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ORIGINAL PAPER

Novel human papilloma virus (HPV) genotypes in children with recurrent respiratory papillomatosis

Ioannis N. Mammas · George Sourvinos · Elena Vakonaki · Panagiota Giamarelou · Catherine Michael · Demetrios A. Spandidos

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Abstract
Introduction Recurrent respiratory papillomatosis (RRP) is characterized by the presence of benign virally induced tumors of the larynx and respiratory epithelium that may obstruct the airway and tend to recur frequently. RRP is caused by the human papilloma virus (HPV), most frequently by HPV types 6 and 11. In this study, we present four cases of children with RRP in whom HPVs other than HPV-6 or HPV-11 were found.
Material and methods In all four cases, HPV typing was performed by polymerase chain reaction (PCR) followed by restriction digestion (RFLP) in biopsy samples collected during surgery.
Results In the first case, simultaneous HPV infection with types 13 and 39 was detected, while in the second case HPV-40 and HPV-56 were found. In cases 3 and 4, the biopsy samples were positive for unidentified 'low-risk' HPVs.
Conclusions The presence of novel HPV genotypes in children with RRP emphasizes the need for further investigation of the implication of these genotypes in the disease.

Keywords HPV · Recurrent respiratory papillomatosis · Childhood · Genotypes

I. N. Mammas · G. Sourvinos (✉) · E. Vakonaki · D. A. Spandidos
Department of Virology, Faculty of Medicine, University of Crete, Heraklion,
Crete, Greece
e-mail: sourvino@med.uoc.gr

P. Giamarelou · C. Michael
Department of Pathology, 'Aglaia Kyriakou' Children's Hospital, Athens, Greece

Abbreviations
HPV human papilloma virus
RRP recurrent respiratory papillomatosis

Introduction

Childhood recurrent respiratory papillomatosis (RRP) occurs at an incidence of 0.3–3.9/100,000 and is considered the most common benign tumor affecting the larynx [26]. It is characterized by the recurrent growth of benign papillomas along the epithelium of the upper respiratory tract, including the larynx, vocal cords, arytenoids, subglottis, and trachea. RRP is potentially life-threatening as it has the tendency to grow in size and number, causing complete airway obstruction [24]. The diagnosis of RRP is confirmed by direct laryngoscopy and biopsy for tissue diagnosis. Currently, RRP is primarily treated with multiple surgical debridements, while medication recently has been used as adjuvant therapy [3, 6].

The human papilloma virus (HPV) has been identified as the etiologic agent of RRP [13]. This small DNA-containing non-envelopedicosahedralcapsidvirusis7,900-basepairslong andnearly120differenttypeshavebeenidentified[5, 28]. RRP is almost universally induced by HPV types 6 and 11, the same types that cause genital warts [26]. Compared to HPV-6, HPV-11 is more likely to precipitate severe disease and is associated with earlier presentation [19, 27, 28]. It tends to require more frequent debridement, has a higher risk of bronchopulmonary spread, and more often necessitates tracheotomy [26]. Although several researchers have described the presence of multiple HPV-6 and HPV-11 infections [1], or co-infections with the oncogenic types

HPV-16 and HPV-18 in RRP [10–12], data on the presence of other HPV types in RRP is limited.

This study presents four cases of children diagnosed with RRP at the Department of Pathology of the "Aglaia Kyriakou" Children's Hospital in Athens during the period 1996 to 2006. In the first case, simultaneous HPV infection with HPV-13 and -39 was detected, while the second case was co-infected with HPV-40 and HPV-56, and the final two cases with unidentified 'low-risk' HPVs.

Materials and methods

We retrospectively reviewed all cases of children diagnosed with RRP at the Department of Pathology of the "Aglaia Kyriakou" Children's Hospital in Athens, Greece, during the period of 1996–2006. Clinicopathological data (age, sex, origin, and residence) were available for all children included in the study. We investigated the medical history of the children in detail, including their prenatal, birth, and past medical history, as well as their parental and social history. Information regarding follow-up was also documented. The children were surgically managed, and pathologic analysis was performed according to standard protocols at the Department of Pathology of the 'Aglaia Kyriakou' Children's Hospital in Athens, Greece. Representative tissue sections exhibiting the presence of koilocytes and morphologic features characteristic of HPV-associated papillomatosis are depicted in Fig. 1.

