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Original Article

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

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ABSTRACT

Recurrent Respiratory Papillomatosis (also known as Laryngeal Papillomatosis) is a benign, sporadic tumor primarily affecting children, caused by the papillomavirus. The estimated prevalence of this condition is approximately four cases per 100,000 children and two cases per 100,000 adults. Human papillomavirus types 6 and 11, commonly associated with genital warts, are the predominant strains implicated in the disease. The most common symptoms include airway obstruction, voice disturbances, and difficulty speaking. These lesions are typically integrated and rarely appear in isolation; in rare cases, they may progress toward malignancy. A total of thirty-one laryngeal samples from patients with a positive pathological diagnosis of 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. A form containing detailed patient information accompanied the samples. After confirming the presence of the β-globulin gene in the DNA of the samples, specific primers (MY09/11 and GP5+/6+) were employed to detect human papillomavirus (HPV). Among the 31 samples, 29 contained the HPV genome, with HPV-6 identified in 13 samples and HPV-11 in 16 samples. The phylogenetic tree of the isolated HPV strains was subsequently plotted. Statistical analyses revealed no significant difference in the incidence of HPV 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 status, and the incidence of RRP. Additionally, the research indicated that RRP lesions predominantly affect pediatric patients, with only a small proportion of adults affected. Further extensive studies are necessary to elucidate the main risk factors associated with RRP patients.

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1. 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 most frequent cause of voice disorders in this age group. Its prevalence is estimated at four cases per 100,000 children and two 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 infect epithelial tissues in humans and other animals. To date, over 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 HPV types 6 and 11, as well as types 16, 18, 31, and 33 (They are contributing to the 99% of the cases), 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 throughout the airways and frequently recur (4, 5). The primary treatment for this surgical condition involves the use of a CO_2 laser for precise removal of affected areas while preserving the integrity of the vocal cords. However, due to the recurrent nature of the disease, frequent or periodic surgeries are often necessary. On average, children with RPP undergo approximately 13 surgical stages throughout the duration of the disease. Additionally, treatment with interferon alpha, cidofovir, and bevacizumab-a humanized anti-HPV monoclonal antibody-can help reduce lesion growth and shorten the duration of therapy (6, 7).

In some cases, administration of the HPV vaccine following surgery is recommended 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 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, exhibiting 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; therefore, the occurrence of these lesions is rare in children born via cesarean surgery.

In addition to maternal history of genital warts, other risk factors for RRP include firstborn status, younger maternal age, low maternal socioeconomic status, and the presence of Class II HLA antigen polymorphisms in the child (10). In comparison, HPV-6 is more frequently associated with genital warts, while HPV-11 is more likely to induce laryngeal lesions in cases of RRP. Children under the age of three tend to exhibit more extensive and severe lesions. Observations indicate that intervals of up to five years may occur between the initial exposure to the virus 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,

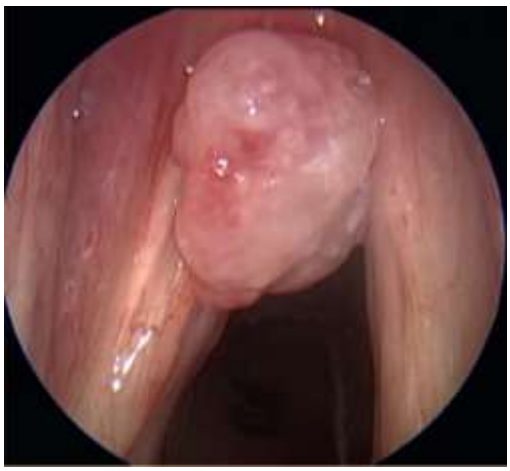

Figure 1. HPV-induced RRP lesion in the larynx.

