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Expression of vimentin (VIM) and metastasis-associated 1 (MTA1) protein in laryngeal squamous cell carcinoma are associated with prognostic outcome of patients

Sotirios Karamagkiolas[a,*], Ioannis Giotakis[a], Efthimios Kyrodimos[a], Evangelos I. Giotakis[a], Agapi Kataki[b], Fani Karagianni[b], Andreas M. Lazaris[c]

[a] First Department of Otolaryngology, Athens General Hospital "Hippokration", Faculty of Medicine, National and Kapodistrian University of Athens, Athens, Greece
[b] First Department of Propaedeutic Surgery, Athens General Hospital "Hippokration", Faculty of Medicine, National and Kapodistrian University of Athens, Athens, Greece
[c] Department of Vascular Surgery, University General Hospital "Attikon", Faculty of Medicine, National and Kapodistrian University of Athens, Athens, Greece

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ABSTRACT

Purpose: Laryngeal squamous cell carcinoma (LSCC), a common type of head and neck cancer, is associated with high rates of metastasis and recurrence. In this study, we investigated the potential combinatorial prognostic value of NOTCH1, Vimentin (VIM), and Metastasis-associated 1 (MTA1) protein in LSCC, using immunohistochemistry.

Materials and methods: Tissue specimens from 69 patients with LSCC were immunohistochemically evaluated for the protein expression of NOTCH1, VIM, and MTA1. Then, biostatistical analysis was performed, in order to assess the prognostic value of the expression of each one of these proteins.

Results: NOTCH1 expression status was not a significant prognosticator in LSCC, as shown in Kaplan-Meier survival analysis. On the contrary, both VIM and MTA1 seem to have an important prognostic potential, independently of TNM staging and histological grade of the tumor. In fact, positive VIM expression was shown to predict patients' relapse and poor outcome regarding patients' overall survival, in contrast with MTA1, the positive expression of which predicts higher disease-free survival (DFS) and overall survival (OS) rates in LSCC.

Conclusions: VIM and MTA1 constitute potential tumor biomarkers in LSCC and could be integrated into a multiparametric prognostic model. Undoubtedly, their prognostic value needs further validation in larger cohorts of LSCC patients.

1. Introduction

Malignant head and neck neoplasms are mostly squamous cell carcinomas (SCC), originating from the mucosal lining epithelium of hypopharynx, larynx, trachea, oral cavity, and oropharynx [1]. SCC includes about 95% of laryngeal cancer cases, the majority of which begin at the supraglottic and glottic regions. Laryngeal SCC (LSCC) is most common in people after the age of sixty years; however, some LSCC cases have also been observed in childhood. During the last decades, the incidence of LSCC both in men and in women has increased [2]. This augmentation is probably related to changes in tobacco and alcohol consumption.

Head and neck SCC (HNSCC) is the sixth leading cancer by incidence, since 500,000 new cases per year are diagnosed globally, with LSCC being the second most common type of HNSCC [3]. LSCC is characterized by high rates of metastasis and relapse. In the near future, accurate prognosis of patients, based not only on the current clinical staging system but also on novel biomarkers, and treatment response monitoring are expected to lead to a better clinical management of this malignancy [4,5]. Therefore, the discovery and validation of novel molecular biomarkers in LSCC represent an urgent need for the scientific community [6].

Notch signaling is a highly conserved intercellular signaling pathway that tunes interactions between physically adjacent cells through binding of Notch family receptors to their respective ligands [7]. The NOTCH1 preproprotein is processed *via* proteolysis in the trans-Golgi network to give birth two polypeptide chains forming heterodimers, in order to form the mature cell-surface receptor. This

* Corresponding author at: First Department of Otolaryngology, Athens General Hospital "Hippokration", Faculty of Medicine, National and Kapodistrian University of Athens, 114 Vasilissis Sofias Ave., Athens GR-11527, Greece.
E-mail address: sotiriskar13@windowslive.com (S. Karamagkiolas).

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receptor participates in the development of numerous cell and tissue types [8]. The pivotal role of NOTCH1 has already been highlighted in carcinogenesis [9,10].

