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BRIEF REPORT

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p16^{INK4a} and pRb expression in laryngeal squamous cell carcinoma with and without infection by EBV or different genotypes of HPV: a retrospective study

Jose Manuel Vazquez-Guillen[1], Gerardo C. Palacios-Saucedo[2*], Alondra Yamileth Alanis-Valdez[1], Andrea Huerta-Escobedo[1], Angel Zavala-Pompa[3], Lydia Guadalupe Rivera-Morales[1], Ana Carolina Martinez-Torres[1], Vianey Gonzalez-Villasana[4], Julio Cesar Serna-Hernandez[2], Silvia Judith Hernandez-Martinez[2], Edmundo Erbey Castelan-Maldonado[2], Martha Socorro Montalvo-Bañuelos[5], Cesar Alejandro Alonso-Tellez[2], Ethel Corinthia Sanchez-Fresno[2], Reyes S. Tamez-Guerra[1] and Cristina Rodriguez-Padilla[1]

Abstract

Background Laryngeal squamous cell carcinoma (LSCC) represents one of the principal tumors of the head and neck. Human papillomavirus (HPV) and Epstein–Barr virus (EBV) are considered risk factors for the development and the clinical prognosis of LSCC. High levels of p16^{INK4a} are suggested as a surrogate marker of HPV or EBV infection in some head and neck tumors but in LSCC is still controversial. Furthermore, pRb expression may be considered an additional biomarker but it has not been clearly defined. This work aimed to compare the expression of pRb and p16^{INK4a} as possible biomarkers in tumor tissues with and without infection by EBV or different genotypes of HPV from patients with LSCC.

Methods Tumor samples from 103 patients with LSCC were previously investigated for the presence and genotypes of HPV using the INNO-LiPA line probe assay and for the infection of EBV by qPCR. p16 ^{INK4a} and pRb expression was assessed by immunohistochemistry.

Results Of the 103 tumor samples, expression of p16^{INK4a} was positive in 55 (53.4%) and of this, 32 (56.1%) were positive for HPV whereas 11 (39.3%) were EBV positive but both without a significantly difference ($p > 0.05$). pRb expression was positive in 78 (75.7%) and a higher frequency of this expression was observed in HPV negative samples (87.0%) ($p = 0.021$) and in high-risk HPV negative samples (85.2%) ($p = 0.010$). No difference was observed when comparing pRb expression and EBV infection status ($p > 0.05$).

Conclusion Our results support the suggestion that p16^{INK4a} is not a reliable surrogate marker for identifying HPV or EBV infection in LSCC. On the other hand, most of our samples had pRb expression, which was more frequent in tumors without HPV, suggesting that pRb could indicate HPV negativity. However, more studies with a larger

*Correspondence:
Gerardo C. Palacios-Saucedo
palsaugc@gmail.com
Full list of author information is available at the end of the article

number of cases are required, including controls without LSCC and evaluating other molecular markers to determine the real role of $p16^{INK4a}$ and pRb in LSCC.

Keywords p16 ^{INK4a}, pRb, Human papillomavirus, Epstein–Barr virus, Laryngeal squamous cell carcinoma

