Maspin Nuclear Localization is Related to Reduced Density of Tumour-associated Micro-vessels in Laryngeal Carcinoma

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Abstract. *Background: MASPIN, a tumour suppressing serpin, is a potent inhibitor of angiogenesis. CD105 is a proliferation-associated protein acting in endothelial cells of angiogenic tissues. For the first time the relation between nuclear MASPIN expression and CD105-assessed micro-vessel density (MVD) in laryngeal carcinoma was investigated. Patients and Methods: The sub-cellular distribution of MASPIN and nuclear MASPIN expression were immuno-histochemically determined in 35 cases of laryngeal carcinoma. The percentage of the fields occupied by CD105-assessed micro-vessels was also calculated. Results: MVD was significantly lower in laryngeal carcinomas with MASPIN nuclear staining than in carcinomas with cytoplasmic staining (p=0.02). The mean nuclear MASPIN expression was higher in patients without carcinoma recurrence than in those with recurrence (p=0.06). The mean MVD was significantly higher in patients with recurrence of carcinoma (p=0.024). Conclusion: The crucial role of MASPIN nuclear localization in reducing the MVD has been demonstrated. Nuclear MASPIN re-expression should be investigated as a potential therapeutic option in the treatment of laryngeal carcinoma.*

MASPIN, a tumor suppressing serpin (serin protease inhibitors), has been isolated from mammary epithelial cells (1). In the past few years, evidence consistently showed that MASPIN suppresses tumor growth, invasion, metastasis, and induces tumor cell apoptosis (2). Zhang *et al.* (3) have demonstrated that MASPIN is a potent inhibitor of angiogenesis. Tumour angiogenesis can be understood as a disruption of normal control mechanisms of endothelial cell growth and function (4). *In vitro*, MASPIN acts directly on cultured endothelial cells by stopping their migration towards basic-fibroblast growth factor and vascular endothelial growth factor (VEGF), and reducing endothelial cell proliferation and tube formation (3). Li *et al.* (5) have concluded that MASPIN induces endothelial cell apoptosis *in vitro*; targeting *in vivo* MASPIN to the endothelial cells in mammary tumors disrupted neo-vasculatures and caused vessel leakage.

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, shorter overall and relapse-free survival rates. Although a marker that is strictly specific for tumor vasculature probably does not exist, a variety of potential candidates are under investigation. Endoglin (CD105) is a proliferation-associated and hypoxia-inducible protein abundantly expressed in angiogenic endothelial cells. Tanaka *et al.* (6) have demonstrated that antibodies against CD105 reacted preferentially with active endothelial cells of angiogenic tissues, but not with stable vessels which were just trapped in the tumor. CD105 seems to be superior to other commonly used panendothelial markers (*e.g.,* CD31, CD34, Factor VIII) in evaluation of tumor angiogenesis in clinical studies (7). The available information regarding the expression and the prognostic role of CD105 in laryngeal SCC are extremely limited but very promising. In 2005, Martone *et al.* (8) studied CD105 expression in 127 consecutive cases of head and neck squamous cell carcinoma (SCC); 107 were laryngeal malignancies. CD105-assessed MVD was significantly higher in N+ carcinomas. Patients with a high MVD had a significantly shorter disease-free and overall survival. Marioni *et al.* (9) considered CD105-assessed MVD in 43 cases of laryngeal SCC. All the measures were performed using a computer-based image analysis system. The mean CD105-assessed MVDs were 11% and 6% in laryngeal SCC with and without malignancy recurrence, respectively. Multivariate logistic regression

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determined that CD105-assessed MVD was significantly related to disease recurrence.

The aim of the present retrospective study was to investigate for the first time the relation between nuclear MASPIN expression and CD105-assessed micro-vessel density in 35 consecutive cases of laryngeal SCC.

