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Nuclear expression of onco-suppressors nm23-H1 and maspin are associated with lower recurrence rate in laryngeal carcinoma

Gino Marioni[a,*], Giancarlo Ottaviano[a], Cosimo de Filippis[b], Elena Fasanaro[c], Benedetto Randon[a], Stefano Meneghesso[a], Luciano Giacomelli[d], Laura Astolfi[e], Stella Blandamura[d]

[a] Department of Neuroscience DNS, Otolaryngology Section, University of Padova, Padova, Italy
[b] Department of Neuroscience DNS, Audiology Unit, University of Padova, Treviso, Italy
[c] Radiotherapy Unit, Veneto Institute of Oncology IOV–IRCCS, Padova, Italy
[d] Department of Medicine DIMED, University of Padova, Padova, Italy
[e] Department of Neuroscience DNS, Bioacoustics Research Laboratory, University of Padova, Padova, Italy

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ABSTRACT

Purpose: The main aim of the study was to preliminarily investigate the possibly related role of nuclear onco-suppressors maspin and nm23-H1, a metastasis suppressor, in laryngeal squamous cell carcinoma (LSCC).
Materials and methods: Maspin expression pattern and nuclear nm23-H1 expression were ascertained in 62 consecutive LSCCs.
Results: Recurrence rate was significantly lower in patients with a nuclear maspin pattern of expression; nuclear nm23-H1 expression was significantly lower in patients who experienced disease recurrence. Disease free survival (DFS) was significantly longer in patients with maspin nuclear pattern or with nuclear nm23-H1 expression ≥10%. A significant association was found between nuclear nm23-H1 expression and maspin pattern of expression in LSCC. KNN discriminant analysis considered N status, maspin sub-cellular localization and nuclear nm23-H1 expression. The selected variables' accuracy in terms of relapse was 82%. Positive predictive accuracy was 100%, and negative predictive accuracy 79%.
Conclusions: Nuclear nm23-H1 expression and maspin pattern, also in association, show promise as recurrence indicators in LSCC. Further studies are needed to shed more light on the nm23-H1 mechanism of action in LSCC and thus find ways to restore nm23-H1 loss. These preliminary findings suggest that re-activating maspin functions might represent an important goal in the treatment of advanced LSCC.

1. Introduction

Tumor suppressor genes play critical roles in orchestrating anti-cancer programs. In addition to canonical tumor suppression programs that control cell division, cell death and genome stability, other tumor suppressor gene activities have recently emerged that regulate metabolism, immune surveillance and the epigenetic landscape [1]. The role of tumor suppressor genes in laryngeal squamous cell carcinoma (LSCC) is still unclear. Improving knowledge in this field could have significant clinical applications: there is an undeniable need for new, more effective therapeutic strategies to improve overall survival for patients with LSCC, and in particular for advanced cases.

Metastasis suppressors can act by inhibiting one or more steps in the metastatic cascade and regulate a wide range of biochemical signaling pathways [2]. The non-metastatic gene 23 (NM23) is located on chromosome 17q21, which encodes for a 17 kDa protein with 152 amino acids. Nm23-H1 was the first metastasis suppressor to be identified and it has been the most studied because of its negative correlation with the development, progression and metastasis of various malignancies [3]. In LSCC, Marioni et al. [4] reported that nuclear nm23-H1 levels were lower in patients with disease recurrence. By multivariate analysis, nuclear nm23-H1 expression was prognostically significant in terms of disease free survival (DFS). Wang et al. [5] reported that in patients with cervical metastases receiving postoperative cisplatin-based chemoradiation, low nm23-H1 expression in head and neck carcinoma correlated with locoregional recurrence.

Maspin, also known as serpinB5, is a member of the ovalbumin serpin (ov-serpin or serpinB) family. Maspin is usually expressed in epithelial cells and has been described as a tumor suppressor in cancer. It inhibits cell motility and invasion *in vitro*, and angiogenesis, tumor

* Corresponding author.
E-mail address: gino.marioni@unipd.it (G. Marioni).

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formation, and metastasis of different cancer cell lines *in vivo*. Recent evidence indicates that the nuclear localization of maspin in cancer cells is necessary for its tumor suppressor activity [6]. Considering LSCC, in a series of 108 cases treated surgically, DFS was significantly longer in patients with a nuclear maspin expression pattern, and the rates of lymph node metastases and disease-recurrence were significantly lower [7].

