Genotyping of human papillomaviruses in patients with Recurrent Respiratory Papillomatosis in Firouzgar Hospital, Tehran, Iran

Abstract

Recurrent Respiratory Papillomatosis (also known as Laryngeal Papillomatosis) is a benign and sporadic tumor that primarily affects children and is caused by the papillomavirus. The estimated prevalence of this condition is approximately 4 cases per 100,000 children and 2 cases per 100,000 adults. Human papillomavirus types 6 and 11, which are commonly associated with genital warts, are the predominant strains implicated in this disease. The most common symptoms include airway obstruction, voice disturbances, and difficulty speaking. These lesions are typically integrated and are rarely observed individually; they sometimes progress toward malignancy. A total of thirty-one laryngeal samples from patients with a positive pathological response to recurrent respiratory papillomatosis RRP were collected from the hospital's ear, nose, and throat department. Each sample was preserved in formaldehyde and embedded in paraffin blocks. Accompanying the samples was a form containing detailed patient information. After analyzing the presence of the β-globulin gene in the DNA of the samples, specific primers (MY09/11 and GP5+/6+) were employed to detect the presence of human papillomavirus (HPV). Among the 31 samples, 29 were found to contain the HPV genome, with HPV-6 identified in 13 samples and HPV-11 in 16 samples. The phylogenetic tree of isolated HPV viruses was subsequently plotted. Statistical analyses revealed no significant difference in the incidence of HPV viruses between men and women, nor in the incidence of RRP. However, a significant correlation was identified between residing in suburban areas, low income, and welfare levels, and the incidence of RRP. Additionally, the research indicated that RRP lesions predominantly affect pediatric patients, with only a

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small percentage of adults being affected. Further extensive studies are necessary to elucidate the main risk factors associated with RRP patients.

Keywords: recurrent respiratory papillomatosis, papillomavirus, larynx, tumor, types 6 and 11

Introduction

There are two types of RRP, also known as peripheral papillomatosis or laryngeal papillomatosis: 1. Juvenile Onset RRP (JORRP) and 2. Adult Onset RRP (AORRP). RRP is the most common benign laryngeal tumor in children and the second leading cause of voice disorders in this age group. Its prevalence is estimated at 4 cases per 100,000 children and 2 cases per 100,000 adults. The highest incidence rates have been reported in the United States, Canada, Norway, Denmark, South Africa, and Australia (1). The disease is caused by the human papillomavirus (HPV), which belongs to the *Papillomaviridae* family. *Papillomaviridae* is a family of small, non-enveloped DNA viruses with an icosahedral capsid. The viruses in this family primarily target epithelial tissues in humans and other animals. To date, more than 200 genotypes of human papillomavirus have been identified based on the L1 gene sequence, which encodes the major capsid protein. These genotypes are classified into five genera: Alpha, Beta, Gamma, Mu, and Nu (2, 3). More than 90% of RRP cases are attributed to types 6 and 11, as well as types 16, 18, 31, and 33, all of which carry a high carcinogenic risk. Other types are rarely associated with this condition. The lesions associated with the disease appear as white to pinkish tumors. Despite their benign histological characteristics, they have a tendency to spread to the airways and recur (4, 5). The primary treatment for this surgical disease involves the use of a CO_2 laser for the precise removal of affected areas while preserving the integrity

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of the vocal cords. However, due to the recurrent nature of the disease, frequent and periodic surgeries are often necessary. On average, many children undergo approximately 13 surgical stages throughout the duration of the disease. Additionally, the use of interferon alpha, cidofovir, and bevacizumab (a humanized anti-HPV monoclonal antibody) can help reduce lesion growth and shorten the duration of treatment (6, 7). In some cases, the administration of the HPV vaccine following surgery is advised to decrease the likelihood of lesion recurrence. The incidence of RRP is higher in children and men. Additionally, juvenile-onset RRP tends to be more aggressive, with a higher likelihood of recurrence. Due to the non-specific symptoms and the rarity of the disease, many patients are often misdiagnosed and treated inappropriately for extended periods. In affected children, a maternal history of genital warts is a significant risk factor, as it is believed that the infection typically develops during passage through the birth canal, where contact with infected genital areas occurs (8, 9). In a study conducted in Denmark, nearly 1% of children whose mothers had a history of genital warts developed RRP and exhibited a 231-fold increased risk of lesions compared to children whose mothers had no history of genital warts. Although intrauterine transmission of RRP-associated papillomaviruses is possible, it is uncommon; consequently, the occurrence of this lesion is rare in children born by cesarean surgery. In addition to maternal history of genital warts, other risk factors for RRP include being a firstborn child, low maternal age, low socioeconomic status of the mother, and polymorphisms of Class II HLA antigens in the child (1). In comparison, HPV-6 is more commonly associated with genital warts, while HPV-11 is more likely to induce laryngeal lesions in cases of RRP. Children under the age of three exhibit a greater extent and severity of lesions. Observations indicate that intervals of up to five years may occur between the initial exposure to the virus