Vimentin (VIM) is a type-III intermediate filament protein. Intermediate filaments, along with microtubules and actin microfilaments, compose the cytoskeleton [11]. The role of VIM is to maintain cell shape and integrity of the cytoplasm, and to stabilize cytoskeletal interactions. It is implicated in neuritogenesis and cholesterol transport, and also operates as an organizer of a number of other crucial proteins involved in cell attachment, migration, and signaling [12,13]. Mutations in the *VIM* gene have been linked to several human diseases [14].

Metastasis-associated 1 (MTA1) protein is abundantly expressed in metastatic cells, particularly mammary adenocarcinoma cell lines [15,16]. Expression of this protein is associated with the metastatic potential of at least two types of carcinomas, although it is also expressed in many normal tissues [17]. The role it plays in metastasis has not been fully elucidated, yet [18]. The profile and activity of this gene product suggest that it is involved in regulating transcription and that this may be accomplished by chromatin remodeling [18–20].

In this study, we sought to investigate the potential combinatorial prognostic value of NOTCH1, VIM, and MTA1 proteins in LSCC using immunohistochemistry (IHC) in malignant laryngeal tumors of LSCC patients.

2. Materials and methods

2.1. Collection of LSCC tissue specimens

This study included 69 cancerous laryngeal tissue specimens obtained from patients with LSCC as pretreatment tissue biopsy or as surgical treatment sample, at the First Department of Otolaryngology, Athens General Hospital “Hippokration”, Faculty of Medicine, National and Kapodistrian University of Athens (Athens, Greece) collected between 2005 and 2012. All tumors were histologically characterized by a pathologist. Detailed follow-up information was available for all LSCC patients and included disease-free survival (DFS) status (disease-free or relapse) and overall survival (OS) status (deceased or alive), the dates of the events and the cause of death. This original research study was approved by the institutional Ethics Committee of the National and Kapodistrian University of Athens, in accordance with the ethical standards of the 1975 Declaration of Helsinki, as revised in 2000.

2.2. Expression analysis using IHC

Paraffin sections, mounted on superfrost glass slides, were used for the IHC detection of NOTCH1, VIM, and MTA1 proteins. Sections were deparaffinized in xylol, rehydrated in a graded ethanol series, and then subjected to microwave antigen retrieval. Endogenous peroxidase activity was blocked using 0.1% hydrogen peroxide. Tissue sections were then incubated at 4 °C overnight with one of the following primary antibodies at the specified dilution: anti-NOTCH1 (Novus Biologicals, dilution 1:600), anti-VIM (Dako/Agilent, dilution 1:200) and anti-MTA1 (Santa Cruz Bitoechnology; dilution 1:100). IHC staining was performed using the ChemMate™ EnVision™ system (Dako K5007), according to instructions provided by the manufacturer. Sections were then counterstained with Mayer hematoxylin, dehydrated, cleared, and mounted. Appropriate positive controls were also used in IHC, following the guidelines of the manufacturer of each antibody.

After staining, sections were evaluated under a light microscope. Ten fields at x200 were randomly selected and independently examined by two experienced pathologists. All markers were separately evaluated in the cancer tissues, adjacent epithelia, and stroma. The percentage of immunopositive cells (below 30% were considered as weakly stained, between 31% and 69% as moderately stained, and above 70% as strongly stained) and a four-scale intensity scheme (0: negative; 1: weak, 2: moderate, and 3: high staining intensity) were combined and a semi-quantitative score was attributed to each section, as previously described [21].

2.3. Biostatistical analysis

Descriptive statistics analysis was initially performed. Due to the small number of cells not expressing these three proteins, all samples were further categorized into negative (IHC staining score: 0 or 1) or positive (IHC staining score: 2 or 3). Associations between IHC score of NOTCH1, VIM, and MTA1 proteins with clinicopathological variables of LSCC patients were analyzed using the chi-square (χ^2) test or the Fisher's exact test, where appropriate. Kaplan-Meier disease-free survival (DFS) and overall survival (OS) analysis was performed and differences between the survival curves were assessed using the log-rank (Mantel-Cox) test. Cox regression models were also built to assess the proportional hazard for patients' relapse or death. The level of significance for all statistical tests was defined at a probability value of < 0.050 ($P < 0.050$).

