Laryngeal Papillomatosis: Molecular, Ultrastructural and Clinical Evaluation

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Abstract

Our study was designed to correlate the prevalence of human papilloma viruses (HPV) types 6 and 11 in laryngeal papillomatosis specimens using PCR to the clinical and pathological characteristics of these patients as a trial to evaluate the usefulness of HPV typing in identifying which patients are at a higher risk for more frequently recurring aggressive disease or future malignant. This study was conducted on 36 adult patients who were identified clinically and histopathologically as laryngeal papilloma. An index of the number of procedures (laryngoscopy and carbon dioxide laser ablation) needed per year for each patient was calculated to assess the frequency of recurrence and disease aggressiveness. They were subjected to the detection of HPV6 and 11 DNA by PCR and electron microscopic examination of biopsies that were taken from laryngeal papillomas and non diseased sites as controls. We found that all papillomas (100%) were positive for HPV, among them the positive rate of either HPV6 or HPV11 was 25% (9/36) and 41.7% (15/36) respectively, the positive rate of mixed types of HPV6 + 11 was 33.3% (12/36). Percentage of Patients needed >3 Pr/y were significantly increased (p>0.05 & 0.01) in cases with mixed infection of both HPV6+11 in comparison to those infected with HPV 11 or HPV6 respectively and in HPV 11 infected cases in comparison to those infected with HPV 6 (p<0.05).

Percentage of cases revealed dysplastic features (cellular atypia, invasion, keratinization and necrosis) were significantly increased (p<0.05 & 0.01) in mixed infection of both HPV6+11 in comparison to those infected with HPV 11 or HPV6 respectively and in HPV 11 infected cases in comparison to those infected with HPV 6 (p<0.05).

Correlation study revealed direct correlation between percentage of patients needed >3 Pr/y and the appearance of dysplastic features.

Our findings suggest that viral typing is one marker that may be useful to identify patients at a higher risk for aggressive disease and, possibly, to malignant potential but more studies with a larger number of cases are needed to assess their diagnostic and prognostic value.

Key Words: Laryngeal papillomatosis – HPV – Electron microscope.

Introduction

THE papilloma is one of the most common benign neoplasms of the larynx. Laryngeal papillomatosis is a disease of widespread papilloma formation that most commonly affects the larynx but may involve multiple areas of the aerodigestive tract. Typically, onset of disease during childhood is associated with much more aggressive disease. The etiology of this disease is the human papillomavirus especially types 6 and 11 [1]. Little is known about immunological mechanisms involved in laryngeal HPV infection, but in defense against HPV cellular immunity is considered a more important mechanism than humoral immunity [2].

The diagnosis is often delayed because the symptoms mimic a variety of other diseases [3].

The clinical course is unpredictable; spontaneous regression may occur following a few treatments. However, in other cases, the disease has a more aggressive course, recurs more frequently, and can extend well into adult life, requiring repeated surgical procedures. In a small number of patients, the disease extends to the tracheobronchial tree and may develop into squamous cell carcinoma [4].

Despite the low incidence of these diseases, both juvenile onset and adult onset laryngeal papillomatosis continue to concern otolaryngologists because treatment is often prolonged and unsatisfactory. Over many years various treatments have met with varied claims of success, yet no treatment today is universally accepted [8].

Recurrent Respiratory Papillomatosis is a rare viral disease characterized by multiple benign growths (papillomas) in the middle and lower respiratory tract. Symptoms usually begin with hoarseness and/or a change in voice. The growths
Laryngeal Papillomatosis may be surgically removed, but frequently recur and may require additional surgery. Affected individuals may experience long periods without recurrence (remission) and/or the disease may disappear completely. Children under five years of age are most commonly affected, although adults represent about one-third of all documented cases. People with Recurrent Respiratory Papillomatosis may have difficulty breathing (dyspnea) and/or experience other life-threatening complications if the papillomas block the airway [6].

Among the numerous methods for detecting HPV DNA sequences in tissue biopsies, the in vitro gene amplification technique of PCR was chosen because it represents the most sensitive and specific method [7].

The goal of this study was to evaluate the usefulness of HPV typing in identifying patients with a higher risk for recurrence of laryngeal papillomatosis and malignant potential.

