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Clinical significance of epithelial-to-mesenchymal transition in laryngeal carcinoma: Its role in the different subsites

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Abstract

Background: During epithelial-to-mesenchymal transition, cancer cells lose adhesion capacity gaining migratory properties. The role of the process on prognosis has been evaluated in 50 cases of laryngeal carcinoma.

Methods: E-cadherin, N-cadherin, β-catenin, α-catenin, γ-catenin, caveolin-1, and vimentin immunohistochemical expression were evaluated using a double score based on staining intensity and cellular localization.

Results: Cytoplasmic E-cadherin and α/γ catenin staining were associated with a decrease in survival, cytoplasmic β-catenin was associated with advanced stage, and N-cadherin and vimentin expression were associated with poor differentiation and tumor relapse. On the basis of cancer cells, epithelial or mesenchymal morphological and immunophenotypic similarity we identified 4 main subgroups correlated with a transition to a more undifferentiated phenotype, which have a different pattern of relapse and survival.

Conclusion: The negative prognostic role of epithelial-to-mesenchymal transition has been confirmed and a predictive role in glottic tumors has been suggested, leading us to propose epithelial-to-mesenchymal transition as an additional adverse feature in laryngeal carcinoma.

KEYWORDS

β-catenin, E-cadherin, epithelial-to-mesenchymal transition, laryngeal carcinoma, N-cadherin

1 | INTRODUCTION

Laryngeal squamous cell carcinoma (SCC) is a relatively rare cancer burdened by a high morbidity and mortality rate,[1] strongly correlated with tobacco smoking and alcohol intake.[2] Disease stage is the main validated prognostic factor[3,4] because a molecular classification is still not available in laryngeal SCC. Moreover, the site of the laryngeal tumor significantly affects prognosis[5] as well as the pathologic features of the tumor[6] and the Ki-67 labeling index.[7] However, innovative biomarkers that are able to define the biological behavior of the tumor are urgently needed in order to assess the risk of recurrence and tailor the patient's therapy, but they need to be validated and translated into clinical practice.[8,9] It was established by several studies that epithelial-to-mesenchymal transition (ie, the rapid and often reversible changing from the epithelial morphology to a mesenchymal phenotype) is linked to unfavorable prognostic markers and poor prognosis in several cancers, including laryngeal SCC.[10,11] During epithelial-to-mesenchymal transition, cancer cells lose their mechanisms of adhesion and acquire the anchorage-independent growth ones, which lead to migration, invasion, increased metastatic potential, immunosuppression,[12] and drug resistance.[13] The critical step during epithelial-to-mesenchymal transition is the

"cadherin switch,"[14] which is the decrease in the expression of E-cadherin, a transmembrane receptor, essential in the formation of intercellular junctions,[15] associated to a simultaneous increase of the expression of the transmembrane cell-cell adhesion protein N-cadherin. The "cadherin switch" provides a mechanism for transendothelial migration, anoikis resistance, and increased invasiveness.[16] Others proteins involved in the epithelial-to-mesenchymal transition are the α, β, and γ catenins, which colocalizes with E-cadherin at the cytoplasmic domain, connecting by linking the β-catenin/cadherin complex to the intracellular cytoskeleton actin fibers, as well as the mesenchymal marker vimentin, a cytoskeletal intermediate filament type III.[17] β-catenin in the cytoplasm, freed from its assembling with E-cadherin,[18] is also involved in the canonical Wnt signaling pathway, taking part in modulation of cell proliferation and signal transduction.[19,20] Several studies have reported its translocation into the nucleus, where β-catenin acts as a cofactor for the transcription T-cell factor leading to epithelial-to-mesenchymal transition[21] and poor prognosis.[22] Finally, the scaffolding protein caveolin-1 is involved in the growth factor-induced process of disassembling of adheren junctions and desmosomes.[23–25]

The purpose of this study was to highlight a possible association between mesenchymal phenotype acquisition and adverse clinicopathological prognostic factors as well as the recurrence rate, the patient's disease-free survival (DFS), and overall survival (OS) by evaluating the expression of a panel of different epithelial-to-mesenchymal transition markers in 50 patients affected by laryngeal SCC. The possibility that epithelial-to-mesenchymal transition could identify, in the context of tumors with the same pathological stage, a subset of cancers that have different prognosis has also been investigated. In this report, we confirm that epithelial-to-mesenchymal transition is a critical process strongly correlated with an aggressive clinical behavior in laryngeal SCC.

2 | MATERIALS AND METHODS

2.1 | Clinical data and tissue samples

Formalin-fixed paraffin-embedded tissue of laryngeal SCC retrieved from the archived specimens of the Division of Pathology, Sapienza University of Rome, were obtained from 46 patients undergoing surgery with radical intent for laryngeal SCC from March 2006 to October 2013. In addition, 4 early-stage laryngeal SCC tumors treated without lymph node dissection (Nx) on the basis of a negative baseline imaging for lymph node involvement (cN0) were included. The study has been submitted and approved by the Bioethics Committee of Policlinico Umberto I, Rome, and was compliant with the reporting recommendations for tumor marker prognostic study (REMARK) recommendations.[26] The patients, informed during the follow-up, gave their consent for the study. Primary surgical treatment and any standard adjuvant strategies[27] are listed in Table 1. All patients underwent follow-up as advised.[27] The main exclusion criteria of the study were a previous multimodal treatment intended to preserve the larynx, as well as the presence of multifocal or multicentric cancers, positive margins, metastatic disease, and nonadherence to the follow-up program. For each laryngeal carcinoma, all the hematoxylin-eosin–stained tissue slides obtained from a suitable sampling of surgical specimen, used for histological diagnosis (evaluation of microscopic features, and grading and staging of the neoplasia), were reexamined and the more representative slides (1 or 2 sections) were selected for the immunohistochemical study. Sections of normal epithelium adjacent the carcinomas of each patient was used as the control. On the basis of the risk of relapse, we classified the patients as: (i) low risk (stages I and II); (ii) intermediate risk (T3 with adverse pathologic primary site features and/or N1), further divided in low grade (G1/G2) or high grade (G3/G4); and (iii) poor risk (T4 and/or N2). The DFS and OS were recorded as well as the site of relapse (regional or systemic). The occurrence of second primary tumors was also remarked.

