

ORIGINAL STUDY

The relationship between human papilloma virus genotyping and antiviral treatment in recurrent respiratory papillomatosis

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ABSTRACT

BACKGROUND. Recurrent respiratory papillomatosis (RRP) is a disease caused by Human Papilloma Virus (HPV), characterized by the development of papillomas in the respiratory tract. About 95% of the papillomas have laryngeal localization, involving HPV genotypes 6,11,30,16,18, and the rest are distributed in the nasopharynx, oropharynx, lungs, bronchi and trachea, with involvement of HPV genotypes 13,32,57,16,18. It is well known that genotypes 16, 18 and 31 are associated with oncogenic high-risk.

MATERIAL AND METHODS. We performed a prospective clinical study (October 2010 – December 2012) on 29 patients with RRP positive diagnosis, confirmed by anatomo-pathological examination. 23 patients received non-specific antiviral therapy and six patients associated surgery with specific antiviral treatment with Cidofovir or Interferon 2alfa. The HPV genotyping was done in all cases and the biological material consisted of biopsy samples. Total cellular DNA isolation was performed with High Pure Template Kit (Roche). HPV DNA genotyping was performed with Linear Array Kit (Roche).

RESULTS. The results showed predominance – 51.72% – of genotype 6 both in the juvenile and adult forms. Genotypes 11 and 32 were found in equal percentage, 20.69%, and genotypes 16 and 18 in 3.45% of the cases. The p-value for the group of patients who have received specific antiviral treatment was of 0.008 and 0.02 in those patients who have undergone non-specific antiviral treatment, which reflects the fact that the observed differences are due to the treatment followed by patients, treatment which has had a statistically significant effect.

CONCLUSION. Viral genotyping knowledge is a helpful method for identifying the cases with high risk of malignancy and shift to the best association between surgery and specific antiviral therapy; we observed that juvenile forms are more aggressive; in this study, genotype 6 prevailed. It's a fact that the combination of antiviral and surgical treatment reduces relapses. We noticed a high frequency of laryngeal location-genotype 6 and 11 (low risk) and multiple location of viral lesions can be associated with genotype 16/18 (high risk).

KEYWORDS: Human Papilloma Virus, recurrent respiratory papillomatosis, JORRP - juvenile-onset RRP, AORRP - adult-onset RRP

INTRODUCTION

Recurrent respiratory papillomatosis (RRP) is a disease caused by Human Papilloma Virus (HPV), characterized by the development of papillomas in the respiratory tract¹. Papillomas are verrucous lesions, often with multiple warty excrescences that occur primarily in the larynx (almost 95%)², and involve HPV genotypes 6, 11, 30, 16, 18; the rest are distributed in the nasopharynx, oropharynx, lungs, bronchi and trachea, with involvement of HPV 13, 32, 57, 16, 18^{3,4}.

RRP is a benign lesion with frequent recurrences and sometimes with malignant potential, especially for genotypes 16,18,31^{5,6}.

The disease tends to recur and spread throughout the entire aero-digestive tract. RRP is classified, based on the age of onset of symptoms, in two forms: JORRP (juvenile-onset RRP) - onset in children younger than 5 years and AORRP (adult-onset RRP), which usually is diagnosed in the fourth decade of life⁷.

Since the larynx is the most affected site, the most common symptoms of the disease are progressive

Laryngoscopic and Clinical Assessment Scale for RRP A. Clinical Score	
1. Describe the patient's voice today: normal ___(0), abnormal ___(1), aphonic ___(2)	
2. Describe the patient's stridor today: absent ___(0), present with activity ___(1), present at rest ___(2)	
3. Describe the urgency of today's intervention: scheduled ___(0), elective ___(1), urgent ___(2), emergent ___(3)	
4. Describe today's level of respiratory distress : none ___(0), mild ___(1), moderate ___(2), severe ___(3), extreme ___(4)	
Total Clinical Score (Questions 1 through 4) = <u> (0-11) </u>	