Patients and Methods

Patients. A total of 35 cases of laryngeal SCC were evaluated. Thirty patients were male and 5 female, with a mean age of 65.7 years (standard deviation (SD) 10.2 years). All patients underwent partial or total laryngectomy at the Section of Otolaryngology of Padova University, Italy. Neck dissection and radiotherapy were also performed in 15 and 12 cases, respectively. According to the TNM Classification of Malignant Tumours of the International Union Against Cancer (10), the pathological staging of primary laryngeal lesions (pT) was T1 in 19 cases, T2 in 7 cases, T3 in 4 and T4 in 5 cases. Pathological regional lymph node staging was N0 in 9 cases, N1 in 1, and N2 in 5. Mean follow-up time was 39.3 months (median 42 months; SD 20.6 months). Considering pathological grading, 13 out of 35 cases were staged as G1, 14 as G2 and 8 as G3.

Immunohistochemistry. For immunohistochemical evaluation formalin-fixed, paraffin-embedded 5 μm sections were cut for all 35 cases. For each sample, MASPIN and CD105 reactivity were evaluated.

(i) Immunohistochemistry for MASPIN. The antibody used was Maspin, mouse monoclonal antibody, clone G167-70 (BD-Biosciences, PharMingen Int., San Diego, CA, USA), diluted 1:500. At first the sections were microwaved (750 W) for antigen unmasking for 20 min in a citrate buffer (10 mM pH 6.0). The slides were incubated for 45 min at room temperature with the primary antibody. The slides were then incubated with EnvisionSystem HRP mouse (Dako, Denmark) for 30 min. The colour was developed using 3.3-diaminobenzidine (DAB) (Dako) for 4 min. Sections were counterstained with Mayer's haematoxylin. As positive control normal breast tissue was used; a negative control the antibody was substituted by phosphate-buffered solution (PBS).

(ii) Immunohistochemistry for CD105. The CD105 mouse monoclonal antibody, clone Sn6h (Dako) was used, diluted 1:10. 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, Newcastle-upon-Tyne, UK) 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; 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; the antibody was replaced with PBS for negative control.

Field selection. The sections were scanned by the senior pathologist (S.B.) at x100 in order to select from the less differentiated areas of tumor the three areas with the highest degree of vascularisation (hot spots), free of necrosis or haemorrhage. The percentage of the fields examined which were occupied by CD105-assessed micro-vessels (area fraction) was determined for each specimen and a smaller portion of the same area used to determine MASPIN reaction.

Image analysis determinations. The expression of MASPIN and CD105 were evaluated using the "CIRES" workstation image analysis system (Zeiss, Jena, Germany), consisting of a conventional light microscope (Axioskop model, Zeiss, Jena, Germany) connected to a 3CCD colour video camera (KY-F55BE JVC, Japan). The images were captured by a frame grabber (Kontron, Eching, Germany) and then analyzed. The frame grabber and the image analysis (I.A.) program, operating on line with the camera, were hosted by a PC. During all measurement sessions, illumination was kept constant at a fixed value and the stray light effect was reduced using Koehler's illumination setting (11). 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 Shannon-Nyquist sampling theorem (12). 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 in the nucleus and cytoplasm. All measured objects were in the range of 60 to 117 of the grey scale for CD105 and between 10 to 90 for MASPIN evaluation.

For MASPIN reactivity, a double count was performed: the first assessing the percentage of stained nuclei (a minimum of 600 cells at x400), the second evaluating the MASPIN subcellular distribution pattern creating three categories: prevalently nuclear (>5% positive nuclei; cytoplamic reactivity almost totally absent), nuclear/cytoplasmic (cytoplasmic staining together with >25% positive nuclei), and prevalently cytoplasmic (cytoplasmic positivity >5% of counted cells; nuclear reactivity almost totally absent). 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.

To assess the reproducibility of 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 91%, $p<0.001$.