**Material and Methods**

This study was conducted on twelve patients admitted to ENT department of Kasr El Aini hospital, their ages ranged from 16 to 27 years with a mean of (18±3.61). They were 24 males & 12 females and diagnosed clinically by different symptoms and/or signs include hoarseness, stridor, dyspnea and cyanosis, laryngoscopic examination and then histopathologically of biopsies. An index of the number of procedures (laryngoscopy and carbon dioxide laser ablation) needed per year for each patient was calculated to assess the frequency of recurrence and disease aggressiveness. Molecular and ultrastructural studies were carried out on biopsy specimens and cellular scrapes that were taken from either laryngeal papillomas or the non diseased sites as controls.

**Sample collection:** Biopsies were divided into two pieces: One piece was kept in glutaraldyde and cacodylate for EM examination and the other piece was directly stored at -70°C till used to be examined by PCR technique for the presence of HPV6,11 DNA.

1- Detection of HPV6,11 DNA by PCR Technique in Laryngeal Biopsies:

**I- DNA Extraction:**

From biopsies according to Ausubel et al. [8] using proteinase K in presence of 1% SDS, phenol-chloro-form extraction and ethanol precipitation.

II- **PCR Amplification:**

- Primers used: The two sets of primers specific for E6 region of HPV-6,11 DNA would amplify and detect a specific band of the expected size 130bp supplied at concentration of 100pmol/µl (Biometra. Germany). Their sequences according to the previously published data [9] are as follow:
  - P6,11 F: (5'-AAGGGCGTAACCCGAATCGGT-3')
  - P6, 1 1R: (5'-GTTTGCAGGCTCTGTG CATA-3')
  - P6, 1 1F and p6, 1 1R correspond to the E6 sense sequence and to the E6 antisense sequence of HPV-6,11 respectively.

- Amplification: It was carried out in a programmable Thermal Cycler 480 (Perkin Elmer-Norwalk, CT) with the following protocol: After one cycle of denaturation at 94°C for 10min. 30 cycles of 94°C for 1min. 55°C for 2min. and 72°C for 2min. were carried out followed by extension at 72°C for 10min.

III- **Electrophoretic separation and identification:**

Ten microliters of PCR product was electrophoresd at constant current 100 volt for 45min. through 2.5% agarose gel stained with ethidium bromide (0.5mg/ml). Bands were visualized under ultraviolet illumination and photographed in parallel with a negative control in which PCR reaction was performed without a template DNA.

2- Electron microscopic studies for detection of HPV affection:

Biopsies were put in fixative solution 4% glutaraldehyde with sodium cacodylate, fixed in 2% osmium tetraoxide, dehydrated with ascending concentration of alcohol and embedded in epoxy resin according to the technique of [10]. Semi-thin section one-micron thickness were performed by ultra microtome from the made capsules and stained with methylene blue azur II and examined by light microscopy to choose the areas of ultra thin section which were mounted on copper grids and double stained with uranyl acetate and lead citrate and examined by EM.

**Statistical methods:**

Version 10 Statistical Package for Social Science software (SPSS Inc., Chicago, IL) was used to analyze the data. Univariate analysis was performed using Chi-square to find out the relation between various qualitative data. Pearson’s $\chi^2$ test with continuity correction and Mann-Whitney test was used to assess correlations between percentage of patients needed >3 Pr/y and the appearance of dysplastic features.
Results

Laryngoscopic examination revealed multiple lesions that have cauliflower-like appearance in the air passages. Solid or cystic granular parenchymal lesions could be detected Fig. (1). Relation between percentage of patients needed >3 Pr/y, appearance of dysplastic features and Human Papilloma virus type is detected in Table (1).

HPV6 & 11 DNA analysis:

Representatives of PCR results for HPV6,11 in laryngeal papillomas biopsies are illustrated in Figs. (2,3) and Table (1).

All papillomas (100%) were positive for HPV, among them the positive rates of HPV6 and HPV 11 were 25% (9/36) and 41.7% (15/36) respectively, the positive rate of mixed types of HPV6+11 was 33.3% (12/36).

All patients had mixed infection of both HPV6+11 (33.3%) and 60% of patients had HPV-11 (25%) needed more than 3 Pr/y.

So, percentage of patients needed >3 Pr/y were significantly increased (p<0.05 & 0.01) in cases with mixed infection of both HPV6+11 in comparison to those infected with either HPV11 or HPV 6 respectively and in HPV 11 infected cases in comparison to those infected with HPV6 (p<0.05).