The main aim of this study was to conduct a preliminary clinicopathological investigation into the possibly related role of nuclear onco-suppressors maspin and nm23-H1 in LSCC.

2. Materials and methods

2.1. Patients

The study was conducted in accordance with the principles of the Helsinki Declaration. Data was examined in agreement with the Italian privacy and sensible data laws (D. Lgs 196/03) and the Otolaryngology Section's University of Padova internal rules. Before undergoing surgery, all patients included in the study signed a detailed informed consent form.

The study involved 62 consecutive cases of LSCC treated with primary surgery. As in the recommendations adopted for LSCC at our institution [8,9], all patients (55 males, 7 females; mean age 63.1 ± 8.7 years) had undergone microlaryngoscopy with laryngeal biopsy, upper aero-digestive tract endoscopy, neck ultrasonography (with or without fine needle aspiration cytology), head and neck contrast-enhanced computerized tomography (CT), and/or magnetic resonance imaging, chest X-ray and liver ultrasonography.

All patients underwent laryngeal surgery at the Otolaryngology Section of Padova University, with unilateral or bilateral cervical lymph node dissection in 50 cases. Pathological staging, and the characteristics of primaries and metastases warranted postoperative RT in 13 cases according to current guidelines. Table 1 provides details of patients' clinicopathological features, based on the 7th edition of the TNM Classification of Malignant Tumors [10]. No distant metastases (M) were detected at diagnosis. As previously reported [9], in our institution the clinical follow-up after treatment (adjustable to patients' individual characteristics) was scheduled as follows: (i) once a month for the 1st year; (ii) every 2 months in the 2nd year; (iii) every 3 months in the 3rd year; (iv) every 4 months in the 4th year; (v) every 6 months in the 5th year; and (vi) every 12 months thereafter. Neck ultrasonography and chest X-rays were also performed at least yearly. Contrast-enhanced CT of the neck, total body positron emission tomography, chest CT, and liver ultrasonography were repeated as necessary. The mean follow-up was 78.6 ± 52.3 months.

2.2. Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections using a fully automated system (Bond-maX; Leica, Newcastle Upon Tyne, UK). Sections were dewaxed and rehydrated, then incubated in retrieval buffer solution (Leica) pH 6.0 for antigen unmasking. The antibodies used were: maspin (monoclonal mouse antibody, clone EAW24, diluted 1:100, Leica), and nm23-H1 (monoclonal mouse antibody, clone 37.6, diluted 1:800, Santa Cruz Biotechnology, Santa Cruz, CA). Specimens were then washed with phosphate-buffered saline (pH 7.0) and incubated with a Bond Polymer Refine Detection Kit (Leica) in accordance with the manufacturer's protocols. Staining was visualized with 3,3′ diaminobenzidine, and the slides were counterstained with Mayer's hematoxylin. Three areas were chosen by the pathologist (S.B.), irrespective of their position in the carcinoma tissue. In these areas, 500 carcinoma cells were considered: maspin subcellular pattern of expression and the percentage of nm23-H1 positive nuclei were assessed.

2.3. Statistical analysis

The statistical tests applied were Fisher's exact test, the Mann-Whitney *U* test, and Rank sum test, as appropriate. The log-rank test was used to compare DFS (in months), stratified by the variables analyzed. The chosen cutoff for binarization coincided with the median values for the nuclear nm23-H1 as it was not subjective, and analytically achieved the best fit.

For multivariate analysis, clinicopathological parameters and/or biomarkers with a p-value < 0.05 for prognostic purposes (carcinoma relapse occurrence) in univariate setting were considered. KNN discriminant analysis [11] was preferred. Kth nearest neighbor multivariate discriminant analysis for binary data was performed to assess the efficiency of the selected variables in discriminating the patient outcomes. The following procedure was applied: $K = 1$ (number of the nearest), prior probabilities reflecting the composition of the group investigated, Dice similarity measure, standard method for dissimilarity conversion. The results have been displayed by classification and error rate tables (L.G.).