3. Results

3.1. Clinicopathological characteristics of the study cohort

The current study comprised 69 cancerous laryngeal tissue specimens from patients who were diagnosed with LSCC. Patients biological and clinicopathological characteristics are summarized in Table 1. Regarding the treatment of LSCC patients included in the current study, most of them [52 out of 69 patients; (75.4%)] received a multimodal therapy. Moreover, 55 (79.7%) patients were subjected to radiotherapy, while 28 (40.6%) patients were treated with chemotherapy. During the accrual follow-up period, recurrence was recorded for 40 (58.0%) out of the 69 LSCC patients of the study. The median DFS was 66.0 months (range: 1.0–120.0), while the estimated mean DFS time was 96.0 months (95% confidence interval [95% CI] = 51.9–140.1). Moreover, 34 patient deaths (49.3%) occurred during the respective follow-up period; all of them were due to causes related to LSCC. The median OS was 83.0 months (range: 1.0–120.0), while the estimated mean 0S time was 117.0 months (95% CI = 95.1–138.9).

3.2. NOTCH1, VIM, and MTA1 protein expression in LSCC tissue specimens and their association with patients' clinicopathological variables

Protein expression analysis using IHC was performed for NOTCH1, VIM, and MTA1 in all samples (Table 2). No significant associations were observed between NOTCH1, VIM, or MTA1 protein expression and TNM stage, tumor invasion, nodal status, or histological grade.

Table 1
Clinical and biological characteristics of LSCC patients.

Total number of patients	69
	Median (range)
Age (years)	64 (36–90)
Disease-free survival (months)	66 (1 − 120)
Overall survival (months)	83 (1–120)
	Number of patients (%)
Histological grade	
I	9 (13.0%)
II	42 (60.9%)
III	18 (26.1%)
TNM stage	
I	18 (26.1%)
II	14 (20.3%)
III	20 (29.0%)
IV	17 (24.6%)

Table 2
IHC results, as distributed among the 69 LSCC tissue specimens.

	Number of patients (%)
NOTCH1	
0	13 (18.8%)
1	30 (43.5%)
2	16 (23.2%)
3	10 (14.5%)
VIM	
0	2 (2.9%)
1	21 (30.4%)
2	14 (20.2%)
3	32 (46.5%)
MTA1	
0	30 (43.5%)
1	19 (27.5%)
2	9 (13.0%)
3	11 (16.0%)

3.3. VIM immunopositivity and negative MTA1 expression predict LSCC patients' relapse

NOTCH1 expression status was not a significant prognosticator in LSCC, as shown in Kaplan-Meier DFS analysis (Suppl. Fig. 1A). On the other hand, Kaplan-Meier survival curves illustrated that LSCC patients with VIM-positive tumors had inferior DFS rates than those with negative VIM expression ($P = 0.032$) (Fig. 1A). In accordance with this finding, univariate Cox regression analysis revealed that VIM immunopositivity predicts a significantly higher risk of patients' relapse (hazard ratio [HR] = 2.20, 95% CI = 1.04–4.67, $P = 0.039$).

With regard to MTA1, Kaplan-Meier survival curves showed that LSCC patients with MTA1-positive tumors had higher DFS rates than those with negative MTA1 expression ($P = 0.017$) (Fig. 1B). In accordance with this finding, univariate Cox regression analysis revealed that MTA1 immunopositivity predicts a significantly lower risk of patients' relapse (HR = 0.39, 95% CI = 0.17–0.88, $P = 0.023$).

Interestingly, the ability of MTA1 immunopositivity to predict LSCC recurrence is superior to VIM protein expression, TNM staging, and histological grade of the laryngeal tumor. Thus, multivariate Cox regression analysis revealed that positive MTA1 expression predicts a significantly lower risk of patients' relapse (HR = 0.43, 95% CI = 0.19–0.98, $P = 0.044$), independently of VIM immunostaining, TNM staging, and tumor histological grade (Table 3).