Introduction

Laryngeal squamous cell carcinoma (LSCC) is one of the most common type of head and neck cancer and, although tobacco smoking and alcohol abuse are the principal risk factors, some viral infections are implicated [1, 2]. Human papillomavirus (HPV) is a group of oncoviruses that have been widely associated with the development and clinical prognosis of LSCC tumors [3]. According to their oncogenic potential, HPV are categorized into low-risk (LR-HPV) or high-risk (HR-HPV) genotypes [4]. LR-HPV are associated with warts or skin papillomas, whereas HR-HPV are directly associated with the development of invasive carcinomas [5, 6]. Prevalence studies have reported that HPV-16 and -18 are the most common HR-HPV genotypes found in LSCC although, other HR-HPV, such as 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 are also important [7, 8]. Epstein–Barr virus (EBV) is strongly associated with onset and progression of several types of epithelial cell tumors such as nasopharyngeal carcinoma or gastric adenocarcinoma [9, 10]. The role of EBV in LSCC is controversial since difficulties have been reported in detecting its presence in this kind of cancer or it has shown a low prevalence [11]. Both HPV and EBV produce oncoproteins that have the potential to induce processes of carcinogenesis and tumor progression [12]. E7 is an oncoprotein coded by HPV that promotes retinoblastoma protein (pRb) degradation by the ubiquitin–proteasome pathway [13]. Degradation of pRb consequently induces the overexpression of the tumor suppressor protein $p16^{INK4a}$, which is essential in cell cycle progression [14]. Latent membrane protein 1 (LMP1) is one of the major EBV oncoproteins that inactivates transcription factors essential for the expression of $p16^{INK4a}$ [15]. The alteration of p16 ^{INK4a} expression leads to hyperphosphorylation of pRb that promotes uncontrolled cellular proliferation [16]. High levels of $p16^{INK4a}$ expression have been suggested as a surrogate marker of HPV and EBV infection in pharyngeal, nasopharyngeal, and oropharynx tumors, whereas in laryngeal tumors are still controversial; therefore, pRb expression may be considered as an additional biomarker, particularly in tumors with infection by HPV and EBV [12, 17–19]. This work is aimed to compare the expression of pRb and $p16^{INK4a}$ as possible biomarkers in tumor tissues with and without infection by EBV or different genotypes of HPV from patients with LSCC attending a third-level care referral hospital.

Materials and methods

Samples

Formalin-fixed and paraffin-embedded (FFPE) tissue specimens from patients with LSCC were included in a retrospective study. Samples were taken from a previous study evaluating the HPV and EBV prevalence in patients with LSCC [20]. All patients had undergone surgical resection of the laryngeal tumor between 2012 and 2015 at the Unidad Medica de Alta Especialidad (UMAE) No. 25, a tertiary referral hospital of the Instituto Mexicano del Seguro Social (IMSS) located in northeastern Mexico. The information obtained from clinical records included age, sex, history of alcohol and tobacco consumption, and laryngeal subsite location of the tumor (subglottic, glottic, or supraglottic). Histological and pathological grading of tumors was made using the Broder's classification [21]. The National Committee of Scientific Research of the IMSS granted ethical approval to carry out the study (R-2014-785-055). Individual informed consent was not obtained as specimens were retrospectively collected.

HPV genotyping and EBV detection

Tumor sections from each FFPE sample were carefully punched off and conducted to a DNA isolation using the NucleoSpin DNA FFPE (Macherey–Nagel, Düren, Germany). The presence and genotype of HPV were evaluated using the INNO-LiPA HPV Genotyping Kit Extra II Amp (Innogenetics, Gent, Belgium), a line probe assay designed for the identification of 32 genotypes of the HPV, including thirteen HR-HPV (HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, and HPV-68), six possible HR-HPV (HPV-26, HPV-53, HPV-66, HPV-70, HPV-73, and HPV-82), nine LR-HPV (HPV-6, HPV-11, HPV-40, HPV-42, HPV-43, HPV-44, HPV-54, HPV-61, and HPV-81), and other four not yet classified HPV genotypes (HPV-62, HPV-67, HPV-83, and HPV-89) [22]. To detect DNA of EBV, a 69 bp fragment flanking the 679–748 position of the viral LMP2 glycoprotein was amplified using the primers 5′-AGC TGT AAC TGT GGT TTC CAT GAC-3′ and 5′-GCC CCC TGG CGA AGA G-3′ with the probe 6FAM5′-CTG CTG CTA CTG GCT TTC GTC CTC TGG-3′TAMRA. All the reactions for EBV detection were performed after the HPV genotyping assays using the TaqMan Universal PCR MasterMix (Applied Biosystems, Foster City CA, USA) in the Light Cycler (Roche). Synthetic DNA fragment (gBlocks Gene

Fragment, IDT) containing the 69 bp sequence of the amplicon of EBV was used as amplification control and to evaluate the efficiency and detection limits of the assay.