Data analysis. When required, the following statistical tests and model were applied: *t*-test corrected for differences in variances, Fisher exact test, Kruskal-Wallis test for trend, Mann-Whitney *U*-test, Spearman's rank correlation test. The receiver operating curve (ROC) approach was applied to determine the analytically best-fitting cut-off values of nuclear MASPIN and CD105-assessed MVD to be used for a Kaplan-Meier survival analysis. Log-rank test was used to analyse disease-free interval differences (in months) in patients stratified according to nuclear MASPIN expression and MVD. We considered $p<0.05$ to be significant; values in the following range $0.10>p\geq0.05$ were considered as indicating a statistical trend. The STATA 8 (StataCorp, College Station, TX, USA), statistical package, was used for all evaluations.

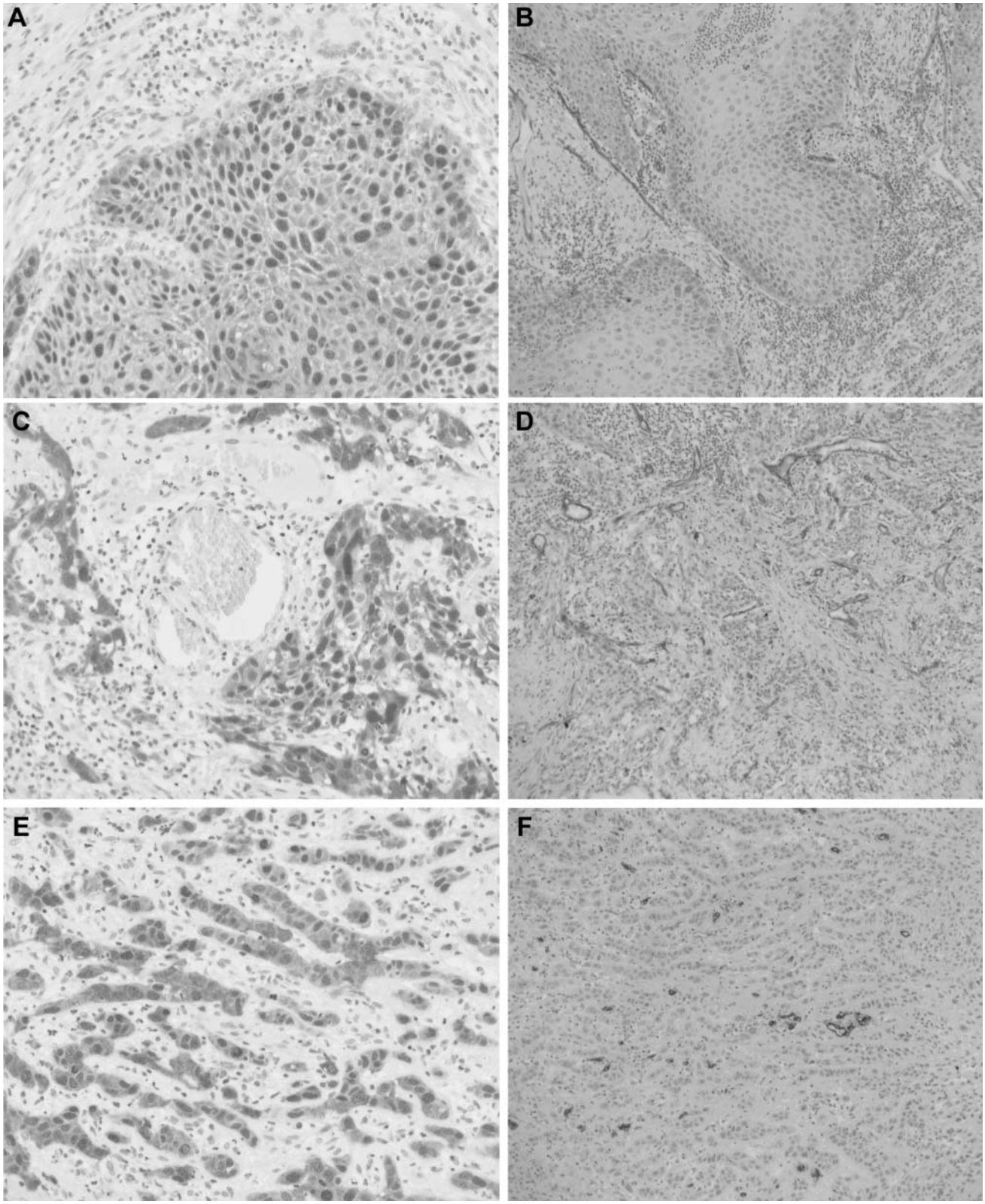

Figure 1. *(A) MASPIN (original magnification x200) nuclear pattern of expression and corresponding cytoplasmic CD-105 (original magnification x100) (B); (C) MASPIN nuclear-cytoplamic pattern of expression (x200) and corresponding CD-105 staining (x 100) (D); (E) MASPIN cytoplasmic pattern of expression (x200) and corresponding CD-105 staining (x100) (F).*

Table I. *CD105-assessed MVD according to the MASPIN subcellular localization pattern in laryngeal SCC.*

MASPIN localization pattern	Number of patients	Mean CD105-assessed MVD (sd)
Nuclear or nuclear-cytoplasmic	11	4.9% (2.3%)
Cytoplasmic	24	9.2% (8.2%)
p (*t*-test corrected for differences in variances)		0.02

MVD: micro-vessel density; sd: standard deviation.

Table II. *Mean nuclear MASPIN expression and CD105-assessed MVD in different clinico-pathological sub-groups of the series of laryngeal SCCs examined.*

TNM status	Number of patients	Mean nuclear MASPIN (sd)	Mean CD105-assessed MVD (sd)
pT1	19	12.8% (26.4%)	7.1% (3.4%)
pT2	7	15.0% (23.4%)	10.3% (5.4%)
