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Co-Detection of Low-Risk HPV 6/11 and the Tumor Suppressor Protein pRb in Benign and Malignant Laryngeal Lesions: A Retrospective Molecular Study

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Abstract **Background:** Many low-risk human papillomavirus (lrHPVs) genotypes have been detected in a large number of benign and malignant head and neck lesions using different molecular techniques. **The Study Aims:** To detect low-risk human papillomavirus (lrHPV) genotypes and investigate their association with the overexpression of the Rb protein. It has been reported that increased rates of lr HPV prevalence and expression of pRb might have possible role in pathogenesis and development of laryngeal lesions. **Methods:** Various (157) formalin – fixed, paraffin –embedded (FFPE)laryngeal lesions tissue blocks specimens were collected. Molecular detection and genotypes of low-risk human papillomaviruses (lrHPVs) using a modern version of in situ hybridization with type-specific probes, alongside the assessment of Rb protein overexpression via immunohistochemistry. **Results:** The current results show no statistically significant association between HPV6/11 DNA in situ hybridization (ISH) scores or signal intensity and pRb protein immunohistochemistry (IHC) scores or staining intensity in laryngeal nodules, polyps, or malignant lesions as following; 2(25.0%) in moderate score and high intensity 2 (25.0%) for malignant lesions while in polyps benign lesions in 1 case (25%) in moderate score and high intensity 1 (25%) .In nodules benign lesions, the score was in low and moderate in 1 case (20%) for each whereas the intensity detected in 2 case. Overall, integrated HPV 6/11 DNA signals showed the highest mean percentage in malignant lesions (12.78±23.40%), followed by episomal (3.87±11.44%) and mixed patterns (1.91±8.12%). Integrated patterns showed the highest mean percentage in polyps (10.14±22.90%), whereas episomal patterns showed the highest mean percentage in nodules (6.62±11.10%). Mixed signals were also reported in the control group, although differences among signal patterns in this group were not statistically significant (p = 0.3710). **Conclusion:** The heterogeneity of HPV6/11 DNA-ISH patterns and pRb expression may reflect underlying cellular and tissue alterations associated with benign and malignant laryngeal lesions. These findings suggest a potential, indirect role of low-risk HPV in laryngeal lesion development; however, the lack of significant associations between HPV 6/11 signals and pRb expression in the lesion groups precludes establishing a causal relationship. Further studies are warranted to clarify the biological significance of these findings.

Key Words Low risk HPV6/11, Laryngeal Malignant and Benign Lesions,P RB , Signal Patterns, Dr Tamara JPMSCISH, IHC

INTRODUCTION

Globally, Human Papillomaviruses (HPVs) rank among the most widespread viral infections [1]. Over 450 types of Human Papillomaviruses have been identified. HPVs are small viruses, non- enveloped with a circular double strand DNA genome belonging to the Papillomaviridae family and epithelial and mucosal tropic viruses at different anatomical regions across diverse higher vertebrate species, exhibiting species-specific activity that promotes cellular proliferation [2].Virus transmission may occur through direct and skin to skin contact ,particularly during sexual intercourse or other forms of intimate physical contact [3].

The clinical manifestation of human papillomavirus (HPV) infection varies substantially according to specific viral genotype and includes benign clinical lesions such as cutaneous and anogenital warts, premalignant abnormalities as intraepithelial neoplasia, and anogenital cancers. Roughly, 70% to 90% of HPV infections are subclinical and lack overt visible clinical signs [4,5]. Depending on its possiple cancer relation, scientist divided them to high oncogenic risk HPVs (HPV16 the most oncogenic type) and low oncogenic risk HPVs [6]. Functional characterization of HPV early gene products E6 and E7 serve pivotal roles in oncogenesis and malignant transformation. High oncogenic

risk HPV6 as well as E7 have a high affinity to integrated to host cell genome more than low oncogenic risk E6 and E7 through binding to two tumor suppressor genes P53 and Retinoblastoma RB respectively result in disruption of physiological cell cycle checkpoints and machinery. These transcription factors drive environmental adaptation and organismal survival via gene expression regulation [7].

Under environmental stress factors including biotic stressors like freezing, anoxia, dehydration and pathogens the retinoblastoma (Rb)-E2F pathway controls progression of cell cycle [8]. Post-translational alterations of Rb (phosphorylation or acetylation) repress E2F-driven proliferation during metabolic or osmotic stress. Concurrently, canonical cell cycle regulators—including cyclins, CDKs, and checkpoint proteins—detect DNA damage or replication errors to arrest cell division. This review examines the molecular mechanisms governing cell cycle control, highlighting the roles of the Rb-E2F network and Cyclin-CDK complexes in mediating survival under extreme cellular stresss [9].

Laryngeal lesions are abnormal growths or tissue changes in the voice box (larynx). They include non-cancerous nodules, polyps, cysts, and inflammatory conditions, as well as pre-cancerous changes and malignant tumors like squamous cell carcinoma [10]. Common signs are hoarseness, throat pain, and breathing changes. These lesions frequently appear on the inferior vocal cords and could exhibit distinct micro vascular features [11].So, the presence study represents an important contribution to understanding the potential role of low risk HPV6,11 in pathogenesis and tumorigenesis of laryngeal malignant and benign lesions in Iraq.

METHODS

Histopathology Tissues Specimens

The study was retrospective one. One hundred and fifty seven archived laryngeal tissue block specimens (formalin fixed, paraffin embedded) were collected randomly from many private laboratories as well as Baghdad medical city and Baqubah learning hospitals . Biopsy specimens of histologically normal- appearing laryngeal tissues that are suspected free from significant pathological changes were retrieved the archival records Forensic Medicine Institute in Baghdad province. All samples assembled during the period from January 2024 till February 2026. The diagnosis were depend on the recoded histopathalogical documents of the corresponding laryngeal lesions blocks after confirmatory histopathalogical re-examination by histopathologist for each obtained tissue blokes. All tissues blocks were subjected to cut as serial thin sections of 4 µm thickness. All tissues were diagnosed based on the original pathology reports stocked on charged slides except one for hematoxylin and eosin examinations for each laryngeal tissues lesions and apparently healthy control tissues. Lesions were classified as benign non-neoplastic lesions, including vocal-fold polyps and nodules, or malignant lesions according to their documented histopathological features. These laryngeal tissue lesions blocks composed of 45 laryngeal malignant lesions ,72 laryngeal benign lesions (35 polyps and 37 nodules) were subjected to laboratory testing.Ages of tissue blocks specimens range from 2 to83.

