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Study of The Role of Human Papilloma Virus and Laryngopharyngeal Reflux in Adult Vocal Fold Polyps

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ABSTRACT

Background: There are several possible causes of vocal fold polyps in adults**.** The mechanical damage is the main cause for polyp formation. Laryngopharyngeal reflux and human papilloma virus are possible co-factors.

Objective: The aim of this study was to find out the associated roles of laryngopharyngeal reflux and human papilloma virus in the development of adult vocal fold polyps.

Patients and Methods: This cross-sectional observational study included 50 adult patients presented with vocal fold polyps. Patients were preoperatively assessed for the presence of laryngopharyngeal reflux using the validated nine-item reflux symptom index and eight-item reflux finding score. All patients were subjected to excision of the laryngeal polyp by microlaryngoscopic surgery. The excised samples were sent for human papilloma virus (HPV) detection by PCR.

Results: Laryngopharyngeal reflux (LPR) was presented in 34 patients (68%). Reflux symptom index ranged from 6 to 25 (mean 13.06 ± 4.95). Reflux finding score ranged from 3 to 18 (mean 10.08 ± 4.32). Seventeen patients (34%) had positive HPV-6. Fifteen patients had positive HPV-6 out of 34 patients diagnosed with LPR (44.11%), compared to only two patients had positive HPV-6 among 16 patients without LPR (12.5%) (P = 0.027). There were significant relations between presence of HPV-6 and both reflux symptom index and reflux finding score; both were significantly higher among patients with positive HPV-6 (P = 0.028) and (P < 0.001) respectively. **Conclusion:** Both laryngopharyngeal reflux and human papilloma virus play important associated roles in the development of vocal fold polyps in adults.

Keywords: Human papilloma virus; Laryngopharyngeal reflux; Vocal fold polyp.

INTRODUCTION

Adult vocal fold polyps are generally unilateral benign lesions affecting larynx, usually causing hoarseness of voice, breathiness and/or voice fatigue [(1)]. However, rare cases have been reported with large or giant polyps causing airway obstruction [(2)].

The mechanical damage is the main cause for polyp formation either vocal over-and misuse or strong coughing. Smoking, gastroesophageal reflux, chronic/recurrent upper respiratory infections, and allergy are considered as co-factors [(3)].

There are several possible causes of vocal fold polyps which can lead to increased vocal fold blood vessel permeability, local edema, hypoxia, degeneration, and fibrosis [(4)]. Laryngopharyngeal reflux (LPR) is an inflammatory condition of the upper aerodigestive tract related to effect of gastric or duodenal content reflux, which induces morphological changes in the upper aerodigestive tract [(5)]. The laryngopharynx is more susceptible than the esophagus to epithelial damage caused by reflux, since the laryngopharynx has a thin epithelium compared to the esophagus [(6)]. The higher prevalence of laryngopharyngeal reflux episodes in patients with benign true vocal fold lesions supporting the concept of a direct damaging effect of acidic gastric juice on the true vocal cords [(7)].

Human papilloma viruses are species-specific viruses, which show strict tropism for the stratified squamous epithelium. Over 170 types have been identified so far that can infect the skin or the mucosal surface of the aerodigestive tract, those viruses can be classified as low-risk and high-risk types [(8)]. Laryngeal papilloma can be developed and caused by the human papilloma virus (HPV), most commonly of low-risk subtypes 6 and 11, which infiltrates and proliferates within epithelial tissue. This condition can manifest in either childhood or adulthood [(9)]. The genotype HPV-11 appears to be associated with more severe forms, with an increased risk of airway obstruction [(10)]. However, only 20% of patients with laryngeal papilloma have measurable levels of human papilloma viral DNA [(11)]. Also, human papilloma virus can be latent in vocal folds without laryngeal papillomas and viral DNA can be detected by polymerase chain reaction (PCR) in the epithelium of the vocal cords in patients suffering from chronic laryngitis, nodules, or polyps reflecting the possible prevalence of latent HPV infections in the vocal cord mucosa [(12)].

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We aimed in this study to find out the important roles of laryngopharyngeal reflux and human papilloma virus in the development of vocal fold polyps in adult patients and to study the possible association between LPR and HPV in adult vocal fold polyps.

PATIENTS AND METHODS

This study was carried out as a cross-sectional observational study at Benha University Hospital, Benha Faculty of Medicine, Benha, Egypt; during the period from May 2018 to August 2020. Fifty adult patients were included, suffering from hoarseness of voice due to presence of vocal fold polyp and presented to the out-patient clinic of the hospital.

