

Biomarkers predicting malignant progression of laryngeal epithelial precursor lesions: a systematic review

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Abstract Some laryngeal epithelial precursor lesions progress to invasive carcinoma and others do not. Routine light microscopic classification has limited value in predicting the evolution of these lesions. This article reviews the experience to date with the use of molecular markers for the prognostic evaluation of laryngeal epithelial precursor lesions. We conducted a thorough review of the published literature to identify those studies using biomarkers to predict malignant progression of laryngeal epithelial precursor lesions. Of the 336 studies identified in this systematic search, 15 met the inclusion criteria and form the basis of this review. Limited studies suggest that

certain biomarkers are potentially reliable predictors of malignant progression including various regulators of cell adhesion and invasion (e.g. FAK, cortactin, osteopontin, and CD44v6) and proliferation-associated markers such as TGF- β R2 and Kv3.4. The predictive value of these markers, however, has yet to be confirmed in large-scale prospective studies. Although the cell cycle-related proteins are the most frequently studied markers, none have been consistently reliable across multiple studies. The absence of standardization in methodologies, test interpretation, and other parameters may contribute to study inconsistencies. Various biomarkers have proved to have

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potential prognostic value and could be clinically relevant. The utility and prognostic power of these biomarkers should be confirmed in large, well-designed, standardized prospective studies.

Keywords Larynx · Epithelial precursor lesions · Dysplasia · Carcinoma in situ · Biomarkers

Introduction

As in other epithelial malignancies, the development of laryngeal squamous cell carcinoma is a multistep process driven by an accumulation of genetic alterations in the surface epithelium [1–3]. The accumulation of these genetic alterations, in turn, induces a stepwise progression of morphologic changes that is referred to as laryngeal squamous dysplasia, laryngeal intraepithelial lesion, laryngeal intraepithelial neoplasia, or laryngeal epithelial precursor lesion.

Various classification systems have been crafted in an attempt at describing and quantitating the histologic features of laryngeal epithelial precursor lesions [4]. In the most recent edition of the World Health Organization (WHO) Classification of laryngeal epithelial precursor lesions, three grading systems are recognized: the dysplasia system, the Squamous Intraepithelial Neoplasia (SIN) system, and the Ljubljana classification [5, 6]. Of these, the most frequently used grading system is the dysplasia system [6], but the value of this and other grading systems has been severely hampered by two factors. First, histological grading of laryngeal epithelial precursor lesions is not highly reproducible. Both inter-observer and intra-observer reproducibility is poor, usually associated with a low non-weighted or weighted kappa statistic (κ value) [7–9]. Second, the presence and degree of dysplasia do not reliably predict biological behavior. Some lesions with only mildly dysplastic changes progress to invasive carcinomas; and conversely, the majority of severely dysplastic lesions never progress to invasive carcinomas. The histologic irreproducibility and the biological unpredictability of grading schemes has limited the usefulness of morphology alone as the primary means of directing therapeutic strategies [5, 10–12].

Consequently, novel markers are needed that can be reproducibly analyzed from one laboratory to the next, and that can reliably identify those precursor lesions that are most likely to progress into invasive carcinomas. A comprehensive identification and characterization of the molecular alterations associated with the development of laryngeal squamous cell carcinoma might provide valuable diagnostic tools for cancer risk assessment of precursor lesions, and could ultimately guide the construction of a more unified classification of laryngeal epithelial precursor lesions than those presently available.

The present article reviews the evidence and conclusions related to the use of molecular markers for the prognostic evaluation of laryngeal epithelial precursor lesions.

Materials and methods

A literature search was performed using PubMed on the 10th June 2011 for English language publications between the years 1980–2011 using the following search criteria in the title or abstract: ‘larynx’ or ‘laryngeal’, coupled with ‘dysplasia’ or ‘pre-malignant’, and each of the four combinations was then combined with the terms ‘markers’ and ‘prognosis’. When there was any statement in the abstract on follow-up data and outcomes of the laryngeal pre-malignant lesions, the full text article was searched and all review articles were also checked in full. References from any full text articles were cross-checked to ensure inclusion in this review if appropriate.

Only publications that included patients with a histological diagnosis of laryngeal dysplasia using a recognized classification grading system and that analyzed biomarkers in relation to histologic and clinical data were considered. In the case of multiple series from the same institution, the most up to date or largest series was selected.