3.4. Positive VIM expression and loss of MTA1 expression are unfavorable prognosticators of LSCC patients' OS

NOTCH1 expression status was not a significant predictor of LSCC patients' OS, as shown in Kaplan-Meier survival analysis (Suppl. Fig. 1B). On the other hand, Kaplan-Meier survival curves illustrated that LSCC patients with VIM-positive tumors had shorter OS time intervals than those with negative VIM expression ($P = 0.001$) (Fig. 2A). Accordingly, univariate Cox regression analysis revealed that VIM immunopositivity predicts a significantly higher risk of patients' death (HR = 4.29, 95% CI = 1.65–11.16, $P = 0.003$).

Regarding MTA1, Kaplan-Meier survival curves demonstrated that LSCC patients with MTA1-positive tumors had higher OS rates than those with negative MTA1 expression ($P = 0.004$) (Fig. 2B). Accordingly, univariate Cox regression analysis revealed that MTA1 immunopositivity predicts a significantly lower risk of patients' death (HR = 0.27, 95% CI = 0.10–0.70, $P = 0.007$).

Most importantly, the prognostic significance of VIM and MTA1 was shown to be independent of TNM staging and histological grade of the tumor, using multivariate Cox regression analysis, in which the multiparametric models were adjusted for VIM expression, MTA1 expression,

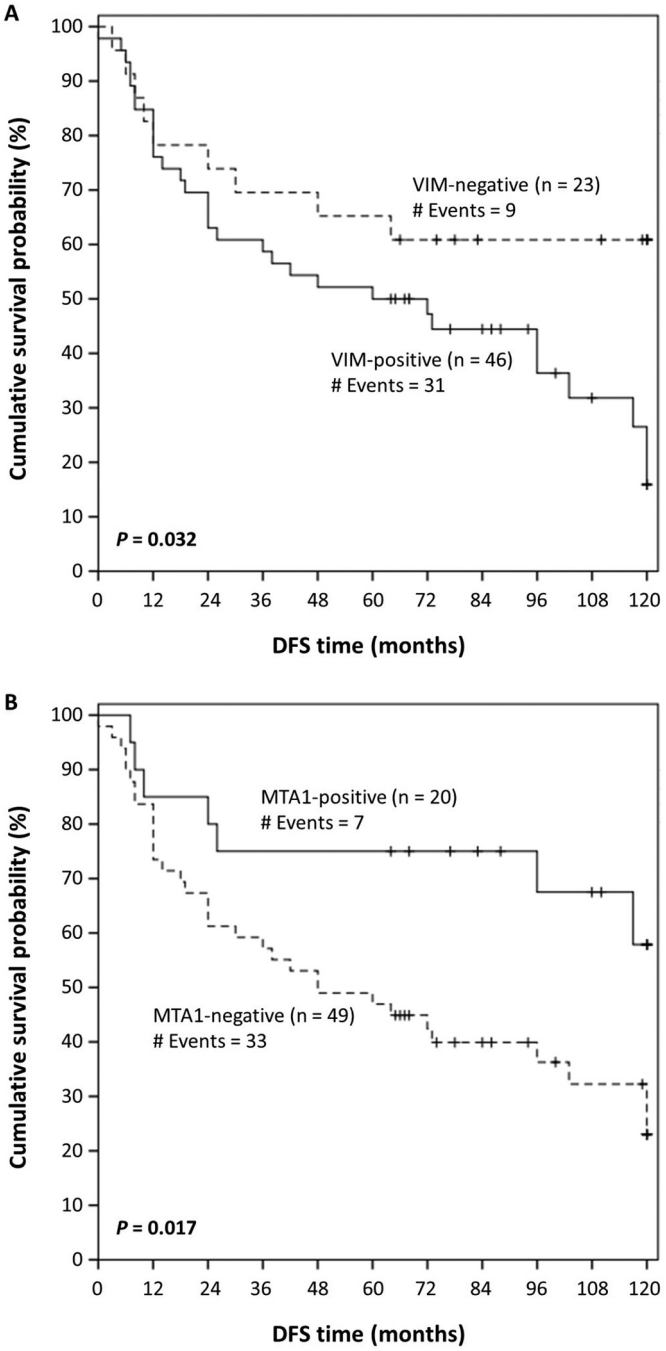

Fig. 1. Kaplan-Meier curves for the disease-free survival (DFS) of LSCC patients with tumors exhibiting positive and negative expression of VIM and MTA1 proteins. **(A)** VIM immunopositivity predicts unfavorable prognosis in LSCC, as patients with VIM-positive tumors have significantly shorter DFS, compared to LSCC patients with VIM-negative tumors. **(B)** On the contrary, MTA1 immunopositivity is indicative of a lower probability of tumor recurrence in LSCC patients.