TABLE 1 Surgical primary treatment and adjuvant strategies

	No. of cases	%
Surgery	50	
Partial laryngectomy	24	48
Total laryngectomy	26	52
Neck dissection	46	92
Adjuvant radiotherapy[a]	4	8
Adjuvant radiochemotherapy[b]	24	48

[a]Radiotherapy was administered in pT3-negative lymph node or pT3-positive single lymph node cancers with at least one adverse features (perineural, lymphatic, or vascular invasion).
[b]Adjuvant radiochemotherapy was administered in pN2 or pN3 nodal disease, extracapsular nodal spread, adverse pathologic primary site features (pT4 primary, perineural, lymphatic, or vascular invasion). Cisplatin (100 mg/m^2 every 21 days i.v.) was the concurrent systemic therapy administered.

2.2 | Immunohistochemistry

From selected paraffin tissue blocks, consecutive tissue sections (2 microns) of tumor and normal samples were cut and used for the immunohistochemical (IHC) study. The sections were deparaffinized and rehydrated and used for IHC study. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and antigen retrieval was made by boiling them in citrate buffer (0.01 mol/L, pH 6) with microwaves (750 W). Then the sections were incubated for 1 hour at room temperature (RT) with primary antibodies: anti-caveolin-1

TABLE 2 Characteristics of the patients, histological features, and survival parameters

Characteristics	No. of cases
Race	
White	50
Sex ratio M:/F	5:1
Female	8
Male	42
Mean age, year (range)	64.6 ± 10 (range 47-87)
<65	23
>65	27
Smoking status	
Never smoker	2
Ex-smoker[a]	12
Former smoker[b]	21
Still smoker[c]	15
Alcohol	
Alcohol user	33
No alcohol	17
Tumor localization	
Glottic	37
Supraglottic	13
Microscopic type tumors	
Keratinizing SCC	3
Nonkeratinizing SCC	42
Sarcomatoid carcinoma (Spindle-cell carcinoma)	5
T and N classification distribution	
T1-T2	20
T3-T4	30
N0	22
N1-N2	24
Risk classes	
Low risk	10
Intermediate risk	13
Poor risk	27
Tumor grading	
G1-G2	24
G3-G4	26
Proliferation index	
Ki67 < 15	25
Ki67 ≥ 15	25
Relapse	
Relapsed	27
No Relapse	23
DFS	
5-y	24
OS	
5-y	24

Abbreviations: DFS, disease-free survival; OS, overall survival; SCC, squamous cell carcinoma.

[a]Patients who had discontinued tobacco use at least 5 years before the diagnosis of laryngeal SCC.

[b]patients who continued to smoke up to laryngeal SCC diagnosis.

[c]patients who continued to smoke after laryngeal SCC diagnosis.

(clone N-20, sc-894, 1:50), anti-E-cadherin (clone H-108, sc-7870, 1:100), anti-α-catenin (clone H-297, sc-7894, 1:50), anti-β-catenin (clone H-102, sc-7199, 1:50), anti-γ-catenin (clone H-80, sc-7900, 1:100), and anti-N-cadherin (clone H-63, sc-7939, USA, 1:50), were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-vimentin (clone V9, M0725, 1:100) and anti-Ki67 (clone M7240, 1:50) were obtained from Dako, Glostrup, Denmark. Universal LSAB2 System-HRP (Dako) was used to label the primary antibody. The reaction product was visualized with 3,3-diaminobenzidine (Dako). The sections were counterstained with Mayer's hematoxylin. Negative control was obtained by omitting the primary antibody. The immunostainings were evaluated using a semiquantitative double score based on staining intensity (0 = negative, 1 = weak, 2 = moderate, and 3 = strong) and localization (I = membrane, II = membrane and cytoplasmic, III = cytoplasmic, IV = membrane, cytoplasmic, and nuclear, V = cytoplasmic and nuclear, and VI = nuclear).[28] Tumors with a value ≥15 positive cells for Ki67 immunostaining were classified as high kinetic index. The IHC evaluation was performed by 2 experienced investigators (C.R.d.G. and C.R.), not informed about the antibody used. Each investigator analyzed all the tumor areas in the slides (magnification × 1.5, × 4, and × 20) for each antibody staining for 3 times and the scoring of the staining was calculated as weighed mean (interobserver agreement 0.81). Laryngeal SCCs were classified in accordance to morphological and immunophenotypic characteristics as: (i) epithelial-like, when similar to the normal epithelium; (ii) in transition, when tumor cells are losing epithelial characteristics but have not yet acquired a mesenchymal phenotype; and (iii) mesenchymal-like, when the tumor cells have lost their epithelial phenotype and acquire mesenchymal features.