Methods

Human papillomaviruses DNA were detected and genotyped by advanced molecular chromogenic in situ Hybridization technique employing a cocktail long Digoxigenin-labeled DNA probes (T-1144-400; ZytoVision GmbH, Bremerhaven Kit, Germany) to target specifec DNA sequences in tissue specimens in order to screen HPV genotypes : 6,11,16,18,31,33,35,39,45,51,52,56,58,59,66,68,and 82). Concurrently, low-risk HPV6/11 genotypes were identified using particular DNA probes tagged with Digoxigenin (T-1055-400), ZyoVision GmbH., Bremerhaven, Germany.

Methodology

Chromogenic in situ hybridization (CISH) analysis was conducted in accordance with the protocol and guidelines provided by the manufacturer. Firstly, pre-treatment processes (by dewax, rehydration and proteolysis digestion) ,then the second steps included; Denaturation and Hybridization and the third steps was Post- hybridization and Detection through using Housekeeping gene-specific molecular probes labeled with digoxigenin (DIG) were targeted to elicited reactive staining. Except for the diluted probe, which was omitted from the negative control solution, all reagents were added as specified in the experimental protocol. Within the framework of HPV screening, positive signals [(T-1151-40: ZytoFast Plus Implementation kit AP-Permanent red)] Positive signals were detected as intense bright-red staining with Red Permanent Solution, followed by hematoxylin counterstaining. Whereas the signals detected by CISH for HPV6/11 [(ZytoFast Plus Implementation kitHRP-green: T-1073-40)] were visualized as bright-green staining, with nuclear counterstaining at the sites of complementary sequence hybridization. In situ hybridization signals for the different molecular markers were quantitatively assessed using a light microscope at 10× and 40× magnifications. The number of positive cells was determined by microscopic examination at 1000× magnification. Negative controls processed under the same conditions were used, where available, to assess assay performance and signal specificity. Housekeeping-gene probes, if used, served to assess nucleic acid preservation and assay adequacy. Negative controls were used to assess nonspecific background signals. Control outcomes and predefined criteria for accepting or rejecting assay results were documented. Specimens with inadequate tissue preservation or insufficient control performance were considered uninterpretable according to the laboratory's established criteria.

The percentage of positive signals and staining intensity scores, reflecting the proportion of positive cells and the intensity of the staining reaction respectively. It used to

detect the in-situ hybridization signals. The staining intensity scores ranged from no staining to high staining. Positive cells were counted in ten randomly selected microscopic fields, with 100 cells evaluated per field. Cases were subsequently classified into three categories according to the Score 1 (1–25%), Score 2 (26–50%), and Score 3 (>50%) [12]. The proportion of positive cells was determined by calculating the mean percentage of positive cells across the ten randomly selected microscopic fields.

Immunohistochemically (IHC) staining was performed to evaluate pRB protein overexpression in various laryngeal lesions. The HRP/DAB-based Exposure Detection System for mouse and rabbit antibodies (Abcam, product no. ab80436), in combination with the monoclonal anti-P RB1/2 antibody (EPR7927 ab166611), was used for immunohistochemically staining according to the manufacturer's instructions. The intensity of pRB staining in the nucleus and cytoplasm was assessed and classified as either high or absent. The staining scores were calculated based on the percentage of cells exhibiting positive reactivity. Specimens exhibiting weak to moderate nuclear staining in 10–75% of cells were assigned a score of +1, whereas specimens showing strong nuclear staining in more than 75% of cells were assigned a score of +2[13]. Specimens lacking nuclear staining were assigned a score of 0. Statistical differences among the variables of interest were assessed using the chi-square test, independent-samples t-test, and analysis of variance (ANOVA), as appropriate.

Ethical Certification

Ethical approval, also referred to as ethical clearance, is formal authorization granted by an accredited Research Ethics Committee (REC) or Institutional Review Board (IRB) following an ethical review of the proposed research. Such approval confirms that the study has been designed and conducted in accordance with internationally recognized ethical principles, applicable regulations, and institutional guidelines. For research involving human participants, human-derived biological specimens or data, or laboratory animals, documentation of ethical approval is an essential component of the research process.

The permission criteria established by the Institutional review board and local ethics committee permission for the project was obtained and followed. On July, 28, 2026, a local ethics commission accepted this project under project number CEPEC/12 after reviewing and approving the study protocol and subject information.

Statistical Analysis

The Chi-square test, T test, ANOVA test and Tukey's multiple comparisons test were used to evaluate the variables under investigation in this study. The SPSS software, Version 24, was used for the statistical analyzes, and a p-value of less than 0.05 was considered statistically significant.

RESULTS

Age Distribution Across the Study Groups

The overall age distribution of the research participants and control groups that underwent larngectomy or endoscopic biopsy. Table 1 presents the selected demographic characteristics of the study patients tissues and the control groups. In regard to correlation age with study groups, all groups ages range from 2-83 years with range and mean age of each group as following; control group; 2-66 (27.25±17.15), nodule group; 20-75(42.49±13.03), polyp group; 8-75 (40.57±18.39), malignant group 8-83(56.91± 17.12)]. Patients tissues groups and controls group showed statically high significant differences ($p<0.0001$). Also highly significant differences when used Tukey's multiple comparisons test among study groups except between polyp and nodule groups $p = 0.9612$ (Table 2).

Table 1: Age Distribution Across the Study Groups

Age Groups	N	Mean±SD	SE	Range		p-value
				Min	Max	
Control	40	27.25±17.15	2.71	2.0	66.0	<0.0001 **
Nodule	37	42.49±13.03	2.14	20.0	75.0	
Polyp	35	40.57±18.39	3.11	8.0	75.0	
Malignant	45	56.91±17.12	2.55	8.0	83.0	

Table 2: Tukey's Multiple Comparisons Test

Tukey's Multiple Comparisons Test	Below threshold?	Summary	Adjusted p-Value
Control vs. Nodule	Yes	**	0.0005
Control vs. Polyp	Yes	**	0.0037
Control vs. Malignant	Yes	**	<0.0001
Nodule vs. Polyp	No	ns	0.9612
Nodule vs. Malignant	Yes	**	0.0008
Polyp vs. Malignant	Yes	**	0.0001

** $p < 0.01$

Table 3: Gender Distribution Across the Study Groups

Gender	No. (Percentage)				Chi-square	p-Value
	Control	Nodule	Polyp	Malignant		
Male	24 (60.0%)	27 (72.97%)	20 (57.14%)	39 (86.67%)	10.78	0.0130 *
Female	16 (40.0%)	10 (27.03%)	15 (42.86%)	6 (13.33%)		
Total	40 (100%)	37 (100%)	35 (100%)	45 (100%)		

*p<0.05

Gender Distribution Across the Study Groups

From Table 3, the gender distribution results among study groups that underwent laryngeal lesions reveled higher percentage of male more than female with statically differences (p<0.0130) as in following groups male; malignant, polyp, nodule and control 39(86.67%),20 (57.14%),27 (72.97%),24 (60.0%) higher than female 6 (13.33%), 15 (42.86%),10 (27.03%), 16 (40.0%) respectively.

