence after treatment and shorter disease free interval. The CD105 expression may be useful to detect cervical node-negative patients with a higher risk of early laryngeal carcinoma recurrence.*

Tumour angiogenesis, the formation of peri-tumour and intra-tumour new blood vessels, is crucial for the growth of solid malignancies. An avascular tumour rarely grows larger than 2 to 3 mm^3, but, once vascularized, tumour progression is rapid. Neovascularization allows tumour growth by supplying nutrients and oxygen, disposing of metabolites and releasing growth factors that promote tumour cell proliferation (1). The angiogenic process includes cell migration, proliferation, micro-vessel differentiation and anastomosis, extracellular matrix degradation and structural reorganization (2, 3). Tumour angiogenesis is the result of an inbalance between pro- and anti-angiogenic factors produced by both the malignancy and the normal cells. Pro-angiogenic factors include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-2), transforming growth factor (TGF-β1), placenta growth factor and angiopoietins (4).

CD105 (endoglin) is a proliferation-associated and hypoxia-inducible protein abundantly expressed in angiogenic endothelial cells. CD105 is a disulfide-linked homodimeric cell membrane glycoprotein of 180 kDa. In humans, the CD105 gene is located on chromosome 9q34 and it is arranged into 14 exons. CD105 is a component of the receptor complex of TGF-β1, a pleiotropic cytokine that modulates angiogenesis by the regulation of different cellular functions including proliferation, differentiation and migration (5). In the absence of TGF-β1, CD105 shows an anti-apoptotic effect in endothelial cells under hypoxic stress, suggesting for a protective role of CD105 against pro-apoptotic factors (6).

VEGF plays a crucial role in tumour angiogenesis. VEGF production is induced *via* cytokines, activation of oncogenes, and loss of tumour suppressor genes (7). VEGF expression is up-regulated in most human malignancies stimulating proliferation, migration, chemotaxis and survival of endothelial cells (8, 9). This protein binds to the tyrosine kinase receptors VEGFR1 (also known as flt-1), VEGFR2 (also known as Flk-1/KDR), and VEGFR3. These receptors differ in their signalling properties. In particular, VEGFR2 activation is associated with both endothelial cell migration and proliferation and is followed by activation of the mitogen-activated protein kinase cascade (10). VEGFR2 is a type 3

Correspondence to: Gino Marioni, MD, Department of Medical and Surgical Specialties, Section of Otolaryngology, University of Padova, Via Giustiniani 2, 35100 Padova, Italy. Fax: +39 0 49 8752266, e-mail: gino.marioni@unipd.it

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membrane bound tyrosine kinase receptor consisting of an extracellular region with seven immunoglobulin-like domains, a transmembrane domain and a tyrosine kinase domain with an insert of approximately 70 amino acids (11). VEGFR2 belongs to the known high-affinity VEGF receptors and is presumed to play a substantial role in tumour neoangiogenesis. Initially, the expression of VEGFR2 was reported to be restricted to the classic endothelium (12). Later, it was found to stimulate migration, proliferation and survival of non-endothelial cells, including monocytes, neurons, chondrocytes, retinal epithelium, and smooth muscle cells (13). The identification of VEGF receptors in carcinoma cell lines and in a variety of solid tumours (pancreatic, prostatic and head and neck) has generated recent interest (8, 9, 14). The biological significance of VEGF receptors on cancer cells is unclear, but associated up-regulation of anti-apoptotic genes has been hypothesized (13).

The aim of the present study was to investigate the expression and cellular localization of CD105 and VEGFR2 (Flk-1/KDR) in 62 cases of laryngeal squamous cell carcinoma (SCC). The relationship between these immunohistochemical parameters and other clinico-pathological parameters and the disease-free interval of this series was also studied.

Patients and Methods

Patients. A total of 62 consecutive cases of laryngeal SCC were retrospectively evaluated. Fifty-four patients were male and 8 female, with a mean age of 65.4±10.0 years. All the patients had undergone partial or total laryngectomy at the Section of Otolaryngology of the University of Padova. Modified radical neck dissection and post-operative radiotherapy had also been performed in 44 and 18 cases, respectively. According to the TNM Classification of malignant tumours of the International Union Against Cancer (15), the pathological staging of the primary laryngeal lesions (pT) was T1 in 26 cases, T2 in 18, T3 in 9, and T4 in 9. Pathological regional lymph node staging (pN) was N0 in 35 cases, N1 in 1, N2 in 7 (3 N2b, 4 N2c) and N3 in 1. Eighteen patients were cN0. The mean follow-up time was 38.6±21.0 months. For pathological grading, 21 out of the 62 cases were staged as G1, 28 as G2, and 13 as G3.