pT3	4	21.8% (38.9%)	15.0% (17.9%)
pT4	5	36.7% (32.1%)	5.5% (2.1%)
p (Kruskal-Wallis test for trend)		0.34	0.74
pN+	6	1.2% (2.0%)	13.1% (14.2%)
pN0	9	17.5% (24.3%)	8.2% (6.2%)
p (Mann-Whitney *U*-test)		0.54	0.60
Pathological grading			
G1	13	12.7% (27.8%)	7.8% (5.1%)
G2	14	18.3% (27.5%)	10.0% (9.5%)
G3	8	18.4% (30.1%)	6.5% (3.0%)
p (Kruskal-Wallis test for trend)		0.65	0.83
Without L-R Rec	21	21.3% (32.3%)	6.1% (2.6%)
With L-R Rec	14	8.7 (16.4%)	11.9% (9.6%)
p (*t*-test corrected for differences in variances)		0.06	0.024

L-R Rec: loco-regional recurrence; MVD: micro-vessel density; sd: standard deviation.

Results

Seven out of 35 cases were characterized by a nuclear pattern of MASPIN compartmental localization, 4 cases by a nuclear-cytoplasmic pattern and 24 cases by a cytoplasmic pattern. The mean nuclear MASPIN expression was 42.9% (SD 35.3%) in the cases with nuclear pattern, 57.9% (SD 17.1%) in the cases with nuclear-cytoplasmic pattern, 1.6% (SD 4.3%) in the cases with cytoplasmic pattern. (Figure 1 A, C, E) CD105 expression was absent from SCC cells and tumor stromal components. The mean CD105-assessed MVD in laryngeal SCCs was 20.2% (SD 7.2%). Considering the recent evidence that MASPIN tumor-suppressor activity in laryngeal SCC is due to MASPIN localized in cellular nuclei (13), cases with nuclear or nuclear-cytoplasmic patterns were combined for statistical analysis. A *t*-test, corrected for differences in variances, showed that CD105-assessed mean MVD was significantly lower in the group of laryngeal SCCs with MASPIN nuclear localization (nuclear/nuclear-cytoplasmic pattern) than in the group of SCCs with a prevalently cytoplasmic MASPIN pattern ($p=0.02$) (Table I); (Figure 1 B, D, F). Spearman's rank correlation test failed to identify a significant inverse correlation between nuclear MASPIN positivity and CD105-assessed MVD ($\varrho=-0.17$, $p=0.32$).

