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HPV GENOTYPING IN RECURRENT RESPIRATORY PAPILLOMATOSIS

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Recurrent respiratory papillomatosis is a disease cause by Human papillomavirus (HPV) characterized by the development of papillomas in the aero-digestiv tract. About 95% of the papilloma have laryngeal localization involving HPV genotypes 6 and 11 and the rest are distributed in the nasopharynx, oropharynx, lungs, bronchi and trachea. Our research consists of a prospective clinical study on 65 patients. Inclusion criteria were: children and adult patients, positive diagnosis of RRP, patients with a history and treatment records, patients who have not performed viral analysis for RRP. Exclusion criteria were: immunodeficient patients, pregnancy and lactation, associated diseases with contraindication treatment. The principle of genotyping kit is based on the amplification of a consensus sequence of the viral genome -L1 gene. Viral genotyping was performed in all patients: 20 had genotype 6; 11 patients had genotype 11; 2 patients had genotype 16; 2 patients had genotype 18; 2 patients had genotype 30; 2 patients had HPV 32; 3 patients had genotype 40; 1 case is with HPV 55; 1 person with HPV 2 and 21 patients were HPV negativ. Identification of genotype help us to classify "high-risk" disease on progression to malignancy and to choose the optimal therapeutic scheme - surgery and antiviral therapy.

Key words: HPV–Human Papilloma Virus, RRP- Recurrent respiratory Papillomatosis, PCR- polymerase chain reaction, viral genotyping.

INTRODUCTION

Recurrent respiratory papillomatosis (RRP) is a disease caused by Human papillomavirus (HPV), characterized by the appearance of papillomas in the airways.[1] Papillomas are verrucous and exophytic lesions, often with multiple warty excrescences that occur primarily the larynx and involves genotypes HPV 6,11,30, 16.18[2]; the rest are distributed in the nasopharynx, oropharynx, lungs, bronchi and trachea[3], with involvement of HPV 2,13,32,40, 57,16,18. RRP is a benign lesion with frequent recurrences and sometimes with malignant potential.[4]

The disease is a benign disease but tends to recur and spread throughout the entire aero-digestiv tract.[5] The evolution of the disease is variable, with spontaneous remission or aggressive papillomatous growth or malignant transformation.[6]

RRP is classified based on age of onset of symptoms in two forms: JORRP (juvenile-onset RRP)- debut in children younger than 5 years and AORRP (adult-onset RRP) debut in persons in the fourth decade of life.[7]

RRP has a predilection for anatomic sites that are junctions between squamous and ciliated epithelium[8]; the most frequent localisations for papillomas are the limen vestibuli, the nasopharyngeal surface of the soft palate, the midline of the laryngeal surface of the epiglottis, the upper and lower margins of the ventricle, the undersurface of the vocal folds, the carina, and at bronchial spurs. In patients with tracheostomy, RRP is often seen at the stoma and in the mild-thoracic trachea.

The coexistence of two different epithelia creates squamo–columnar junctions (SCJs)[8] at multiple sites in the respiratory tract, entities that are thought to be a prerequisite for the spread of human papillomavirus (HPV) infections in this region.

RRP is a rare disease, and adult patients may have symptoms for months or longer before the disease is recognized.

The most common symptoms of the disease are related to airway obstruction and they are reprezented by progressive hoarseness, respiratory distress; upper airway obstruction may be life threatening and may be the presenting symptom. Other symptoms include the following: stridor, voice change, choking episodes, dyspnea, wheezing and cough.[9] Characteristic triad in children with RRP is relentlessly progressive hoarseness, stridor, and respiratory distress.[10]

HPV-6 and HPV-11 are the most common types associated with RRP, but, rarely, affected tissues contain HPV-16 and HPV-18. HPV infection is the most common sexually transmitted disease in the world; as many as 75% of women have genital HPV during their lifetime. Thirty to 60% of mothers of children affected with JORRP have genital HPV, compared with 5% of mothers of unaffected children.[11]

The true incidence and prevalence of RRP are unknown.