Immunohistochemistry. For immunohistochemical evaluation formalin-fixed, paraffin-embedded 5 μm sections were cut for all 62 cases. For each sample, VEGFR2 and CD105 reactivity were evaluated.

Immunohistochemistry for VEGFR-2. The Flk-1/KDR/VEGFR2 epitope specific rabbit antibody (Lab Vision Corporation, Fremont, CA, USA) was used, 1:50 dilution. Formalin-fixed sections were boiled in 10 mM citrate buffer, pH 6.0 for 20 min, followed by cooling at room temperature for 20 min. The sections were pre-incubated with protein block (Novocastra Laboratories Ltd, Newcastle-Upon-Tyne, UK) for 5 min to block non-specific background staining and then incubated for 30 min at room temperature with the primary antibody. Post primary block (Novolink Polymer Detection System; Novocastra) was applied for 20 min and the specimens were then washed with PBS (pH 7.0) for 3 min and incubated with Novolink Polymer for 20 min. The colour was developed using 3.3'-DAB (DAKO, Glostrup, Denmark) for 4 min. As positive control a ductal breast carcinoma sample was used and the antibody was replaced with PBS for the negative control.
Immunohistochemistry for CD105. The CD105 mouse monoclonal antibody, clone Sn6h (DAKO, Glostrup, Denmark) was used, 1:10 dilution. The slides were pre-treated for antigen retrieval by incubation for 10 min with protease XIV (Sigma Chemical Co., St Louis, MO, USA) at room temperature. The sections were pre-incubated with protein block (Novocastra Laboratories Ltd) for 5 min to block non-specific background staining and then incubated for 45 min at room temperature with the primary antibody. Post primary block (Novolink Polymer Detection System; Novocastra) was applied for 20 min and the specimens were then washed with PBS (pH 7.0) for 3 min and incubated with Novolink Polymer for 20 min. The colour was developed using 3.3'-DAB (DAKO) for 4 min. Sections were counterstained with Mayer's haematoxylin. As positive control an angiosarcoma sample was used andthe antibody was replaced with PBS for the negative control.

Image analysis determinations. The sections were scanned by the senior pathologist (S.B.) at x100 in order to select from the less differentiated areas of tumour the three areas with the highest degree of vascularisation (hot spots), free of necrosis or haemorrhage. The expressions of CD105 and endothelial VEGFR2 were evaluated using the "CIRES" workstation image analysis system (Zeiss, Jena, Germany), consisting of a conventional light microscope (Axioskop model, Zeiss) connected to a 3CCD colour video camera (KY-F55BE, JVC, Yokohama, Japan). The images were captured by a frame grabber (Kontron, Eching, Germany) and then analysed. The frame grabber and the image analysis (I.A.) program, operating on line with the camera, were computer hosted. During all measurement sessions, illumination was kept constant at a fixed value and the stray light effect was reduced using Koehler's illumination setting (16). The online segmentation and measurement routine allowed rejection of artefacts and revision of all selected areas after measurements. Spatial calibration was also performed using a stage micrometer giving a value of 0.25 micron/pixel, in agreement with the Shannon-Nyquist sampling theorem (17). The red-grey-blue (RGB) grabbed pixels corresponding to the images were processed and coded by the software in a grey intensity scale according to colour luminance (from 0 to 256 grey values [8 bit coding] in our measurements). The green channel, the more sensitive, was used to identify each epitope. All the measured objects were in the range of 60 to 117 of the grey scale for CD105 evaluation. Cytoplasmic CD-105 staining was determined in endothelial cells of laryngeal SCC. The percentage of the fields occupied by CD105-assessed micro-vessels (area fraction) was determined at x400 for each specimen. For VEGFR2 endothelial expression evaluation all the measured objects were in the range from 71 to 161 of the grey scale. To assess the reproducibility of the I.A. against conventional determinations based on light microscopy, the senior pathologist blindly scored 50% of the total and randomly selected slides, using the same strategy (except I.A.) irrespective of protein type. The results of both evaluation modalities correlated fairly: Spearman's 88%. For each case, the senior pathologist evaluated at x400 magnification VEGFR2 expression in SCC tumour cells in the same tumour areas previously investigated for endothelial VEGFR2 expression.