TNM stage (I/II *vs.* III/IV), and histological grade (I/II *vs.* III) (Table 4).

3.5. VIM and MTA1 protein levels retain their prognostic significance in early-stage LSCC patients and are able to further stratify these patients in different prognostic subgroups

As early-stage LSCC patients are substantially different from advanced-stage patients, respectively, in terms of their prognosis and postoperative treatment, Kaplan-Meier survival analysis was carried out

Table 3
Cox regression analysis for the prediction of disease-free survival of LSCC patients.

Variable	DFS					
	HR[a]	95% CI[b]	*P* value	HR[a]	95% CI[b]	*P* value
	Univariate analysis			Multivariate analysis[c]		
NOTCH1 expression status						
Negative	1.00			–		
Positive	0.80	0.41–1.55	0.51	–	–	–
VIM expression status						
Negative	1.00			1.00		
Positive	2.20	1.04–4.67	**0.039**	1.72	0.79–3.74	0.17
MTA1 expression status						
Negative	1.00			1.00		
Positive	0.39	0.17–0.88	**0.023**	0.43	0.19–0.98	**0.044**
TNM stage						
Early (I/II)	1.00			1.00		
Advanced (III/IV)	1.82	0.95–3.49	0.070	1.51	0.77–2.97	0.24
Histological grade						
I/II	1.00			1.00		
III	1.65	0.83–3.25	0.15	1.32	0.65–2.70	0.44

[a] Hazard ratio, estimated from Cox proportional hazard regression model.
[b] Confidence interval of the estimated HR.
[c] Multivariate models were adjusted for TNM stage and histological grade.

separately in each group of patients stratified according to their tumor stage, in order to evaluate the additive effect of VIM and MTA1 protein expression on DFS and OS. As depicted in Fig. 3A, early-stage LSCC patients with VIM1-positive tumors tended to relapse significantly earlier than early-stage patients with VIM1-negative laryngeal tumors ($P = 0.012$). Importantly, VIM1 immunopositivity in LSCC predicts an unfavorable outcome in both early-stage and advanced-stage patients with regard to their OS ($P = 0.016$ and $P = 0.041$, respectively) (Fig. 3B and Fig. 3C, respectively). Furthermore, as clearly illustrated in Fig. 3D, MTA1 immunopositivity in laryngeal tumors predicts longer OS in early-stage LSCC patients ($P = 0.026$).

4. Discussion

LSCC represents the second commonest type of HNSCC in the United States, as more than ten thousand people are yearly diagnosed with LSCC. The incidence of this cancer type is higher among males, especially among those of age between fifty and seventy years [22]. Although the survival of LSCC patients has been prolonged during the last two decades, mainly due to an increase in the effectiveness of treatment offered to patients [23], the prognosis of those with metastatic disease and/or relapse is rather poor, even after having received a combination of radiotherapy and chemotherapy [24]. The discovery of novel biomarkers and the development of new targeted therapies that can be applied in LSCC patients could lead to even longer survival [25,26].

The clinical potential of numerous proteins as prognostic markers in LSCC has been thoroughly investigated during the last twenty years. Particular attention has been drawn to molecules with key role in cancer-related cellular processes including cell cycle, growth, proliferation, and apoptosis [5,27–29]. Some of these molecules could represent novel biomarkers in head and neck malignancies [30,31], including LSCC [32], nasopharyngeal carcinoma [32,33], and other cancer types [34]. In fact, alternative splicing and subsequent generation of different isoforms has increased the repertoire of such potential biomarkers [35–47]. From a clinical aspect, the most interesting and well-studied molecule in LSCC has been the tumor suppressor *TP53* gene and its respective protein p53 [48,49]. Proteins with prognostic power in LSCC also include cell cycle regulators like cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors [29].