Paraffin-embedded biopsy specimens of laryngeal tissues were collected, and genomic and viral DNA was extracted and stored at −20°C. DNA purity was assessed by a UV/VIS spectrophotometer estimating the A_{260}/A_{280} ratio, and DNA was titrated to 200 μg/ml. The presence of amplifiable DNA was verified by polymerase chain reaction (PCR) using primers specific for b2-microglobulin. The presence of HPV DNA was evaluated by PCR using the Biotypap Kit (Biotools, B&M Labs, S.A., Spain) followed by the restriction fragment length polymorphisms (RFLP) technique according to the manufacturer's instructions. This method detects thirty-two HPV types, including types 6, 11, 13, 16, 18, 30, 31, 32, 33, 34, 35, 39, 40, 42, 43, 44, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 64, 66, 67, 68, and 69. All PCR reactions included the appropriate negative controls. PCR-RFLP products were analyzed on 2% agarose gel and photographed on a UV light transilluminator.

Results

Case 1 A 5-year-old male child presented with a 6-month history of snoring and 1.5 months of hoarseness. He was delivered by cesarian section after a 37-week gestation, and his past medical history was free. Upon examination, his respiratory sounds were normal. Direct laryngoscopy showed multiple papillomas located on both vocal cords that were expanding to the subglottic area. The papillomas were removed surgically, and biopsy samples were collected. Pathologic analysis of the lesions revealed the presence of koilocytes and morphologic features characteristic of HPV-associated papillomatosis. The child was re-operated twice, at the ages of 5 and 6 years, due to relapse. Two years after his third operation, the child was progressing well and was free of symptoms. HPV typing of the biopsy sample collected during the first surgery revealed the presence of the HPV-13 and HPV-39 genotypes (Fig. 2).