Data analysis. As the data deviated from normal distribution, when required, the following distribution-free statistical tests have been applied: Spearman's ϱ correlation coefficient, Fisher's exact test, Kruskal-Wallis and Kruskal-Wallis for trend tests. The Kaplan-Meier survival function and the log-rank test were applied to display and evaluate the different disease-free intervals of the patients stratified according to the selected variables. The receiver operating curve (ROC) approach was applied to determine the analytically best-fitting cut-off points of the variables selected for the subsequent survival analysis. A multivariate analysis was not performed as there were many variables relative to the number of patients. A p-value <0.05 was considered to be significant; values in the range $0.10>p\geq0.05$ were considered as indicating a statistical trend. The STATA 8 (Stata Corp, College Station, TX, USA) statistical package was used for all evaluations.

Results

Thirty-eight out of the 62 cases of laryngeal SCC did not experience malignancy recurrence after treatment. Twenty-six developed locoregional malignancy recurrence after a mean period of 15.7±11.2 months (median 12.5 months). The mean follow-up periods were 40.9±20.8 months (median 44.5 months) and 35.0±21.3 months (median 25.5 months) in patients without and with recurrence of disease, respectively. The Kruskal-Wallis test did not disclose a significant difference between mean follow-up periods ($p=0.29$). The Fisher exact test showed that carcinoma recurrence was associated to pN-staging ($p=0.02$) and stage grouping ($p=0.01$). Statistical analysis failed to disclose a significant relationship between T-stage or grading and recurrence of disease (Fisher exact test, $p=0.08$ and $p=0.28$, respectively). Considering laryngeal carcinoma treatment modalities, the Fisher exact test showed that the recurrence rate was not significantly different between the groups of patients who had undergone or not undergone post-operative radiotherapy ($p=0.29$).

The log-rank test showed a significant difference in the disease-free interval (in months) in patients stratified according to N-stage ($p=0.001$) and stage-grouping ($p=0.001$) but not according to pT-stage ($p=0.20$) or pathological grading ($p=0.27$)

CD105 expression. Only rare vessels in the normal healthy mucosa adjacent to the SCC showed a reaction to CD105. CD105 stained intensively intratumoral vessels (Figure 1 A, B), but CD105 expression was absent from the laryngeal SCC cells and in the tumour stromal components.

The CD105 mean expression stratified according to pT-stage, N-stage, stage-grouping and pathological grading is shown in Table I.

The Kruskal-Wallis test showed a significant association between CD105 expression and locoregional recurrence ($p=0.009$). Interestingly, in the N0 patients, CD105 expression was significantly associated with locoregional recurrence of SCC (Kruskal-Wallis test, $p=0.03$).

Statistical analysis ruled out an association between CD105 expression and pT-staging (Kruskal-Wallis for trend test, $p=0.25$), N-stage (Kruskal-Wallis test, $p=0.64$), stage-grouping (Kruskal-Wallis test for trend, $p=0.37$) and pathological grading (Kruskal-Wallis test for trend, $p=0.90$).

The calculated cut-off value identified by ROC for the Kaplan-Meier survival analysis was 7% (sensitivity 71%, specificity 69%). The log-rank test showed a significant difference in the disease-free interval (in months) in patients stratified according to CD105 expression ($p=0.02$) (Figure 2).

VEGFR2 expression in endothelial cells. Cytoplasmic and membrane staining in laryngeal malignant endothelium was observed in all our samples (mean expression 11.6%±9.8%), but the intensity of the staining was heterogeneous and often weak (Figure 1C).

Table I summarises the vessel endothelial cell VEGFR2 expression in laryngeal carcinoma stratified according to pT-stage, N-stage, stage-grouping and pathological grading. Statistical analysis ruled out an association between vessel endothelial cell VEGFR2 expression and pT-stage (Kruskal-Wallis test for trend, $p=0.84$), N-stage (Kruskal-Wallis test, $p=0.84$), stage-grouping (Kruskal-Wallis test for trend, $p=0.83$) and pathological grading (Kruskal-Wallis test for trend, $p=0.30$). The Kruskal-Wallis test found a trend towards a significant association between endothelial cell VEGFR2 expression and locoregional carcinoma recurrence ($p=0.09$).

The cut-off value identified by ROC for Kaplan-Meier survival analysis was 19% (sensitivity 50%, specificity 29%) The log-rank test showed no significant difference in the disease-free intervals (in months) in patients stratified according to VEGFR2 endothelial expression ($p=0.48$).