2.3 | Statistical analysis

Statistical analysis was performed using SPSS software (SPSS, Chicago, IL). Association between parameters was

evaluated by the chi-square (and Fisher's exact) 2-sided test. DFS was calculated from diagnosis to disease progression, and OS was calculated from diagnosis to the date of death with the Kaplan-Meier method. Finally, a log-rank test was performed to evaluate the statistical significance of difference in survival. A P value $< .05$ was considered statistically significant. The probability that a mesenchymal-like patient developed a relapse (the positive predictive value of mesenchymal-like patients), was calculated by the ratio between the number of mesenchymal-like patients who developed a relapse and the total of mesenchymal-like patients.

3 | RESULTS

The characteristics of patients and the histological features of the tumor, including TNM staging sec Union for International Cancer Control 2009[29] and World Health Organization grading, as well as tumor localization and survival parameters are listed in Table 2. The median follow-up of patients was 35 months. The overall relapse rate was 54% (27/50) with a local relapse rate of 33% (9/27). The predominant sites of metastasis were the lungs (13/18; 72%) and mediastinal lymph nodes (5/18; 28%), which was associated in 4 cases with locoregional recurrence. The 3-year and 5-year DFS were 25 of 50 = 50% and 24 of 50 = 48%, respectively, whereas the 3-year and 5-year OS were 28 of 50 = 56% and 24 of 50 = 48%, with a cancer-specific survival of 25 of 50 = 50%. Survival has also been affected in 3 patients by the onset of a second metachronous primary tumor. The DFS ($P < .002$), OS ($P < .001$) and the relapse rate ($P < .019$) were significantly different in the 3 classes of risk (Supporting Information Figure S1). Moreover, disease relapse negatively affected 5-year OS ($P < .001$; Table 3).

TABLE 3 Patient risk, laryngeal subsite, disease-free survival, and overall survival in relation to the disease relapse

Variables	No. of cases	Relapse	No Relapse	*P* value
Low risk	10	2 (20%)	8 (80%)	**.0194**
Intermediate risk	13	6 (46%)	7 (54%)	
Poor risk	27	19 (70%)	8 (30%)	
Glottic	37	17 (46%)	20 (54%)	
Supraglottic	13	10 (77%)	3 (23%)	
5-y DFS	24	1 (4%)	23 (96%)	
5-y OS	24	5 (21%)	19 (79%)	**<.001**

Abbreviations: DFS, disease-free survival; OS, overall survival.
p-value in bold indicates statistical significance.

Immunohistochemical expression and correlation with outcome

Mesenchymal-like behavior of caveolin-1

In our study, all laryngeal SCCs examined did not exhibit the normal laryngeal mucosa expression pattern of caveolin-1 (1-I). In fact, 9 of 50 cases (18%) of laryngeal SCC revealed a strong membrane and cytoplasmic staining (3-II) corresponding to the initial step of caveolin-1 deregulation. The 41 of 50 cases (82%) presented a strong and exclusive cytoplasmic staining (3-III), which matches a further step of caveolin-1 deregulation (Supporting Information Figure S2 - 1a,b,c). The ubiquitary alteration of caveolin-1 highlighted in laryngeal SCC did not allow us to detect any significant difference between the protein expression or its translocation and any of the prognostic factors considered in conjunction with survival parameters (Table 4).

E-cadherin quantitative and qualitative alteration in laryngeal squamous cell carcinoma

In the control samples, the expression pattern of the E-cadherin was strong, homogeneous, and mainly limited to the cell membrane (3-I) as expected. An intense E-cadherin staining on cell membrane (3-I) has only been evidenced in 3 tumors, whereas 23 cancers (46%) showed the simultaneous localization of E-cadherin on the membrane and in the cytoplasm (3-II). An abnormal and exclusive cytoplasmic staining (3-III) was highlighted in 48% of tumor samples (Supporting Information Figure S2 - 2a,b,c). We report that DFS and the OS were significantly different in the 3 different patterns of E-cadherin expression with a significant decreased OS and DFS in tumors with an abnormal cytoplasmic E-cadherin accumulation (respectively, $P < .068$ and $P < .010$; Table 4).

Intracytoplasmic α, β, and γ catenin storage

The normal mainly limited cell membrane expression pattern of β-catenin was rare (6/50; 12%) in the tumor samples analyzed (3-I). An abnormal cytoplasmic localization associated with membrane staining (3-II; 68%) or even exclusively cytoplasmic (3-III; 20%) was highlighted in the majority of laryngeal SCCs. The relocation of the β-catenin was consistent with the late stages in the developmental process of epithelial-to-mesenchymal transition (Supporting Information Figure S2 - 3a,b,c). We report a significant association between the abnormal storage of β-catenin in cancer cell cytoplasm and the T3-T4 group ($P < .017$; Table 4). The DFS and OS were different in the 3 patterns of expression of β-catenin, but the worst survival curve was observed in tumors with initial β-catenin deregulation ($P < .001$ and $P < .002$) compared with tumors with abnormal advanced β-catenin cytoplasmic accumulation. In addition, the abnormal

TABLE 4 Antibody cellular localization versus T and N classifications, grade, laryngeal subsite relapse, disease-free survival, and overall survival