Distribution of LrHPV6/11 DNA-ISH Signal Intensities

The present results of positive -ISH signal of intensity for LrHPV-DNA was reported high staining intensity percentage in malignant group, polyps group, nodules group and control group 13/45 (28.9%), 8/35 (22.9%), 7/37 (19%) and 1/40 (2.5%) respectively, with no intensity staining signal in the same groups 32/45 (71.1%), 27/35 (77.1%), 30/37 (81%) and 39/40 (97.5%) respectively. Significant differences were found among groups (X^2 = 10.47, p = 0.0150) as Table 4.

Distribution of LrHPV6/11 DNA-ISH signal scores

The distribution of positive ISH staining signal scores for LR-HPV DNA varied across the study group, In the malignant group, high staining scores were observed in 8 cases (17.78%), moderate scores in 3 (6.67%), and low scores in 2 (4.44%). In the polyp group, 5 cases (14.29%) showed high scores, 1 (2.86%) showed a moderate score, and 2 (5.71%) showed low scores. In the nodule group, high scores were observed in 4 cases (10.81%) and low scores in 3 (8.11%), with no cases classified as moderate. Among controls, 39 specimens (97.5%) were negative, while 1 (2.5%) showed a moderate score. The reported differences among the four groups were not statistically significant (χ^2 = 14.63, p = 0.1015), as shown in Table 5.

Distribution of LrHPV6/11 DNA-ISH Signal Patterns (Integrated, Episomal and Mixed)

The presence of low-risk human papillomavirus (HPV) DNA in laryngeal lesions was investigated using histological specimens. Low-risk HPV DNA was detected at a significantly higher frequency in laryngeal malignant tissues following by polyps and nodules groups with absent of integrated and episomal signal pattern in control group that is indicating a significant association between low-risk HPV infection and laryngeal malignancy. The HPV-DNA signals detected by in situ hybridization (ISH) appeared as distinct red-colored signals within the nuclei, corresponding to the sites of complementary HPV-DNA sequences. The distribution of LR-HPV 6/11 DNA signal patterns varied among the different lesion groups. In malignant lesions, the integrated signal pattern showed the highest mean percentage, at 12.78±23.40 (range: 0–80%), followed by the episomal pattern, at 3.87±11.44 (range: 0–50%), and the mixed pattern, at 1.91±8.12 (range: 0–50%). The differences among the three signal patterns were statistically significant (p = 0.0030). Similarly, in polyp lesions, the integrated pattern exhibited the highest mean percentage, at 10.14±22.90 (range: 0–70%), followed by the episomal pattern, at 1.43±5.89 (range: 0–25%). No mixed signal pattern was observed in this group. The differences among the signal patterns were statistically significant (p = 0.0046). In nodule lesions, the episomal pattern showed the highest mean percentage, at 6.62±11.10 (range: 0–40%), followed by the integrated pattern, at 2.84±10.96 (range: 0–60%), and the mixed pattern, at 0.14±0.82 (range: 0–5%). These differences were statistically significant (p = 0.0097). In the control group, only the mixed pattern showed a non-zero mean percentage, at 1.25±7.91 (range: 0–50%), whereas the integrated and episomal patterns had mean percentages of 0.00±0.00. However, the differences among the three signal patterns in the control group were not statistically significant (p = 0.3710) as in Table 6.

Comparison of HPV 6/11 DNA-ISH Signal and pRB-IHC Expression among the Studied Groups

LR-HPV6/11DNA/pRB protein -IHC scores: In patients with laryngeal malignancie, Positive HPV 6/11 DNA-infected cells demonstrated detectable ISH staining signals and positive pRB immunoreactivity was noticed in 2/45 case (25.0%) in moderate score. Conversely, absent staining was exhibit in 26/45cases (70.3%). Within benign polyp's tissue cases demonstrated positive lrHPV6/11 DNA–pRB IHC staining, with moderate score (++) staining noted in 1/35 case (25.0%), while absent staining was recorded in 24/35 cases (77.4

Table 4: Distribution of Signal Intensities HPV6/11 DNA-Positive Cases among the Studied Groups, as Detected by in Situ Hybridization (ISH).

	No. (Percentage)				Chi-square	p Value
Intensity	Control	Nodule	Polyp	Malignant	10.47	0.0150 *
High	1 (2.5%)	7 (19%)	8 (22.9%)	13 (28.9%)		
No Intensity	39 (97.5%)	30 (81%)	27 (77.1%)	32 (71.1%)		
Total	40 (100%)	37 (100%)	35 (100%)	45 (100%)		

Table 5: Distribution of Signal scores HPV6/11 DNA-Positive Cases among the Studied Groups, as Detected by in Situ Hybridization (ISH)

	No. (Percentage)				Chi-square	p-Value
Score	Control	Nodule	Polyp	Malignant	14.63	0.1015 NS
-ve	39 (97.5%)	30 (81.08%)	27 (77.14%)	32 (71.11%)		
Low	0 (0.0%)	3 (8.11%)	2 (5.71%)	2 (4.44%)		
Moderate	1 (2.5%)	0 (0.0%)	1 (2.86%)	3 (6.67%)		
High	0 (0.0%)	4 (10.81%)	5 (14.29%)	8 (17.78%)		
Total	40 (100%)	37 (100%)	35 (100%)	45 (100%)		

Table 6: Distribution of HPV 6/11 Signal Patterns According to Their Mean Values and Ranges in Laryngeal Lesions Tissue Samples