VEGFR2 expression in laryngeal carcinoma cells. Cytoplasmic and membrane staining was present in laryngeal carcinoma cells in 19 out of the 62 samples (30.6%) (Figure 1D) with a mean VEGFR2 expression of 17.3%±17.5%. Table I summarises the laryngeal carcinoma cell VEGFR2 expression stratified according to pT-stage, N-stage, stage-grouping and pathological grading. Statistical analysis showed no significant association between laryngeal carcinoma cell VEGFR2 expression and pT-stage (Kruskal-Wallis test for trend, $p=0.84$), N-stage (Kruskal-Wallis test, $p=0.47$), stage-grouping (Kruskal-Wallis test for trend, $p=0.67$), pathological grading (Kruskal-Wallis test for trend, $p=0.57$), or locoregional recurrence of disease (Kruskal-Wallis test for trend, $p=0.52$).

The cut-off value identified by ROC for Kaplan-Meier survival analysis was 30% for VEGFR2 expression in carcinoma cells (sensitivity 25%, specificity 81%). The log-

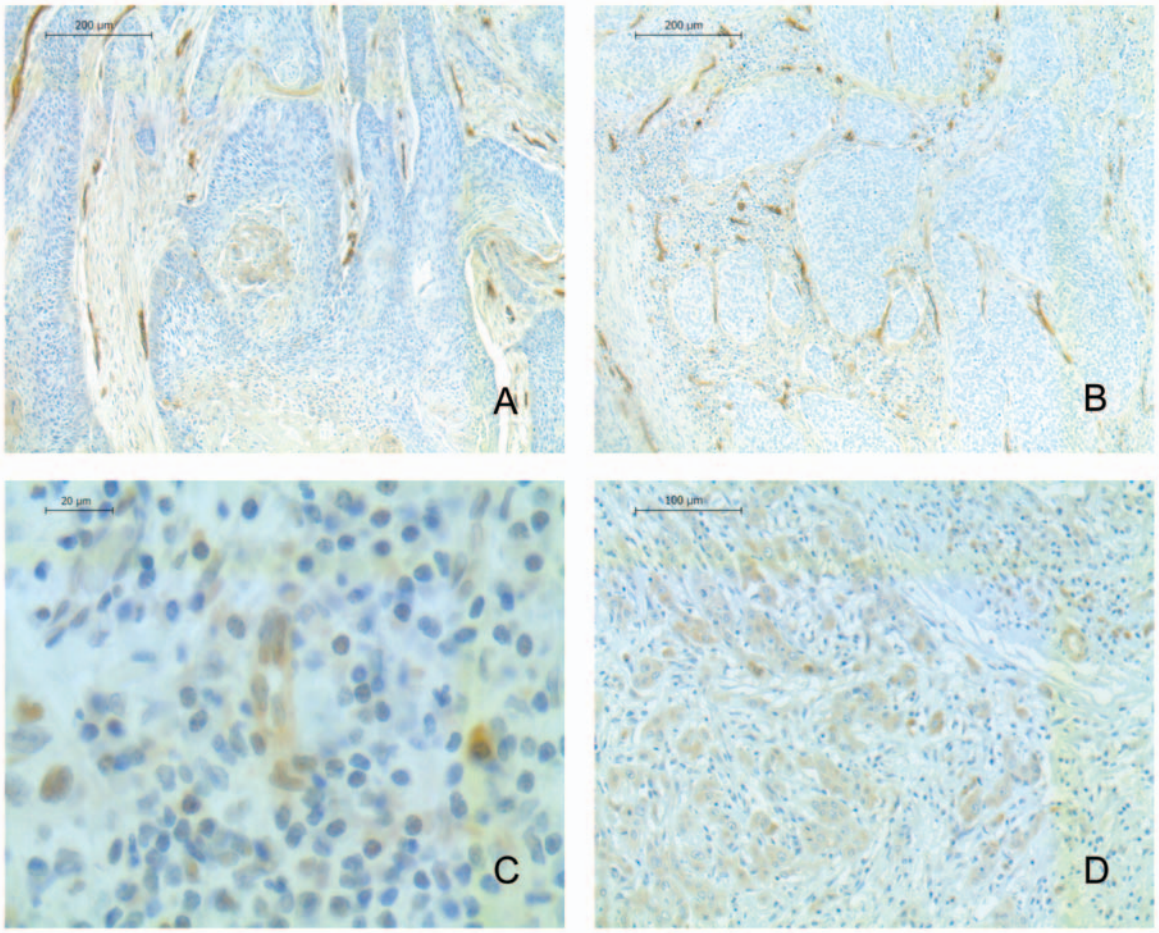

Figure 1. *A. CD105 expression in poorly differentiated laryngeal carcinoma; B. CD105 expression in well-differentiated laryngeal SCC; C. cytoplasmic VEGFR2 expression in vessel endothelial cells of laryngeal carcinoma; D. cytoplasmic VEGFR2 expression in laryngeal carcinoma cells.*