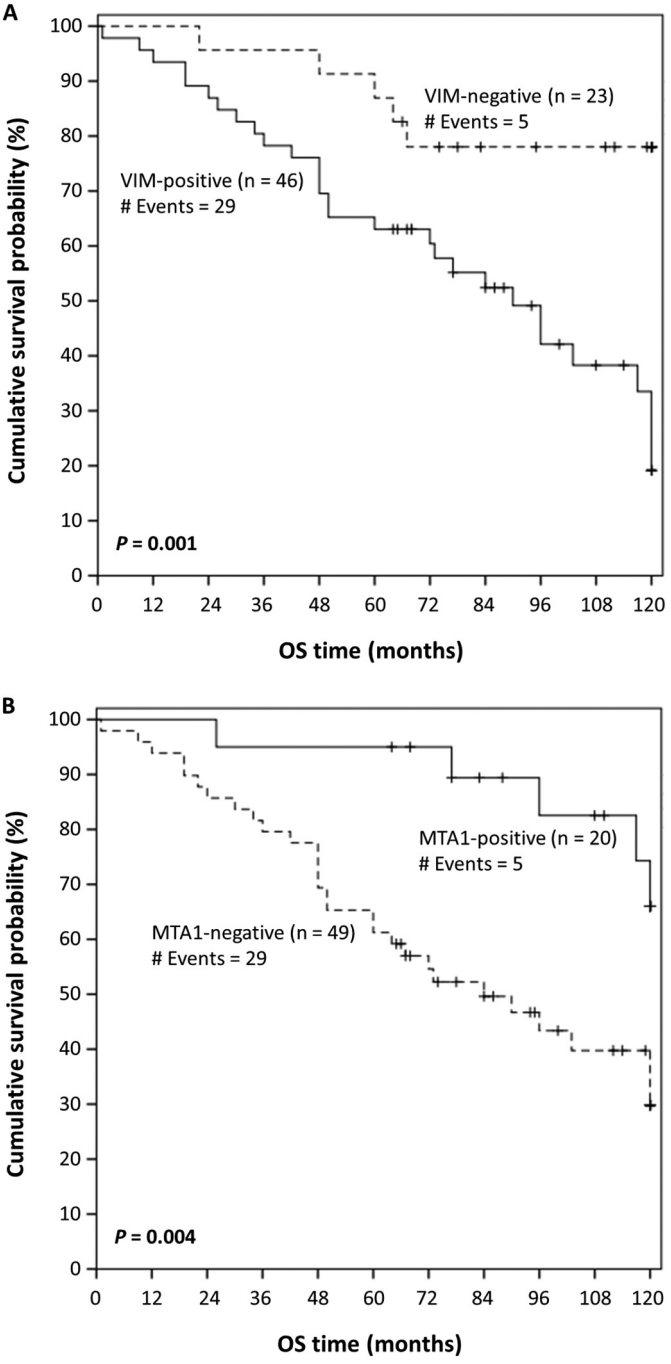

Fig. 2. Kaplan-Meier curves for the overall survival (OS) of LSCC patients with tumors showing positive and negative expression of VIM and MTA1 proteins. **(A)** VIM immunopositivity predicts inferior OS in LSCC, compared to LSCC patients with VIM-negative tumors. **(B)** On the contrary, MTA1 immunopositivity predicts longer OS of LSCC patients.

Furthermore, cyclin D3 (*CCND3*) mRNA overexpression as well as gene amplification of cyclin D1 (*CCND1*) have been described as independent prognosticators of poor OS in this malignancy [50,51]. Many researches have focused on the elucidation of the diagnostic and/or prognostic potential of Ki-67 (MKI67) and proliferating cell nuclear antigen (PCNA), cathepsins B and D, E-cadherin, the epidermal growth factor receptor (EGFR) family members, and vascular endothelial growth factor (VEGF) subfamily members [29]. Non-coding RNAs, including microRNAs [52–66] and long non-coding RNAs represent another class of promising molecular biomarkers in human malignancies

Table 4
Cox regression analysis for the prediction of overall survival of LSCC patients.