Figure 1 Derkay method of staging

B. Anatomical Score	
For each site, score as: 0 = none, 1 = surface lesion, 2 = raised lesion, 3 = bulky lesion	
LARYNX	
• Epiglottis:	Lingual surface.....Laryngeal surface....
• Aryepiglottic folds:	Right....Left....
• False vocal cords:	Right....Left....
• True vocal cords:	Right....Left....
• Arytenoids:	Right....Left....
• Anterior commissure.....	
• Posterior commissure.....	
• Subglottis.....	
TRACHEA	
• Upper one-third.....	
• Middle one-third.....	
• Lower one-third.....	
• Bronchi:	Right....Left....
• Tracheotomy stoma....	

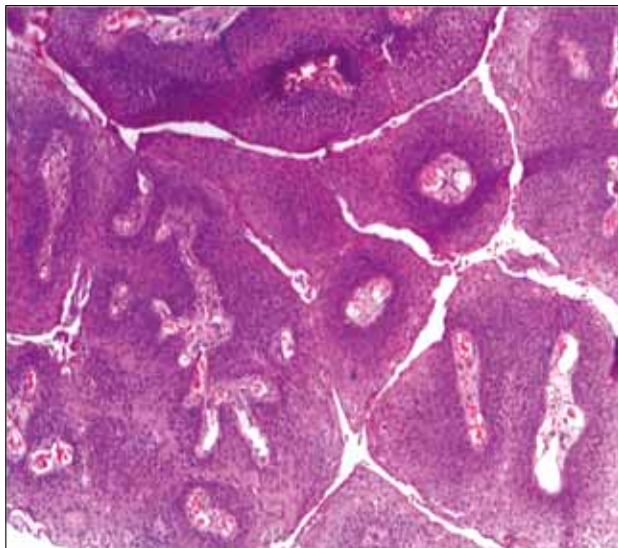


Figure 2 Papillomas histological aspect

hoarseness and respiratory distress; upper airway obstruction may be life-threatening and also may be the main presenting symptom. Other complaints include the following: stridor, voice change, choking episodes, dyspnea, wheezing and cough⁸.

Based on clinical symptoms and the laryngofibrosopic aspect of the lesions, the Derkay score is calculated⁹ (Figure 1).

Microscopically, the papillomas appear as exophytic projections of keratinized squamous epithelium overlying a fibrovascular core, with koilocytes, dyskeratosis, parakeratosis, metaplasia and dysplasia (Figure 2)¹⁰.

Spontaneous remission of the disease is rare and is related to endocrine, hormonal or immunologic influences. Papillomas may recur after years of remission. Children with RRP frequently experience remission after several years, which may be related to puberty. By this time, the patient may have undergone more than 20 sur-

gical procedures. Disease in adults tends to be milder.

The purpose of the therapy is to release the airways, to improve voice and quality of life and to reduce the frequency of relapses or to facilitate remission. The primary treatment involves repeated surgical debulking, usually by means of microdebridement, angiolytic laser, carbon dioxide laser or cryotherapy¹¹. Associated medical treatment may consist of: intralesional cidofovir, oral indole-3-carbinol, interferon or photodynamic therapy. The quadrivalent HPV vaccine protects against HPV types 6, 11, 16, and 18 and therefore has a potential for prevention and decreasing the incidence of RRP¹².

MATERIAL AND METHODS

A clinical prospective study was performed on 29 patients (13 female patients and 16 male), from October 2010 until December 2012. All patients were histologically diagnosed with recurrent respiratory papillomatosis.

Study inclusion criteria were:

1. children and adult patients;
2. positive diagnosis of RRP;
3. patients who have not completed antiviral treatment;
4. patients who have not performed viral analysis.

Exclusion criteria were:

1. patients with immune deficiencies (HIV/AIDS, tuberculosis, malignancies);
2. pregnancy and lactation;
3. associated diseases with contraindication for antiviral treatment.