rank test ruled out a significant difference in the disease-free intervals (in months) in patients stratified according to VEGFR2 expression in carcinoma cells ($p=0.52$).

Correlations between CD105 expression and VEGFR2 expressions in endothelial cells and in laryngeal carcinoma cells. Spearman's rank correlation test identified a trend towards significant correlation between VEGFR2 endothelial expression and carcinoma cell VEGFR2 expression ($p=0.05$) and between VEGFR2 endothelial expression and CD105 expression ($p=0.08$). Significant correlation between carcinoma cell VEGFR2 expression and CD105 expression was ruled out (Spearman's rank correlation test, $p=0.79$)

Discussion

Micro-vessel density (MVD) has been reported to be an independent prognostic indicator of outcome in a variety of human malignancies, with increased MVD correlating with malignancy progression and shorter overall and disease-free survival rates (18). In head and neck SCCs there have been contradictory results concerning the clinical significance of MVD. These discrepancies may reflect the differences in treatment protocol and in the endothelial markers used for immunohistochemical staining.

In the present study, to reduce the possibility of significant bias due to series heterogeneity in a retrospective setting, only surgical specimens (not biopsies) of carcinoma of a specific structure (larynx) consecutively treated in a defined period (2000-2004) by the same surgical staff were investigated. Regardless of other available information on head and neck carcinoma MVD determination, for most of the determinations a computer-based image analysis system was preferred for the accuracy, precision and reproducibility of immunostained slide analysis allowed by this device.

The CD105 expression in oral and oropharyngeal SCC has been studied by several groups (19-24) but the available information regarding the expression and the prognostic

Table I. *CD105 expression, VEGFR2 expressions in vessel endothelial cells and laryngeal carcinoma cells.*

	Nr. cases	Mean CD105 expression	Mean VEGFR2 expression in vessel endothelial cells	Mean VEGFR2 expression in carcinoma cells
pT1	26	6.2%±4.4%	9.7%±7.5%	5.1%±14.7%
pT2	18	9.9%±6.4%	14.9%±13.5%	6.7%±12.7%
pT3	9	11.5%±13.2%	14.6%±13.2%	4.4%±10.1%
pT4	9	10.0%±9.0%	11.8%±8.0%	3.9%±7.0%
N+(pN+)	9	13.3%±11.8%	11.1%±9.4%	1.7%±3.5%
N0 (cN0 or pN0)	53	7.7%±5.4%	11.7%±9.9%	5.9%±13.3%
Stage I	26	6.2%±4.3%	9.7%±7.5%	5.1%±14.7%
Stage II	17	9.2%±5.9%	14.9%±13.9%	7.1%±13.0%
Stage III	6	10.1%±8.2%	11.2%±4.2%	5.0%±12.2%
Stage IV	13	11.4%±10.3%	11.4%±8.9%	3.5%±6.2%
G1	21	6.6%±5.3%	9.5%±6.8%	3.1%±7.1%
G2	28	11.1%±8.2%	11.1%±8.0%	5.5%±11.0%
G3	13	6.1%±4.0%	16.2%±15.2%	8.2%±20.3%
Without L-R Rec	38	6.6%±4.8%	12.1%±7.3%	5.6%±13.8%
With L-R Rec	24	11.5%±8.6%	10.8%±12.9%	4.8%±10.2%

L-R Rec: locoregional recurrence.

role of CD105 in laryngeal SCC is still limited. Out of 127 cases of head and neck primary SCCs described by Martone *et al.* (25), 107 were laryngeal malignancies and the CD105-assessed MVD was significantly higher in the N+ carcinomas. The patients with high MVD had a significantly shorter disease-free and overall survival and that was the only independent marker of carcinoma recurrence or death by multivariate analysis. In our previous study (26), 13 out of 43 cases of laryngeal SCC developed locoregional recurrence after treatment and multivariate logistic regression confirmed that CD105-assessed MVD was significantly related to disease recurrence but not to disease relapse time.