Electron microscopic study of laryngeal papilloma:

Examination of semi thin section of the biopsies of laryngeal papilloma showed well-differentiated, non-keratinizing squamous epithelium covering a delicate, fibrovascular core in all cases infected with HPV-6 (25%) and in 40% of cases infected with HPV-11 (16.6%) Fig. (4). Features of dysplasia, such as cellular atypia, invasion, keratinization and necrosis could be detected in 60% of cases infected with HPV-11 (25%) and in all cases had mixed infection (33.3%) Fig. (5).

Ultrathin section revealed fibrovascular core of laryngeal papilloma infected with the viral particles Figs. (6,7). Viropathic changes (koiocytic cells) which suggest the diagnosis could be detected in all the cases Fig. (8). Three of the patients who had HPV-11 (16.6%) and all the patients had mixed infection with HPV-6&11 (33.3%) showed cell necrosis Fig. (9) and reactive fibrosis Fig. (10).

Percentage of cases revealed dysplastic features were significantly increased (p <0.05 & 0.01) in mixed infection of both HPV6+11 in comparison to those infected with HPV 11 or HPV6 respectively and in HPV 11 infected cases in comparison to those infected with HPV6 (p<0.05).

Correlation study revealed direct correlation between percentage of patients needed >3 Pr/y and the appearance of dysplastic features.

Fig. (1): The lesions are multiple and have a cauliflower-like appearance in the air passages as detected by laryngoscopic examination.

Fig. (2): Electrophoretic analysis of HPV-6 amplification products. The 120bp product was obtained. Lanes 1,12: DNA standard size marker. Lanes 10,11: Positive and negative controls respectively. Lanes 2-9: Laryngeal biopsies specimens positive for HPV-6.

Fig. (3): Electrophoretic analysis of HPV-11 amplification products. The 130bp product was obtained. Lanes 1,12: DNA standard size marker. Lanes 10,11: Positive and negative controls respectively. Lanes 2-9: Laryngeal biopsies specimens positive for HPV-11.
Fig. (4): Semithin section of laryngeal papilloma infected with HPV-6. Laryngeal papilloma is sessile (P) with well-differentiated, non-keratinizing squamous epithelium (arrow) covering a delicate, fibrovascular core (C) (stained with methylene blue azur II) (X100).

Fig. (7): A-Ultrastructure of the fibrous core of laryngeal papilloma infected with viral like particles (arrow) (X5000).

Fig. (5): Semi thin section of laryngeal papilloma infected with mixed infection with the both HPV-6&11. Features of malignancy, such as cellular atypia, invasion, keratinization and necrosis could be detected. (stained with methylene blue azur II) (X100).

Fig. (8): Electon micrograph of koilocytic cell (KC) (viropathic effect) in laryngeal papilloma infected with HPV which suggests the diagnosis (X2800).

Fig. (6): Electron micrograph of fibrovascular core of laryngeal papilloma infected with HPV-6 (X5000).

Fig. (9): Necrotic cell in laryngeal papilloma infected with viral like particles (arrow) (X2800).
Fig. (10): Reactive fibrosis (F) and viral particle (arrow) in laryngeal papilloma infected with HPV-11 (X2800).

Table (1): Relation between percentage of patients needed >3 Pr/y, appearance of dysplastic features and Human Papilloma virus type.

<table>
<thead>
<tr>
<th></th>
<th>HPV-6 n=9/36 (25%)</th>
<th>HPV-11 n=15/36 (41.7%)</th>
<th>Mixed n=12/36 (33.3%)</th>
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<tr>
<td></td>
<td>Number of cases</td>
<td>Percent</td>
<td>Number of cases</td>
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<tr>
<td>&gt;3 Pr/y (%) (n=36)</td>
<td>0</td>
<td>0</td>
<td>9</td>
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<tr>
<td>Dysplasia (%) (n=36)</td>
<td>0</td>
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<td>9</td>
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** Significant increase in comparison to HPV-6 p<(0.01).
+ Significant increase in comparison to HPV-11 p<(0.05).
* Significant increase in comparison to HPV-6 p<(0.05).

**Discussion**

Laryngeal papillomatosis is the most common benign tumor of the larynx in children [11]. The disease is induced by human papillomavirus (HPV), especially types 6 and 11 [12] and is characterized by its recurrence. The clinical course is unpredictable and spontaneous regression may occur following a few treatments. However, in other cases, the disease may have a more aggressive course, recurs more frequently and can extend well into adult life, requiring repeated surgical procedures. In a small number of patients, the disease extends to the tracheobronchial tree and may develop into squamous cell carcinoma [4].