The biological material consisted of intraoperative biopsies embedded in paraffin blocks sectioned at 50µm. Deparaffinization was performed with xylene followed by washing in graded alcohol (100%, 80%, 60% and 40% and double distilled water). Samples

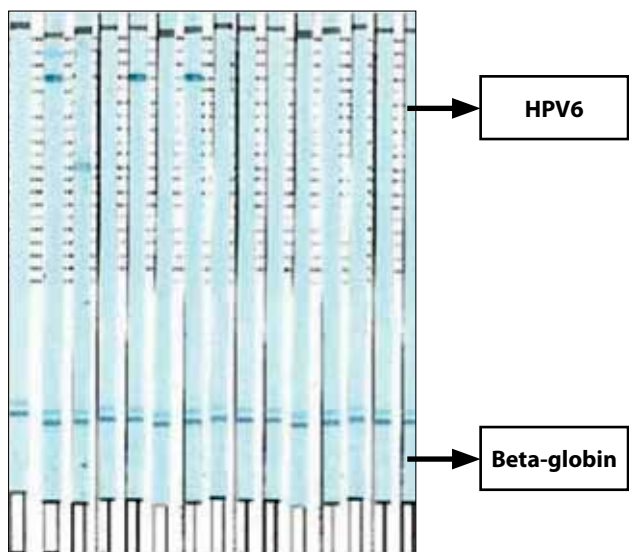


Figure 3 HPV genotype detection

were taken in 300µl phosphate saline (PBS).

Total DNA isolation was performed with the High Pure Template kit (Roche). Cell lysate, obtained by treating dewaxed sections or biopsies chopped with proteinase K, was passed through the column. Resin column retained DNA and cellular contaminants were removed by washing. Total DNA was eluted with 200µl Elution Buffer, preheated to 70°C. The purified DNA was stored at 4°C.

HPV DNA detection and genotyping was performed by Linear Array kit (Roche). Selection of primers of Master Mix allows amplification of 37 HPV genotypes, including 13 high-risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). Another pair of primers aimed β-globin as a control marker for DNA isolation and amplification.

The kit principle is based on the amplification of a consensus sequence of the viral genome (gene L1) and on denatured amplicons; genotyping relies on