p16^{INK4a} and pRb expression

Immunohistochemical (IHC) staining method for p16^{INK4a} and pRb was performed on 5-µm-thick FFPE tissue cross-sections using the mouse and rabbit specific HRP/DAB (ABC) detection IHC kit (Abcam, Cambridge, UK) with the monoclonal anti-CDKN2A/p16INK4a antibody (clone EPR1473, ab108349, dilution 1:100; Abcam, Cambridge, UK) and the monoclonal anti-Rb antibody (clone EPR17512, ab181616, dilution 1:250; Abcam, Cambridge, UK). Hematoxylin–eosin (HE) stains were also prepared for histological evaluation of tumors. Two independent pathologists who were blinded to the clinical information and to the HPV or EBV status of the samples evaluated all the IHC and HE stains. Evaluations were made based on staining intensity as other previous reports have done. Specimens were considered positive for p16^{INK4a} expression if they showed ≥ 75% of stained cells, whereas for pRb, ≥ 25% of stained cells were interpreted as a positive expression [23–26].

Statistical analysis

We used the Mann–Whitney U test, Pearson's X^2 test, and Fisher's exact test. A p value < 0.05 was considered statistically significant. All analyzes were performed using the IBM SPSS Statistics program (Version 20, SPSS. Inc., Chicago, USA).

Results

One hundred and three FFPE specimens from the previously published study with sufficient tumor tissue for IHC were eligible for the present work. The age range of the included patients was 40 to 89 years, with a median of 64, and most were 60 years or older (73.8%). Most samples were from men (96.1%), and tobacco smoking was common (98.0%). In contrast, alcohol intake frequency was low (6.8%). The glottis subsite was the most frequent location of tumors (49.5%). We had 57 (55.3%) tumor samples positive for HPV DNA, and 42 (40.8%) of these had at least one HR-HPV genotype. EBV DNA was present in 28 (27.2%) samples. Of these, 16 (15.5%) were in co-infection with HPV, 11 (10.7%) corresponded to co-infections with at least one HR-HPV, and 12 (11.7%) cases had only EBV infection (Table 1). Clinical characteristics of patients with LSCC tumor samples positive for at least one HR-HPV are summarized in Table 2. HPV-52 was the most frequently HR-HPV genotype, with 34 cases corresponding to 59.6% of all the HPV-positive samples. Immunohistochemistry were assessed considering a positive expression of p16^{INK4a} if ≥ 75% of tumor cells were stained and for pRb if ≥ 25% of stained cells were observed (Fig. 1).

Of the 103 tumor samples, p16^{INK4a} expression was positive in 55 (53.4%). According to the surgical source of the sample, biopsies (64.5%) had a higher frequency of expression of this protein than laryngectomies ($p = 0.009$ by the Pearson's chi-squared test). Samples cataloged as Grade III by Broder's classification showed a higher frequency of p16^{INK4a} expression (80.9%) compared with those of grade I (50.0%) and II (42.1%) ($p < 0.05$ by the Mann–Whitney U test). Only one of the seven alcohol consumers showed p16^{INK4a} expression (14.3%) ($p = 0.048$ by the Fisher's Exact Test). No significant difference was observed when comparing p16^{INK4a} expression with the presence of HPV or EBV ($p > 0.05$ by the Fisher's Exact Test). Regarding pRb expression, 78 (75.7%) samples were positive. Specimen's obtained from biopsies (84.2%) showed a higher frequency of pRb expression than laryngectomy samples ($p = 0.025$ by the Pearson's chi-squared test). The frequency of pRb expression was higher in HPV negative samples (87.0%) ($p = 0.021$ by the Fisher's Exact Test). A higher frequency of pRb expression was also observed in the group of HR-HPV negative samples (85.2%) ($p = 0.010$ by the Fisher's Exact Test). No significant differences ($p > 0.05$) were observed in the expression of p16^{INK4a} or pRb by age and sex of patients, laryngeal tumor subsite, and tobacco consumption habits of the patients (Table 1).