Additional statistics derived from the model were calculated, i.e. sensitivity, specificity, positive predictive accuracy, negative predictive accuracy and accuracy, which indicated the ability of the selected variables in the model to detect the outcome (relapse [1] *vs* no relapse [0]).

A p-value < 0.05 was considered significant. The STATA™ 10 (Stata Corp, College Station, TX, USA) statistical package was used for all analyses.

3. Results

3.1. Clinical outcome

Forty-two of the 62 LSCC patients experienced no disease recurrence after treatment, while 20 relapsed after a mean 12.8 ± 10.4 months. The Mann-Whitney *U* test ruled out any significant difference between the mean follow-up (in months) of patients

Table 1
Maspin expression pattern and nm23-H1 nuclear expression in relation to classical clinicopathological and prognostic variables in LSCC.

Clinicopathological variables	No. of cases	MASPIN pattern of expression Nuclear/non-nuclear	Mean nm23-H1 nuclear expression % ± SD	Mean disease-free survival in months ± SD	Recurrence rate (%)
pT1–pT2	37	9/28	15.8 ± 12.1	68.9 ± 49.2	11/37 (30%)
pT3–pT4	25	9/16	11.2 ± 10.8	44.8 ± 48.2	9/25 (36%)
G1–G2	47	16/31	13.4 ± 11.2	67.1 ± 51.2	12/47 (26%)
G3	15	2/13	15.5 ± 13.5	34.3 ± 37.1	8/15 (53%)
pN+	17	1/16	8.6 ± 9.0	30.9 ± 38.4	11/17 (65%)
N0 (cN_0 + pN_0)[a]	45	17/28	15.9 ± 12.1	69.8 ± 49.9	9/45 (20%)
Without cancer recurrence	42	16/26	17.4 ± 11.6	81.3 ± 46.4	
With cancer recurrence	20	2/18	6.7 ± 8.4	12.8 ± 10.4	

[a] cN_0 12 cases and pN_0 33 cases.

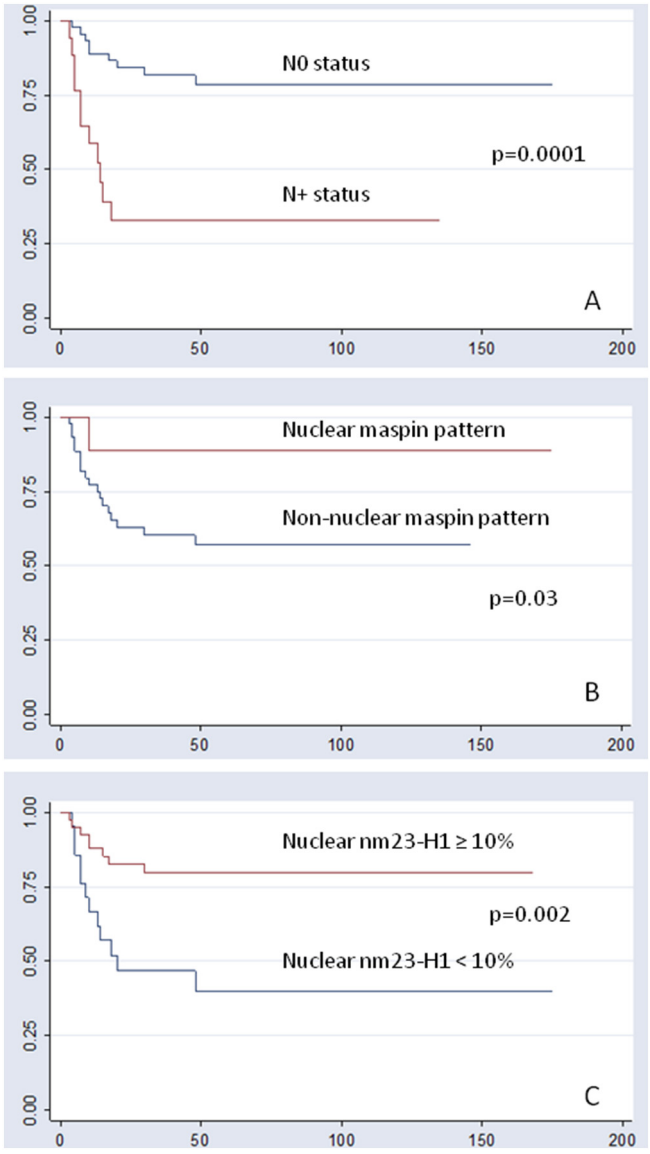

Fig. 1. Disease-free survival in laryngeal squamous cell carcinoma patients estimated on the basis of N status (A), maspin expression pattern (nuclear vs non-nuclear) (B), and nuclear nm23-H1 expression (c); time interval (abscissa) in months.

with and without disease recurrence (p = 0.28).