Results

According to our search criteria, 336 papers were initially identified. After sorting and removal of duplicates, all the remaining abstracts were reviewed, and 23 papers were retrieved and reviewed in detail. At this stage, a total of 8 studies were further excluded, mainly due to cross-sectional design, lack of follow-up data or histological diagnosis; or if the studies included patients with cancer or dysplastic lesions in the presence of cancer or dysplasias from sites other than the larynx.

Of the 15 selected articles, ten were retrospective studies (13–22; Table 1), four were case-control studies (23–26; Table 2), and only one was a prospective study (27; Table 3). It is worth noting that the prospective study only included moderate ($n = 14$) and severe ($n = 13$) dysplasias, and that patients received an experimental treatment (interferon- α , 13-*cis*-retinoic acid and α -tocopherol) that could influence the natural progression of the lesions. Nevertheless, the progression rate was 37% (10/27), close to the expected cancer incidence in this setting.

All the studies employed immunohistochemistry to determine the expression of one or more biomarkers in paraffin-embedded biopsies of the lesions. Additionally, three studies also analyzed various different genetic markers: cellular DNA content (DNA index or ploidy)

Table 1 Results of retrospective studies on biomarkers in laryngeal epithelial precursor lesions

Study	Number of patients	Histology	Biomarker (s)	Biological role	Association of histology with prognosis	Progression to carcinoma (%)	Progression positive cases (%)	Progression negative cases (%)	Comments
Munck-Wikland et al. [13]	38	38 sev dys	PCNA P53 Aneuploidy	Proliferation Cell cycle	NA	9/38 (24%)	PCNA: 26% P53: 36% High DNA aberration:33%	PCNA: 18% P53: 18% Low DNA aberration: 0%	PCNA, p53, and aneuploidy related with progression
Zhao et al. [14]	44	14 hyperplasia 10 mild dys 11 mod dys 9 sev dys	PCNA	Proliferation	Yes	7/44 (16%)	37%	0%	Higher PCNA staining significantly associated with transformation
Pignataro et al. [15]	32	14 mild dys 18 mod dys	P53 Cyclin D1	Apoptosis Cell cycle	No	10/32 (31%)	P53: 20% Cyclin D1: NA	P53: 33% Cyclin D1: 31%	Higher Ki67 expression significantly associated with transformation.
Krecicki et al. [16]	45	20 mild dys 18 mod dys 7 sev dys	MDM-2 Ki67 PCNA P53 BCL-2	Cell cycle Proliferation Proliferation Cell cycle Apoptosis	No	10/45 (22%)	MDM2: 0% Ki67: 82% NA	MDM2: 33% Ki67: 9% NA	None of the cases expressed cyclin D1
Jeannon et al. [17]	114	23 mild dys 65 mod dys 26 sev dys	P21 P27 P53 Ki67	Cell cycle Cell cycle Cell cycle Proliferation	Yes	28/114 (24%)	P21: 24% P27: 17% P53: 18% 29%	P21:25% P27:38% P53:37% 20%	No prognostic significance
Mirza et al. [18]	69	14 mild dys 38 mod dys	Ki67	Proliferation	Yes	20/80 (25%)	29%	20%	No prognostic significance
Staibano et al. [19]	82	17 sev dys 21 mild dys 8 mod dys 53 sev dys	Osteopontin (OPN) CD44v6	Cell adhesion Cell adhesion	NA	37/82 (45%)	OPN: 33% CD44v6: 38%	OPN: 91% DFS CD44v6: 94% DFS	OPN and CD44v6 expression in full-thickness lesion associated with transformation
Rodrigo et al. [20]	84	14 mild dys 26 mod dys 44 sev dys	Podoplanin	Stem cell marker	No	33/84 (39%)	51%	14/47 (30%)	Higher podoplanin expression associated with transformation