We excluded from the study patients younger than 18 years, patients with history of laryngeal surgery, patients with history of laryngeal cancer, and/or patients with history of trauma to larynx.

Detailed history was obtained including the demographic data and history of the condition; onset, course, duration, frequency of exacerbations, and development of any complications. Full otorhinolaryngology examination of ear, nose, and throat was completed. Then examination of the larynx by video laryngostroboscopy was performed to detect the presence of laryngeal polyps and to document the physical findings of laryngopharyngeal reflux (LPR).

Subjectively, all patients were evaluated for the presence of symptoms of LPR such as intermittent dysphonia, vocal fatigue, voice breaks, chronic throat clearing, excessive throat mucus, chronic cough, and/or chronic airway obstruction. The nine-item reflux symptom index (RSI) was applied, it is a developed and validated questionnaire to quantify patient's symptoms of LPR and evaluate treatment efficacy. The scores of this questionnaire range from 0 (normal) to 45 (most severe). This validated questionnaire has displayed excellent reproducibility and criterion-based validity [(13)].

Objectively, the laryngeal examination findings were recorded, the possible signs of LPR can range from vocal fold hyperemia or edema, subglottic edema or diffuse laryngeal edema, ventricular obliteration, posterior commissure hypertrophy and/or granuloma. The eight-item reflux finding score (RFS) was applied, it is a validated tool to document the physical findings severity of LPR. The scores of this tool range from 0 (normal) to 26 (most severe) [(14)].

A score of reflux symptom index (RSI) greater than 13, or reflux finding score **(**RFS) above 7 points, were considered positive and clinically diagnostic and indicative of LPR [(15, 16)].

Operative procedure:

All patients were subjected to excision of the vocal fold polyp by direct microlaryngoscopy under general anesthesia. The excision samples were sent to microbiology department to search for human papilloma virus by polymerase chain reaction (PCR).

Detection of HPV by PCR:

Human papilloma virus genome detection was done by a multiplex PCR-based HPV typing assay using type-specific primers and agarose gel electrophoresis as described by **Romero-Pastrana [(17)]**.

DNA extraction from paraffin sections:

The formalin-fixed, paraffin-embedded (FFPE) tissue specimens from fifty patients were analyzed. After cutting deep into the block, eight 10 μm-sections of formalin-fixed paraffin embedded tissue were transferred to Eppendorf vials. Total DNA was extracted using QIAamp DNA FFPE Tissue (Qiagen, Hilden, Germany).

Deparaffinization of the sections were done by vortexing and incubation with xylene. Then xylene was decanted and its residue was removed by two pure ethanol washes. The air-dried pellet was then incubated for 10 hours with 25 μL of proteinase K and 250 μL of ATL lysis buffer in a heat block at 56°C. The lysed emulsion was further purified with the spin-column kit. Finally, DNA was eluted in 50 μl of AE buffer (0.5 mM EDTA) and then stored at -20ºC for further use.

DNA amplification:

Amplification was performed using forward primer (5′-ACG TGG CCT TGT GCG GTA CAG TC -3′) and reverse primer (5′-AGA GAC GAG TCA GGC AAT GC -3′). The reaction was performed in a total volume of 25 μL composed of 1 μL of each primer, 5 μL of extracted DNA, 12.5 μL of Easy Taq® Universal PCR SuperMix (TransGen Biotech, Haidian District, Beijing, China), and 5.5 μL of nuclease free water.

Amplification was done according to the protocol of: initial denaturation step at 95°C for 15 min, 10 cycles of 30 s at 94°C, 90 s at 65°C, and 90 s at 72°C, followed by 30 cycles of 30 s at 94°C, 90 s at 63°C, and 90 s at 72°C, with a final extension at 72°C for 10 min.

Detection of PCR amplification products:

The amplified samples were then run in on 2% agarose gel, in the presence of a DNA marker, using gel electrophoresis and visualized on a UV transilluminator to detect presence of amplified product and to type the HPVgenome.

Ethics and Consent:

Informed written consent was obtained from all participants in the study. **The study was performed in accordance with the Helsinki declaration and its amendments. The study protocol was approved by the Research Ethics Committee at Faculty of Medicine, Benha University (REC-FOMBU), Egypt.**

Statistical analysis

Obtained data were statistically analyzed using SPSS version 16 software (SPSS Inc, Chicago, IL, USA). "Chi-square test" was used to analyze categorical data, which were presented as number and percentage. While "two sample t-test" was used to analyze quantitative data, which were presented as mean, standard deviation (SD), and range. P-value ≤ 0.05 was considered the accepted level of significance in this work.