	No. of cases	Normal pattern	Initial deregulation	Late deregulation	*P* value
Caveolin-1					
T1-T2	20	0	3 (15%)	17 (85%)	
T3-T4	30	0	6 (20%)	24 (80%)	
G1-G2	24	0	4 (17%)	20 (83%)	
G3-G4	26	0	5 (19%)	21 (81%)	
N0	22	0	4 (18%)	18 (82%)	
N1-N2	24	0	4 (17%)	20 (83%)	
Glottic	37	0	8 (22%)	29 (78%)	
Supraglottic	13	0	1 (8%)	12 (92%)	
Relapse	27	0	4 (15%)	23 (85%)	
No relapse	23	0	5 (22%)	18 (78%)	
5-y DFS	24	0	6	18	
5-y OS	24	0	4	20	
E-cadherin					
T1-T2	20	1 (5%)	10 (50%)	9 (45%)	
T3-T4	30	2 (7%)	13 (43%)	15 (50%)	
G1-G2	24	1 (4%)	14 (58%)	9 (38%)	
G3-G4	26	2 (8%)	9 (34%)	15 (58%)	
N0	22	2 (9%)	10 (45%)	10 (45%)	
N1-N2	24	1 (4%)	10 (42%)	13 (54%)	
Glottic	37	2 (5%)	18 (49%)	17 (46%)	
Supraglottic	13	1 (8%)	5 (38%)	7 (54%)	
Relapse	27	2	11	14	
No relapse	23	1	12	10	
5-y DFS	24	1	13	10	**<.010**
5-y OS	24	1	11	12	**<.006**
β-Catenin					
T1-T2	20	4 (20%)	15 (75%)	1 (5%)	**<.017**
T3-T4	30	2 (7%)	19 (63%)	9 (30%)	
G1-G2	24	3 (12.5%)	18 (75%)	3 (12.5%)	
G3-G4	26	3 (12%)	16 (61%)	7 (27%)	
N0	22	3 (14%)	15 (68%)	4 (18%)	
N1-N2	24	3 (12.5%)	15 (62.5)	6 (25%)	
Glottic	37	5 (13%)	24 (65%)	8 (22%)	
Supraglottic	13	1 (8%)	10 (77%)	2 (15%)	
Relapse	27	3	18	6	
No relapse	23	3	16	4	
5-y DFS	24	3	17	4	**<.001**
5-y OS	24	3	17	4	**<.002**
α-Catenin					
T1-T2	20	2 (10%)	3 (15%)	15 (75%)	
T3-T4	30	1 (3%)	11 (37%)	18 (60%)	
G1-G2	24	1 (4%)	7 (29%)	16 (67%)	
G3-G4	26	2 (8%)	7 (27%)	17 (65%)	
N0	22	1 (4%)	5 (23%)	16 (73%)	
N1-N2	24	2 (8%)	7 (29%)	15 (63%)	
Glottic	37	2 (5%)	10 (27%)	25 (68%)	
Supraglottic	13	1 (8%)	4 (31%)	8 (61%)	

(Continues)

TABLE 4 (Continued)

	No. of cases	Normal pattern	Initial deregulation	Late deregulation	*P* value
Relapse	27	1	8	18	
No relapse	23	2	6	15	
5-y DFS	24	2	7	15	**<.001**
5-y OS	24	1	8	15	**<.001**
γ-Catenin					
T1-T2	20	1 (5%)	10 (50%)	9 (45%)	
T3-T4	30	2 (7%)	9 (30%)	19 (63%)	
G1-G2	24	1 (4%)	12 (50%)	11 (46%)	
G3-G4	26	2 (8%)	7 (27%)	17 (65%)	
N0	22	0	9 (41%)	13 (59%)	
N1-N2	24	3 (12.5%)	6 (25%)	15 (62.5%)	
Glottic	37	2 (5%)	16 (43%)	19 (51%)	
Supraglottic	13	1 (8%)	3 (23%)	9 (69%)	
Relapse	27	3	8	16	
No relapse	23	0	11	12	
5-y DFS	24	0	12	12	**<.045**
5-y OS	24	1	12	11	**<.004**
N-cadherin					
T1-T2	20	17 (85%)	3 (15%)		
T3-T4	30	25 (83%)	5 (17%)		
G1-G2	24	24 (100%)	0		**<.043**
G3-G4	26	18 (69%)	8 (31%)		
N0	22	20 (91%)	2 (9%)		
N1-N2	24	18 (75%)	6 (25%)		
Glottic	37	31 (84%)	6 (16%)		
Supraglottic	13	11 (85%)	2 (15%)		
Relapse	27	19	8		**<.005**
No relapse	23	23	0		
5-y DFS	24	23	1		
5-y OS	24	21	3		
Vimentin					
T1-T2	20	18 (90%)	2 (10%)		
T3-T4	30	19 (63%)	11 (37%)		
G1-G2	24	22 (92%)	2 (8%)		**<.092**
G3-G4	26	15 (58%)	11 (42%)		
N0	22	18 (82%)	4 (18%)		
N1-N2	24	15 (62.5%)	9 (37.5%)		
Glottic	37	26 (70%)	11 (30%)		
Supraglottic	13	11 (85%)	2 (15%)		
Relapse	27	15	12		**<.013**
No relapse	23	22	1		
5-y DFS	24	22	2		
5-y OS	24	17	9		

Abbreviations: DFS, disease-free survival; G, grade; OS, overall survival.
p-value in bold indicates statistical significance.

cytoplasmic accumulation of γ and α-catenin were predominant (respectively, 3-III and 1-III; Supporting Information Figure S2 - 4a,b,c, and 5a,b,c). This pattern of expression was associated with worse DFS (respectively, $P < .045$ and $P < .001$) and OS (respectively, $P < .004$ and $P < .001$; Table 4).