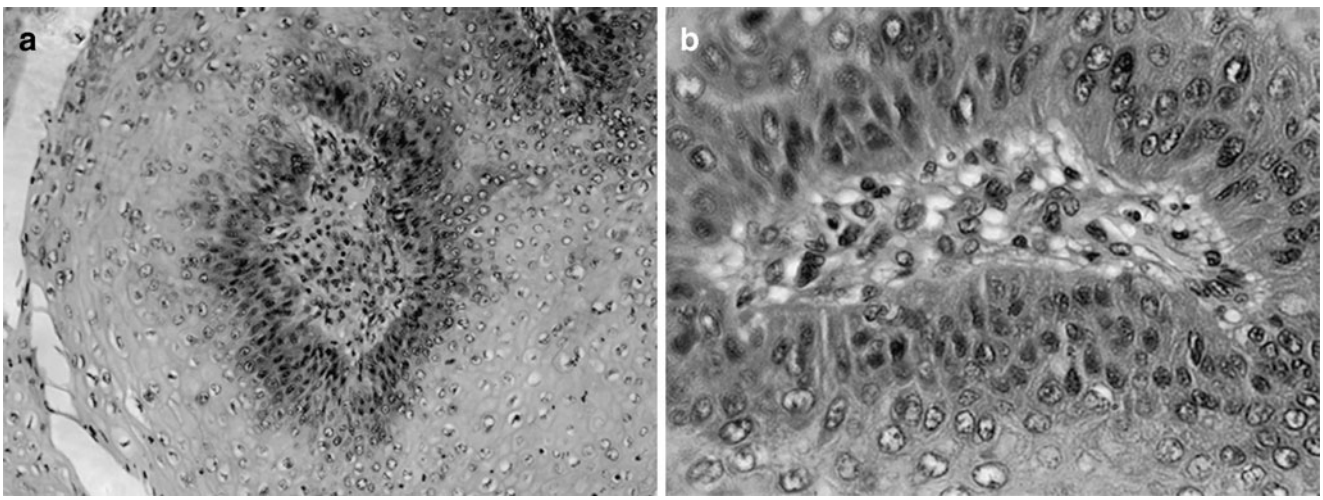

Fig. 1 Biopsy sample of recurrent respiratory papillomatosis in a 5-year-old child (Case 1). Numerous fragments of papilloma can be observed. Koilocytosis is a prominent feature of the cytoplasm, and the nuclei are enlarged, wrinkled, and frequently multiple. **a** ×20 magnification; **b** ×63 magnification

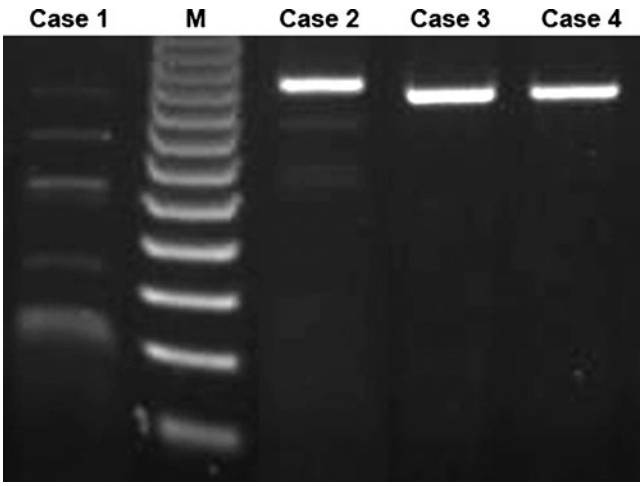

Fig. 2 HPV detection by polymerase chain reaction (PCR) followed by restriction digestion (RFLP). According to the HPV kit employed, the electrophoretic pattern of Case 1 corresponds to the genotypes HPV-13 and HPV-39, case 2 to co-infection with HPV-40 and HPV-56, and cases 3 and 4 to unidentified 'low-risk' HPVs

Case 2 A 6-year-old male child presented with a 2-year history of wheezing that was non-responsive to inhaled corticosteroid and short-acting inhaled β_2-agonist. The child was born by normal vaginal delivery, and his past medical history was free. There was no family history of asthma or atopy. Examination revealed mild inspiratory and expiratory wheezing and an oxygen saturation of 96%. Direct laryngoscopy showed the presence of papillomas on both vocal cords that were treated surgically. Pathologic analysis was used to confirm the presence of HPV-associated papillomatosis. Anti-asthmatic medications were discontinued, and the child was discharged. One month after discharge the child was asymptomatic; however, no further follow-up data were available for this patient. PCR-RFLP determined that the sample collected during surgery was concurrently positive for HPV-40 and HPV-56 (Fig. 2).

Case 3 A 3-year-old male child was admitted to the 'Aglaia Kyriakou' Children's Hospital with a 10-month history of hoarseness. The child was born by normal vaginal delivery with a birth weight of 2,850 g, and his past medical history was free. Upon examination, his respiratory sounds were normal. Direct laryngoscopy showed laryngeal papillomatosis, which was confirmed by pathologic analysis. The papillomas were surgically managed and the child was discharged. One year after his discharge, the child was asymptomatic and free of hoarseness or wheezing. HPV typing of the collected biopsy sample revealed the presence of 'low-risk' HPV other than HPV-6 or HPV-11 (Fig. 2).

Case 4 A 2.5-year-old male child presented at the 'Aglaia Kyriakou' Children's Hospital with a tracheostomy of unknown etiology, which he had received at the age of 7 months. The child was delivered by normal vaginal delivery, and his past medical history could not be determined. Direct laryngoscopy showed the presence of glottic masses that were expanding to the subglottic area, causing glottis obstruction. Biopsy samples were obtained, and pathologic analysis was indicative of the presence of papillomas. The papillomas were surgically removed, and at 1 and 2 months after surgery the larynx remained free of papillomas, with excellent mobility of the vocal cords. The tracheostomy was closed, and the child was discharged with no respiratory problems. One year after the operation, the child was progressing well and was free of symptoms. HPV typing of the collected biopsy sample revealed the presence of 'low-risk' HPV. PCR-based analysis provided no further evidence of any particular HPV genotypes for this case, but did clearly exclude HPV genotypes 6 and 11 (Fig. 2).