Table 1 continued

Study	Number of patients	Histology	Biomarker (s)	Biological role	Association of histology with prognosis	Progression to carcinoma (%)	Progression to positive cases (%)	Progression to negative cases (%)	Comments
Menendez et al. [21]	67	7 mild dys 26 mod dys 34 sev dys	KV3.4	Potassium channel	No	24/67 (36%)	17/35 (48%)	7/32 (22%)	KV3.4 expression associated with transformation
Rodrigo et al. [22]	82	12 mild dys 26 mod dys 44 sev dys	Cortactin (CTTN) FAK CCND1 (Expression and amplification)	Cell motility Cell adhesion Cell cycle	No	28/82 (34%)	Expression: CTTN: 12/21 (57%) FAK: 14/23 (61%) CCND1: 16/41 (39%) Amplification: CTTN: 6/10 (60%) FAK: 10/26 (38%) CCND1: 9/13 (69%)	Expression: CTTN: 16/61 (26%) FAK: 14/59 (24%) CCND1: 12/40 (30%) Amplification: CTTN: 22/72 (30%) FAK: 18/56 (32%) CCND1: 19/67 (28%)	FAK and/or CTTN expression, and CTTN or CCND1 amplification associated with transformation

Mod Moderate, *Sev* severe, *Dys* dysplasia, *NA* not available, *DFS* disease-free survival

Table 2 Results of case-control studies on biomarkers in laryngeal epithelial precursor lesions

Study	Number of patients	Histology	Biomarker (s)	Biological role	Positive expression in cases	Positive expression in controls	Significance
Gallo et al. [23]	14 cases	Cases and controls:	P53	Cell cycle	P53: 66%	P53: 60%	Higher Ki67 expression in non-transformed controls
	14 controls	7 hyperplasia 4 mild dys 2 mod dys 1 sev dys	Ki67	Proliferation	Ki67: 17.4 (Labeling index)	Ki67: 37.7 (labeling index)	
Uhlman et al. [24]	18 cases (63 samples)	89 hyperplasia	EGFR	Proliferation	EGFR: 14/18 (78%)	EGFR: 6/20 (30%)	Positive expression of EGFR, P53, and Cyclin D1 significantly higher in cases that progressed to carcinoma
	20 controls (71 samples)	29 mild dys	P53	Cell cycle	P53:10/18 (55%)	P53:0/20	
		25 mod dys	Cyclin D1	Cell cycle	Cyclin D1:11/18 (61%)	Cyclin D1:1/20 (5%)	
		27 sev dys (Total biopsies)					
Gallo et al. [25]	16 cases	Cases:	P16	Cell cycle	P16: 60%	P16: 46%	Simultaneous positive expression of P16 and P53 more frequent in transformed cases
	32 controls	8 hyperplasia 4 mild dys 2 mod dys 2 sev dys	P53	Cell cycle	P53: 33%	P53: 60%	
Franchi et al. [26]	15 cases 30 controls	7 hyperplasia	TGFβ-RII	Proliferation inhibitor	Loss of TGFβ-RII: 11/15 (73%)	Loss of TGFβ-RII: 5/30 (17%)	Loss of TGFβ-RII significantly more frequent in cases
		4 mild dys					
		2 mod dys					
		2 sev dys					
		Controls:					
		14 hyperplasia					
		8 mild dys					
		4 mod dys					
		2 sev dys					

Mod Moderate, *Sev* severe, *Dys* dysplasia, *NA* not available

Table 3 Results of prospective studies on biomarkers in laryngeal squamous intraepithelial lesions

Study	Number of patients	Histology	Biomarker	Biological Role	Association of histology with prognosis	Progression to carcinoma (%)	Progression Positive cases (%)	Progression negative cases (%)	Significance
Papadimitrakopoulou et al. [27]	27	14 mod dys	CCND1/cyclin D1	Cell cycle	No	10/37 (27%)	AG/AA genotype: 9/15 (60%) Cyclin D1 expression: 7/9 (78%)	GG genotype: 1/9 (11%) Cyclin D1 expression: 3/15 (20%)	AG,AA genotype and Cyclin D1 positive expression associated with progression to carcinoma

Mod Moderate, Sev severe, Dys dysplasia

[13], *CCND1*, *CTTN* and *FAK* gene amplification [22], and cyclin D1 genotype [27].

The longitudinal studies included only dysplastic lesions with the exception of the study by Zhao et al. [14] that also included hyperplasias, whereas all case-control studies included both patients with hyperplasias and dysplasias.

In general the most frequently studied biomarkers are proteins associated with proliferation and cell cycle control (PCNA, Ki67, p53, p16, p21, p27, and cyclin D1) and only a few studies have investigated molecules involved in cell adhesion and invasion (Osteopontin, CD44, Focal Adhesion Kinase [FAK], cortactin). Since there are no universally accepted scoring systems, most studies differ in the method used to quantify biomarker staining thus precluding a pooling of the results available for each biomarker.