Group	SP Percentage	N	Mean±SD	SE	Range		p-value
					Min	Max	
Control	Integrated	40	0.0±0.0	0.0	0.0	0.0	0.3710 NS
	Episomal	40	0.0±0.0	0.0	0.0	0.0	
	Mixed	40	1.25±7.91	1.25	0.0	50.0	
Nodule lesions	Integrated	37	2.84±10.96	1.80	0.0	60.0	0.0097 **
	Episomal	37	6.62±11.10	1.82	0.0	40.0	
	Mixed	37	0.14±0.82	0.14	0.0	5.0	
Polyp lesions	Integrated	35	10.14±22.9	3.87	0.0	70.0	0.0046 **
	Episomal	35	1.43±5.89	1.00	0.0	25.0	
	Mixed	35	0.0±0.0	0.0	0.0	0.0	
Malignant lesions	Integrated	45	12.78±23.4	3.49	0.0	80.0	0.0030 **
	Episomal	45	3.87±11.44	1.71	0.0	50.0	
	Mixed	45	1.91±8.12	1.21	0.0	50.0	

Table.7: Comparison of HPV 6/11 DNA-ISH Signal Scores and pRB-IHC Expression Scores among the Studied Groups

Studied Groups				Rb scores		Pearson Chi-Square (p-value)
				Negative	+	
A.H. Control	HPV6 / 11 scores	Negative	No.	37	2	p = 0.00 Highly Sign. (p<0.01)
			%	100.0%	66.7%	
		+	No.	0	1	
			%	0.0%	33.3%	
	Total		No.	37	3	
			%	100.0%	100.0%	
Nodules lesions	HPV6 / 11 scores	Negative	No.	27	3	p = 0.411 Non Sign. (p>0.05)
			%	84.4%	60.0%	
		+	No.	2	1	
			%	6.2%	20.0%	
		++	No.	3	1	
			%	9.4%	20.0%	
	Total		No.	32	5	
			%	100.0%	100.0%	
Polyps lesions	HPV6 / 11 scores	Negative	No.	24	3	p = 0.809 Non Sign. (p>0.05)
			%	77.4%	75.0%	
		+	No.	2	0	
			%	6.5%	0.0%	
		++	No.	5	1	
			%	16.1%	25.0%	
	Total		No.	31	4	
			%	100.0%	100.0%	
Malignant lesions	HPV6 / 11 scores	Negative	No.	26	6	p = 0.797 Non Sign. (p>0.05)
			%	70.3%	75.0%	
		+	No.	2	0	
			%	5.4%	0.0%	
		++	No.	9	2	
			%	24.3%	25.0%	
	Total		No.	37	8	
			%	100.0%	100.0%	

S* = Significant, NS*: Non-Significant

%). Laryngeal benign nodules, positive moderate score (++) and low score (+) staining for lrHPV6/11 DNA–pRB IHC signal were identified in 1/37 case (20.0%) and 1/37 case (20.0%), respectively, whereas negative staining was recorded in 25/37 cases (84.4%.)

Finally, regarding non-tumor control tissues which appeared normal upon gross examination low-score staining (+) for low-risk human papillomavirus (lrHPV6/11) DNA and pRB protein was observed in one case /40(33.3%), whereas absent staining results were recorded in 29/40 cases (100%). Statistical analysis revealed significant differences just in control group amongthe studied groups (p<0.01), as shown in Table 7.

B. LR-HPV6/11DNA/pRB protein -IHC Intensities

Strong staining signal intensity of LR-HPV 6/11 DNA-positive infected cells in relation to PRB were observed in two/45 cases (25.0%), while no staining signal were detected in 26/45(70.3%) cases with laryngeal malignant tumors. In the laryngeal polyp group, strong staining intensity for (LRHPV 6/11) DNA/PRB protein -IHC expression was recorded in one/35 case (25.0%), whereas no staining signal was found in 24/35 cases (77.4%). In the laryngeal nodule group, strong positive staining intensity was noted in two/37 cases (40.0%), while no staining signal was observed in 27/37 cases (84.4%). In apparently healthy individuals (control group), strong positive staining intensity was

Table 8: Comparison of HPV 6/11 DNA-ISH Signal Intensities and pRB-IHC Expression Intenseties among the Studied Groups

Studied Groups				Rb intensity		Pearson Chi-Square (p-value)
				No Intensity	High intensity	
A.H. Control	HPV6 / 11 intensity	No Intensity	No.	37	2	p = 0.00 Highly Sign. (p<0.01)
			%	100.0%	66.7%	
		High intensity	No.	0	1	
			%	0.0%	33.3%	
	Total		No.	37	3	
			%	100.0%	100.0%	
Nodules Lesions	HPV6 / 11 intensity	No Intensity	No.	27	3	p = 0.196 Non Sign. (p>0.05)
			%	84.4%	60.0%	
		High intensity	No.	5	2	
			%	15.6%	40.0%	
	Total		No.	32	5	
			%	100.0%	100.0%	
Polyps lesions	HPV6 / 11 intensity	No Intensity	No.	24	3	p = 0.914 Non Sign. (p>0.05)
			%	77.4%	75.0%	
		High intensity	No.	7	1	
			%	22.6%	25.0%	
	Total		No.	31	4	
			%	100.0%	100.0%	
Malignant lesions	HPV6 / 11 intensity	No Intensity	No.	26	6	p = 0.789 Non Sign. (p>0.05)
			%	70.3%	75.0%	
		High Intensity	No.	11	2	
			%	29.7%	25.0%	
	Total		No.	37	8	
			%	100.0%	100.0%	

S* = Significant, NS*: Non-Significant

observed in one/40 case (33.3%), while no staining signal was detected in 37/40 cases (100%)of control group in regard to lrHPV 6/11 DNA/pRB-IHC. Statistical analysis revealed no significant differences among the studied groups p (> 0.05) except control group (p = 0.0) as in Table 8, the HPV 6/11 intensity categories were dichotomized into no intensity and high intensity by combining the positive score categories (+ and ++), as defined in the study scoring protocol.

DISCUSSION

Laryngeal lesions can develop across all age groups; however, their histopathological and clinical characteristics vary substantially with age and stage of life [14]. Laryngeal carcinoma is an invasive malignant neoplasm arising from the epithelial tissues of the larynx, characterized by progressive cellular proliferation and local tissue infiltration. It constitutes a significant proportion of head and neck malignancies, accounting for approximately 1–5% of cancers encountered within otolaryngology practice [15]. The pathogenesis of laryngeal carcinoma is influenced by multiple well-established risk factors, with tobacco exposure, alcohol consumption, and long-term occupational exposure to carcinogenic agents representing the principal contributors [16,17]. Human papillomavirus (HPV) infection is associated with various benign and malignant upper respiratory tract lesions and may contribute to cellular transformation and tumorigenesis. Laryngeal tumors associated with High and Low oncogenic risk HPV genotypes such as 16,18,6,11 respectively [18]. In laryngeal tumors particularly laryngeal squamous cell carcinoma (LSCC) the retinoblastoma protein (pRB) functions as a key regulator of the cell cycle and a tumor suppressor; alterations in its expression levels or its dysfunction via viral pathways play a pivotal role in tumorigenesis, the determination of patient prognosis, and the response to treatment [19].