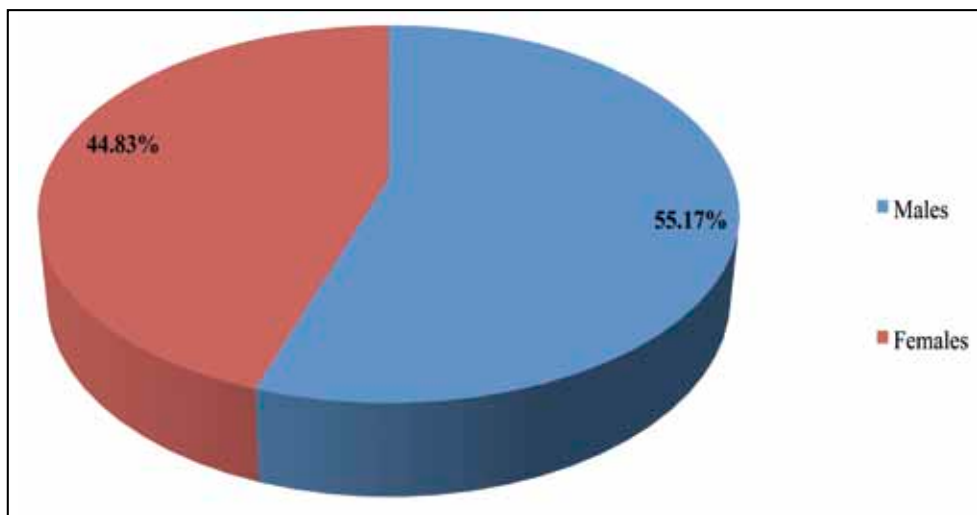


Chart 1 Patients' sex distribution

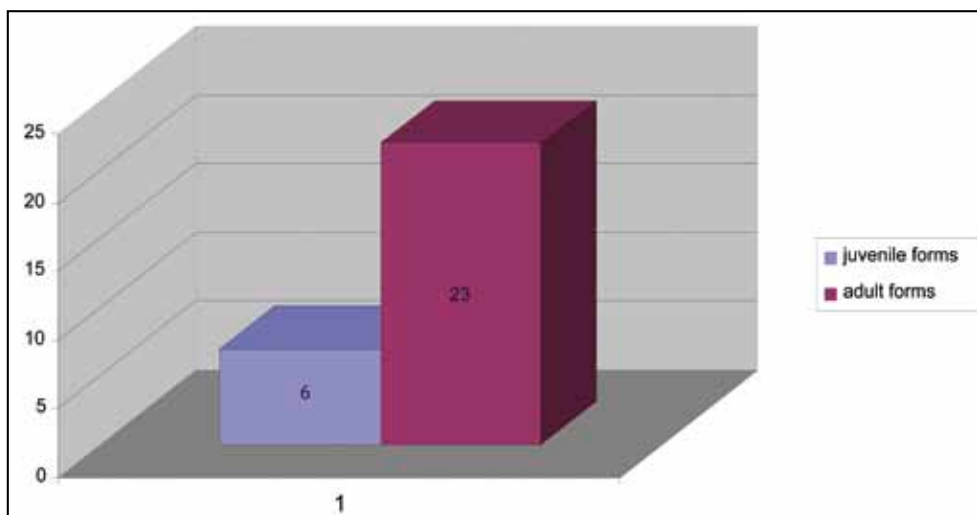


Chart 2 Respiratory recurrent papillomatosis types - juvenile and adult forms



Figure 4 Juvenile laryngeal papillomatosis – JORRP – intraoperative aspect



Figure 5 Adult form of laryngeal papillomatosis – laryngofibrosopic examination



Figure 6 Nasal papilloma located in the left middle nasal meatus – nasal endoscopic examination



Figure 7 Nasal papilloma originating from the anterior part of the nasal septum – left nasal fossa (nasal endoscopic examination)

specific molecular probes, immobilized on solid support¹³. Since the primers used are biotinylated, hybrids formed are detected using Streptavidin conjugated with alkaline phosphatase (Figure 3). Adding substrate allows visualization of immobilized hybrids. High sensitivity (93%) and specificity (93%) recommend this kit for genotyping.

RESULTS

The mean age of the 29 patients diagnosed with recurrent respiratory papillomatosis was 43 years. The sex ratio was 1.2, with a predominance of the disease in female patients (Chart 1).

26 of the patients included in this study were adults (89.66%) – three of them were diagnosed in childhood and they represent juvenile forms of papillomatosis; three patients included in the study, a girl and two boys, were paediatric patients with juvenile recurrent papillomatosis (10.34%) (Chart 2).

The patients presented different location sites of

their papillomas: 62.07% had laryngeal papillomatosis (18 cases) (Figure 4, Figure 5), 27.59% oropharyngeal papillomatosis (8 cases) and 10.34% nasal localization (3 cases) (Figure 6, Figure 7) (Chart 3).

Viral genotyping was performed in all patients. 55.17% of them were HPV positive (16 patients) and approximately 45% were HPV negative (13 patients) (Chart 4).

In paediatric patients with laryngeal papillomatosis – 2 had genotype 6 and one genotype 11. Patients with adult form had the following distribution: 7 with genotype 6 and 5 with genotype 11; in those with juvenile form genotype 6 was identified. Three patients were found with nasal papilloma genotype 6 and six patients with tonsillar papilloma genotype 32. Genotype 16 was found in the jugal mucosa localization of the papillomas (1 patient) and genotype 18 in the only case of tongue tumor (Chart 5).