Variable	OS					
	HR[a]	95% CI[b]	*P* value	HR[a]	95% CI[b]	*P* value
	Univariate analysis			Multivariate analysis[c]		
NOTCH1 expression status						
Negative	1.00			–		
Positive	0.96	0.49–1.99	0.51	–	–	–
VIM expression status						
Negative	1.00			1.00		
Positive	4.29	1.65–11.16	**0.003**	3.59	1.36–9.48	**0.010**
MTA1 expression status						
Negative	1.00			1.00		
Positive	0.27	0.10–0.70	**0.007**	0.31	0.12–0.82	**0.018**
TNM stage						
Early (I/II)	1.00			1.00		
Advanced (III/IV)	2.18	1.08–4.41	**0.029**	1.91	0.93–3.94	0.079
Histological grade						
I/II	1.00			1.00		
III	2.05	0.99–4.25	0.054	1.40	0.66–2.95	0.38

[a] Hazard ratio, estimated from Cox proportional hazard regression model.
[b] Confidence interval of the estimated HR.
[c] Multivariate models were adjusted for TNM stage and histological grade.

[67–73], including LSCC. Nevertheless, none of all these potential tumor biomarkers have been validated in studies of large cohorts of LSCC patients and established in clinical practice, so far [29,74,75].

In this original research study, we investigated the potential combinatorial prognostic value of NOTCH1, VIM, and MTA1 proteins in LSCC, using immunohistochemistry (IHC). Although the objectiveness of RNA quantification by a validated quantitative real-time PCR approach has generally more advantages than the immunohistochemical assessment of protein expression [76], the results of IHC are more easy to exploit, since the large diversity of alternatively spliced variants – usually non-coding RNA molecules that are eliminated through nonsense mediate mRNA decay – may complicate their accurate quantification [77–79]. A limitation of this study was that the IHC analysis was based on a subjective high-power field scoring system, rather than a quantifiable digital subtraction analysis. In fact, the use of a 4-point scale is less reliable, thus introducing a fair amount of error into the data analysis. However, the use of a dichotomous cut-off value and the subsequent categorization of patients into negative or positive with regard to the expression of each studied molecule are likely to alleviate the need for a more objective scoring.

With regard to DFS, univariate Cox regression analysis showed that positive VIM expression status in LSCC predicts an increased risk of patient relapse, and Kaplan-Meier survival analysis revealed significantly lower DFS rates for patients with malignant neoplasms exhibiting VIM immunopositivity, in contrast with MTA1 immunopositivity. Regarding OS, Cox regression demonstrated again that