Discussion

To assess the expression of p16 ^{INK4a} and pRb in LSCC specimens with and without infection by EBV or different genotypes of HPV, 103 FFPE samples retrospectively collected from patients were evaluated by IHC analysis. The epidemiological characteristics of the studied sample are consistent with those previously described for this disease [11, 19, 27–33]. p16^{INK4a} status has been well characterized in HPV-positive head and neck tumors such oropharyngeal carcinoma and has been suggested as a surrogate marker for infection of HPV and progression, but this has not been definitively established for LSCC [34, 35]. In this study, 55 (53.4%) of 103 patients were positive for p16^{INK4a}. Tong et al. found that 115 (54%) of 211 LSCC specimens were positive for p16^{INK4a} [36]. Elhadj et al. show that the expression positive of p16^{INK4a} was found in 36 (51.43%) of 70 LSCC cases [37]. Expression of p16^{INK4a} might be affected by genetic or epigenetic mechanisms [38]. Some studies suggest hypermethylation may represent an early event in carcinogenesis of different neoplasms, such as endometrial cancer [39]. In LSCC, expression level of p16^{INK4a} is significantly reduced and hypermethylation has been shown to be a common mechanism causing this downregulation [40].

Table 1 Correlation of $p16^{INK4a}$ and pRb expression with clinicopathological characteristics of 103 patients with laryngeal squamous cell carcinoma

	Total (n = 103)	$p16^{INK4a}$ positive[a] (n = 55)	$p16^{INK4a}$ negative[b] (n = 48)	*p*	pRb positive[c] (n = 78)	pRb negative[d] (n = 25)	*p*
Age (years)	64 (40–89)	64 (43–87)	63.5 (40–89)	0.086	64 (40–89)	64.5 (46–81)	0.216
Age ≥ 60 years	76 (73.8%)	40 (52.6%)	36 (47.4%)	0.793	59 (77.6%)	17 (22.4%)	0.456
Sex				1.000			0.570
Male	99 (96.1%)	53 (53.5%)	46 (46.5%)		74 (74.7%)	25 (25.3%)	
Female	4 (3.9%)	2 (50.0%)	2 (50.0%)		4 (100%)	0 (0.0%)	
Surgical sample				0.009			0.025
Biopsy	57 (55.3%)	37 (64.9%)	20 (35.1%)		48 (84.2%)	9 (15.8%)	
Laryngectormy	46 (44.7%)	18 (39.1%)	28 (60.9%)		30 (65.2%)	16 (34.8%)	
Anatomic location				0.575			0.436
Glottic	51 (49.5%)	28 (54.9%)	23 (45.1%)		42 (81.4%)	9 (17.6%)	
Subglottic	4 (3.9%)	2 (50.0%)	2 (50.0%)		3 (75.0%)	1 (25.0%)	
Supraglottic	15 (14.6%)	10 (66.7%)	5 (33.3%)		11 (73.3%)	4 (26.7%)	
Transglottic	33 (32.0%)	15 (45.4%)	18 (54.6%)		22 (66.7%)	11 (33.3%)	
Hitopathological grade				0.010			0.421
Grade I	44 (42.7%)	22 (50.0%)	22 (50.0%)		35 (79.5%)	9 (20.5%)	
Grade II	38 (36.9%)	16 (42.1%)	22 (57.9%)		26 (68.4%)	12 (31.6%)	
Grade III	21 (20.4%)	17 (80.9%)	4 (19.1%)		17 (81.0%)	4 (19.0%)	
Smoking	101 (98.0%)	54 (53.5%)	47 (46.5%)	1.000	76 (75.2%)	25 (24.8%)	1.000
Alcoholism	7 (6.8%)	1 (14.3%)	6 (85.7%)	0.048	6 (85.7%)	1 (14.3%)	1.000
HPV infection				0.557			0.021
Postitive	57 (55.3%)	32 (56.1%)	25 (43.9%)		38 (66.7%)	19 (33.3%)	
Negative	46 (44.7%)	23 (50.0%)	23 (50.0%)		40 (87.0%)	6 (13.0%)	
HR-HPV infection				1.000			0.010
Positive	42 (40.8%)	22 (52.4%)	20 (47.6%)		26 (61.9%)	16 (38.1%)	
Negative	61 (59.2%)	33 (54.1%)	28 (45.9%)		52 (85.2%)	9 (14.8%)	
EBV infection				0.119			1.000
Positive	28 (27.2%)	11 (39.3%)	17 (60.7%)		21 (75.0%)	7 (25.0%)	
Negative	75 (72.8%)	44 (58.7%)	31 (41.3%)		57 (76.0%)	18 (24.0%)	
Coinfections							
HPV + EBV	16 (15.5%)	11 (68.8%)	5 (31.2%)	0.529	9 (56.3%)	7 (43.7%)	1.000
HR-HPV + EBV	11 (10.7%)	7 (63.6%)	4 (36.4%)	0.455	6 (54.5%)	5 (45.5%)	1.000