According to our results, type 6 was predominant, both in the juvenile and adult forms (51.72%). Genotypes 11 and 32 were found in equal percentage (20.69%) among the patients from our study group.

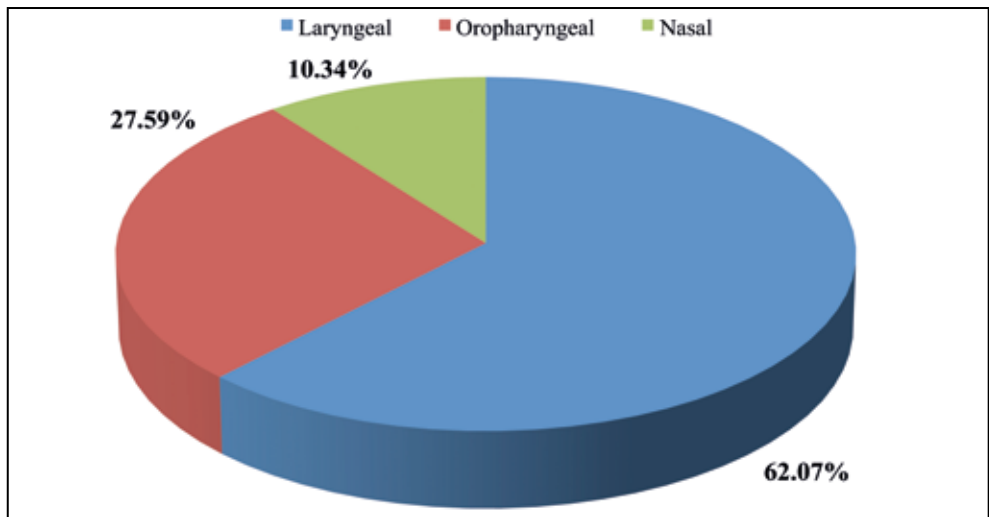


Chart 3 Papillomas localization

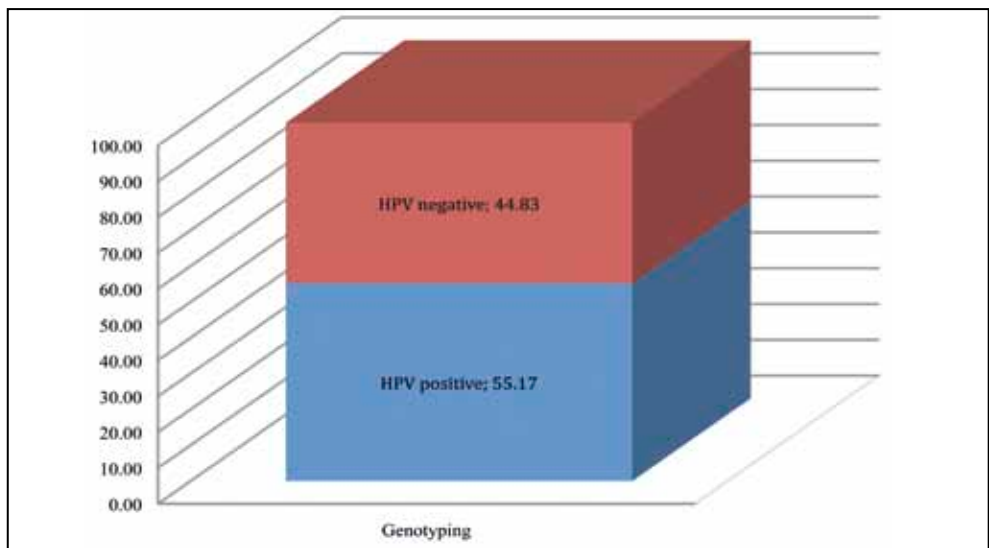


Chart 4 HPV genotyping

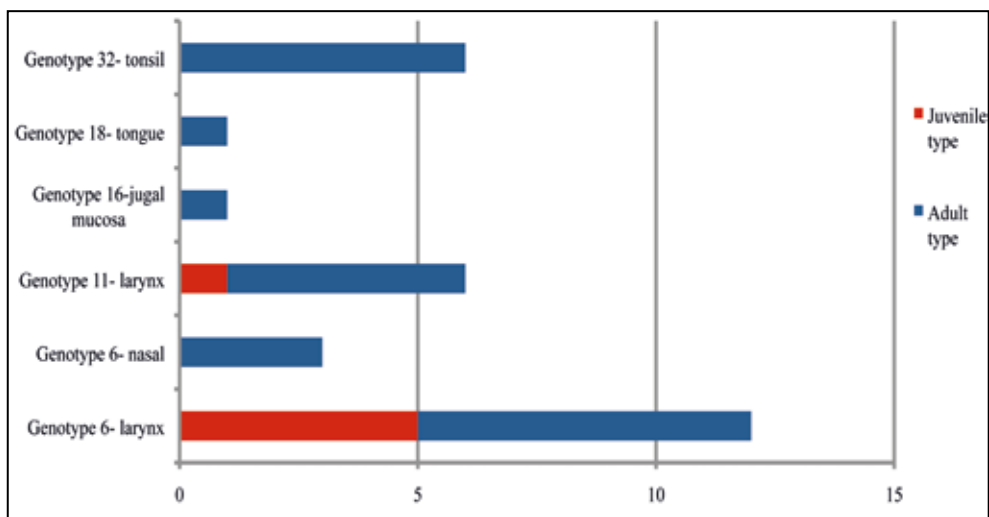


Chart 5 HPV genotyping

Regarding genotypes 16 and 18, each of them represented 3.45% of the analyzed cases.

All patients underwent surgical treatment. The patients included in the study were divided into two groups: 1st group – six patients (representing 20.69%) – received combined therapy - surgery and intralesional injection with Cidofovir or Interferon 2-alfa (Chart 6, Figure 8); 2nd group – 23 patients – were treated with surgery and nonspecific antiviral treatment (Isoprinosine). Regarding the Cidofovir concentration, we used 0.3 mg/ml for children and 0.75 mg/ml for adults; the volume of each injection depended on the extension of the lesions; the average number of the procedures in the 24-month study was 3.3. The Isoprinosine dose used in the study was 10 mg per kg body weight and was administered 7 days in the first two months after surgery and, then, every 3 months for 2 years.

Derkey score was used to assess the patients included in this study at their first admission, or in case of recurrences. In the first study group, we had 2 remissions and 4 recurrences after one year of treatment and at the end of 2012, 3 remissions and 3 recurrences. In the second group, after two years of treatment, we had 3 remissions and 9 relapses (2 patients developed carcinoma on the pre-existing lesion) in laryngeal papillomatosis; in those cases of oropharyngeal papillomatosis we had 6 remissions and 2 relapses, and in case of nasal papillomatosis no recurrences were registered.