Twenty-one out of 35 previously having laryngeal SCC did not experience malignancy recurrence. Fourteen developed loco-regional malignancy recurrence after a mean period of 16.3 months (SD 10.6 months). The mean nuclear MASPIN expressions (percentage positivity) were 21.3% (SD 32.3%) and 8.7% (SD 16.4%) in patients with and without recurrence of SCC, respectively. Mean nuclear MASPIN was higher in the group of patients without SCC recurrence than in the group with (*t*-test, $p=0.06$). The mean MVD in laryngeal SCC without recurrence of disease was 6.1% (SD 2.6%). The mean CD105-assessed MVD in laryngeal SCC with malignancy recurrence was 11.9% (SD 9.6%). A *t*-test corrected for differences in variances disclosed that mean MVD was significantly higher in those patients who developed recurrence of SCC ($p=0.024$) (Table II).

In the present series, the Fisher exact test showed no statistical associations between MASPIN pattern (nuclear/nuclear-cytoplasmic pattern *vs.* cytoplasmic pattern) and pT-staging ($p=0.72$). Mean nuclear MASPIN expressions (%) and MVD according to pT-staging are given in Table II. The Kruskal-Wallis test for trend failed to identify any significant relation between nuclear MASPIN expression or CD105-assessed MVD and pT-stage ($p=0.34$ and 0.74, respectively).

Statistical analysis showed no relation between the MASPIN pattern and lymph-node status (pN0/pN+) (Fisher exact test, $p=0.71$). The mean nuclear MASPIN expression (%) and CD105-assessed MVD in pN0/pN+ cases are shown in Table II. The Mann-Whitney *U*-test found no significant differences in mean nuclear MASPIN expression (%) or MVD values between pN+ and pN0 patients ($p=0.54$ and 0.60, respectively).

Statistical analysis showed no relation between MASPIN pattern and stage-grouping (Fisher exact test, $p=0.60$). Mean nuclear MASPIN expressions (%) and MVD in laryngeal SCCs according to stage grouping are shown in Table II. The Kruskal-Wallis test showed no significant relation between nuclear MASPIN expression (%) or CD105-assessed MVD and laryngeal SCC stage-grouping ($p=0.61$ and 0.87, respectively).

Fisher's exact test failed to disclose any statistical associations between MASPIN pattern (nuclear/nuclear-cytoplasmic pattern vs. cytoplasmic pattern) and pathological grading ($p=0.56$). Table II also summarizes mean nuclear MASPIN expression (%) and CD105-assessed MVD according to pathological grading. The Kruskal-Wallis test for trend failed to disclose any significant relation between nuclear MASPIN expression (%) or MVD and laryngeal SCC pathological staging ($p=0.65$ and 0.83, respectively).

We used the ROC approach to identify the analytically best-fitting cut-off values of nuclear MASPIN and CD105-assessed MVD to be used for a Kaplan-Meier survival analysis. The calculated cut-off values were 2.00% and 6.96% for nuclear MASPIN and MVD, respectively (nuclear MASPIN: sensitivity 52.32%, specificity 57.14%, area under ROC curve 0.58; CD105-assessed MVD: sensitivity 71.43%, specificity 71.43%, area under ROC curve 0.73). The log-rank test showed no significant differences in the disease-free interval (in months) in patients stratified according to nuclear MASPIN expression (%) ($p=0.85$). On the other hand, the log-rank test showed a significant difference in the disease-free interval (in months) in patients stratified according to the CD-105-assessed MVD ($p=0.03$).

Discussion

Angiogenesis is essential for malignant tumor growth: most solid tumors cannot grow beyond a few millimetres without neovascularization. Studies from animal models and clinical experiments have shown a close correlation between tumor vascular density and a negative prognosis with respect to malignancy invasiveness and metastasis (5). Although MASPIN has been found to be an inhibitor of angiogenesis *in vitro* and *in vivo* and MASPIN clinical relevance in human cancers has been extensively investigated since its discovery in 1994, the relation between MASPIN expression and tumor-associated microvascular density has not been determined.