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during childbirth and the subsequent development of lesions in children. The likelihood of RRP lesions disseminating to the bronchi and lungs, progressing to severe dysplasia, or leading to cancer remains low. The risk of developing RRP lesion malignancy is estimated to be less than one percent in children and less than five percent in adults. The risk of malignancy increases with tobacco use, the use of cytotoxic drugs, and exposure to X-rays. Most HPV-related laryngeal cancers occur in patients who do not have RRP but are infected with high-risk human papillomaviruses (HPVs), particularly HPV-16 (11). Due to the limited information and research on this disease in Iran, this study was conducted to investigate its history in the country.

2.Materials and methods

2.1. Sample Collection

Thirty-one laryngeal samples (Figure 1) from patients with a positive diagnosis of RRP were collected from the Ear, Throat, and Nose Department of Firozgar Hospital in Tehran between 2002 and 2009. These samples, preserved in formaldehyde and embedded in paraffin blocks, were examined in this study. Patient information, including age, gender, initial symptoms, age at first surgery, number of surgeries, intervals between surgeries, affected areas, history of tracheostomy, treatment with interferon, and recovery status, was also retrieved from the hospital archives.

2.2. Sample Preparation

Samples were separately taken from each of the paraffin blocks, and the paraffin degradation process was conducted using xylenol and pure ethanol.

2.3. DNA extraction

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Using the FavorPrep FFPE Tissue DNA Extraction Micro Kit (Taiwan), genomic DNA was extracted from tissue samples and stored in a -80°C freezer. The Thermo Scientific NanoDrop 1000 (Waltham, USA) was utilized to measure the purity of the extracted DNA, specifically by assessing the OD 260/280 ratio.

2.4. Quality control of extracted genomic DNA

For internal control of extracted DNA, the B-globin gene was amplified using proprietary primers PCO3: 5' - ACACAACTGTGTTCACTAGC-3 'and PCO4: 5' - CAACTTCATCCACGTTCACC - 3' (12, 13). PCR was performed in a final reaction volume of 12 microliters, containing 100-500 nanogram of extracted DNA, six microliters of Master Mix (Sigma Aldridge, St. Louis, USA), one microliter of each primer and DDW (Double Distilled Water). The PCR cycling conditions were 95°C for 4 minutes, followed by 38 cycles of 95°C for 60 seconds, 58°C for 45 seconds, and 72°C for 45 seconds, and a 10 minute final extension at 70°C. The products of PCR were electrophoresed on a 1.2% agarose gel, and visualized using the gel documentation system - Image capture (Biometra, Germany).

2.5. Molecular detection of HPV by Nested PCR

A nested PCR method was employed to enhance the sensitivity of HPV-DNA detection from samples. The MY09/11 primer pairs (external primers) used were MY09: 5' - CGTCCAAGGAACTGATC - 3' and MY11: 5' - GCCAGGGCTATAAAATGC - 3', along with the GP5+/6+ primer pairs (internal primers) GP5+: 5' - TTTGTTACTGTGTAGATACTACTACTAC - 3' and GP6+: 5' - AAAAATAAACTGTAAATCATATTC - 3'. These primers were utilized for the detection of the HPV L1 gene (12, 13). They are general consensus primers that targeted a conserved segment of the L1 gene of the viral capsid and detecting all HPV types. The MY09/11 primer pairs amplify a 450 bp fragment, while the

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GP5+/6+ primer pairs amplify a 150 bp fragment located within the inner part of the MY09/11 amplicon. Both outer and inner PCR reactions were carried out in a total content of 12 microliters containing 100-500 nanogram of extracted DNA, six microliters of Master Mix, one microliter of each primer and sterile DDW. For the second step of amplification (inner reaction), 0.5 microliters of the first PCR product was utilized as DNA template. The PCR cycling conditions were 94°C for 4 minutes, followed by 35 cycles of 95°C for 60 seconds, 56°C for 60 seconds, and 72°C for 60 seconds, and a 10 minute final extension at 70°C. A same thermal program was used for nested round using GP5+/6+ primers, except the annealing temperature that changed to 58°C. The products of PCR were electrophoresed on a 1.2% agarose gel, and visualized using the gel documentation system - Image capture (Biometra, Germany).