The prevalence of RRP is estimated of 4.3 per 100,000 children and 1.8 per 100,000 adults in the United States.[12]

There are three risk factors for JORRP firstborn child, vaginal delivery, and mother younger than 20 years.[13] Presence of this allele, HLA-DRB1*0102 in white patients, predisposes patients to RRP.[14] The risk factors for JORRP do not apply to adult-onset cases. This suggests that adult disease does not represent reactivation of latent disease. The mode of transmission of HPV in AORRP is not known. Child-to-parent transmission by cough has never been documented. Patients who had AORRP also were more likely than controls to have more lifetime sexual partners and a higher frequency of oral sex.[15]

Macroscopically, papillomas can be pedunculated or sessile, spread over the mucosal surface. They tend not to be friable. Microscopically, the papillomas appear as exophytic projections of keratinized squamous epithelium overlying a fibrovascular core, with Koilocytes (vacuolated cells with clear cytoplasmic inclusions), dyskeratosis, parakeratosis and dysplasia.

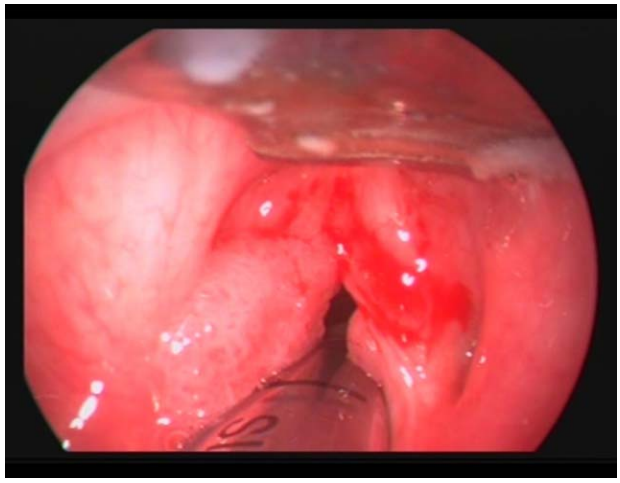

Figure 1. Papillomas- laryngofibroscopic exam.

The goal of therapy is to release the airways, to improve voice and quality of life and to reduce frequency of relapses. The primary treatment involves repeated surgical debulking, by microdebridement, cold excision, CO2 laser ablation, pulsed dye laser (PDL) ablation or cryotherapy.[7] This may be followed by an injection of cidofovir into the resection site in patients with moderate-to-severe disease. Medical treatment is associated and consists in intralesional cidofovir, oral indole-3-carbinol or interferon, and photodynamic therapy.[16]The quadrivalent HPV vaccine protects against HPV types 6, 11, 16, and 18 and therefore has promise for prevention and decreasing the incidence of RRP.[17]

Papillomaviruses are nonenveloped viruses with icosahedral symmetry with 72 capsomeres that surround a genome containing double-stranded circular DNA with approximately 8000 base pairs.[6]

HPV genome has three regions: The early (E) region codes for 6 nonstructural genes involved in cellular transformation.

The late (L) region codes for 2 structural proteins, L1 and L2 which form the capsid.

The long control region is a noncoding region that regulates replication and gene function.

Papillomaviruses has 2 modes of replication:

1. Stable replication of the episomal genome in basal cells.

2. Runaway, or vegetative, replication in more differentiated cells to generate progeny virus.

The virus is established in the basal layer of mucosa where enters viral DNA and will produce viral RNA which will encode proteins that cause changes in differentiation of normal mucosa and will induce papillomas transformation.