The critical β-catenin nuclear translocation

A nuclear and cytoplasmic staining (3-V) could be revealed in one locally advanced and poorly differentiated tumor sample. This glottic cancer fell into the high-risk category,

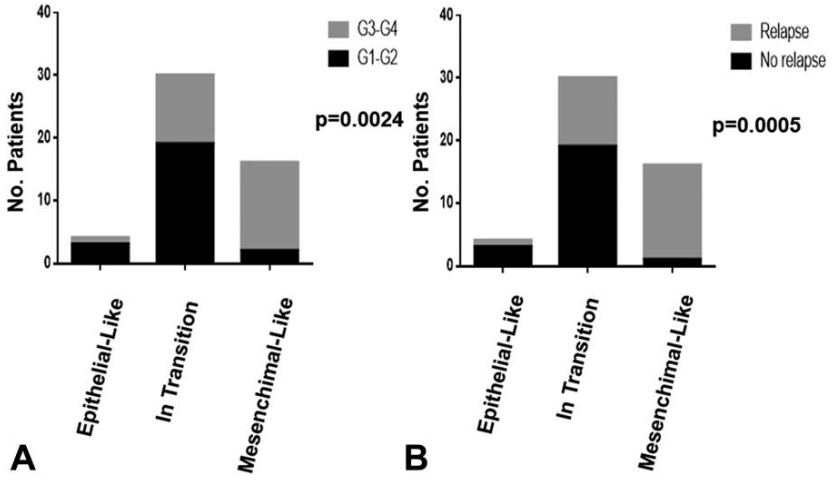

FIGURE 1 Epithelial-to-mesenchymal transition status influence on differentiation A and relapse B

and a metastatic lung progression after 15 months of diagnosis was recorded (Supporting Information Figure S2 - 3d).

The "Cadherin switch"

Normal laryngeal mucosa did not express N-cadherin (0), an aberrant expression of the protein on the cell membrane (2-I) was highlighted in 8 tumor samples (16%; Supporting Information Figure S2 - 6a,b). Small neoplastic N-cadherin positive clusters were exclusively demonstrated in undifferentiated/poorly differentiated tumors ($P < .043$) and in patients whose disease had relapsed ($P = .005$). We did not find a significant different N-cadherin expression in the 2 laryngeal subsites (Table 4).

Upregulation of vimentin and a mature mesenchymal phenotype acquisition

Vimentin staining was moderately positive in the cell submembrane cytoplasm (2-I; 15%) and in the cytoplasm (2-III; 85%) of elongated cells with mesenchymal shape in 13 laryngeal SCCs (26%; Supporting Information Figure S2 - 7a,b, Supporting Information Figure S3). The vimentin expression was statistically associated to the tumor grade ($P < .092$) and with tumor relapse ($P < .013$). Surprisingly, the detection of vimentin-positive tumors was more common in glottic (30%) rather than in supraglottic cancers (15%; Table 4).

Concordance of epithelial-to-mesenchymal transition markers expression between each other

We found that all the markers analyzed were concordant between each other and highly consistent with the information provided, allowing us to detect the framework of different steps of the epithelial-to-mesenchymal transition stepwise process. The detection of abnormal caveolin-1 expression as well as the cadherin/catenin complex breakdown and the pathological accumulation of the proteins in the cytoplasm up to the expression of the mesenchymal markers, represent sequential stages recognizable in the neoplastic samples. The combination of multiple features provides an advantage over the information provided by every single marker on the state of development of the epithelial-to-mesenchymal transition (Supporting Information Figure S4). Only a minority (4/50; 8%) of laryngeal SCCs retain an epithelial-like immunophenotype, whereas a mesenchymal-like phenotype was achieved in 16 of 50 (32%) tumor samples (3 N-cadherin-positive cancers, 8 vimentin-positive cancers, and 5 N-cadherin and vimentin-positive cancers). Most of laryngeal SCC tumors showed an immunophenotype in transition (30/50; 60%) characterized by a broad continuous spectrum of alterations ranging from tumors that have an initial loss of their epithelial characteristics (17/30; 34%) up to cancers characterized by deep premesenchymal alterations (13/30; 43%).

Association among epithelial-to-mesenchymal transition status and clinical data, histopathological features, smoking status, proliferation index, and patient risk classes

We found an association between epithelial-to-mesenchymal transition status with the male sex ($P < .041$), but the different weight between the sexes must be taken into account (Table 5), and with the degree of differentiation characterized by a significant increase of anaplasia in mesenchymal tumors ($P < .024$; Figure 1A). No correlation could be demonstrated between epithelial-to-mesenchymal transition status and the clinical data, including the classes of risk (Table 5).