In the group of patients who have followed specific antiviral treatment, Derkey score decreased approximately 4.9 points after the first year of treatment and another 0.6 points in the second year (13.7 in 2010 to 8.8 in 2011 to 8.2 in 2012) (Chart 7). For patients with non-specific antiviral treatment, Derkey score was nearly 7.83 in 2010 and decreased to 5.74 in 2011 and 5 in 2012 (Chart 8).

Chart 9 represents the dynamic evolution and difference of Derkey score between patients who have followed specific antiviral treatment and those with non-specific antiviral treatment. Based on the data, we calculated the threshold value of the confidence coefficient p taken before the beginning of the treatment as $\alpha=0.05$. By multivariate analysis, the covariates (Derkey score, number of injections with Cidofovir or doses of Isoprinosine, time and tumor recurrences) are put in relation with efficacy, as a binary target variable. If the p-value calculated by the model for testing significance is greater than alpha, then the null hypothesis is accepted, meaning that parameters are not significantly different from 0; hence the result is by chance, with a probability of 1-alpha (95%). The study conducted on the 2nd group patients, those who have undergone non-specific antiviral treatment (Isoprinosine), showed a p-value less than the threshold amount (p-value = 0.02). Regarding the 1st group, who has followed specific anti-

viral treatment (Cidofovir), p-value was 0.008. The differences appeared in the study are due to the treatment followed by patients, treatment which has had a statistically significant effect in the 1st study group.