Fisher's exact test identified significant differences in the distributions for lymph node status (N0 *vs* N+) (p = 0.002), but not for pT (pT1–T2 *vs* pT3–T4) (p = 0.78), pathological grade (G1–G2 *vs* G3) (p = 0.06), or stage grouping (stage I–II *vs* stage III–IV) (p = 0.28), between the two subgroups of patients with and without loco-regional carcinoma recurrences. The log-rank test showed a significant difference in DFS (in months) when patients were stratified by N status (p = 0.0001) (Fig. 1A) or pathological grade (p = 0.018), but not if they were stratified by pT (p = 0.48), or stage grouping (p = 0.15).

3.2. Nuclear maspin sub-cellular location in LSCC, its clinicopathological features, and prognosis (Table 1)

Four of the considered specimens were maspin-negative. Twelve out of 62 cases were characterized by a nuclear pattern of maspin compartmental localization, 6 cases by a nuclear-cytoplasmic pattern and 40 by a cytoplasmic pattern. Considering the evidence that maspin tumor-suppressor activity in laryngeal SCC is due to maspin localized in cellular nuclei [7,12,13], cases with nuclear or nuclear-cytoplasmic patterns were combined (nuclear pattern) for statistical analysis.

Fisher's exact test revealed a significant difference in the distribution of the maspin expression pattern (nuclear *vs* non-nuclear) vis-à-vis the variables pT (p = 0.04) and N-status (p = 0.01), but not vis-à-vis stage (p = 0.78) or grading (p = 0.19). The recurrence rate was significantly lower among LSCC patients with a nuclear maspin pattern of expression (Fisher's exact test, p = 0.04). DFS was significantly longer in the sub-cohort of patients with maspin nuclear pattern of expression than in the non-nuclear one (log-rank test, p = 0.03) (Fig. 1B).

3.3. Nuclear nm23-H1 expression in LSCC, its clinicopathological features and prognosis

Epithelial cells from normal laryngeal mucosa showed scattered staining, the cylindrical respiratory-type cells being stained more often than normal squamous cells. Staining was weak and cytoplasmic in both cell types, and distributed through all the epithelial layers. Granular nm23-H1 cytoplasmic staining predominated in LSCC specimens considered, but carcinoma cells expressing nuclear nm23-H1 were also found.

The Mann–Whitney *U* test identified significant differences in mean nuclear nm23-H1 expression when LSCCs were distributed by lymph node status (N0 *vs* N+, p = 0.004) and pathological staging (stage I–II *vs* III–IV, p = 0.04), but not by pT (T1–2 *vs* T3–4, p = 0.11) or pathological grade (G1–2 *vs* G3, p = 0.74). The nuclear nm23-H1 expression was significantly lower in LSCC patients who experienced disease recurrence than in those who did not (Mann–Whitney *U* test, p = 0.0006). The DFS was significantly longer for patients whose nuclear nm23-H1 expression was ≥10% (median value for the cohort) than for those whose nuclear nm23-H1 expression was < 10% (log-rank test, p = 0.002) (Fig. 1C).

3.4. Association between nuclear nm23-H1 expression and maspin location in LSCC

Mean nuclear nm23-H1 expression was 19.9% ± 11.5% in the sub-cohort of LSCCs with maspin nuclear pattern and 11.5% ± 10.9% in the sub-cohort with maspin non-nuclear pattern (Figs. 2A, B, 3A, B). Rank sum test found a significant association between nuclear nm23-H1 expression and maspin subcellular pattern of expression (nuclear *vs* non-nuclear) (Hazard Ratio 0.92, Confidence Interval 0.87–0.97, p = 0.01).