To the best of our knowledge, this is the first study in Iraq to investigate the association between LR-HPV 6/11 gene products and pRB expression using CISH and IHC, respectively, in archived FFPE laryngeal tissues from benign and malignant lesions.

1-Age and Gender Distribution among Patients with Benign and Malignant Laryngeal Lesions

In the present study, as demonstrated in Table 1, the age of the study participants ranged from 8.0 to 83.0 years. Patients with malignant laryngeal tumors had a mean age of 56.91±17.12 years, which was significantly higher than that observed among patients with benign laryngeal lesions, including polyps and nodules. The mean age of patients with benign lesions was within the fourth decade of life whereas in control group was 27.25±17.15 with range age 2-66 years. While gender findings showed males proportion was higher than female proportion in all study groups (control, nodules, polyps and malignant as following male /female 24(60.0%)/16(40.0%), 27(72.97%)/10(27.03), 20(56.14%)/ 15(42.86%) and 39(86.67%)/ 6(13.333%) with highly statically differences among groups.

The age distribution observed in the present study is in agreement with findings reported by several previous studies, as demonstrated by Ghosh *et al.*,(2023)who found the highest frequency of age over 50 years in patents with HRHPV infection in Squamous Cell Carcinoma of larynx[20].Also consist with Shalarah *et al.* who evaluate

the role of p53,p16 and cyclinD1 as well as detection of HPV in patient with laryngeal SCC, found increased laryngeal cancer with age range from 31-80 and in mean age (58.93 ± 11.18) years[21]. Gomaa and his college's (2017) found the age range of patients infected with HPV and undergo laryngeal cancer was 38 – 70 years old with mean 57.06 years that consist with the present study [22]. Also agreed with Megahed *et al.* study that showed the range and mean of age were 43-73 and 56.9 ±7.2) respectively in patients with HPV and different grades of laryngeal cancer [23]. And disagreed with many studies that carried to detect different types of HPV infections in laryngeal cancers in Nassef *et al.* who found the range and mean of age from 42-76 and (60.8± 9.8) years respectively in 8%of biopsies taken from the tumor sites and no HPV was detected in biopsies taken from normal tissues [24]. The present investigation compatible with Jiang *et al.* whom use PCR technique to detect HPV types including low risk HPV genotypes 6 and 22 found range and median age were 36-78 and 65 years old in patients with positive HPV16 and laryngeal SCC [25]. Also the current study in line with Laskaris *et al.* that showed the LSSC increased with age and the range and mean of patients age were from 21-88 and (62.6) respectively when detect HPV DNA infection in Greek patients with SCC of larynx [26]. In addition to the aforementioned prior studies, the current study aligns with those of Gallus *et al.* and Vazquez-Guillen *et al.* which identified the presence of Human papillomavirus (HPV) in cases of laryngeal squamous cell carcinoma in separate studies, the range and mean of both studies 39.88.5 (64.2)and 34-89(64) years [27,28].In regard to laryngeal malignancy, reported that percentage of male higher than female as in [20-28].

In regard of age distribution of benign tumor (Polyps and Nodules), the range and mean of this study were in 8-75 and 40 years respectively. The present study concordant with Iravani *et al.* study that reported range and mean of patients with (HPV) DNA in benign laryngeal lesions were 18-80((40.57) years [29]. Also match with study of Brooks *et al.* and Ikegami *et al.* Studies have shown that the age range and mean age of individuals diagnosed with laryngeal papilloma and human papillomavirus (HPV) ranged from 44 years to over 60 years [30,31]. The present study also in accordance with Omland *et al.*(2014)and Hirakawa *et al.* reported the age of patients was matched forties [32,33].This finding in disagreement with many studies as Manjarrez *et al.* who found most laryngeal papillomatosis samples contain low-risk HPV determined by both PCR and *in situ* PCR with age range from (5-15) years [34].Reccurrent laryngeal squamous in Mujic *et al.* who identified HPV DNA and in laryngeal lesions in Northeast Bosnia and Herzegovina with range and median age from 39-80(61) years [35]. Allstudy above demonstrated that male prominence more than female [29-35].

The observed differences in age distribution among patients with various laryngeal lesions may be attributable to the distinct biological and etiological characteristics of these lesions. In malignant laryngeal tumors, the higher age observed in affected patients may partly reflect the cumulative effects of prolonged exposure to established carcinogens, particularly tobacco smoke, alcohol, and occupational agents, which may progressively contribute to cellular and genomic damage over time [36,37]. Furthermore, aging is associated with the gradual accumulation of somatic DNA damage and genomic alterations, together with alterations in immune function known as immunosenescence [38] The decline in immune surveillance with advancing age may impair the recognition and elimination of abnormal cells, thereby facilitating the accumulation and persistence of potentially malignant cellular changes [39]. Conversely, the occurrence of benign HPV-associated laryngeal lesions across younger and older age groups may reflect differences in the timing of HPV acquisition, host immune responses, and the biological behavior of individual lesions. Recurrent respiratory papillomatosis, for example, demonstrates a heterogeneous age-of-onset pattern, with distinct pediatric and adult peaks, supporting the influence of age-related and host-specific factors on lesion development [40-42].

In term of gender distribution across present study groups, Although the underlying factors contributing to the increased incidence and observed sex disparity remain incompletely understood, several potential determinants may account for these differences [43].

The pronounced male predominance observed in laryngeal lesions that include malignant and benign tumors were likely multifactorial. It may partly reflect the historically higher prevalence and intensity of tobacco smoking and alcohol consumption among men, both of which are well-established risk factors for laryngeal cancer and may exert synergistic effects when combined [44]. Furthermore, differences in occupational patterns may contribute to this sex disparity, as certain male-dominated occupations involve exposure to potentially carcinogenic agents, including polycyclic aromatic hydrocarbons, engine exhaust, textile dust, and silica. Collectively, differences in lifestyle and occupational exposure between men and women may partly explain the higher burden of laryngeal cancer among males [44,45].