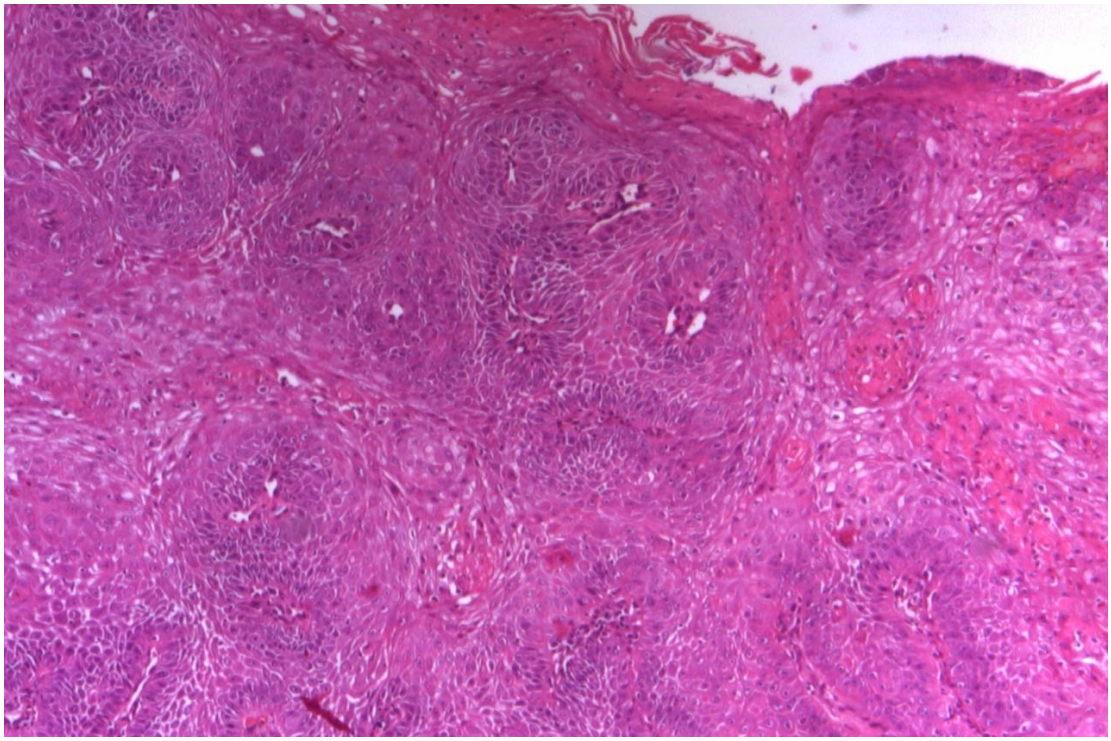

Figure 2. Papillomas histological aspect.

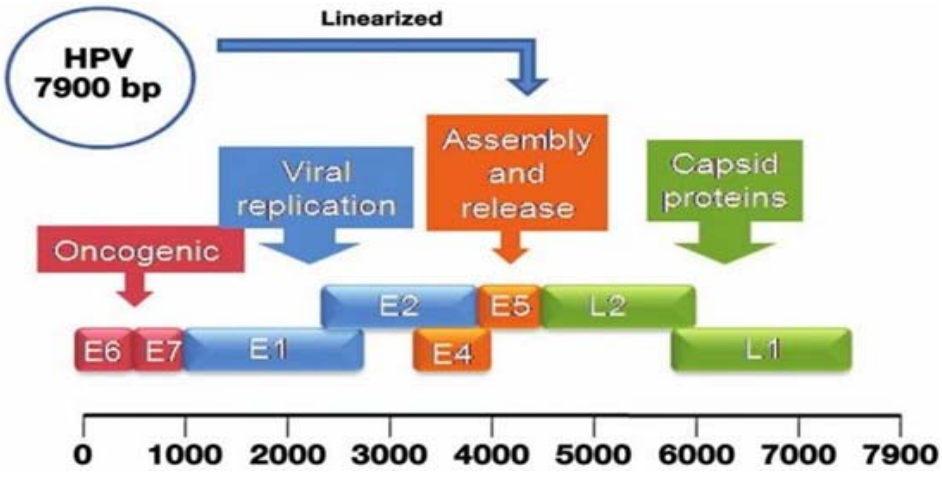

Figure 3. HPV genome.

Nearly 110 different HPV types have been identified.

Specific viral subtypes may be correlated with severity and clinical course of the disease. They are classified:[4]

Low risk types (6, 11, 13, 40, 42–44, 54, 61, 62, 70, 72, 74, 81).

High risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82) risk of progression to malignancy. HPV types 26, 53 and 66 should be considered probably carcinogenic due to the extremely small number of affected patients.

MATHERIAL AND METHODS

A prospective study was performed on 65 patients, from October 2010 until November 2013, 29 female patients (45%) and 36 male (55%). 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 were taken in 300µl phosphate saline (PBS) (Figure 2).

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 (Figure 3).

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.