DISCUSSIONS

RRP is a disease caused by HPV, characterized by the development of papillomas in the respiratory tract. Epidemiological data show that JORRP affects both males and females, in equal numbers, whereas AORRP is more common in males¹⁴. The mean age at diagnosis of JORRP is 3.8 years. The adult form usually manifests in the third or fourth decade of life, but may rarely manifest in patients older than 60 years¹⁵.

Statistical analysis of the relationships among epidemiological factors, HPV type and clinical course revealed that patients with HPV-11 and patients younger than 3 years of age at RRP diagnosis are prone to develop more aggressive disease, as represented by higher severity scores at endoscopic debridement, more frequent operative debridement procedures per year, a greater requirement for adjuvant therapy and greater likelihood of tracheal disease with tracheotomy¹⁶.

Malignant degeneration of papillomatous lesions to squamous cell carcinoma occurs in 3-5% of patients with RRP. Distal airway spread of papillomas is often a forewarning of malignant degeneration. The site of malignancy in JORRP is usually the bronchial or pulmonary parenchyma, whereas the larynx is the usual site in AORRP. Malignant degeneration is more common with the disease caused by HPV-11, HPV-16 and HPV-18 according to the medical literature¹⁷.

In this study, we analyzed patients with juvenile and adult forms of RRP and we observed that juvenile forms are more aggressive and may predispose to more frequent relapses.

23 patients underwent surgery and non-specific antiviral treatment (Isoprinosine), six of them associated surgery with specific antiviral treatment with Cidofovir and Interferon 2-alfa. Cidofovir is a specific antiviral agent used in the therapy of recurrent respiratory papillomatosis¹⁸. Combination of surgical and specific antiviral therapy provided the best results for patients with reduction of recurrent episodes of the disease. Intralesional administration of Cidofovir was associated with partial or complete regression of papillomas, improvement in voice quality and airway status, similar to other studies' results¹⁹.

In this study, we observed an increased frequency of laryngeal location – genotype 6 and 11 (low risk); two cases with laryngeal genotype 6 became malignant after repeated surgical interventions (squamous cell carcinoma), without acquiring high-risk HPV strains. Also, it is well known that multiple sites of viral lesions

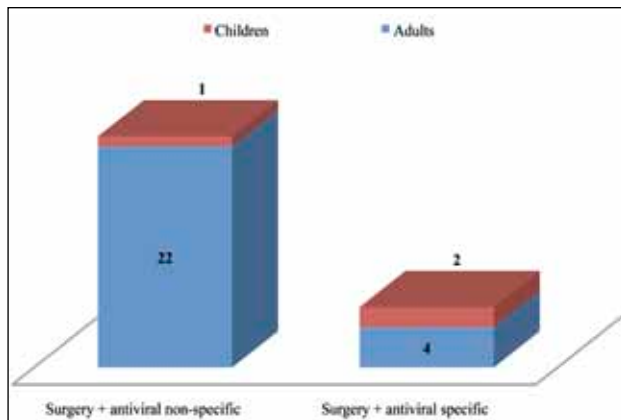


Chart 6 Therapeutic groups



Figure 8 Intralesional Cidofovir injection - intraoperative view.