RESULTS

The age, sex, and other findings in patients are shown in table 1.

Table (1): Findings in total studied patients

Variant		Total patients- n (50)
Age (years):		
Mean ± SD		38.940 ± 5.180
(Range)		(30 - 52)
Sex	n (%)	
Male		22 (44%)
Female		28 (56%)
LPR	n (%)	
Absent		16 (32%)
Present		34 (68%)
RSI		
Mean ± SD		13.060 ± 4.950
(Range)		(6 - 25)
RFS		
Mean ± SD		10.080 ± 4.322
(Range)		(3 - 18)
HPV-6	n (%)	
Negative		33 (66%)
Positive		17 (34%)
HPV-11	n (%)	
Negative		50 (100%)
Positive		0 (0%)

HPV-6: human papilloma virus subtype 6; HPV-11: human papilloma virus subtype 11; LPR: laryngopharyngeal reflux; RFS: reflux finding score; RSI: reflux symptom index

As shown in table 2, there was a statistically significant association between presence of LPR and HPV-6 genome detection.

Table (2): Comparison between laryngopharyngeal reflux present and absent cases

Variant		LPR		Test	
		Present n (34)	**Absent n (16)**	χ^2/t	***P* value**
Age (years):					
	Mean ± SD	39.323 ± 5.649	38.125 ± 4.047	0.759	0.451
	(Range)	(30 - 52)	(33 - 47)		
Sex	n (%)				
	Male	16 (47.05)	6 (37.50)	0.403	0.525
	Female	18 (52.94)	10 (62.50)		
HPV-6	n (%)				
	Negative	19 (55.88)	14 (87.50)	4.847	**0.028**
	Positive	15 (44.11)	2 (12.50)		

HPV-6: human papilloma virus subtype 6; LPR: laryngopharyngeal reflux

There was a statistically significant association between the presence of HPV-6 and both reflux severity index (RSI) and reflux finding score (RFS) (Table 3).

Table (3): Comparison between HPV-6 negative and positive cases

Variant	HPV-6		Test	
	Negative n (33)	Positive n (17)	χ^2/t	*P* value
(years):				
Mean ± SD (Range)	39.151 ± 4.981 (30 - 52)	38.529 ± 5.680 (32 - 49)	0.399	0.692
n (%) Male Female	 15 (45.45) 18 (54.54)	 7 (41.17) 10 (58.82)	0.083	0.773
n (%) Yes No	 19 (57.57) 14 (42.42)	 15 (88.23) 2 (11.76)	4.847	**0.028**
Mean ± SD (Range)	11.969 ± 4.882 (6 - 24)	15.176 ± 4.489 (8 - 25)	2.258	**0.028**
Mean ± SD (Range)	8.636 ± 3.879 (3 - 16)	12.882 ± 3.805 (5 - 18)	3.689	**0.0006**

HPV-6: human papilloma virus subtype 6; LPR: laryngopharyngeal reflux; RFS: reflux finding score; RSI: reflux symptom index

DISCUSSION

The latent and subclinical infections of human papillomavirus (HPV) have gained more interest, yet there is little information about the prevalence of latent HPV infections in the larynx [12]. It is clear that low-risk HPV infection is the predominant causative agent of juvenile laryngeal papillomatosis, but in adult-onset laryngeal papilloma, the condition is usually multifactorial [3,18]. On the other hand, the vocal fold mucosa in adult patients without papillomas (without morphological evidence of a HPV-associated lesion), but suffering chronic laryngitis, vocal cord nodules, or polyps could have detectable human papilloma viral genome by PCR [12].

Chronic laryngeal irritation, irrespective of the specific irritant, may lead to morphologic changes in the larynx including polyp formation. Laryngopharyngeal reflux (LPR) irritates the laryngopharyngeal mucosa causing symptoms and signs of chronic reflux laryngitis. The prevalence of LPR has been constantly increasing nowadays affecting high percentage of the general population [7].

The study of **Formánek *et al.*** [19]**,** stated that the LPR might be a risk factor for adult laryngeal papilloma or polyps by activating or reactivating a latent HPV infection. They recommended additional studies with larger cohorts to confirm their findings and to clear up the mechanisms involved. The aim of the present work was to study the important roles of laryngopharyngeal reflux and human papilloma virus in the development of adult vocal fold polyps and to study the possible association between LPR and HPV in this entity through assessment of the prevalence of human papillomavirus (HPV) in patients presented with adult vocal fold polyp and suffering from laryngopharyngeal reflux (LPR).