Values are shown in absolute frequencies (percent)

[a] ≥ of 25% of staining cells

[b] < of 25% of staining cells

[c] ≥ of 75% of staining cells

[d] < of 75% of staining cells

We found that 80.9% of our specimens classified as grade III presented a higher $p16^{INK4a}$ expression. In contrast to our results, Tong and et al. found that $p16^{INK4a}$ expression was observed more commonly in well-differentiated samples [36]. In our study, only 7 (6.8%) patients were alcohol consumers, and one of them expressed $p16^{INK4a}$. Although this low number of cases precludes reliable statistical comparison, most alcohol consumers were significantly negative for $p16^{INK4a}$ expression ($p = 0.048$). Elhadj et al. found no association between alcohol consumption and $p16^{INK4a}$ expression when evaluating 30 alcohol-consuming patients [37]. Otherwise, we found that 32 (56.1%) of the fifty-five positive samples for $p16^{INK4a}$ were also positive for HPV infection. Stephen et al. reported the expression of $p16^{INK4a}$ in 21 (26.0%) of 80 patients, furthermore, 12 (57.0%) of these were positive for HPV infection [34]. Hernandez et al. observed $p16^{INK4a}$ expression in 8 (7.9%) of 101 cases, and only

Table 2 Clinical data and status of $p16^{INK4a}$ and pRb expression in 42 laryngeal squamous cell carcinoma patients positive for at least one high-risk human papillomavirus