2.6. Genotype determination and genetic analysis

Purification of positive PCR products was done with a PCR purification kit (Favorgen Co., Taiwan) and sent to the Pishgam company for bidirectional sanger sequencing. The sequences were edited, aligned, and analyzed utilizing Chromas (Version 2.5, San Francisco, CA, USA), Bioedit (Version 7.2, Stockport, UK), and EditSeq (Version 7, Madison, WIS, USA) softwares and compared with other HPV sequences submitted in GenBank utilizing the Basic Local Alignment Search Tool (BLAST, NCBI). To examine the phylogenetic relationship between HPV samples and sequences recorded in GeneBank, the sequences were taken from the NCBI site and examined with Bioedit and Lazergene softwares. The Neighbour Joining method was used to generate a phylogenetic tree using the MEGA X software (20) and 1000 bootstrap replicates.

2.7. Statistical analyzes

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Data obtained from patients were statistically examined using SPSS (Version 16, Chicago, IL, USA) statistical software, employing Chi-square and Fisher test.

3. Results

3.1. Quality Control of the extracted genomic DNA

All 31 samples in this experiment tested positive for the presence of the Globulin-β gene using PCO3/4 primers, and their quality was confirmed through proper extraction of genomic DNA.

3.2. Nested PCR result, sequencing, and genotype determination

Altogether, 29 out of 31 quality-confirmed samples were evaluated as positive using the Nested PCR method, indicating the presence of the HPV genome in these samples. The nested PCR products of L1 amplification were used for genotyping through direct bidirectional sequencing. All 29 positive samples were sequenced. The obtained sequences were submitted to the BLASTN server of NCBI to identify the most similar sequences. The homologous HPV types were analyzed using MEGA software for phylogenetic analysis, and the results of the typing are presented in a phylogenetic tree (Figure 2). Additionally, the homology of the isolated HPV sequences (RRP 1-31) compared to GenBank reference sequences is illustrated in Figure 3. Furthermore, the genotype analysis of the 29 HPV samples revealed that 13 samples (44.8%) were identified as HPV-6, while 16 samples (55.2%) were identified as HPV-11 (Figures 3).

3.4. Statistical results of patients

In terms of gender distribution, there were 17 male and 14 female patients. The youngest age at the onset of symptoms was four months, while the oldest was 426 months (approximately 35 years). Due to the wide age range among patients, the

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average age at the time of the first symptoms was approximately 51.37 months (around four years and three months). The earliest age for the first surgery was 12 months, and the latest was 426 months (approximately 35 years), with an average age of 31 months at the time of the first surgery.

Among the patients who underwent tracheostomy, there was an equal distribution of gender, with 50% male and 50% female. In the case of conflict zones within the larynx, among the three patients with isolated glottis conflict, there were two female patients and one male patient. Of the five patients with isolated supraglottic conflict, four were female and one was male.

Among the eight patients who experienced simultaneous glottis and supraglottic conflicts, six were male and two were female. The two patients with simultaneous glottis and subglottic conflicts were both male. Additionally, of the 13 patients who had simultaneous conflicts involving the supraglottis, glottis, and subglottic, eight were female and five were male. In the context of interferon therapy, the highest rate of treatment was observed when all three regions of the patient's larynx were affected, particularly in patients with HPV-11. Similarly, the highest rate of tracheostomy was performed under the same conditions, where all three areas of the larynx were involved. Notably, the highest incidence of tracheostomy occurred in children under the age of three. Furthermore, there was no significant difference in the presence of HPV-6 and HPV-11 concerning the tracheostomy rate. In the analysis of HPV typing by sex distribution, 53.8% of men and 46.2% of women were infected with HPV-6, while 50% of both men and women were infected with HPV-11. For both HPV-6 and HPV-11, the primary complaint among patients was auditory congestion (see Tables 1 and 2).