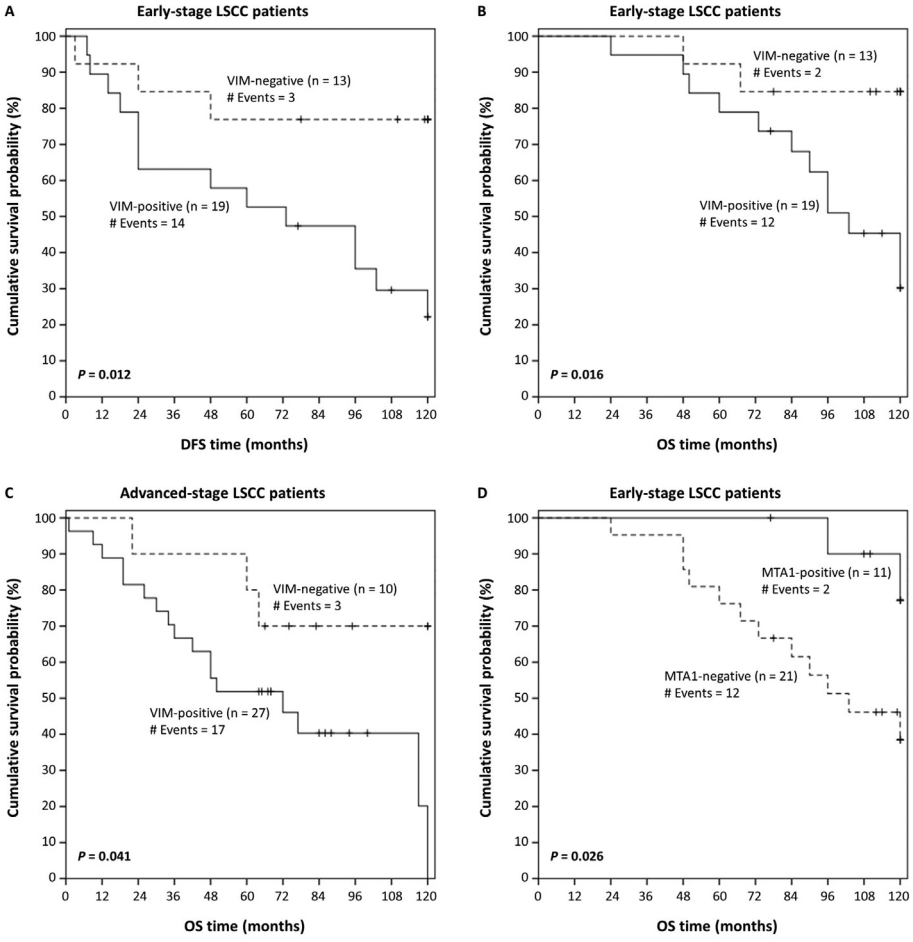

Fig. 3. Kaplan-Meier curves for the disease-free survival (DFS) and overall survival (OS) of distinct prognostic subgroups of LSCC patients with tumors showing positive and negative expression of VIM and MTA1 proteins. **(A)** VIM immunopositivity in laryngeal tumors predicts tumor recurrence in early-stage LSCC patients as well as shorter OS in both **(B)** early-stage and **(C)** advanced-stage LSCC patient. **(D)** On the contrary, MTA1 immunopositivity in laryngeal tumors predicts longer OS in patients with early-stage LSCC.

positive VIM expression status in LSCC is an unfavorable prognosticator in OS; this finding is in agreement with Kaplan-Meier OS analysis results. Therefore, VIM immunopositivity appears as an indicator of poor prognostic outcome in LSCC, in contrast again with MTA1 immunopositivity. Interestingly, our results showed also that early-stage LSCC patients with high intratumoral VIM expression have a worse prognosis regarding both their DFS and OS, compared to early-stage patients with laryngeal tumors being VIM-negative. Thus, the prognostic value of VIM immunopositivity is particularly important in early-stage patients, predicting those whose are more likely to relapse or succumb to their disease. Moreover, advanced-stage patients with laryngeal tumors exhibiting VIM overexpression are predicted to have shorter OS, compared to advanced-stage patients with VIM-negative malignancies. This unfavorable prognostic role of VIM could be partly attributed to a worse response of LSCC patients with VIM-positive tumors to treatment. On the contrary, MTA1 immunopositivity indicates early-stage patients who are less likely to die from LSCC, therefore having a favorable prognostic role in this distinct prognostic subgroup of patients.

The developed multivariate Cox regression models revealed that the prognostic value of VIM and MTA1 expression status is independent of histological grade and – most importantly – of the TNM stage, which is considered as the most reliable prognostic factor in this malignancy. The assessment in this disease is clinical and based on the best possible estimate of the extent of disease before treatment. Staging of the primary laryngeal tumor is based on inspection and palpation when possible, as well as by indirect mirror examination and direct endoscopy when appropriate [80]. However, biomarkers being able to contribute to a more accurate prognosis of LSCC constitute a modern necessity. Under this perspective, VIM and MTA1 could prove very useful as novel tumor biomarkers in LSCC, provided that their validation is successful in other studies using very large cohorts of patients.

In conclusion, this study shows that positive VIM expression is related to unfavorable prognosis in LSCC, in contrast with positive MTA1 expression, which is rather associated with favorable prognosis in this malignancy. Therefore, VIM and MTA1 may represent two novel, useful prognostic tissue biomarkers in LSCC, independent of the clinicopathological features of the malignant laryngeal tumors.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.amjoto.2019.04.002.

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