Patient no.	Sex	Age	Anatomical location	Grade	Smoking	Alcoholism	HPV genotype	EBV	$p16^{INK4a}$	pRb
007	M	≥ 60	Subglottic	II	Yes	Yes	11, 52	–	Negative	Positive
009	M	< 60	Transglottic	I	Yes	No	16	–	Negative	Negative
010	M	≥ 60	Glottic	I	Yes	No	52	–	Negative	Positive
013	M	≥ 60	Supraglottic	II	Yes	No	52	–	Positive	Positive
015	M	≥ 60	Glottic	III	Yes	No	16	–	Negative	Positive
029	M	≥ 60	Supraglottic	II	Yes	No	11, 16	–	Positive	Positive
045	M	≥ 60	Glottic	III	Yes	No	11, 52	–	Positive	Negative
046	M	≥ 60	Glottic	II	Yes	No	11, 16	–	Positive	Negative
047	M	≥ 60	Transglottic	III	Yes	No	6, 11, 52	–	Negative	Negative
049	M	≥ 60	Transglottic	II	Yes	No	11, 52	–	Negative	Negative
051	M	≥ 60	Supraglottic	I	Yes	No	6, 11, 52	–	Positive	Positive
052	M	≥ 60	Glottic	I	Yes	No	16, 52	–	Positive	Positive
053	M	≥ 60	Transglottic	I	Yes	No	11, 52	–	Negative	Negative
056	M	≥ 60	Glottic	II	Yes	No	11, 45	–	Negative	Negative
059	M	< 60	Transglottic	II	Yes	No	52	+	Positive	Negative
060	M	≥ 60	Glottic	II	Yes	No	11, 52	–	Positive	Positive
061	M	≥ 60	Glottic	I	Yes	Yes	11, 16	–	Negative	Positive
062	M	≥ 60	Transglottic	II	Yes	No	6, 11, 52	–	Negative	Positive
063	M	< 60	Subglottic	I	Yes	No	11, 31, 52, 54	+	Positive	Negative
067	M	< 60	Glottic	I	Yes	No	6, 11, 52	+	Positive	Positive
068	M	≥ 60	Transglottic	II	Yes	Yes	11, 16, 52	+	Negative	Positive
069	M	< 60	Transglottic	I	Yes	No	11, 52	–	Positive	Positive
070	M	≥ 60	Transglottic	II	No	No	11, 52	+	Positive	Positive
071	M	≥ 60	Transglottic	I	Yes	No	11, 31, 52, 54	–	Positive	Positive
072	M	≥ 60	Transglottic	I	Yes	No	11, 52	–	Negative	Negative
074	M	≥ 60	Supraglottic	II	Yes	No	11, 31, 52, 54	+	Negative	Negative
075	M	≥ 60	Glottic	III	Yes	No	11, 26, 31, 52, 54	+	Positive	Positive
079	M	≥ 60	Glottic	II	Yes	No	11, 31, 52, 54	–	Positive	Positive
080	M	< 60	Glottic	I	Yes	No	11, 52	–	Negative	Negative
084	M	< 60	Glottic	II	Yes	No	6, 11, 16, 52	–	Positive	Negative
085	M	≥ 60	Supraglottic	II	Yes	No	11, 52	–	Positive	Negative
088	M	≥ 60	Transglottic	II	Yes	No	6, 11, 52	+	Negative	Negative
089	M	≥ 60	Glottic	I	Yes	No	11, 52	–	Positive	Negative
090	M	≥ 60	Transglottic	I	Yes	No	11, 31, 52	+	Positive	Positive
091	M	≥ 60	Glottic	II	Yes	No	11, 52	–	Positive	Positive
092	M	≥ 60	Transglottic	I	Yes	No	11, 31, 52	–	Negative	Positive
097	M	< 60	Glottic	I	Yes	No	52	–	Negative	Positive
099	M	≥ 60	Supraglottic	III	Yes	No	52	–	Positive	Positive
124	F	≥ 60	Glottic	I	Yes	No	45	–	Positive	Positive
141	M	< 60	Glottic	II	Yes	No	52	–	Negative	Positive
148	M	≥ 60	Glottic	II	Yes	No	52	+	Negative	Positive
149	M	≥ 60	Glottic	II	No	No	45	+	Negative	Positive

2 (25.0%) of these were also HPV DNA positive [41]. Sanchez et al. found that 48 (39.0%) of 123 samples were positive for $p16^{INK4a}$ expression, and 14 (29.2%) of these were positive for HPV infection [42]. Dogantemur et al. observed $p16^{INK4a}$ expression in 18 (20.0%) of 90 cases and only 6 (33.3%) of these were also positive for DNA of HPV [43]. The fact that no association between the expression of $p16^{INK4a}$ and HPV infection was observed could support the suggestion that this protein may not be reliable as a surrogate marker for HPV infection [44].