Papillomavirus-Associated Laryngeal Lesions

Distribution of Signal scores HPV6/11 DNA-Positive Cases among the Studied Groups, as Detected by In Situ Hybridization (ISH): In present study results of positive – CISH signal high intensity and high score for LR-HPVDNA were (28.9%)and score in 8 cases (17.78%) in laryngeal malignancy ,while in benign lesions (Polyps and Nodules) was 8cases(22.9%) and 7cases (19%)with high score in5 cases (14.29%)and 4 cases (10.81%) respectively as showed in Table 3 and Table 4.

A review of the available literature revealed no studies specifically assessing staining intensity and score signal of HPV6/11DNA in benign and malignant laryngeal lesions, either independently or in combination, across the different detection methodologies employed. However, several

previous studies have reported the detection of HPV types 6 and 11, showing findings broadly consistent with those of the present study, the findings of the present study are consistent with those reported by Abdulsamad *et al.* who detected HPV 6/11 DNA in 30% of both benign and malignant laryngeal masses. In addition, approximately 26% of non-neoplastic laryngeal lesions, predominantly polyps, showed positive HPV immunohistochemical staining [46]. These results are in line with those of Sun *et al.*, who demonstrated that HPV-6/11 DNA positivity was markedly higher in laryngeal papilloma (33.3%) than in laryngeal squamous cell carcinoma (9.6%), a difference that was statistically significant ($p<0.0005$) [47].The findings of the present study are broadly consistent with those reported by Brooks *et al.*, who investigated HPV-6 and HPV-11 in recurrent laryngeal squamous papillomas using PCR, dot-blot hybridization, and in situ hybridization (ISH). HPV-11 was detected in 2/10 (20.0%) juvenile-onset cases compared with 5/11 (45.5%) adult-onset cases, whereas HPV-6 was detected in 7/10 (70.0%) juvenile cases and 5/11 (45.5%) adult cases. These findings indicate age-related differences in the distribution of HPV genotypes, with HPV-11 occurring more frequently among adults and HPV-6 predominating among juvenile cases [30]. The present findings are also comparable with those of Iravani *et al.* who detected HPV DNA in 23.1% of laryngeal polyps and 7.7% of lesions classified as other benign laryngeal lesions, including nodules and cysts, using PCR [29]. Similarly, Ghallab *et al.* reported HPV-6 DNA in 34% of adult vocal fold polyps; among patients with laryngopharyngeal reflux, HPV-6 positivity reached 44.11%, compared with 12.5% among those without reflux [48].

In contrast, the present results differ from those reported by Manjarrez *et al.* who found the prevalence of HPV6 and HPV11were 60% and 30% respectively in non- malignant laryngeal tissues and it constitute an important risk factor in laryngeal papilloma [49], Hirakawa *et al.*, who detected HPV DNA in 28 of 30 (92.9%) patients with laryngeal papilloma. Among HPV-positive cases, HPV-6 accounted for 89.3%, whereas HPV-11 represented 10.7%. HPV detection and genotyping were performed using molecular methods, while immunohistochemistry was used to assess p16, p21, p53, and Ki-67 expression [33]. The discrepancy between the present study and these previous reports may be related to differences in study populations, lesion characteristics, sample size, geographic variation, and, importantly, differences in HPV detection, lesion types, age at disease onset, geographical distribution, clinical course. and genotyping methodologies including specimen type and sampling site, tissue fixation and preservation, viral load, DNA quality, and the sensitivity and specificity of the molecular assay employed. In particular, differences in PCR primers or ISH probes, the genomic region targeted, and the analytical method used may result in considerable variation in HPV6/11 detection rates. [33-35]. Also, the discrepancies among studies may be partly explained the handling of both HPV6/11 co-infections or with other types of HPV types .Another explanation ,may further contribute to the observed discrepancies. indirect evidence supporting the progressive nature of laryngeal keratosis, particularly observations related to the latency period of HPV infection. The duration of HPV latency may be an important determinant of the timing of initial diagnosis and may contribute to understanding the subsequent progression of laryngeal lesions toward carcinoma [49]. Recurrent benign laryngeal papillomtous lesions most commonly arise in the vocal cords and may extend to other respiratory tract sites, with a clinical course ranging from persistent or latent HPV infection to, in some cases, dysplastic or malignant transformation. They typically originate at the junction of squamous and respiratory epithelium or in areas of squamous metaplasia. HPV DNA detection may be considered in laryngeal mucosal lesions, particularly among patients with prolonged tobacco and alcohol exposure [50].

Distribution of HPV 6/11 Signal Patterns Form According to Their Mean Values and Ranges in Laryngeal Lesions Tissue Samples

As in Table 5 Showed the range and mean percentage of LR-HPV 6/11 DNA exhibiting an integrated pattern were {0-80 (12.78±23.4)} whereas range and mean percentage of episomal signal patterns were {0-50 (3.87±11.44)} then mix signal patterns range and mean were {0-50(1.91±8.12)}. In polyp's lesions, the range and mean percentage of LR-HPV 6/11 DNA exhibiting an integrated pattern were {0-70 (10.14±22.9)} whereas range and mean percentage of episomal signal patterns were {0-25 (1.43±5.89)} and no mixed signal pattern. While in nodule lesions, The range and mean percentage of LR-HPV 6/11 DNA showed an episomal pattern were {0-40 (6.62±11.10)} higher than integrated and mixed pattern {0-60(2.84±10.96)} and {0-5.0(0.14±0.82)} respectively. Whereas in control group, noted absence of integrated and episomal signal pattern of HPV6/11 DNA.