3.5. Multivariate analysis

As mentioned previously, the variable selection procedure using backward elimination for potential clinicopathological and biomarkers prognostic factors (in terms of carcinoma relapse occurrence) was set at p ≥ 0.05 for univariate analysis. N status, subcellular maspin localization, and nuclear nm23-H1 expression were considered. KNN discriminant analysis results are summarized in Tables 2 and 3. The calculated error rate was 0.17 (11 out of 62 cases).

Considering diagnostic effectiveness of the variables in terms of carcinoma relapse occurrence, additional statistics were also calculated: sensitivity (9/20 = 0.45, 95%CI 0.232 < Sens < 0.668); specificity (42/42 = 1, 95%CI 1 < Spec < 1); positive predictive accuracy (9/9 = 1, 95%CI: 1 < pp < 1); negative predictive accuracy (42/53 = 0.79, 95%CI: 0.683 < np < 0.902); and accuracy ([9 + 42] / 62 = 0.82, 95%CI: 0.727 < Acc < 0.904).

4. Discussion

The role of nm23-H1 in LSCC was first investigated in 1996, when Lee et al. [14] demonstrated a direct correlation between strong nm23-H1 protein immunostaining and survival. In 1997, Gunduz et al. [15]

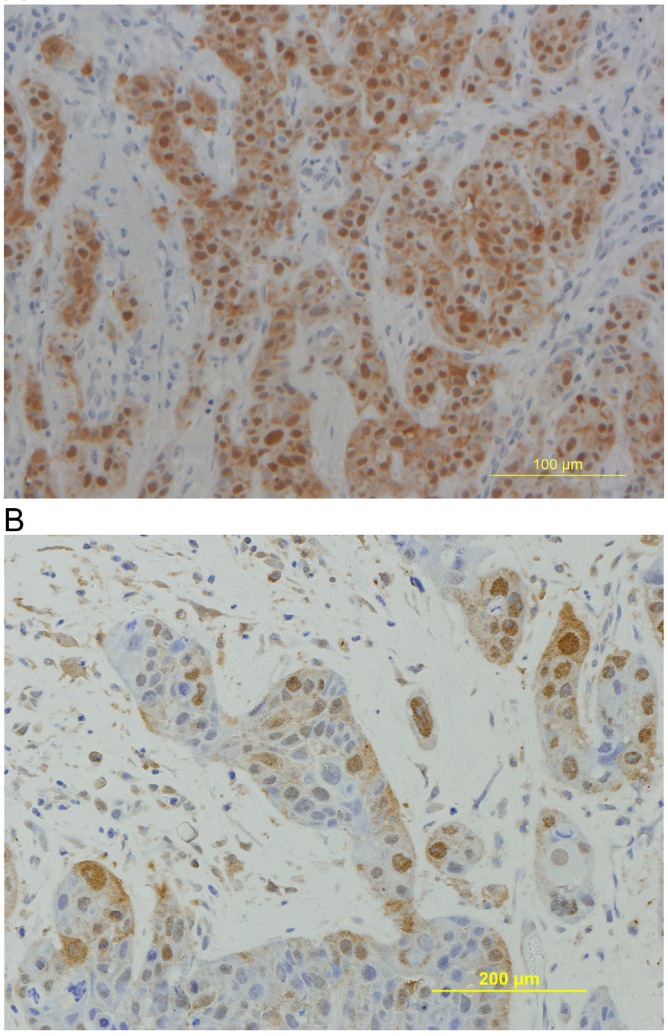

Fig. 2. A case of moderately differentiated laryngeal squamous cell carcinoma showing high nuclear expression of maspin (A) and high nuclear expression of nm23-H1 (B).