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

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 β-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 µL, containing 100-500 ng of extracted DNA, six µL of Master Mix (Sigma Aldridge, St. Louis, USA), one µL 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, with a final extension of 10 minutes at 70°C. The PCR products 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 target a conserved segment of the L1 gene of the viral capsid and detect all HPV types. The MY09/11 primer pairs amplify a 450 bp fragment, while the 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 volume of 12 µL containing 100-500 ng of extracted DNA, six µL of Master Mix, one µL of each primer and sterile DDW. For the second step of amplification (inner reaction), 0.5 µL of the first PCR product was utilized as DNA template. The PCR cycling conditions were 94°C for four 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.

The same thermal program was used for the nested round using GP5+/6+ primers, except for the annealing temperature, which was adjusted to 58°C. PCR products were electrophoresed on a 1.2% agarose gel and visualized using a 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 using Chromas (Version 2.5, San Francisco, CA, USA), Bioedit (Version 7.2, Stockport, UK), and EditSeq (Version 7, Madison, WIS, USA) software and compared with other HPV sequences submitted to GenBank utilizing the Basic Local Alignment Search Tool (BLAST, NCBI). To examine the phylogenetic relationship between HPV samples and sequences recorded in GenBank, the sequences were retrieved from the NCBI site and analyzed using Bioedit and Lazergene software. The Neighbor Joining method was used to generate a phylogenetic tree using the MEGA X software and 1000 bootstrap replicates.

2.7. Statistical analyzes

Data obtained from patients were statistically analyzed 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. Homologous HPV types were analyzed using MEGA software for phylogenetic analysis, and the typing results 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, and 16 samples (55.2%) were identified as HPV-11 (Figure 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). Given the wide age range among patients, the mean age at symptom onset was approximately 51.37 months (around four years and three months). The earliest age for the first surgery was 12 months, while 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, gender distribution was equal, with 50% male and 50% female. In the case of lesion sites 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 involvement, six were male and two were female. Both patients with simultaneous glottis and subglottic lesions were male. Additionally, of the 13 patients who had simultaneous involvement of the supraglottis, glottis, and subglottic, eight were female and five were male. In the context of interferon therapy, the highest treatment rate 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 (Tables 1 and 2).

Statistical analyses conducted in this study revealed no significant difference in incidence of HPV typing viruses between men and women, nor in the development 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 proportion of adults affected 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%; however, pulmonary involvement is rare, occurring in approximately 1% to 3% of cases.