Although comparative studies documenting human papillomavirus (HPV) integration patterns with the host cell chromosome specifically regarding whether the virus integrates, episomal, or mixed patterns within the laryngeal lesions examined in the current study (whether malignant or benign, including polyps and nodules) are scarce, other relevant studies address various benign laryngeal growths and lesions (such as recurrent respiratory papillomatosis) as well as malignant lesions. Notably, the study conducted by Brooks *et al.* aligns closely with the point you aim to establish in your thesis; the researchers examined 35 samples of laryngeal papillomas from both children and adults, utilizing initial and subsequent samples from recurrent cases for comparison. This punctate pattern(integrated) was observed in 7/10 (70%) of juvenile-onset cases and 7/11 (63.6%) of adult-onset cases, as well as in progressive and recurrent disease there for the authors concluded that low-risk HPV integration may be an early and common event in recurrent laryngeal papillomatosis [30].Also the present study highly relevant to study of Huiberts *et al.* which demonstrated the integration of HPV6 and down regulation

of AKR1C3 expression and probable malignant transformation in patient witn juvenile-onset laryngeal papillomatosis episomal patterns The patient suffered from juvenile-onset recurrent respiratory papillomatosis (RRP) since childhood, which later progressed to laryngeal squamous cell carcinoma; HPV6 was detected in both the initial papilloma and the subsequent carcinoma. FISH analysis revealed a diffuse pattern in the papilloma consistent with episomal HPV6, whereas the carcinoma exhibited a single-point integration signal. Furthermore, APOT-PCR and sequencing identified an HPV6–host fusion transcript, mapping the integration site to the AKR1C3 gene at 10p15.1 [51]. Although the study conducted by Lele *et al.* did not directly demonstrate the occurrence of integration, it detected the presence of human papillomavirus type 11 (HPV11) in lesions ranging from papilloma and dysplasia to carcinoma; the researchers concluded that infection with this virus might be an early event associated with the progression of recurrent respiratory papillomatosis (RRP) to cancer. While this finding supports the hypothesis of an early origin of the infection, this study should not be cited in isolation to substantiate that hypothesis [52]. A study by Reddy *et al.* examined nine cases of recurrent respiratory papillomatosis (RRP) originating in the larynx or trachea. Human papillomavirus 11 (HPV11) was detected in the tumors, and where sufficient RNA was available evidence of viral integration was found in three out of seven cases. Thus, these findings support the presence of integrated HPV11 in malignancies associated with recurrent respiratory papillomatosis [53].

In contrast, Deng *et al.* studied 18 samples obtained from 11 patients with adult-onset laryngeal papilloma. Human papillomavirus type 6 (HPV6) was detected in 9 of these patients; 15 samples showed the virus in an episomal (extrachromosomal) state with an intact E2 gene, and no integrated viral sequences were found [54]. Furthermore, a multi-omics study by Sievers *et al.* did not detect HPV-host fusion transcripts in recurrent respiratory papillomatosis samples despite HPV fusions having been identified in certain high-risk head and neck cancers and concluded that HPV-6/11 in recurrent respiratory papillomatosis appears to be predominantly extra chromosomal [55].

The relationship between HPV6/11 infection and the development of benign and malignant laryngeal lesions can be better understood by considering the normal organization and differentiation of the laryngeal epithelium. The larynx contains different epithelial phenotypes according to anatomical location. The mechanically exposed vocal folds are predominantly lined by stratified squamous non-keratinizing epithelium, whereas much of the supraglottic and infraglottic mucosa is lined by respiratory-type pseudostratified ciliated epithelium. Transitional areas and foci of squamous metaplasia may also occur, producing epithelial environments in which HPV infection can be established [56]. Basal cells of both squamous and respiratory-type epithelia express markers such as p63 and integrin-α6, consistent with their proliferative and progenitor-like properties. Experimental and pathological observations suggest that HPV can infect immature laryngeal epithelial cells even in the absence of a classical squamo-columnar junction, while squamous metaplasia of respiratory epithelium may provide an additional permissive environment for papilloma development. E1 functions primarily as the viral replication initiator/helicase, while E2 regulates viral transcription and replication and contributes to the partitioning and maintenance of viral episomes in dividing cells. The continued presence of viral episomes in proliferating basal cells is therefore crucial for persistence and recurrence. Experimental work with HPV11 demonstrates that both E6 and E7 also contribute to stable episomal maintenance, indicating that the functions of these proteins in low-risk HPV infection extend beyond simple transformation [57]. Therefore, the physical status of low-risk human papillomavirus (HPV) types 6 and 11 in laryngeal lesions may occur in episomal, integrated, or mixed episomal/integrated forms. The relative contribution of each viral state to the development of benign laryngeal papillomas and their occasional progression to malignancy remains incompletely understood. Current evidence suggests that HPV6/11 is predominantly maintained as episomal DNA in benign laryngeal papillomas, whereas viral integration has been documented in a subset of malignant lesions. Nevertheless, the detection of integrated viral DNA in some benign lesions indicates that integration may occur before overt malignant transformation in selected case.From the present finding and discrepancy of related other studies findings of signal patterns of HPV6/11DNA ,it suggest the possibility of HPV6/11 integration at an early stage of papilloma development and the accumulation of cellular mutations could transform to malignant statement under certain conditions[54,58].

LR-HPV6/11DNA/pRB protein –IHC

The hypothesis of this retrospective study aimed to analyze the co-localization of low risk human papillomavirus (HPV) DNA specifically types 6 and 11 with pRb protein expression levels in archived samples.

From table 6 and 7. Showed comparison of HPV 6/11 DNA-ISH signal and pRB-IHC expression scores and intensities in study groups as following; 2(25.0%) in moderate score and high intensity 2 (25.0%) for malignant tumor while in polyps benign tumor in 1 case (25%) in moderate score and high intensity 1 (25%) .in nodules benign tumor ,the score was in low and moderate in 1 case (20%) for each whereas the intensity detected in 2 case (40%).

Unfortunately, we did not find studies in the scientific literature that precisely quantify the score and intensity of signal staining associated with the co-presence of human papillomavirus (HPV)6/11 DNA and the tumor-suppressor protein Rb using the specific method described in the current study; however, there are studies closely related to this one.

The present study aligns with the findings of Manjarez *et al.* regarding low-risk human papillomavirus (specifically HPV-6/11) in laryngeal papilloma and laryngeal carcinoma

assessed via in situ hybridization and PCR, in conjunction with pRb measurement via immunohistochemistry (IHC). It was determined that pRb levels were highest in papilloma compared to normal tissue and carcinoma, dropping to their lowest level in the carcinoma cases [34], while in Deng *et al.* found 9 of 11 patients was HPV-positive adult-onset laryngeal papilloma (AO-LP) and pRb positivity was observed in 3 out of 7 cases (42.9%); the case that progressed to carcinoma was pRb-negative. Additionally, no positive result for episomal HPV6 was detected [54].

In contrast, Vazquez-Guillen ertal .,2023 study findings match with present study findings who found Among 103 tumor samples of laryngeal squamous cell carcinoma, 32 samples (56.1%) tested positive for human papillomavirus (HPV), while 78 samples (75.7%) showed positive expression of the pRb protein; a higher rate of this expression was observed in HPV-negative samples (87.0%; p = 0.021) and in samples negative for high-risk viral strains (85.2%; p = 0.010) [28].