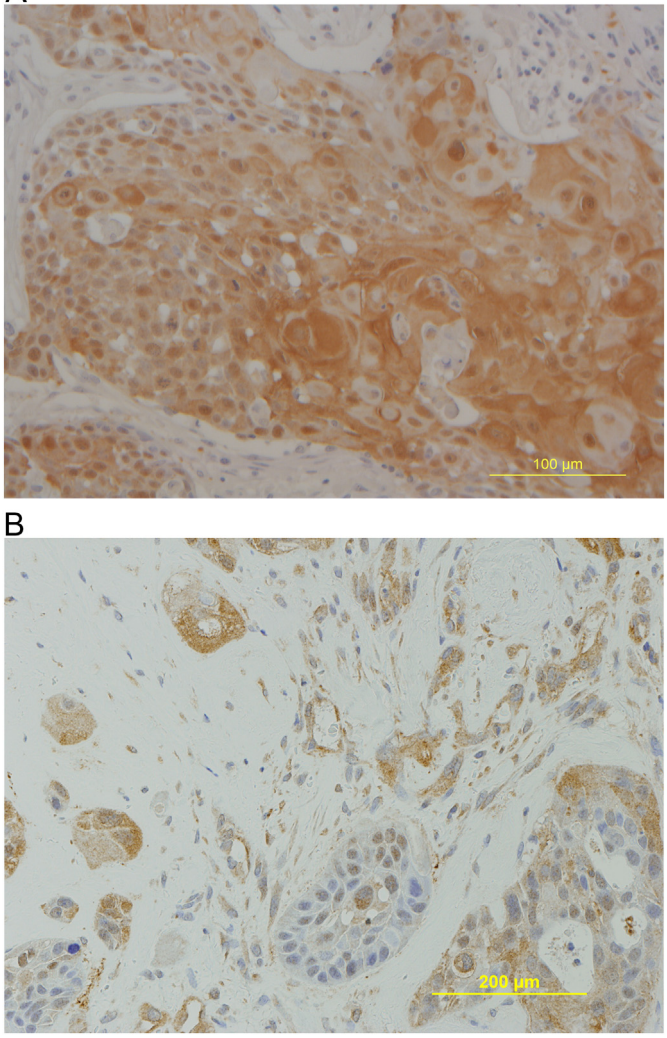

Fig. 3. A case of moderately differentiated laryngeal carcinoma showing cytoplasmic expression of maspin (A) and low nuclear expression of nm23-H1 (B).

studied nm23-H1 expression in a randomly-selected series of 86 surgical specimens of LSCC: the authors concluded that the primary LSCCs with high nm23-H1 levels coincided with fewer distant metastases and/or recurrences, whereas the primary carcinomas with low nm23-H1 expression levels revealed more aggressive behavior. In 2012, the prognostic role of nuclear nm23-H1 was evidenced in a larger series of 104 consecutively-operated LSCCs studied with univariate and multivariate statistical approaches [4]. Very recently, Marioni et al. [16] investigated the role of nuclear nm23-H1 in LSCC epithelial-mesenchymal transition, as determined by E-cadherin, N-cadherin, Snail, zeb1, and zeb2: they found that nuclear nm23-H1 and E-cadherin expressions directly correlated. The role of E-cadherin, N-cadherin and zeb2 expressions has been verified in lymph node metastases, T stage and tumor cell differentiation in LSCC [17].

Marioni et al. [12] investigated the relation between maspin expression and micro-vessel density (MVD) assessed by proliferation-associated CD105 protein that acts in endothelial cells of angiogenic tissues. They found that MVD was significantly lower in laryngeal carcinomas with maspin nuclear staining than in those with cytoplasmic staining. This supports a model in which, in LSCC, maspin may exert anti-angiogenic effects within the carcinoma cell, specifically in the nucleus. Furthermore, preliminary observation supported the hypothesis of an important apoptosis sensitizing effect of nuclear maspin in LSCC. The role of maspin was investigated in the LSCC apoptotic mechanism, determining the relations between maspin, prognosis and cytoplasmic expression of the murine monoclonal antibody M30, expressed by epithelial cells during early apoptosis: M30 expression was significantly higher in the group of malignancies with maspin nuclear

Table 2
Multivariate approach: true outcome.

Classification	No recurrence (no. of cases)	Recurrence (no. of cases)	Total (no.)
0 (no recurrence)	42	11	53[a]
1 (recurrence)	0	9	9[a]
Total	42[b]	20[b]	62
Priors	67.7%	32.3%	100%

[a] Values calculated by the model.
[b] Observed values.

Table 3
Multivariate approach: error rate.

	No recurrence	Recurrence	Total
Error rate	0	0.55	0.17 (11/62)

localization [13].