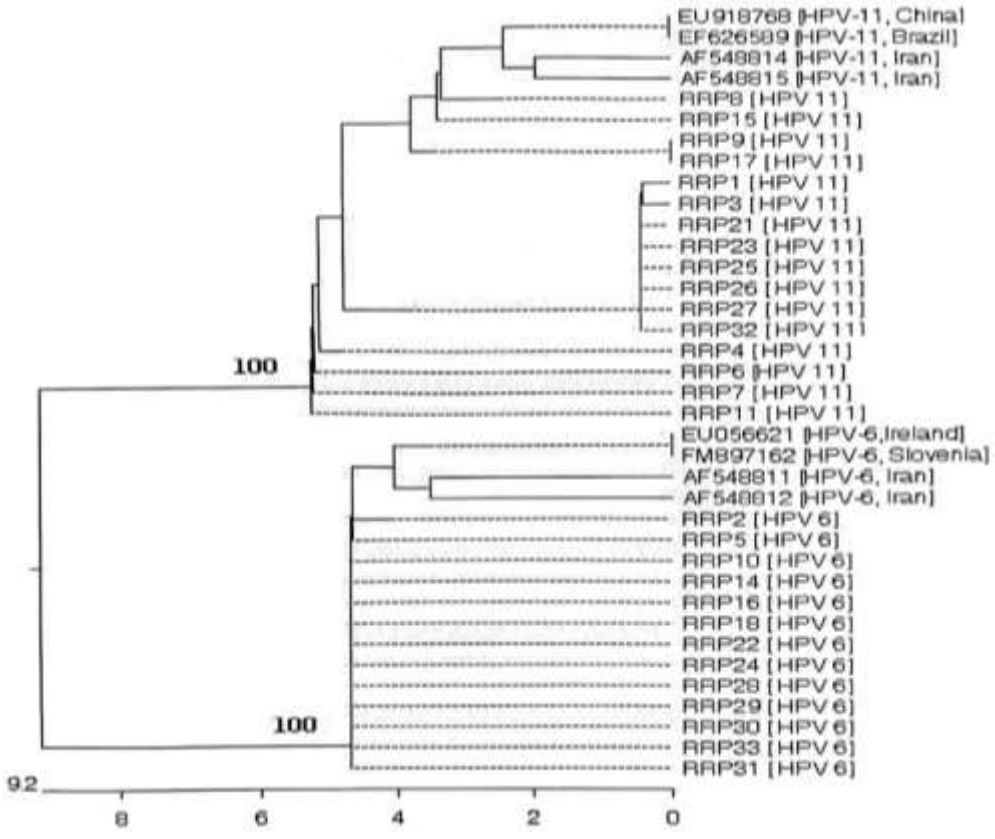

Figure 2. Phylogenetic tree of isolated HPVs (RRP1-31) with GeneBank reference sequences.