TABLE 5 Epithelial-to-mesenchymal transition versus clinical data

	No. of cases	Epithelial-like	In transition	Mesenchymal-like	*P* value
Male	8	0	8 (100%)	0	**<.041**
Female	42	4 (10%)	22 (52%)	16 (38%)	
<65-y of age	23	1 (4%)	15 (65%)	7 (30%)	
>65-y of age	27	3 (11%)	15 (56%)	9 (33%)	
Smoker	48	4 (83%)	28 (58%)	16 (33%)	
Nonsmoker	2	0	2 (100%)	0	
Alcohol use	33	1 (3%)	21 (64%)	11 (33%)	
No alcohol use	17	3 (18%)	9 (53%)	5 (29%)	
Low-risk	10	1 (10%)	8 (80%)	1 (10%)	
Intermediate-risk	13	1 (8%)	9 (69%)	3 (23%)	
Poor-risk	27	2 (7%)	13 (48%)	12 (44%)	
G1-G2	24	3 (12%)	19 (79%)	2 (83%)	**<.024**
G3-G4	26	1 (4%)	11 (42%)	14 (54%)	
T1-T2	20	2 (10%)	14 (70%)	4 (20%)	
T3-T4	30	2 (7%)	16 (53%)	12 (40%)	
N0	22	2 (9%)	15 (68%)	5 (23%)	
N1-N2	24	1 (4%)	12 (50%)	11 (46%)	
Glottic	37	3 (8%)	21 (57%)	13 (35%)	
Supraglottic	13	1 (8%)	9 (69%)	3 (23%)	
Ki67 < 15	25	4 (16%)	13 (52%)	8 (32%)	
Ki67 ≥ 15	25	0	17 (68%)	8 (32%)	
Relapse	27	1 (4%)	11 (41%)	15 (55%)	**<.005**
No relapse	23	3 (13%)	19 (83%)	1 (4%)	

Abbreviation: G, grade.
p-value in bold indicates statistical significance.

Influence of epithelial-to-mesenchymal transition status on relapse and the survival parameters

Among the 27 patients who relapsed, 15 cancers (55%) had a mesenchymal-like immunophenotype, 11 (41%) had a transition phenotype, and only 1 (4%) tumor had an epithelial-like phenotype. The relapse rate was 92% (15/16) in the mesenchymal-like tumors, 25% (1/4) in the epithelial-like tumors, and 40% (11/30) in the tumors in transition. Moreover, the relapse rate was significantly more frequent 54% (7/13) in tumors closer to the mesenchymal than in tumors that have an initial loss of their epithelial characteristics (4/17; 23.5%). This different relapse rate in the different patterns of epithelial-to-mesenchymal transition was statistically significant ($P < .005$; Figure 1B). Moreover, the immunophenotypic pattern of epithelial-to-mesenchymal transition significantly influenced DFS and OS. As shown in Figure 2, the 4 main subgroups identified highlighted 4 different OS and DFS curves. Comparison of survival curves was considered statistically significant (respectively, $P < .047$ and $P < .019$).

The prognostic and predictive role of epithelial-to-mesenchymal transition in the different laryngeal subsites

Despite the aggressive biological behavior of tumors in the supraglottic area, demonstrated by the fact that many cancers

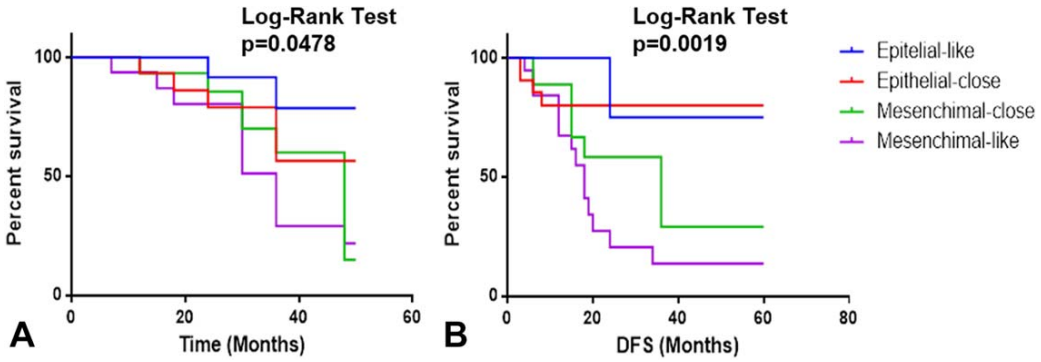

FIGURE 2 Epithelial-to-mesenchymal transition status influence on A, overall survival (OS) and B, disease-free survival [Color figure can be viewed at wileyonlinelibrary.com]

have fallen into the poor-risk group (11/13), we found only 3 supraglottic mesenchymal-like tumors. The tumors were all in the poor-risk category and all of them have relapsed. In addition, all the 5 supraglottic mesenchymal-close tumors have relapsed too. We highlighted that all the 8 supraglottic mesenchymal/mesenchymal-close cancers were: (i) in men; (ii) in the poor-risk group; (iii) associated with predominantly systemic recurrence (lungs 6/8); and (iv) in patients who died during the follow-up.

The relapse rate in supraglottic transition epithelial-close tumors was lower than in the mesenchymal tumors (2/4; 50%) regardless of the risk classes. Unexpectedly, the only supraglottic epithelial-like cancer fell in the poor-risk category and the tumor did not relapse (see Figure 3).