The following sections summarize all published data for the different biomarkers, classified by their biological role.

Proliferation markers

One of the main features of malignancy is the ability of tumor cells to undergo unregulated proliferation. Detection of proliferative activity of laryngeal epithelial precursor lesions has been employed as a prognostic marker in several studies, mainly with immunohistochemical analysis of proliferating cell nuclear (PCNA) and Ki67 antigens [13–16, 18, 23]. However, no study analyzed both proteins simultaneously.

Ki67 is a cell cycle-associated protein and a marker of cell proliferation [28]. Contradictory results have been obtained for this marker (Tables 1, 2): one retrospective study reported a higher rate of malignant progression in cases with elevated Ki67 expression [15], whereas another reported no prognostic significance for this marker [18], and a case-control study showed a higher Ki67 expression in non-progressing controls [23].

PCNA is a 36 kDa, acidic, non-histone, nuclear protein whose expression is associated with the late G1 and S phases of the cell cycle [13]. Three studies analyzed the prognostic significance of PCNA expression (Table 1) [13, 14, 16]. All of them consistently showed higher levels of PCNA positivity in the lesions that progressed to invasive cancer, although this association did not reach statistical significance in one study [16]. Furthermore, PCNA expression significantly correlated with the histological grading in the two studies [14, 16].

Cell cycle regulators

The role of proteins that control the cell cycle has been extensively studied in both the initiation and progression of laryngeal squamous cell carcinoma. In consequence, these

proteins have been investigated as markers of malignant progression in laryngeal dysplasia in several studies (Tables 1, 2, 3).

TP53 is a well-established tumor suppressor gene in head and neck squamous cell carcinoma (HNSCC), as well as in other cancers. Somatic mutations in *TP53* are found in 60–80% of HNSCC cases [3]. When functioning properly, p53 is activated by hypoxia or DNA damage, leading to cell cycle arrest, attempted DNA repair, and, if DNA repair is ineffective, apoptosis [29]. Most studies investigate the inactivation of the *TP53* gene by immunohistochemical analysis of p53 protein levels, in light on the fact that the half-life of the wild-type protein is too short to permit its detection, while the mutant protein is stable. The p53 protein expression as a biomarker of laryngeal cancer risk has been assessed in four retrospective studies [13, 15–17] and three case-control studies [23–25]. These studies used different scoring systems for p53 staining, which prevents a direct comparison of the results. Of the seven studies, only two demonstrated a prognostic significance for p53 expression: a retrospective [13] and a case-control study [24] described an association of p53 expression with progression to malignancy.

In addition to p53, the expression of MDM-2 protein, which is capable of binding and inactivating p53, has also been assessed in one study [15]. However, MDM-2 expression was not associated with progression to malignancy. Contrary to this, cases with negative MDM-2 expression exhibited a higher progression rate.

Cyclin D1 is the second most frequently studied cell cycle-related protein [15, 22, 24, 27]. Its function is to inactivate Rb binding capacity for E2F via phosphorylation, together with cyclin-dependent kinase 4 and 6 and facilitating progression from the G1 phase to the S phase [30]. It is encoded by the oncogene *CCND1*, located on the chromosomal region 11q13, a locus frequently amplified in HNSCC [31]. Elevated expression of cyclin D1 has been associated with malignant progression in a prospective [27] and a case-control [24] study. In contrast, a retrospective study failed to show this association [22], and in another study cyclin D1-positive lesions were not detected [15].

Cyclin D1 gene status has also been investigated. Papadimitrakopoulou et al. [27] examined *CCND1* G/A870 single nucleotide polymorphism and found a correlation between *CCND1* AA and AG genotype and increased cancer risk, compared to *CCND1* GG genotype. The CD1 G/A870 polymorphism is functionally important because it occurs at a splicing donor site; the A870 allele impairs the normal splicing and enhances the production of the splice variant transcript cyclin D1b, which seems to hold oncogenic properties [27].

CCND1 gene amplification was also evaluated in one study [22], and was significantly associated with malignant

progression. In this study, all of the cases with *CCND1* amplification showed cyclin D1 overexpression; however, only *CCND1* gene amplification and not protein overexpression was significantly correlated with progression to malignancy. Since *CCND1* gene frequently was co-amplified with other genes located within the 11q13 amplicon, such as *CTTN* (actin-binding protein cortactin), the prognostic significance attributed to *CCND1* amplification could be actually due to its co-amplification with another gene mapping in this chromosomal region [22].