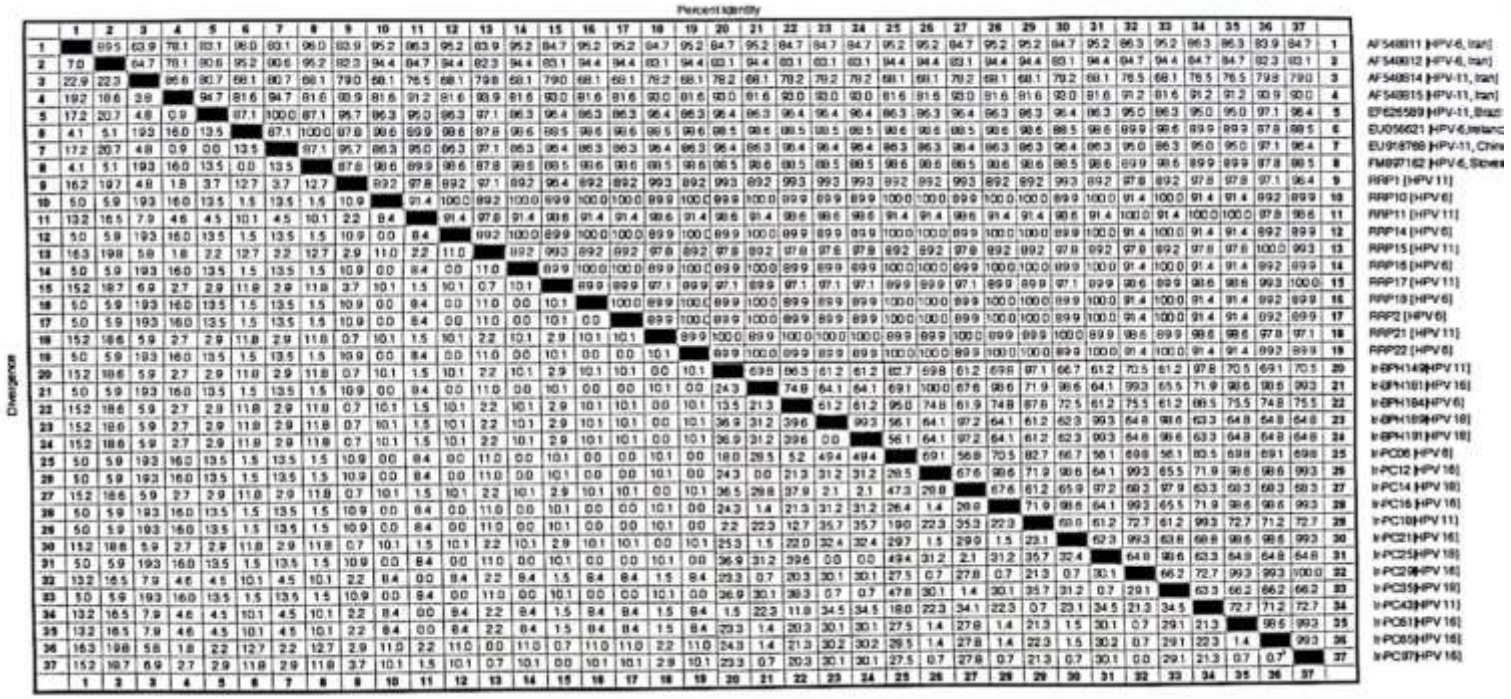

Figure 3. Homology of isolated HPV sequences (RRP 1-31) compared to GeneBank reference sequences.

Table 1. Characteristics of the studied patients.

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

Table 2. Number of surgeries and involved areas.

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

The disease generally affects both sexes; however, 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 during infancy. The overall onset typically ranges from 1 to 17 months of age, and the mean 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, with reported cases of mortality associated with this condition (15, 16).

Shah et al (1), were the first to identify the epidemiological risk factors for RRP, which include Being the first child of a young mother from a low socioeconomic status, and delivery via natural childbirth. Since RRP is directly associated with human papillomavirus (HPV) genital infections in both adults and 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 younger age groups. In the United States, the prevalence of genital HPV infections is estimated to range 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 maternal condyloma is a critical predictor of RRP in the child. The occurrence of condyloma during pregnancy suggests either a recent HPV infection or reactivation of a prior one, potentially triggered by hormonal changes in the mother's body. Additionally, the development and progression of RRP in children 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 at onset of RRP symptoms is a significant factor influencing the lesion severity. 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 require more adjunctive treatments and exhibit 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 to 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 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, yielded similar results. Eftekhaar and Karbalaie Niya (23) examined 12 patients with RRP, aged three to 18 years, between December 2014 and February 2017 at the Iran University of Medical Sciences in Iran. They found that lesions in nine patients contained HPV-6, two patients had HPV-11, and one patient had lesions containing both HPV-6 and HPV-11. Similarly, Yamada and Itoh (16) conducted a study on 29 patients with RRP in Hamamatsu, Japan, between September 2005 and June 2021. They found that lesions in 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 between October 2000 and October 2020 in 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 involving 41 patients with RRP, aged two to 64 years, in São Paulo, Brazil, between 2008 and 2015. They found that 30 patients tested positive for HPV-6, while 11 patients tested positive for HPV-11. Bedard and

Alarcon (25) also conducted a research on 20 patients with RRP, aged two to 17 years, in Cincinnati, Ohio, the USA. They reported that 16 patients tested positive for HPV, with 14 cases involving HPV-6 and two involving 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 findings revealed that 157 patients tested positive for HPV, including 129 with HPV-6, 25 with HPV-11, one with HPV-16, and one patient with co-infection of HPV-6 and HPV-44. Additionally, one patient tested positive for both HPV-6 and HPV-54. Weiss and Heinkele (17) conducted a survey of 44 RRP patients, aged two to 77, in Germany from 2004 to 2013. They found that 32 patients tested positive for human papillomavirus (HPV), including 23 with HPV-6, six with HPV-11, and three with co-infection of HPV-6 and HPV-11.

Lepine and Leboulanger (27) 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, 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 received HPV vaccination.

The present study also confirms 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 (1, 3, 5, 13, 14, 24). Furthermore, previous research conducted both domestically and internationally suggested a clear correlation between the patients' socioeconomic status-including income and welfare levels-and the occurrence of RRP lesions. The disease exhibits higher prevalence in areas characterized by low income and welfare levels, with the majority of affected patients being children under the age of five.

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Authors' Contribution

Study concept and design: H. R.
Acquisition of data: N. M.
Analysis and interpretation of data: N. M.
Drafting of the manuscript: H. A.
Critical revision of the manuscript for important intellectual content: H. A.
Statistical analysis: K. SP.
Administrative, technical, and material support: H. R.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

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.

Data Availability

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

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