In the present study, we included 50 patients with adult focal fold polyps, the age of patients ranged from 30 to 52 years with a mean age of 38.94 ± 5.18 years, twenty-eight of the patients were females (56%) and 22 were males (44%). This matches with **Martins *et al.*** [20] who studied the clinical and morphological characteristics of 76 patients with vocal fold polyps and found that the age ranged between 21 and 60 years with male to female ratio represented 43% to 57%. Our results also matches with **Yun *et al.*** [21] who studied 175 patients with vocal fold polyps, 58.8% were female and 41.2% were male, their age ranged between 24 to 78 years. This means that adult vocal fold polyps do occur in a wide age range and not gender related.

In the present study, the prevalence of laryngopharyngeal reflux (LPR) among the patients with adult vocal fold polyp was (68%) and was not related to age or gender. This goes in line with the studies of **Beltsis *et al.*** [7] and **Chung *et al.*** [22], both used pH monitoring for diagnosis of the reflux and both studies found that LPR presented in (75%) of patients with true adult vocal fold polyps. Also, **Wang *et al.*** [23]

who studied 32 patients submitted to laryngeal surgery for vocal polyps, revealed a significantly higher presence of pepsin (75%) in patients with vocal polyps when compared with the control group (31.25%).

In our study we used the reflux symptom index (RSI) and the reflux finding score **(**RFS) as clinically non-invasive simple diagnostic tools of LPR. Although 24-h double-probe ambulatory pH monitoring is considered the gold standard for LPR diagnosis, but number of studies have been able to validate the RSI and RFS assessments as diagnostic tests of LPR [14,16,24]**.**

Regarding HPV genome detection, we found 17 samples representing (34%) of the studied patients had positive HPV-6 while no one (0%) had positive HPV-11. Our results were higher than **Rihkanen *et al.*** [12] who detected the virus in the epithelium of (19%) of patients operated for chronic laryngitis, nodules, and polypi without laryngeal papillomas. The prevalence of HPV in our study was comparable to previous studies on adult papillomas as **Makiyama *et al.*** [25] who found that (46.1%) of patients with adult laryngeal papilloma were positive for HPV-6 and (7.6%) were positive for HPV-11, but their study included relatively smaller number of participants (13 patients). **Hirai *et al.*** [26] found a higher prevalence of low-risk HPV (66.7%) in adult patients with laryngeal papillomas. The fact that we found the rates of occurrence of the virus in cases of polyps approximates to some extent the rates of occurrence in cases of adult papillomas, this indicates the presence of latent virus in the laryngeal mucosa.

From the results of our study, it seems that there is a strong relation and association between detection of HPV-6 genome and LPR in cases with adult vocal fold polyps. Laryngopharyngeal reflux was present in 15 out of 17 (88.23%) patients with positive HPV-6. Supporting this association in the present study, there were statistically significant higher reflux severity index (RSI) and reflux finding score (RFS) among patients with positive HPV-6. **Formánek *et al.*** [19] noted the association between the laryngopharyngeal reflux (pepsin in tissue) and human papilloma virus detection in adult laryngeal papilloma. They studied 20 recurrent cases of vocal papilloma, HPV was found in all of the biopsies, and pathologic LPR was diagnosed in (40.0%), therefore they might be possible risk factors for this disease.

Previous studies suggested a link between the presence of extraesophageal acid reflux disease and recurrent respiratory papillomatosis in children [27,28]. They hypothesized that the inflammation induced by chronic acid exposure may result in the expression of HPV in susceptible tissues. Our findings in adult vocal fold polyps regarding the significant detection of HPV-6 genome in (44.11%) of patients with LPR, support this hypothesis in adults. Laryngopharyngeal reflux may be one of the factors which lead to human papilloma virus activation. The detection of HPV genome in the excision biopsies of adult vocal fold polyps may be useful as an etiological factor particularly if associated with symptoms and signs of laryngopharyngeal reflux.

CONCLUSION

Both laryngopharyngeal reflux and human papilloma virus play important roles in the development of vocal fold polyps in adults and there is a possible association between LPR and HPV in this entity as the prevalence of human papillomavirus (HPV) in patients presented with adult vocal fold polyp and suffering from laryngopharyngeal reflux (LPR) was significantly higher than that in patients not suffering LPR. There were statistically significant higher reflux severity index (RSI) and reflux finding score (RFS) among patients with positive HPV-6.

Based on these findings and results, future studies are recommended in the direction of possible adjuvant antiviral therapy after excisions of adult vocal fold polyps particularly if associated with symptoms and signs of laryngopharyngeal reflux.

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Availability of data and material: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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