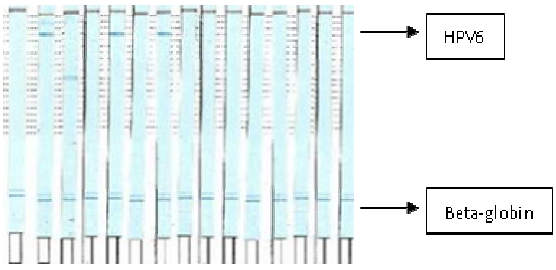

Figure 4. HPV genotype detection.

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 specific molecular probes, immobilized on solid support. Since the primers used are biotinylated, hybrids formed are detected using Streptavidin conjugated with alkaline phosphatase (Figure 4). Adding substrate allows visualization of immobilized hybrids. This method has high sensitivity (93%) and specificity (93% and recommend this kit for genotyping.

RESULTS

In the clinical prospective study, the sex ratio is 1.24, with a predominance of the RRP in male patients – 55% male and 45% female; the mean age it was 42 years (Figure 5).

The patients included in this study presented different location sites of their papillomas: 33 of them (51%) had laryngeal papillomatosis, 25 (38%) oropharyngeal papillomatosis and 7 patients (11%) nasal localization (Figure 6). For the laryngeal localization, 9 patients were diagnosed in childhood and represent JORRP form and 24 patients were adults and they are classified in AORRP form.

Viral genotyping was performed in all patients. The HPV genotype map look like the following:

HPV 6 –20 patients –18 wuth laryngeal localization and 2 with nasal sites.

HPV 11–11 cases in larynx.

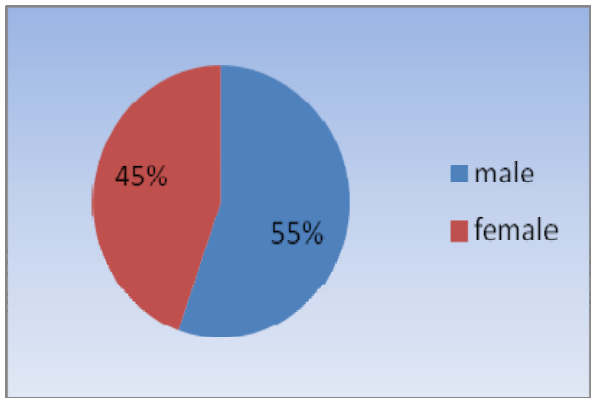

Figure 5. Patients sex distribution.

HPV 16– 2 patients: one with multiple tongue tumors and the other with papillomas on lips and soft palate mucosa.

HPV 18– 2 patients with tongue papillomas.

HPV 30– 2 patients with lueta and tonsillar papillomas.

HPV 32– 2 patients with tonsillar localisations.

HPV 40– 3 patients with oropharyngeal localisations.

HPV 2– 1 person with tumor on the external lip.

HPV 55– 1 case with multiple condilomas on jugal and lips mucosa.

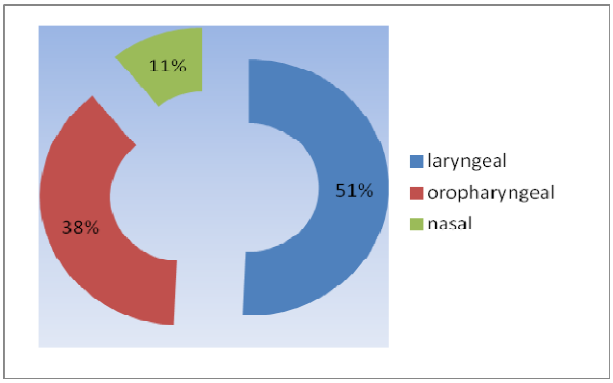

Figure 6. Papillomas localization.

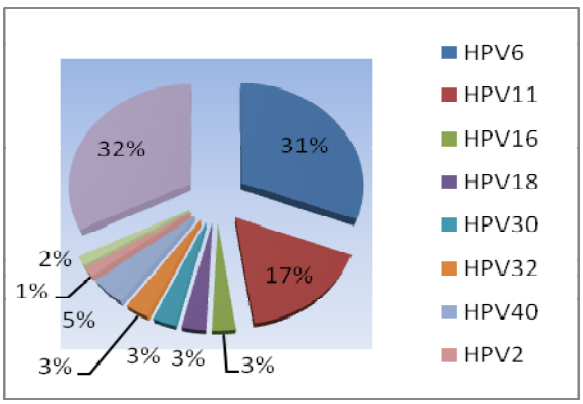

Figure 7. HPV genotyping.