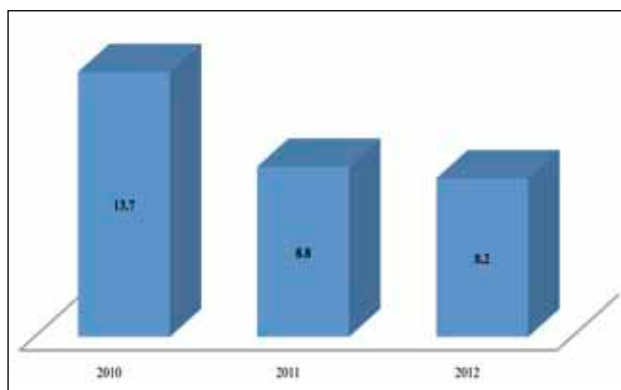


Chart 7 Derkey score for patients with antiviral specific treatment

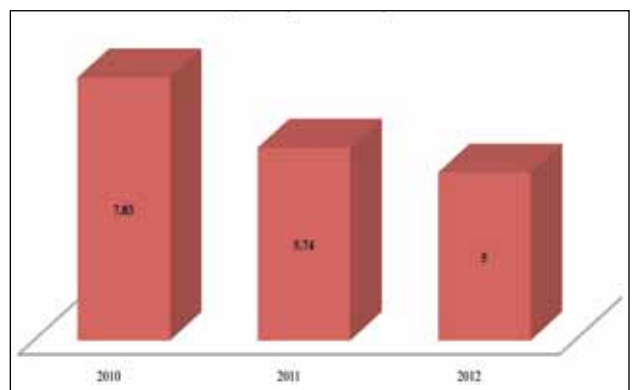


Chart 8 Derkey score for patients with non-specific antiviral treatment

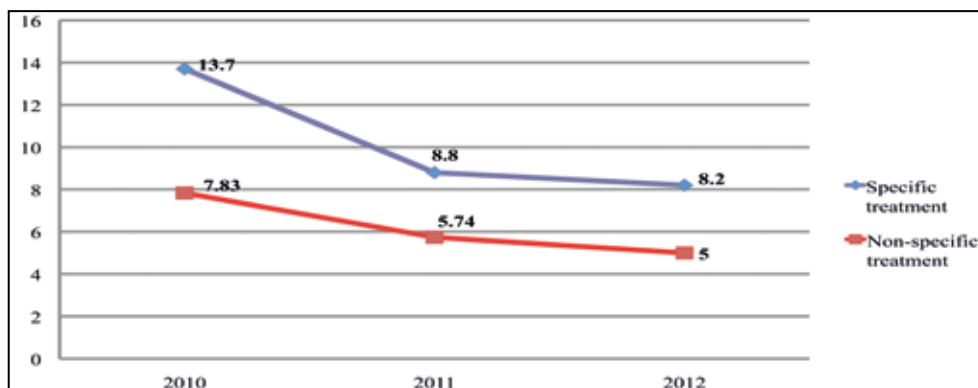


Chart 9 Dynamic difference of Derkey score between patients who have followed specific antiviral treatment and those with non-specific antiviral treatment

are associated with genotype 16/18 (high risk) and require oncologic treatment from the very beginning. The prevalence in the scientific literature is about a quarter for HPV 6, about two-thirds for HPV 11, and about a seventh for combined HPV 6 and 11 infections²⁰. In our study, we found a predominance of genotype 6, both in the juvenile and adult forms.

We conclude that viral genotyping knowledge is a helpful method used for identifying those cases with

high risk for malignancy and shift to the best association between surgery and specific antiviral therapy.

CONCLUSIONS

RRP is a rare disease caused by HPV DNA and most commonly involves genotypes 6 and 11. Our results suggest that HPV 11 infection is associated with a

greater severity of RRP disease, translated as requiring more frequent surgical interventions.

We quantified the efficacy of antiviral specific therapy (intralesional Cidofovir) using the Derkay score and we observed that the score decreased by 3-5 points using this treatment, compared with those who have been administered non-specific antiviral treatment.

We conclude that Cidofovir in combination with surgical debulking reduces the viral load and delays relapses, and seems to be the treatment of choice for RRP.

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