Discussion

Increased understanding of the role of human papilloma virus (HPV) has demonstrated that HPV types 6 and 11 are the principal causes of recurrent respiratory papillomatosis (RRP) in childhood [8, 9, 19, 21]. HPV-11 is considered as the most common cause of RRP, while HPV-6 is detected less frequently [8, 19]. The present study demonstrated the presence of HPV genotypes other than HPV-6 or HPV-11 in children with RRP. Based on phylogenetic and epidemiological criteria, HPV-39 and HPV-56 are considered 'high-risk' HPVs, while HPV-13 and HPV-40 are considered 'low-risk' [17]. To the best of our knowledge, this is the first study to demonstrate the presence of HPV types 13, 39, 40, and 56 in children with RRP.

In cases 1 and 2, simultaneous HPV infection with HPV-13 and HPV-39 (case 1), and HPV-40 and HPV-56 (case 2) was detected. Multiple HPV infection in patients with RRP has been reported by several researchers, with HPV-6 and HPV-11 co-infection detected most frequently [1, 10, 11, 23]. In laryngeal premalignant lesions studied by Azzimonti et al. [1], multiple HPV infections containing two or three HPV types were detected in 60% of 28 HPV-positive lesions. Lin et al. [10] reported the case of a 58-year-old female patient who presented with laryngeal squamous papillomas with severe dysplasia and was co-infected with HPV-11 and HPV-16. Nicollas et al. [11] reported the case of an 11-year-old girl who presented with RRP as well as HPV-6 and HPV-16 co-infection. Simon et al. [23] reported the case of a 12-year-old child with laryngeal squamous cell carcinoma in whom HPV-18 and HPV-33 simultaneous infection was detected. It has been proposed that the presence of multiple HPV infection is not related to the

clinical severity of RRP [12]. Further research is required to investigate the role of multiple HPV infection in the pathogenesis of RRP.

None of our studied cases was positive for the "oncogenic" HPV types 16 and 18. The two types are considered 'high-risk' HPVs due to their role as the principal etiologic agents of cervical carcinoma and its precursors [28]. HPV-16 and HPV-18, albeit the latter less frequently, have also been detected in precancerous and neoplastic lesions of the oral cavity, pharynx, and larynx [14, 16, 25]. It has been proposed that respiratory infection with "high-risk" HPVs introduces a long-term risk of squamous cell carcinoma development [15]. Moreover, the DNA integration of HPVs into the host-cell genome may contribute to the occurrence of severe dysplasia in RRP [11]. HPV-39 and HPV-56 are also considered 'high-risk' due to their presence in cervical carcinoma. However, their role in the malignant transformation of RRP, as well as in head and neck carcinoma progression has yet to be investigated and elucidated. Our results do not only corroborate the implication of HPV in the development of the disease, but furthermore, we report HPV types, which are described for the first time in this pathological condition. Moreover, the detection of non-oncogenic HPV types is also of interest and it has been previously hypothesized [1]. The variations in HPV types detected could be explained by geographic or ethnic variations.

Since 2006, the two multivalent HPV vaccines approved by the European Union have already been introduced into the national vaccination programs of several European countries, including Greece [18]. The two vaccines offer protection against HPV-16 and HPV-18, while the quadrivalent HPV vaccine also offers protection against HPV-11 and HPV-6 [2]. It has been proposed that HPV vaccination against HPV-6 and HPV-11 offers protection against RRP in children [2]. Recently, Chesson et al. [4] have estimated the health and economic benefits of preventing RRP through the quadrivalent HPV vaccination. However, the finding of our study that additional HPV types can be involved in the pathogenesis of RRP indicates that this protection may be especially limited to geographical areas with a high prevalence of other non-HPV-6 or HPV-11 types.