In 21 cases we did not determined HPV. The results showed predominance of genotype 6 (31%). Genotype 11 was found in 17% of cases. Genotypes 16, 18, 30 and 32 were found in equal percentage, .3%, among the patients from our study group; HPV 40 represented 5% and HPV 2 just 1% in our cohort (Figure 7).

DISCUSSION

RRP is a disease caused by HPV, characterized by the appearance of papillomas in the respiratory tract, from the nose to the lung; however, >95% of cases involve the larynx. In JORRP, 52% of children have only laryngeal involvement. The trachea is the next most commonly involved site. However, 31.8% of children had papillomas in areas outside of the trachea and larynx (*e.g.*, oropharynx, nasopharynx, mouth, bronchi, lung parenchyma)[9].

Epidemiological data show that JORRP affects males and females in equal numbers, whereas AORRP is more common in males.

The mean age at diagnosis of JORRP is 3.8 years. In the adult form, symptoms are installed in the third or fourth decade of life but may rarely manifest in patients older than 60 years.

Statistical analysis of the 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 surgery procedures per year, a greater demand for adjuvant therapy, and greater presumption of tracheal disease with tracheotomy.[18]

By the use of highly sensitive real-time PCR, HPV has been found in 100% of papillomas from RRP patients.[19] It is clear that the sensitivity of technique used for HPV detection is relevant although it is also important to analyse the viral subtype.

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.

Malignant degeneration of papillomatous lesions to squamous cell carcinoma occurs in 3–5% of patients with RRP. Distal airway spread of papillomas often is a forewarning of malignant degeneration.[20] The site of malignancy in JORRP usually is the bronchial or pulmonary parenchyma, whereas the larynx is the usual site in AORRP. Malignant degeneration is more common with disease caused by HPV-11 and HPV-16 and HPV 18 according to the medical literature.

All the patients have performed surgery and nonspecific antiviral treatment (Isoprinosine), nine of them associated surgery with specific antiviral treatment with Cidofovir and interferon 2 alfa. Combination of surgical and specific antiviral therapy provided the best results for the patients with the reduction of recurrent episodes of the disease.21 Intralesional administration of cidofovir directly into the site of papillomas was associated with partial-to-complete regression of papillomas, improvement in voice quality and airway status, and decreased need for surgery.[22]

Our results suggest that HPV 11 infection is associated with a greater severity of JO RRP disease, translated as requiring more frequent surgical interventions (one patient with 11 laryngeal genotype requires frequent tumoral debulking - every 6 months.

In this study, we observed an increased frequency of laryngeal location – genotype 6 and 11 (low risk). Despite this, we had thre cases with laryngeal location and genotype 6 which became malignant – after repeated interventions, papillomas transformed into squamous cell carcinoma, without acquiring high-risk HPV strains. Also, the multiple localisation of viral lesions are associated with genotype 16 or 18 (high risk) and require oncogenic treatment from the very beginning.

We conclude that viral genotyping knowledge help us to identify cases with high-risk for malignancy and relegate on the best therapeutic strategy between surgery and specific antiviral therapy.

CONCLUSIONS

RRP is a rare disease. It's caused by HPV DNA and most commonly involves genotypes 6 and 11. 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 infections; in our study we found a predominance of genotype 6 (62%), both in the juvenile and adult forms.

There are around 110 different HPV types and these are characterised into the low risk (benign

disease) or high-risk (malignant disease potential) types where risk implies the potential for malignant transformation. HPV types are classified by genotype which is defined by the sequence of the viral L1 gene which schows the greatest conservation. The major HPV types contains within it a family of "variants" that are characterised by differences in nucleotide sequence of approximately 2% in conserved and 5% in less conserved regions.

RRP involve frequent genotypes 6 and 11, well as 2, 13, 32, 40, 57, 16, 18 in a small rate.

We conclude that identification of genotype help us to classify disease on progression to malignancy and to choose the optimal therapeutic scheme - surgery and antiviral therapy. Knowing the" high risk" genotypes, we will increse surveillance of patients, based on screening progrmmes, and we will reduce the incidence of HPV-induced cancers.

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