As part of the family of cell cycle-related proteins, the cyclin-dependent kinase inhibitors (CDKI) p16, p21 and p27 are important in mediating the stepwise progression from the resting G0 phase into G1 in mitosis [30]. The expression of these proteins has been reported in two articles (Tables 1, 2). More precisely, p16 (p16INK4A) was analyzed in a case-control study where reduction of p16 expression tended to be more frequent in cases than in controls [25]. p21 and p27 expression was assessed in a retrospective study, but none of these proteins were associated with malignant progression [17].

Apoptosis regulatory proteins

Tissue growth depends on a balance between the rates of both cell proliferation and cell death. The Bcl-2 family genes have a key role in controlling programmed cell death, or apoptosis, with some proteins in the family promoting and others (such as Bcl-2) inhibiting apoptosis [32]. A retrospective case series evaluated Bcl-2 expression (Table 1), but the authors did not find any diagnostic or predictive value for this protein [16].

Transmembrane receptors

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine-kinase, which influences cell division, migration, adhesion, differentiation and apoptosis. EGFR is overexpressed in many cases of HNSCC [3]. EGFR has been investigated in a case-control study (Table 2) and its expression was found to be significantly higher in cases that progressed to malignancy [24].

Another important, but inhibitory, growth factor pathway associated with HNSCC is the transforming growth factor- β (TGF- β) pathway. TGF- β 1 signals through specific receptor complexes formed by two different transmembrane proteins that belong to the serine-threonine kinase receptor family, the TGF- β type I and II receptors (TGF- β RI and TGF- β RII) [3]. Various studies have demonstrated that TGF- β RII expression is frequently lost in HNSCC and this may result in resistance to TGF- β mediated growth suppression [33]. The expression of TGF- β RII was analyzed in a case-control study (Table 2)

and the loss of TGF- β RII expression was significantly more frequent in cases that progressed to carcinoma than in controls [26], suggesting that the loss of TGF- β mediated growth inhibition could facilitate the progression of laryngeal precancerous lesions to invasive carcinoma.

Cell adhesion molecules

Both cell-cell interactions and cell-stroma interactions play an important role during carcinogenesis. Alterations in proteins implicated in the regulation of these interactions are frequent in HNSCC and have also been explored as markers of cancer risk in laryngeal epithelial precursor lesions (Table 1).

Osteopontin (OPN) is a 33-kDa highly acidic calcium-binding glycosylated phosphoprotein that can function both as a cell adhesion molecule and as a cytokine. It binds to the integrin cell surface receptors α v or β 1-containing integrins as well as exon v6-containing CD44 isoforms (CD44v6), thereby supporting proliferation, chemotaxis, attachment, and migration of many cell types [34]. CD44 is a cell surface glycoprotein that is involved in regulating cell-cell and cell-matrix interactions, migration, and tumor growth and progression. It is considered a marker of stemness [35]. In addition to CD44, several tissues also express larger CD44 isoforms, encoded by alternatively spliced transcripts called CD44 variants (CD44v). CD44v6 is necessary for OPN binding [36]. The OPN/CD44v6 axis has been assessed in one retrospective study [19]. This study showed that the disease-free survival rate in patients affected by laryngeal dysplasia negatively correlated with intense OPN staining and full-thickness CD44v6 positivity [19]. These results suggest that the up-regulation of the OPN/CD44v6 axis is an early event during the progression of laryngeal dysplasia and could be used as predictive marker in these lesions.

A key factor involved in the regulation of cell-extracellular matrix interactions is the focal adhesion kinase (FAK), an intracellular tyrosine-kinase protein that localizes to cellular focal contact sites. Evidence also suggests that FAK is a key component of growth factor receptor signaling pathways such as those activated by platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) [37]. FAK overexpression has been observed in both HNSCC and laryngeal dysplasias [38]. FAK protein expression and gene amplification has been determined in a retrospective study [22]. The results showed that FAK overexpression correlated significantly with increased cancer risk and was an independent predictor of laryngeal cancer development in multivariate analysis. *FAK* gene amplification, however, did not exhibit any prognostic significance.