We have found that 81% (13/16) of tumors that exhibit a mesenchymal-like phenotype were localized in the glottic area. As opposed to supraglottic cancer, in the glottic area, we highlighted the mesenchymal acquisition in tumors of all risk classes, although the incidence of mesenchymal-like tumors in the poor-risk group prevailed (9/12). We found mesenchymal-like tumors both in the low-risk group (1 case) and in the intermediate-risk group (3 cases), as shown in Figure 3. We reported that 12 of 13 glottic mesenchymal-like tumors were recurrent, regardless of the risk stratification, including the

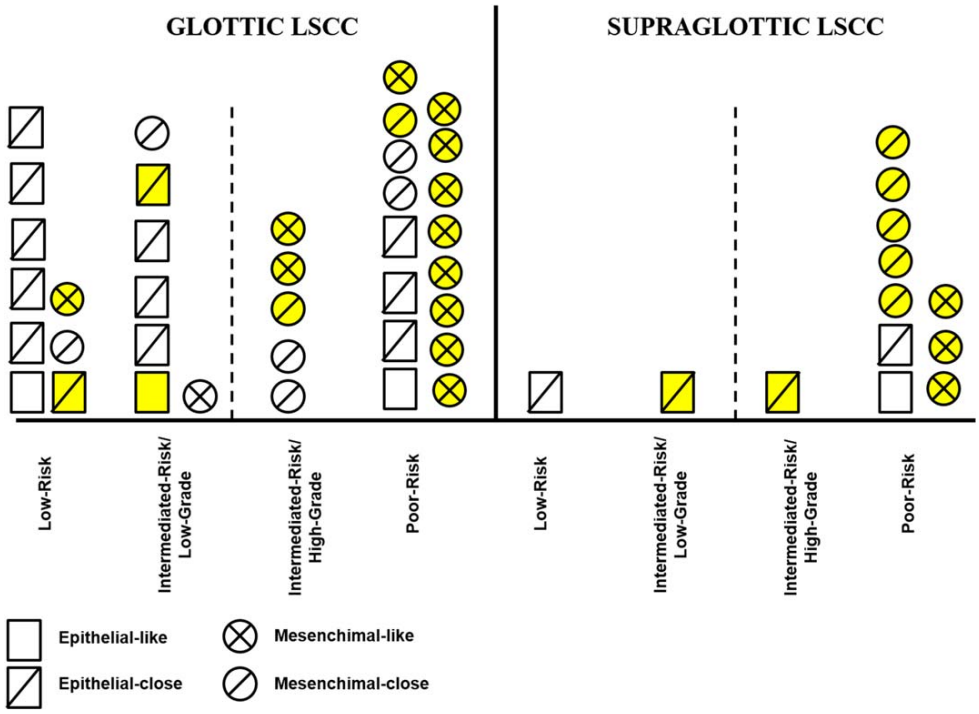

FIGURE 3 The prognostic and predictive role of epithelial-to-mesenchymal transition in the different laryngeal subsites (yellow = relapse) [Color figure can be viewed at wileyonlinelibrary.com]

low-risk mesenchymal glottic cancer. We stress a positive predictive value of relapse in the mesenchymal phenotype acquisition of 94%. Moreover, glottic mesenchymal-like tumors seem to have a more indolent behavior compared with supraglottic tumors showing an equal locoregional and systemic recurrence (6/12; 50%) with 4 of 13 patients (31%) currently alive. In addition, the recurrence rate in glottic tumors classified in transition (4/21) was higher in mesenchymal-close (2/8; 25%) versus the epithelial-close cancers (2/13; 15%), regardless of the risk stratification. Among the 3 glottic tumors that expressed the epithelial-like phenotype, 1 experienced recurrence (1/3; 33%; see Figure 3).

Epithelial-to-mesenchymal transition status in tumors treated without lymph node dissection

In 3 of 4 cases, the glottis was the primary site of the small group of early tumors that underwent neck dissection, and all were in the low-risk group. One tumor was classified as epithelial-like and 3 tumors as in transition epithelial-close. None of the patients treated without lymph node dissection experienced local, nodal, or systemic recurrence. They were all disease free and alive.