Statistical analyses conducted in this study revealed no significant difference in the incidence of HPV typing viruses between men and women, nor in the development

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of RRP lesions in either gender. Additionally, there was no difference in the presence of HPV-6 and HPV-11 in the laryngeal regions of patients. The statistical results indicated a significant correlation between residing in suburban areas and lower income and welfare levels with the incidence of RRP, corroborating findings from previous studies. Furthermore, consistent with both past internal and external research, RRP lesions predominantly affected pediatric populations, with only a small percentage of adults being impacted in this study.

4. Discussion

RRP is a sporadic condition; however, it is the most common benign tumor of the upper respiratory tract in children. RRP primarily affects the larynx but can also involve other areas of the respiratory tract. The prevalence of RRP ranges from 3 to 26 percent, although pulmonary involvement is rare, occurring in approximately 1 to 3 percent of cases. The disease generally affects both sexes equally, but it is observed to be twice as common in men compared to women (14).

Most cases of RRP occur before the age of five, with approximately 20% of cases arising in infancy. The overall incidence typically ranges from 1 to 17 months of age, and the average age at diagnosis is around 3.3 years. To manage RRP in children, multiple surgical interventions are often necessary, averaging about 13 procedures over the course of the disease. The onset of RRP in infants younger than six months can be life-threatening, and there have been reported cases of mortality associated with this condition (15, 16).

Shah and his colleagues first identified the epidemiological risk factors for RRP, which include: 1. Being the first child of a young mother with a low socioeconomic status, and 2. Having a natural childbirth (1). Since RRP is directly associated with human papillomavirus (HPV) genital infections in both adults and

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children, its prevalence is also influenced by the prevalence of HPV genital infections. Unfortunately, in Iran, there is no comprehensive estimate of the prevalence rate of genital HPV infection in the adult population. However, scattered reports indicate a high and increasing prevalence among adults, particularly among young people. In the United States, the prevalence of genital HPV infections is estimated to be between 10% and 20%. However, the prevalence of clinical manifestations, such as condyloma lesions, is significantly lower, estimated at approximately 1% among sexually active individuals (17). Epidemiological risk factors indicate that the presence of condyloma in the mother is a critical predictor of RRP in the child. The occurrence of condyloma during pregnancy suggests either a recent HPV infection or the reactivation of a previous infection, potentially triggered by hormonal changes in the mother's body. Additionally, the development and progression of RRP in a child are influenced by the mother's immunological response to HPV, her ability to transfer adequate antibodies to the child, and the child's genetic predisposition (18, 19). The age of the patient at the onset of RRP symptoms is a significant factor influencing the severity of lesions. Studies indicate that patients who exhibit manifestations of the disease before the age of five require more surgical interventions and have a higher incidence of tracheostomy. Additionally, these patients often necessitate more adjunctive treatments and experience elevated rates of pulmonary spread (20, 21).

HPV-6 and HPV-11 are the most common causes of RRP and belong to a low-risk carcinogenic group. A rare trend has been observed in some RRP lesions caused by HPV-11, which may progress toward malignancy. This progression has been associated with the integration of the viral genome into the host genome and mutations in the P53 gene. Additionally, lesions are more likely to spread to the

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lower respiratory tract during infections with HPV-11 compared to HPV-6 and other types (22).

Studies conducted by various researchers, both domestically and internationally, have yielded similar results. Eftekhaar and Karbalaie Niya (23) examined 12 patients with RRP aged 3 to 18 years between December 2014 and February 2017 at the University of Medical Sciences in Iran. They found that the lesions of nine patients contained HPV-6, two patients contained HPV-11, and one patient had lesions containing both HPV-6 and HPV-11. Similarly, Yamada and Itoh (16) studied 29 RRP patients in the Japanese city of Hamamatsu from September 2005 to June 2021, discovering that the lesions of 12 patients contained HPV-6, while seven patients had lesions containing HPV-11. Bertinazzi and Gheit (3) conducted a study involving 20 patients with RRP from October 2000 to October 2020 in the Italian provinces of Treviso and Belluno. They observed the following human papillomavirus (HPV) infections among the patients: four patients had HPV-6, three had HPV-11, three had both HPV-6 and HPV-11, three had both HPV-16 and HPV-11, one had both HPV-6 and HPV-17, one had both HPV-6 and HPV-19, two had both HPV-6 and HPV-111, one had all three HPV-6, HPV-11, and HPV-111, one had all three HPV-6, HPV-11, and HPV-100, and one patient had four types: HPV-6, HPV-11, HPV-21, and HPV-100. Nogueira and Küpper (24) conducted a survey of 41 patients with RRP aged 2 to 64 years in São Paulo, Brazil, from 2008 to 2015. They found that 30 patients were positive for HPV-6, while 11 patients tested positive for HPV-11. Bedard and Alarcon (25) studied 20 RRP patients aged 2 to 17 years in Cincinnati, Ohio, concluding that 16 patients were positive for HPV, with 14 specifically having HPV-6 and 2 having HPV-11. Amiling and Meites (26) examined 215 RRP patients with an average age of 4.5 years across 26 medical centers in 23 U.S. states from January 2015 to August 2020. Their