In the present study, a significant association between CD105 expression and locoregional recurrence was shown ($p=0.009$). Interestingly, although carcinoma recurrence was associated with pN-staging ($p=0.02$), in the N0 patients CD105 expression was significantly associated to locoregional recurrence of SCC ($p=0.03$). A significant difference in the disease-free intervals (in months) in patients stratified according to CD105 expression was also shown ($p=0.02$). Thus the CD105 results were extremely promising for detecting patients with laryngeal SCC at risk of early locoregional disease recurrence. The potential of vascular targeting as a cancer therapeutic approach has been firmly established in experimental studies. The vascular targeting agents lead to rapid reductions in tumour blood flow and extensive necrosis in experimental tumours (27). CD105 may represent an ideal target for anti-angiogenic therapies also for laryngeal carcinomas.

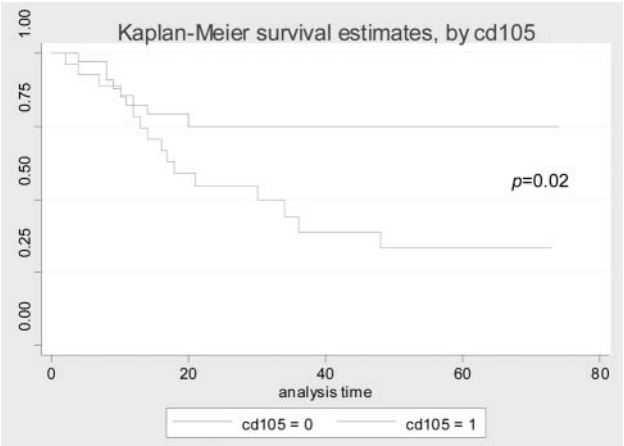

Figure 2. *Disease-free interval estimates by CD105 endothelial expression in laryngeal carcinoma (CD105 expression <7% (0) or ≥7% (1)). Time calculated in months.*

VEGFR2 expression in head and neck SCC has only recently been studied. Giatromanolaki *et al.* (28) found no association between VEGF/flk-1 complex activated MVD and response to chemo-radiotherapy or patient's survival in a series of 104 inoperable locally advanced

head and neck SCCs. Neuchrist *et al.* (12) found major VEGFR2 expression in the stromal cells and endothelium of frozen sections, whereas the tumour cells showed modest VEGFR2 immunoreactivity. Furthermore, 2 out of 4 head and neck SCC tumour cell lines revealed positive VEGFR2 immunoreactivity while RT-PCR showed synthesis of VEGFR2 mRNA in all four lines. Lalla *et al.* (8) found that VEGFR1-2 and 3 were consistently expressed by both tumour cells and vascular endothelial cells. Kyzas *et al.* (14) observed cytoplasmic and membrane VEGFR2 staining in all of the tumour endothelium samples and in 57% of the tumour cell samples. Kyzas and colleagues (14) found no significant association of VEGFR2 expression with histological grade or lymph node status, whereas a strong correlation was observed for higher clinical stage and for tumours located in the oral cavity and larynx (9 cases). The VEGFR-2 overexpression and VEGF/VEGFR-2 co-overexpression were significantly correlated with worse survival but VEGFR-2 expression was not correlated with disease recurrence.

The evidence of VEGFRs on vascular endothelial cells in head and neck SCC strongly supports the hypothesis that the VEGF family is an important paracrine mediator of angiogenesis. Interestingly, the presence of VEGFRs on head and neck SCC tumour cells suggests an autocrine regulatory function for VEGF in carcinoma growth.

In the present study, cytoplasmic and membrane VEGFR2 endothelial staining was observed in all samples. While CD105 stained the complete vessel outlines, VEGFR2 stained some cells. Cytoplasmic and membrane VEGFR2 staining was present also in the carcinoma cells in 30.6% of the samples. A trend towards significant correlation between endothelial VEGFR2 expression and carcinoma cell VEGFR2 expression ($p=0.05$) and between endothelial cell VEGFR2 expression and CD105 expression ($p=0.08$) was demonstrated..

Conclusion

In laryngeal carcinoma CD105 but not VEGFR2 expression correlated with carcinoma recurrence after treatment and shorter disease-free interval. The CD105 expression in activated endothelial cells can be considered as potentially useful for detecting cervical node-negative patients with higher risk of early locoregional carcinoma recurrence who might benefit from more aggressive therapy.

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