The same study analyzed also the expression and gene status of the actin-binding protein cortactin (encoded by the

CTTN gene, located at 11q13) that regulates membrane dynamics, actin network assembly, cell-cell adhesion, invadopodia formation and matrix degradation, thereby promoting cell motility and invasion [39]. *CTTN* gene amplification and protein overexpression were found to correlate with poor prognosis and reduced patient survival in HNSCC [31]. Furthermore, the above-mentioned study [22] also showed that both *CTTN* gene amplification and protein overexpression correlated significantly with progression to malignancy. Interestingly, the combination of cortactin and FAK evaluation was statistically significantly superior in terms of prognostic value and also sensitivity, therefore their use as complementary markers was recommended.

Other biomarkers

Podoplanin is a 38 kDa mucin-type transmembrane glycoprotein whose physiological function is still unknown. Despite this, podoplanin expression (D2-40) has been widely used as marker of lymphatic endothelial cells and lymphangiogenesis due to its specific expression by lymphatic but not by blood vessel endothelium [40]. In addition, podoplanin expression has been found to be up-regulated in a number of human cancers, including HNSCC [20, 41] and it has also been described as a marker of malignant progression and poor prognosis in oral cancer [41, 42].

A retrospective study explored the prognostic significance of podoplanin in laryngeal dysplasias (Table 1), and the results showed a higher risk of malignant progression in laryngeal dysplasias with elevated podoplanin expression [20], similar to the observations in oral premalignancies, although in the larynx the differences did not reach statistical significance.

Kv3.4 is an A-type potassium (K⁺) channel (also called KCNC4, shaw III or Raw3) that belongs to the shaker-related Kv subfamily. Numerous studies have implicated voltage-gated K⁺ (Kv) channels in the proliferation of a variety of malignant cells and also in tumorigenesis [43]. Aberrant expression of several Kv channels has been reported in multiple human cancers [43]. The expression of a Kv3.4 channel subunit was determined in a set of 67 laryngeal dysplasias [21]. In this retrospective study, patients with Kv3.4-positive lesions had a significantly higher laryngeal cancer incidence than those with negative lesions (Table 1).

Discussion

Laryngeal squamous cell carcinoma usually develops in a multistep process: normal mucosa—dysplasia—carcinoma

in situ—invasive carcinoma [1–3]. Dysplasia is characterized by increased cell growth, cellular atypia (nuclear and nucleolar abnormalities, an altered nuclear/cytoplasmic ratio, and an altered pattern of cytoplasmic differentiation), and architectural alteration of the epithelium [5]. In the most frequently used classification system (the dysplasia system), the dysplastic changes are graded as mild (dysplasia limited to the basal third of the epithelium, with few mitoses), moderate (dysplasia involving the lower two-thirds of the epithelium, moderate nuclear changes, prominent nucleoli, mitoses in the parabasal, and intermediate layers), and severe (dysplasia involving more than two-thirds of the epithelial thickness, marked nuclear pleomorphism and hyperchromasia, prominent nucleoli, cell crowding, and atypical mitoses) [6]. Carcinoma in situ is a distinct category not included in the dysplasia system. However, many pathologists collapse severe dysplasia and carcinoma in situ into a single category [4, 11]. In this way, Friedmann and Ferlito have used the term laryngeal intraepithelial neoplasia (LIN) to include both dysplasia and carcinoma in situ. In their scheme, LIN I is the equivalent of mild dysplasia; LIN II, moderate dysplasia; and LIN III, severe dysplasia and carcinoma in situ [44].

Laryngeal dysplasias, as a collective group, progress to invasive squamous cell carcinoma at a reported frequency that ranges between 11 and 32% [4, 11, 12]. Ideally, a system for grading the severity of dysplasia should be able to separate those precursor lesions with a high likelihood of malignant progression from those lesions with a low likelihood of progression. Although most studies have generally shown an increasing rate of malignant progression with increasing grades of dysplasia [11], the incidence of progression for each level of dysplasia varies widely among different studies [4]. Transformation rates ranged from 0 to 11.5% for mild dysplasia; from 4 to 24% for moderate dysplasia; from 9.3 to 57% for severe dysplasia. The most prominent discrepancy comes from the latter group, probably because severe dysplasia and carcinoma in situ are often combined together into a single category. In addition, it is difficult to compare data from follow-up studies when different morphological criteria are being used for histological grading of laryngeal epithelial precursor lesions, and it is unlikely that the different classification systems will be unified in the near future. Furthermore, various studies have shown that histology did not have a significant predictive role in assessing laryngeal cancer risk [15, 16, 20, 22, 27]. Under these circumstances, molecular biology emerges as an excellent potential alternative that could be useful to both provide more objective and reliable markers for the identification of high-risk lesions and also to improve the prognostic evaluation of laryngeal epithelial precursor lesions beyond current clinical and histological criteria. Although a genetic progression model for head and neck