Many investigators hypnotized that multiple viral infections could exert synergistic effects that enhance epithelial hyperplasia and facilitate the accumulation of premalignant genetic alterations, potentially accelerating their progression to malignancy [59]. The current results show no statistically significant association between HPV (types 6 and 11) DNA in situ hybridization (ISH) scores or signal intensity and pRb protein immunohistochemistry (IHC) scores or staining intensity in laryngeal nodules, polyps, or malignant lesions. Notably, strong HPV (6/11) DNA signals were observed in the malignant tumor group at very similar rates between pRb-negative and pRb-positive cases (24.3% and 25.0%, respectively). This finding does not support the existence of a consistent inverse relationship between HPV (6/11) viral load and pRb protein expression levels. From a biological perspective, this can be explained by the distinct behavior of low-risk human papillomavirus HPV6 and 11 compared with that of high-risk HPV16 and 18. Although the E7 proteins of HPV6 and 11 are capable of interacting with the retinoblastoma protein family, their effects on the pRb protein are significantly weaker than those of the E7 proteins in high-risk types, whereas their impact on the p130 protein is more pronounced [60]. Consequently, observing pRb protein expression in cells harboring types 6 and 11 does not necessarily rule out dysfunction in the Rb pathway. Signals associated with HPV 6 and 11 detected via in situ hybridization (DNA-ISH/CISH) in certain lesions may suggest focal viral integration; however, these signals should be interpreted as indicators rather than definitive evidence of chromosomal integration, as directly demonstrating the linkage of viral DNA to the host genome requires molecular techniques such as integration-specific PCR or sequencing [61]. Previous studies have indicated the presence of HPV 6 and 11 in an apparently integrated state within a sample of benign and malignant laryngeal lesions. Brooks and colleagues interpreted the punctate in situ hybridization patterns observed in recurrent laryngeal papilloma tumors as an indication of viral integration, suggesting that integration may occur at an early stage [30]. In contrast, Reidy and colleagues identified both episomal and integrated HPV11 in histologically benign papillomatous lesions, whereas only integrated HPV11 was detected in malignant tissues, accompanied by additional alterations in p53 and IFN-β proteins [53]. Furthermore, Huebbers and colleagues provided direct molecular evidence of HPV6 integration into the host AKR1C3 gene in a laryngeal carcinoma that developed following the early onset of recurrent respiratory papillomatosis (RRP), whereas the preceding papilloma had harbored episomal HPV6 [51]. In contrast, a comprehensive multi-omics analysis of recurrent respiratory papillomatosis (RRP) conducted by Sievers *et al.* failed to identify fusion transcripts between human papillomavirus (HPV) 6/11 and the host genome, supporting the predominance of an extrachromosomal viral state in benign RRP [55]. Collectively, these findings suggest that HPV 6/11 may exist in extrachromosomal, mixed, or focally integrated states, depending on the specific lesion and the stage of the disease. Accordingly, the integrated HPV type 6 and 11 signals detected in this study should not be interpreted as evidence of malignant transformation in themselves; rather, they may represent a localized molecular event occurring in a subset of infected epithelial cells. Malignant transformation likely requires additional genetic and epigenetic alterations affecting cell cycle regulation, DNA repair, apoptosis, and genomic stability. The absence of a statistically significant association between the intensity of HPV 6/11 signaling and pRb protein expression in this malignant cohort suggests that HPV 6/11-associated laryngeal carcinogenesis where it occurs cannot be explained solely by the progressive loss of pRb protein. Therefore, the observed HPV 6/11 and pRb protein patterns are best interpreted as evidence of biological heterogeneity among laryngeal lesions and as potential indicators of distinct stages or mechanisms of HPV persistence, rather than as independent markers of malignant transformation. Furthermore, Alterations in RB and its associated regulatory pathways are also prevalent across a broad range of adult neoplasms. Previous studies have shown that loss of heterozygosity at the RB/p130 locus is a frequent event in laryngeal lesions and give a good explanation in addition to this may be occur defect in RB /P16 INK4a regulatory pathway due to accumulation of somatic genetic alterations potentially leading to dysregulation and, in some contexts, increased accumulation of pRb protein [62]. This does not rule out the presence of a mutant variant, nor does it exclude the possibility of Rb protein inactivation at the post-transcriptional or post-translational level a phenomenon that may be partly attributed to human papillomavirus (HPV) infection [60]. Alternative explanation for the heterogeneity observed in HPV6/11 DNA-ISH patterns and pRb expression may be the coexistence of multiple viral infections and differences in host immune responses. It has been proposed that concurrent viral infections may exert synergistic effects by promoting epithelial hyperplasia, facilitating the accumulation of premalignant genetic

alterations, and potentially accelerating progression toward malignant transformation [63]. Persistent infection with multiple viral types may prolong or intensify viral stimulation of the laryngeal epithelium, thereby sustaining cellular proliferation and increasing genomic instability. Furthermore, persistent HPV infection may reflect an inadequate local immune response to viral clearance rather than a generalized systemic immunodeficiency. In efficient elimination of the virus may permit prolonged persistence within basal and parabasal epithelial cells, thereby increasing the likelihood of accumulating additional genetic and molecular alterations. Accordingly, viral coinfection may represent a potential modifying factor contributing to the persistence and progression of laryngeal lesions, although its specific role in HPV-associated malignant transformation remains to be fully elucidated [64,65].

CONCLUSION

The observed heterogeneity in HPV 6/11 DNA-ISH patterns and pRb expression may reflect the complex interactions between viral infection and alterations in cellular regulatory pathways. Persistent HPV 6/11 infection and incomplete local immune-mediated clearance may potentially allow prolonged viral interaction with basal and parabasal epithelial cells and continued modulation of epithelial proliferation. Such persistence may provide an opportunity for the accumulation of additional molecular and genetic alterations. Furthermore, the coexistence of other viral infections may represent a potential modifying factor influencing viral persistence, epithelial proliferation, and disease progression through interactions with host immune responses and cellular pathways. Nevertheless, the specific contribution of viral coinfection and its role in HPV 6/11-associated malignant transformation remain incompletely understood and warrant further investigation using molecular approaches capable of characterizing concomitant viral infections, host–virus interactions, and functional alterations in the RB pathway.

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