A limited number of clinical investigations studied the influence of MASPIN expression on tumor MVD. Song *et al.* (14) found that MASPIN expression was inversely correlated with CD34-assessed MVD in colon cancer. Wang *et al.* (15) reported a negative correlation between MASPIN expression and CD34-assessed MVD in gastric cancer. In both studies, the immunoreactivity to MASPIN was prevalently localized in the cytoplasms. Considering a cohort of invasive ductal breast cancers, Sopel *et al.* (16) concluded that MVD significantly decreased with an increase in MASPIN expression, whereas in the cases showing c-erbB-2 over-expression, MVD was clearly higher. In 2006, Solomon *et al.* (2) concluded that high MASPIN nuclear expression was associated with significantly lower CD34-assessed MVD in their series of epithelial ovarian carcinomas. On the other hand, Li *et al.* (17) found no significant correlation between the expressions of VEGF and MASPIN in their series of gastric carcinoma. Also Friedrich *et al.* (18) found inconsistent results correlating MASPIN staining pattern with MVD established by CD34 and CD105 immunohistology in a cohort of urothelial carcinomas of the bladder. Very recently, Tsoli *et al.* (19) concluded that, in their series of breast carcinomas, prevalent cytoplasmic MASPIN did not exert a significant effect on CD34-assessed MVD. These discrepancies may reflect the differences in the biological behavior of the malignancies, treatment protocols and pathological methods used to investigate MVD. The controversial results regarding MASPIN role in tumor angiogenesis inhibition may also be due to the different biological properties depending on subcellular localization of MASPIN.

Only a previous study of our group investigated the role played by MASPIN in laryngeal SCC and its prognostic value (13). Our study indicated a significantly higher nuclear MASPIN expression in patients without SCC recurrence after surgery than in those with evidence of SCC recurrence. A significant nuclear localization of MASPIN was statistically associated with a longer disease-free interval in the patient cohort. Nuclear MASPIN expression was inversely correlated with Ki67 expression in laryngeal SCC cells. This result is particularly interesting considering that Ki67 antigen expression has been proven to correlate with cellular proliferation and has been used to determine the growth fraction of neoplastic tissue. All this evidence supports the hypothesis that in laryngeal SCC the specific nuclear localization of MASPIN plays a critical role in its biological function. The above mentioned investigation was one of the first studies taking into account the sub-cellular localization of MASPIN and suggesting that the compartmental localization is indicative of cellular effects, with localization in the nucleus being associated with less aggressive laryngeal SCC properties.

In the current pilot series of laryngeal carcinomas, MASPIN nuclear localization is related to reduced density of tumor-associated CD-105-assessed micro-vessels. CD105-assessed MVD was significantly lower in laryngeal SCCs with a MASPIN nuclear staining (nuclear/nuclear-cytoplasmic pattern) than in SCCs with prevalent cytoplasmic staining ($p=0.02$). Statistical analysis failed in identifying a significant

inverse correlation between nuclear MASPIN expression and CD105-assessed MVD. This was possibly due to the limited size of our series. From a prognostic viewpoint, the mean nuclear MASPIN expression was higher in the patients without than in those with SCC recurrence ($p=0.06$).

Conclusion

The preliminary results indicate the crucial role of subcellular nuclear localization of MASPIN in angiogenesis: CD105-assessed MVD was significantly lower in laryngeal SCCs with a MASPIN nuclear staining than in SCCs with prevalent cytoplasmic staining. Moreover, mean nuclear MASPIN expression was higher in the group of patients that did not develop SCC recurrence. Our results confirmed that CD105-assessed MVD in primary laryngeal SCCs may be a valuable parameter for predicting patients having an increased risk of developing malignancy recurrence after treatment. These observations require verification in larger-scale series.

The use of angiogenesis inhibitors may have widespread application in treatment of cancer. Considering the anti-angiogenetic properties of nuclear MASPIN shown in the present study, nuclear MASPIN re-expression is a potential therapeutic option in the treatment of laryngeal carcinoma.

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