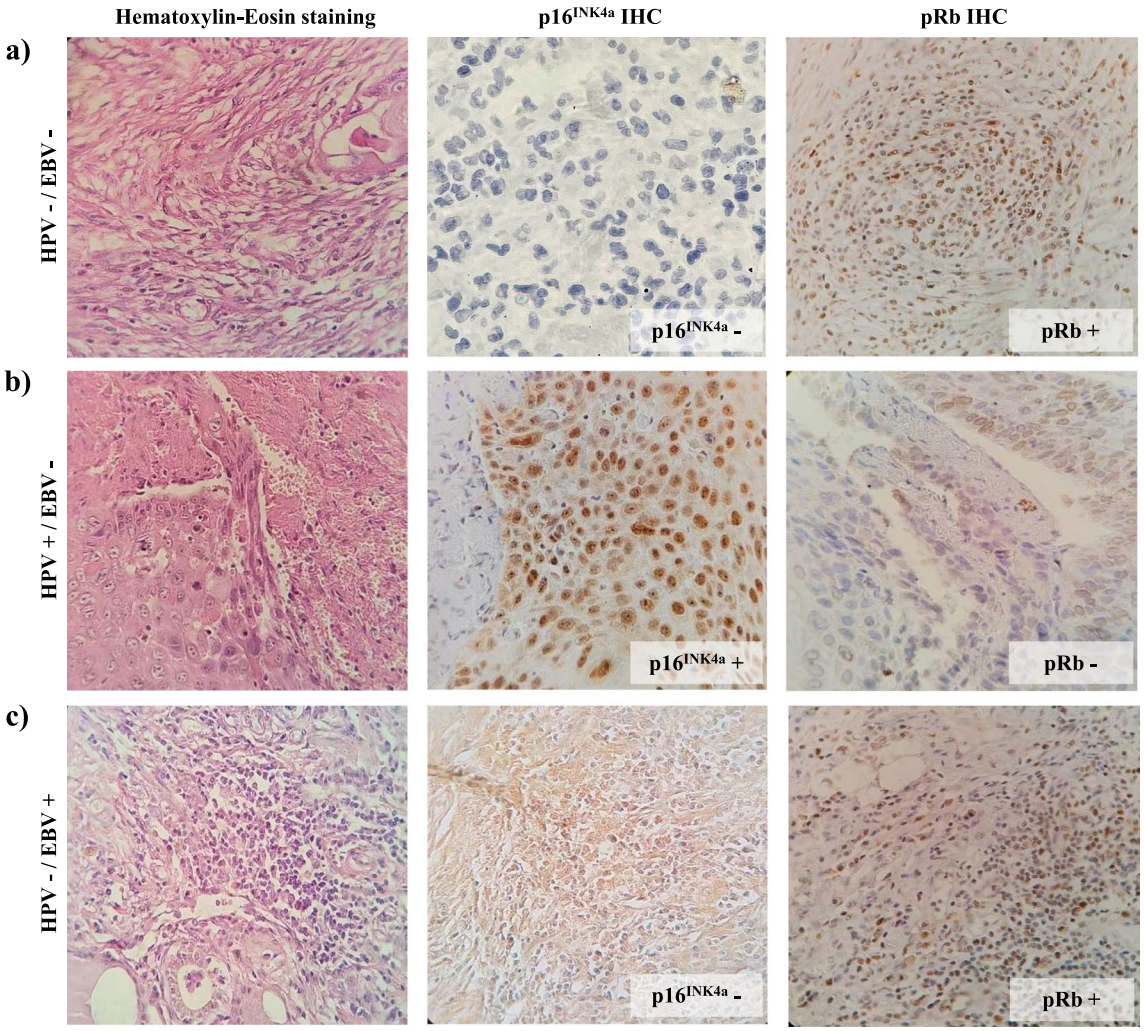

Fig. 1 Representative examples of immunohistochemical (IHC) staining of tumors from patients with laryngeal squamous cell carcinoma. **a** Sample of patient No. 175 without infection of HPV or EBV; **b** sample of patient No. 046 that was positive for the 11 and 16 HR-HPV and negative for EBV; and **c** sample of patient No. 021 that was negative HPV and positive for EBV (×40 microscopic magnification)

Modifications of the $p16^{INK4a}$ and pRb expression have been delineated in several types of human cancers [45, 46]. We found that pRb expression was present in 78 (75.7%) of 103 specimens. Morshed et al. found that 90 (69%) of 130 patients with LSCC had expression of pRb [47]. Krecicki et al. reported that 51(88%) of 58 samples were positive for pRb expression [48]. Some studies suggest that high pRb expression may be due to different factors. Soares et al. suggested that it can be due to an increase in the proportions of proliferating cells, which is supported by the fact that the hyperphosphorylated pRb inactive form increases during the G2/M phases [49]. IHC evaluation using antibodies against phosphorylated and non-phosphorylated pRb forms could solve this. As pRb is a negative regulator of $p16^{INK4a}$, its inactivation results in overexpression of $p16^{INK4a}$. Thus, a positive test for HPV combined with $p16^{INK4a}$ expression has been described as evidence of biologically relevant infection [50, 51]. The frequency of pRb expression was significantly higher in the HPV-negative specimens; we found 40 (87.0%) with positive pRb expression in 46 HPV-negative tumors. However, the clinical and prognostic significance remains poorly described in LSCC. Soares et al. found a significant difference between pRb expression and HPV-positive (9/11, 81.8%) and HPV-negative (15/22, 68.2%) oral squamous cell carcinoma (OSCC) samples [49]. Shaikh et al. demonstrated that pRb expression was predominant in 51 (77.3%) of 66 HPV-negative head and neck cancers cases, but they did not find significant differences [51]. We also observed a significantly