4 | DISCUSSION

Although the best quantification of the epithelial-to-mesenchymal transition score is the evaluation of the epithelial-to-mesenchymal transition key markers by polymerase chain reaction, Western Blot, or flow cytometry, scientific investigation of epithelial-to-mesenchymal transition is hampered by the intrinsic complexity of the process as well as by the transient nature of the transition, which moreover is reversible, furthermore it is not activated simultaneously and is limited to some cluster of cancer cells.[30] The IHC evaluation of the expression of specific markers of epithelial-to-mesenchymal transition has allowed us to identify critical intratumoral areas with loss of epithelial markers and acquisition of the mesenchymal ones, that seem to be able to radically modify the biological behavior of the tumor. Our data confirm that the transition process was characterized by a considerable variability, reaching different maturation levels in different cancers and within different areas of the same tumor. Moreover, the evaluation by IHC staining allowed us to highlight changes in cellular localization of the proteins that seem to be extremely significant from a biological point of view, as well as the morphological finding of epithelial cells that have acquired the mesenchymal shape. In the high prevalence of laryngeal SCCs that we analyzed (46/50), there was an evident and diffuse alteration of epithelial-to-mesenchymal transition markers when compared to surrounding healthy tissue, all indicating a tendency to a rife process of epithelial-to-mesenchymal transition,[31] probably due to a tumor microenvironment especially promoting epithelial-to-mesenchymal transition in laryngeal SCC. Indeed, the extracellular matrix, through different cytokines[32] and growth factors,[33] is able to induce the epigenetic silencing of E-cadherin expression[34] as well as hypoxia[35] and tobacco use.[36] Our data evidenced an impressive predominance of patients who smoked and an alarming number of patients who continued to smoke even after the cancer diagnosis, confirming the "total" evidence that chronic inflammation, hypoxia, and microenvironment modification induced by smoke, contribute in carcinogenesis and tumor progression of laryngeal SCC. Therefore, our findings support the need to implement an education program to remove this bad habit in Italy. In addition, the ubiquity of the caveolin-1 qualitative and quantitative alterations support caveolin-1 as a milestone in the epithelial-to-mesenchymal transition trigger by mediating the growth factor-stimulated disassembly of epithelial cell-cell adhesion complex[23] suggesting indirectly a tumor microenvironment role in epithelial-to-mesenchymal transition control. Our results confirm that the panel of markers correlate with pathologic adverse features of the tumor in agreement with several studies; an aberrant cytoplasm accumulation of the β-catenin was predominantly found in locally advanced tumors,[37,38] the cadherin switch was demonstrated exclusively in poorly differentiated cancers,[39] and the vimentin expression has been significantly associated to the tumor grade.[40] Our results confirm that the epithelial-to-mesenchymal transition markers correlate with clinical outcome; the cadherin switch was demonstrated exclusively in relapsed patients[39] as well as the vimentin expression.[40] E-cadherin, α-catenin, and γ-catenin abnormal cytoplasmic storage were associated with decreased survival rates and a shorter DFS.[41,42] In addition, our data seem to suggest that the canonical Wnt pathway may be inactivated in several laryngeal SCCs. Indeed, β-catenin nuclear staining was highlighted in only 1 glottic tumor, in agreement with the data from the study by Goulioumis et al,[43] allowing us to suggest that alternative signals to the Wnt/β-catenin pathway may be able to negatively influence survival in the late stage of epithelial-to-mesenchymal transition,[44] because both the DFS and OS were more adversely affected in the early stages of the β-catenin dysregulation with respect to the late ones, confirming the Galera-Ruiz et al[12] assumptions.

We especially point out that the full panel of studied markers was overall coherent in the information provided, and that it was more effective in the tumor behavior prediction when compared to the information provided by every single marker of epithelial-to-mesenchymal transition. Only 4 tumors (8%) in our series retained an immunophenotype

similar to the normal epithelium. In others (32%), we described the immunophenotypic completion of epithelial-to-mesenchymal transition associated with the appearance of mesenchymal traits. On the contrary, we found in most of the cases (60%) that some cells were in the phase of the progressive loss of epithelial markers without having yet acquired the mesenchymal ones. In this predominant group of tumors in transition, we identified 2 more subgroups, not preplanned, on the basis of their closeness to be epithelial or mesenchymal. The stepwise process of the transitional status significantly correlated with a transition to a more aggressive undifferentiated phenotype with poorer prognosis and was highly predictive of the disease outcome in laryngeal SCC. The relapse rate within the mesenchymal-like tumors was higher (92%) in respect to every other prognostic factor considered. The recurrence rate was high (53%) in mesenchymal-close cancers also. The lowest recurrence rate has been found in epithelial-like phenotype tumors (20%) and in epithelial-close cancers (23.5%). The different relapse rate among the progressive stage of epithelial-to-mesenchymal transition was statistically significant. In addition, the OS was significantly different in the 4 different patterns of epithelial-to-mesenchymal transition expressions and 4 different survival curves were highlighted by the degree of epithelial-to-mesenchymal transition, overcoming the limited risk prediction based on standard clinical and pathological factors. In effect, only a very small group of glottic and supraglottic tumors (5; 10%) bearing an epithelial immunophenotype (like and close) has experienced disease relapse, probably related to epithelial-to-mesenchymal transition alternative pathways carriers in tumor progression and metastatic potential acquisition. A significant biological difference was stressed in glottic versus supraglottic cancers. Our data evidenced an aggressive biological behavior of supraglottic cancers as reported,[11] but we underline that a poor outcome was present in all patients with supraglottic mesenchymal-like and mesenchymal-close cancers. However, supraglottic epithelial tumors showed a lower aggressive profile, confirming the strongly negative prognostic role of epithelial-to-mesenchymal transition in supraglottic cancers. Paradoxically, tumors with mesenchymal-like phenotypes were more frequent among the usually less aggressive glottic tumors and distributed in all classes of risk, even in the good-risk class. The acquisition of a mesenchymal phenotype in glottic cancers significantly correlates with relapse, allowing us to select the relapse candidates. The high positive predictive value (94%) of the epithelial-to-mesenchymal transition status allows us to consider epithelial-to-mesenchymal transition as a negative prognostic factor and an independent predictor of relapse in glottic cancers.

In addition, we evaluated the small group of early tumors that did not undergo the neck dissection at the time of surgery. Our series is not appropriate to recommend elective lymph node dissection versus therapeutic lymphadenectomy in case of nodal relapse, but we underline how early detection of small tumors was associated with low clinical risk and high curability rate and was able to prevent the activation of the epithelial-to-mesenchymal transition program with a relevant prognostic impact.

In conclusion, our results highlight that the gradual stepwise process of epithelial-to-mesenchymal transition is mainly evident in undifferentiated laryngeal SCC and in relapsing patients and that it negatively influenced DFS and OS. The association between epithelial-to-mesenchymal transition status and adverse prognosis may be important for changing clinical practices, even considering the small number of patients and the limitation of data emerging from retrospective collections.

Therefore, we propose to consider the acquisition of mesenchymal markers as an additional adverse feature by which to get adjuvant treatment decisions for patients with laryngeal SCC.

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SUPPORTING INFORMATION

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