Our study was designed to estimate the prevalence of human papilloma viruses (HPV) types 6 and 11 in laryngeal papillomatosis specimens using PCR and to correlate the presence and type of HPV detected with the clinical and pathological characteristics of these patients as a trial to identify which patients are at a higher risk for either more frequently recurring aggressive disease or future malignant transformation. So, the goal of this study was to evaluate the usefulness of HPV typing in identifying patients with a higher risk for recurrence of laryngeal papillomatosis and malignant potential.

This study was conducted on 36 adult patients admitted to ENT department of Kasr El Aini hospital, who were identified clinically and histopathologically as laryngeal papilloma. They were subjected to the detection of HPV6 and 11 DNA by PCR and electron microscopic examination of biopsies that were taken from either laryngeal papillomas or the non diseased sites as controls.

Typing techniques, such as in situ hybridization and immunohistochemical methods for detection of virus antigen on archival material, are not readily able to differentiate between closely related virus types such as HPV-6 and HPV-11. Typing by polymerase chain reaction eliminates this problem and may be helpful in identifying patients with aggressive recurrent laryngeal papillomatosis [6].

In our study we found that the positive rate of HPV in laryngeal papilloma was 100%, the sequences of HPV6 were detected by PCR in 9 biopsies of the 36 patients (25%), 15 had HPV-11 (41.7%) and 12 biopsies had mixed infection of both HPV-6 and HPV-11 (33.3%). These finding are nearly similar to the results of Smith et al. [13] who found that all papillomas (100%) were positive for HPV either type 6 or 11 with a ratio of 25% to 50% respectively.
There are contradictory reports on the incidence of HPV in laryngeal papilloma. Chen et al. [14] found that the positive rate of HPV in laryngeal papilloma was 91.4% (30/35). Among them the positive rates of HPV6 and HPV11 were 54.2% (19/35) and 25.7% (9/35) respectively, the positive rate of multiple types of HPV6+11 was 11.4% (4/35). While Gabbott et al. [15] found that forty-four of the 47 had HPV-induced papillomas, with type 11 accounting for 24 (55%) and type 6 accounting for 19 (43%); one (2%) was positive for either type 6 or 11.

This study revealed direct correlation between patients needed >3 Pr/y and the appearance of dysplastic features such as cellular atypia, invasion, keratinization and necrosis. Our results in agreement with previous studies which have shown that severe cytologic atypia and dysplasia correlate with frequently recurring lesions [16]. Rahbar et al. [17] suggesting the important role of vascular endothelial growth factor-A in the pathogenesis of recurrent respiratory papillomatosis while Wiatrak [18] found that multiple defects in cell-mediated immunity, may be one of the main causes of its recurrence so the immune system modulation and augmentation may be the potential future treatment to better control this disease process.

In this study percentage of patients needed >3 Pr/y and revealed dysplastic features were significantly increased ($p<0.05$) in HPV11 infected cases in comparison to HPV6 infected ones that may be due to specific HPV-11 genome mutations, such as duplications of the upstream regulatory region [19] or coinfection with other potentially tumorigenic viruses that have been suggested to play a role in HPV-associated cancers [20]. Our results are not in agreement with Lin et al. [21] who found that human papillomavirus (HPV) types 6 and 11 have been associated with benign laryngeal papilloma, while HPV-16 is occasionally associated with laryngeal carcinoma. However, Morshed et al. [22] could not demonstrated any significant correlation between HPV incidence and histological grading and clinical staging. They also found that malignant transformation is, most likely, the result of multiple mutational events and not merely dependent on a single factor such as virus type and other factors are related to HPV-associated malignancy such as persistent infection, radiation exposure, smoking and immunosuppression.

Percentage of patients needed >3 Pr/y and revealed dysplastic features were significantly increased in cases with mixed infection of both HPV6+11 ($p<0.05$ & 0.01) in comparison to those infected with either HPV 11 or HPV 6 respectively. This result goes in parallel with Doyle-Lloyd and Gianoli [23] who reported that the presence of more than one type of HPV in the same tissue associated with more aggressive disease while McClay [24] found that a change in the HPV from a type observed in benign lesions (i.e., type 6) to a type present in malignant lesions occurred when the papilloma converted to an SCC.

Our findings suggest that viral typing is one marker that may be useful to the pathologists and treating physicians attempting to identify patients at a higher risk for aggressive disease and possibly, to malignant potential, but more studies with a larger number of cases are needed to assess their diagnostic and prognostic value.

References