higher frequency of pRb expression in HR-HPV negative specimens. The pRb expression was present in 51 (85.2%) of 61 HR-HPV negative samples. By contrast, Nemes et al. reported no statistical difference in 37 (84%) of 44 HR-HPV negative OSCC samples with pRb expression [52]. Various studies have shown that HPV-positive cancers generally exhibit decreased expression of pRb [53]. In addition, the oncoprotein E7 of HPV is known to participate in the degradation of pRb through a ubiquitin–proteasome and other pathways [54]. Thus, the absence of HPV infection could be related to the pRb expression in our samples.

Conclusion

We detected $p16^{INK4a}$ in half of LSCC cases, and in half of these, HPV was also detected. However, the proportion of samples with $p16^{INK4a}$ expression was similar between those with HPV and EBV. Although the proportion of LSCC samples with $p16^{INK4a}$ expression was higher than reported in several previous studies, our findings suggest little value of this marker as a reliable surrogate for identifying HPV in laryngeal cancer. On the other hand, as in previous studies, most of our LSCC samples had pRb expression, this being more frequent in tumors without the presence of HPV, even in those positive for high-risk HPV. Therefore, pRb expression seems to indicate HPV negativity, which could justify not performing other molecular tests for HPV detection in this type of cases, as previously suggested. However, further studies with a larger number of cases, including controls without laryngeal cancer, involving other tumor suppression pathways or other molecular markers, are necessary to determine the real role of $p16^{INK4a}$ and pRb in LSCC.

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Author contributions
JMVG and GCPS conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft. AYAV and AHE performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper. AZP, LGRM, ACMT, VGV analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft. JCSH, SJHM, EECM, MSMB, CAAT and ECSF participated in the collection and histological reevaluation of the specimens, performed the histopathological evaluations, and analyzed the clinical data. RSTG and CRP contributed to the financing, analyzed the final data, reviewed and approved the final draft of the paper. All authors reviewed the final version of the manuscript.

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Availability of data and materials
The raw data analyzed during the current study are provided in a Supplemental File.

Declarations

Ethics approval and consent to participate
The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers): The National Committee of Investigation of the Instituto Mexicano del Seguro Social granted Ethical approval to carry out the study (Reg. No. R-2014-785-055).

Consent for publication
Not applicable.

Competing interests
The authors declare there are no competing interests.

Author details
[1]Laboratorio de Inmunologia y Virologia, Facultad de Ciencias Biologicas, Universidad Autonoma de Nuevo Leon, San Nicolas de los Garza, Nuevo Leon, Mexico. [2]Division de Investigacion, Departamento de Otorrinolaringologia y Departamento de Anatomia Patologica, Unidad Medica de Alta Especialidad, Hospital de Especialidades No. 25, Instituto Mexicano del Seguro Social, Monterrey, Nuevo Leon, Mexico. [3]Laboratorio Medicina Diagnostica S.A. de C.V., Monterrey, Nuevo Leon, Mexico. [4]Departamento de Biologia Celular y Genetica, Facultad de Ciencias Biologicas, Universidad Autonoma de Nuevo Leon, San Nicolas de los Garza, Nuevo Leon, Mexico. [5]Departamento de Foniatria, Hospital General de Zona No. 6, Instituto Mexicano del Seguro Social, San Nicolas de los Garza, Nuevo Leon, Mexico.

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