Nm23-H1 and maspin have rarely been contemporarily investigated for their roles in clinicopathological studies. Maspin, nm23-H1, and ezrin immunohistochemistry were evaluated by Mhawech-Fauceglia et al. [18] in 120 patients with head and neck carcinoma who underwent an accelerated radiotherapy schedule using the microarray technique. Maspin was the only biomarker found to have a significant association with carcinoma histological grade. The authors reported that the combination of maspin loss and low histological grade was associated with longer DFS in univariate and multivariate analyses. Pasz-Walczak et al. [19] analyzed the expression of both nm23-H1 and maspin proteins in a series of colorectal adenocarcinomas to assess their applicability as prognostic factors in this type of cancer: cytoplasmic medium/high expression level of maspin but no nm23-H1 or presence of maspin nuclear expression was found as independent negative prognostic factor.

The present clinicopathological investigation is the first to examine the possible associated prognostic role of nuclear LSCC onco-suppressors nm23-H1 and maspin. The main strength of this study lies in the homogeneity of the series of patients considered because: (i) they all underwent primary laryngeal surgery alone; (ii) their surgical treatment was performed consecutively by the same team; (iii) only surgical specimens (not biopsies) of LSCC were assessed; (iv) only squamous cell carcinomas located in a single head and neck structure (the larynx) were considered – this is because the role and subcellular localization of the products of onco-suppressors may differ in cancers developing at different head and neck sites (this has already been shown to be the case for maspin in oral *vs* laryngeal carcinomas [20,21]), and for nm23-H1 [4,22], and (v) oncological follow-up criteria were defined. Both univariate and multivariate statistical data analyses were applied. Instead, the main weaknesses of the study concern the retrospective setting of the investigation and the limited number of cases considered. As previously widely reported, higher LSCC recurrence rate and shorter DFS were found in patients with a pN+ lymph node status. Considering the prognostic role of the investigated onco-suppressors, the recurrence rate was significantly lower in patients with a nuclear maspin pattern of expression; moreover, DFS was significantly longer in patients with nuclear maspin pattern of expression. Regarding metastasis suppressor nm23-H1, mean nuclear nm23-H1 expression was significantly higher in N0 than in N+ patients. From a prognostic viewpoint, nuclear nm23-H1 expression was significantly lower in LSCC patients who experienced disease recurrence; the DFS was significantly longer for patients whose nuclear nm23-H1 expression was ≥10%. Interestingly, this investigation firstly found a significant association between nuclear nm23-H1 expression and maspin subcellular pattern of expression in LSCC. In multivariate setting, our KNN discriminant analysis considered N status, sub-cellular maspin localization, and nuclear nm23-H1 expression. The calculated error rate was 0.17. The selected variables' accuracy was 82%; in particular, the positive predictive accuracy amounted to 100% and negative predictive accuracy to 79%.

Metastasis suppressors are a class of genes which, when over-expressed (or re-expressed), cause inhibition of cancer metastasis [2]. Nm23 is one of the over 20 metastasis suppressor genes confirmed *in vivo* [23]. Nm23-H1 is currently proposed as a prognostic marker that can predict a better or worse outcome based on recurrence: our preliminary results seem to support this viewpoint also for LSCC. The relevant Nm23 role in metastasis suppression contributes possible drug targets [3]. Various approaches have been attempted to restore nm23-H1 expression, such as (i) nm23-H1 promoter activation with medroxyprogesterone acetate (MPA) treatment, (ii) activation of downstream gene targets, (iii) gene therapy, or (iv) gene treatment approaches [16]. In particular, available data supported the hypothesis that the exposure to high dose MPA led to decrease in anchorage-independent colonization that is abrogated when cells are transfected with antiserum nm23-H1 [23]. Further studies are needed to shed more light on the mechanism of action of nm23-H1 in laryngeal carcinoma cells and thus find ways to restore nm23-H1 loss of expression/function. In the literature, endogenously expressed maspin has been found in the nucleus and cytoplasm, and detected on the cell membrane surface [24]. Although a better understanding is needed of the mechanisms behind maspin-mediated anti-cancer activity in different sub-cellular sites, these preliminary findings suggest that re-activating maspin functions – in combination with apoptosis-inducing [13] or anti-angiogenic [12] chemotherapeutic agents – might represent an important goal in the treatment of advanced LSCC.

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Conflict of interest

The authors declare that they have no conflict of interest.

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