cancer has been proposed [1, 3], the specific molecular events that promote the evolution of dysplastic lesions to invasive carcinoma are still unknown. To date, no marker or panel of markers has been identified as a reliable predictor of malignant progression in laryngeal epithelial precursor lesions. This article has reviewed and compiled all information currently available on the expression of biomarkers that proved to have potential prognostic value in the studies published to date that could be clinically relevant. Among them were various regulators of cell adhesion and invasion such as FAK, cortactin, osteopontin and CD44v6 [19, 22], and proliferation-associated markers such as TGF- β RII and Kv3.4 [21, 26]. Of note, none of the cell cycle-related proteins, actually the markers most frequently studied, has shown potential as reliable prognostic markers [16, 17, 22–25, 29, 30].

It is also noteworthy that protein expression markers that mirror the changes observed microscopically are not applicable as prognostic markers. This is the case with proliferative markers such as PCNA and Ki67 antigens, markers that can only be used as adjuncts to light microscopy for a more objective and reliable histological grading of laryngeal epithelial precursor lesions but not as independent prognostic markers. Only a marker that can be identified in epithelial precursor lesions whose presence predicts progression would offer any meaningful clinical application. Examples of markers that accomplish this as determined by initial retrospective review studies are cortactin, FAK, osteopontin and CD44v6. It seems reasonable to recommend combining the histopathological diagnosis of laryngeal epithelial precursor lesions, the gold standard in clinical practice for cancer risk assessment and decision-making, with immunohistochemical analysis of protein expression as an adjunct to routine histopathological diagnosis. However, any protein expression analysis requires validation and results may show large inter- and intra-observer variability. Additionally, routine implementation of promising biomarkers for cancer risk assessment will require further confirmation in large prospective, well-standardized studies.

The results of our review also show the lack of good quality studies with adequate longitudinal design, especially prospective studies, evaluating the role of biomarkers in laryngeal epithelial precursor lesions. This problem has also been highlighted in another recent systematic review on this topic [45]. In that review, the authors only included 9 articles. Six of these articles were also included in our review [13, 15–18, 23], and three were excluded because they also included invasive carcinomas in their data analysis. The authors made a meta-analysis with p53, the only biomarker with sufficient data to attempt to apply this type of analysis. However, the five studies that analyzed this

marker used different cut-offs to determine the percentage of p53 positive staining, which emphasizes the need for cautionary remarks on the value of immunohistochemistry as made above. Although a sensitivity analysis was performed for each of the cut-offs used, p53 was not found to significantly predict progression [45]. Only two of the seven studies included in our review that analyzed p53 showed a prognostic significance for this marker. We did not perform a meta-analysis of the biomarkers included in more than one study because of the variability in design, staining technique, marker scoring, and histological grading of the lesions in the different studies. With this heterogeneity in the design and reported data from the different studies, it will continue to be a challenge to draw meaningful results and conclusions of meta-analysis using these data. Therefore, it is imperative that the design and results of tumor marker studies are reported in a comprehensive manner so that they can be evaluated critically. There is now a consensus on how cancer biomarker studies should be reported, as laid out in the REMARK (REporting recommendations for tumor MARKer prognostic studies) guidelines [46]. The use of the REMARK guidelines will allow tumor marker studies to be evaluated critically as well as interpreted appropriately, and the journals' editors must recommend their use.

As highlighted in this review, reported studies of a marker or related markers in laryngeal epithelial precursor lesions (for example proliferation markers or cell cycle markers) yield inconsistent conclusions or stand in direct contradiction. A variety of problems could explain these discrepancies, such as general methodological differences, poor study design, assays that are not standardized or lack reproducibility, and inappropriate or misleading statistical analyses that are often based on sample sizes too small to draw meaningful conclusions [46].

In conclusion, various biomarkers have suggested in preliminary investigations that they might ultimately prove to have prognostic value and could be clinically relevant. FAK and cortactin in particular have shown the strongest association with laryngeal cancer risk. It is mandatory, however, to confirm the utility and prognostic power of these biomarkers in large, well-designed, prospective studies before their implementation in routine clinical practice.

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