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findings revealed that 157 patients were positive for HPV, including 129 with HPV-6, 25 with HPV-11, 1 with HPV-16, and 1 patient who had both HPV-6 and HPV-44. Additionally, one patient was positive for both HPV-6 and HPV-54. Weiss and Heinkele (17) conducted a survey of 44 patients aged 2 to 77 with RRP in Germany from 2004 to 2013. They found that 32 patients tested positive for human papillomavirus (HPV), with 23 patients having HPV-6, six patients having HPV-11, and three patients exhibiting both HPV-6 and HPV-11.

Lepine and Leboulanger published a paper in 2024 indicating that the most promising results were observed regarding the impact of vaccination against HPV on the prevention and reduction of RRP cases in Australia. Since 2007, Australia has implemented a widespread HPV vaccination program using a quadrivalent vaccine, administering at least two doses to both boys and girls. Approximately 80% of girls and 75% of boys have been vaccinated. This vaccination initiative has led to a significant reduction in the RRP rate among young people, decreasing from 0.16 to 0.02 cases per 100,000 children between 2012 and 2016. Notably, no new RRP cases were reported over a two-year period, even from 2016 to 2022. Among the reported RRP cases in children from 2012 to 2016 in Australia, none of the mothers of these children had been vaccinated (27).

The present study also demonstrates the presence of HPV-6 and HPV-11 viruses in patients with RRP in Iran. This finding aligns with results obtained by other researchers in various regions of the world and across different time periods. Furthermore, previous research conducted both domestically and internationally indicates a clear correlation between the patients' income and welfare levels and the occurrence of RRP lesions. The disease is more prevalent in areas characterized by low income and welfare levels, with the majority of affected patients being children under five years of age.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors contribution

Mahsa Nadri performed the experiments, wrote the manuscript, contributed in the design of the study and funding acquisition. Rasoul Hamkar conceived the idea, contributed in the design of the study, funding acquisition and supervised the whole project. Pardis Khorami Shahveh cooperation in carrying out the laboratory process. Adel Hamidi wrote and edited the manuscript.

Data availability

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

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Table 1. Characteristics of the studied patients

Percent	Number of patients	Characteristics	
61.3	19	<3	**Age**
19.36	6	3-5	
9.67	3	6-10	
0	0	10-20	
9.67	3	>20	
54.84	17	Male	**Sex**
45.16	14	Female	
74.19	23	Yes	**Hoarseness**
25.81	8	No	
22.58	7	Yes	**Respiratory distress**
77.42	24	No	
25.81	8	<3	**Age of first surgery**
38.71	12	3-5	
19.36	6	6-10	
3.22	1	10-20	
12.9	4	>20	
70.97	22	Yes	**Interferon treatment**
29.03	9	No	
45.16	14	Yes	**Tracheostomy**
54.84	17	No	
41.94	13	HPV-6	**HPV Type**
51.61	16	HPV-11	
6.45	2	None HPV	
74.19	23	Yes	**Cured**
25.81	8	No	

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Table 2. Number of surgeries and involved areas

Percent	Number of patients		
9.67	3	2	**Surgery frequency**
6.45	2	3	
6.45	2	4	
9.67	3	5	
12.9	4	6	
6.45	2	7	
9.67	3	10	
6.45	2	11	
6.45	2	13	
6.45	2	15	
3.22	1	17	
3.22	1	21	
3.22	1	23	
3.22	1	24	
3.22	1	27	
3.22	1	30	
9.67	3	Glottis	**Involved areas**
16.13	5	Supraglottis	
25.81	8	Supraglottis + Glottis	
6.45	2	Glottis + Subglottis	
41.94	13	Supraglottis + Glottis + Subglottis	

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