Polymerase chain reaction (PCR) is considered the most sensitive method of HPV detection in laryngeal specimens [14]. Maloney et al. [12] have proposed that levels of HPV-6 and HPV-11 viral loads are relatively stable over time in most children with RRP, which suggest that the collection of multiple samples may not be necessary for the detection of HPV. However, childhood RRP is characterized by a relatively low HPV viral load [21] that can potentially cause false negative results. In our study, all samples were HPV positive. This can be attributed to the high sensitivity of the PCR assay employed, as well as to the quality of the samples. Moreover, compared to the standard commercial tests, the employed PCR methodology in our study could detect a wider range of different HPV types. Pathologic analysis in all four cases showed marked keratohyaline granules, non-uniform perinuclear halos, and marked papillomatosis. It has been proposed that RRP samples with these features are associated with productive HPV infection and thus have a higher HPV viral load [21].

The management of RRP depends on the degree of airway involvement and includes surgical removal of the papillomas, though this has a high recurrence rate following treatment [26]. Affected children usually require multiple interventions, which has a considerable impact on patients, their families, and the healthcare system [24]. Factors affecting the time course of RRP include inter-surgeon variability, the extent and severity of papillomas at the time of laryngoscopy, and the use of adjuvant medical therapies [9]. Surgical therapy is considered as the primary treatment of RRP. However, adjunctive drug therapy with cidofovir, interferon, acyclovir, ribivirin, COX-2 inhibitors, retinoids, anti-reflux medications, zinc, and indole-3-carbinol has been used [6] in patients with aggressive disease. Recently, it was proposed that intralesional injections of cidofovir are an effective method of treatment for recurrent respiratory papillomatosis in children [3]. In our study, all cases were managed surgically and no adjunctive drug medication was used before or after the surgical removal of the papillomas.

HPV testing can provide additional information regarding prognosis and can be helpful in the management of RRP during childhood. It has been found that children with RRP infected with HPV-11 are prone to developing more aggressive disease than those with HPV-6 and require more frequent surgical intervention [12, 22]. HPV-11 infection has also been related to more frequent adjuvant therapies, tracheal and pulmonary disease, and tracheostomy [8, 19, 27]. Case 1 of our study, which had simultaneous infection with HPV-13 and HPV-39, required several surgical procedures, while the fourth case, which had 'non-oncogenic' HPV other than HPV-6 and HPV-11, required tracheostomy. The second case, with HPV-40 and HPV-56, and the third case, with 'low-risk' HPV, responded well to surgical management.

Further research is necessary to evaluate the prevalence of these novel HPV types detected in our study in children with RRP as well as their impact on prognosis. It is also important to investigate the prevalence and potential role of these novel HPV types after the introduction of the quadrivalent HPV vaccination into the clinical practice.

In the present cases, there was no maternal history of genital warts or cervical neoplasia. Three children were born by normal vaginal delivery and one child by cesarean section. HPV infection is generally the result of perinatal

transmission, implying that the consideration of sexual abuse is unnecessary in RRP cases [5]. Children with RRP are more commonly delivered vaginally than by caeserian section and are more likely to be the first-born children [7]. Their mothers more frequently have a history of anogenital warts or cytological and histological lesions caused by HPV infection in the genital tract and tend to be under 20 years of age [7]. The mechanism of HPV transmission is believed to occur through viral exposure during the traversal of the birth canal at the time of delivery; however, not all children born to mothers with active genital lesions develop papillomas [20]. Perinatal infection may occur transplacentally, via amniotic fluid during gestation and delivery and by direct exposure to cervical and genital lesions during birth. However, it remains unclear how frequently perinatal infection progresses to clinical lesions, whether genital, laryngeal, or oral. The exact possible modes of HPV transmission and the natural history of HPV infection in childhood remain to be elucidated.

Conflict of interest The authors declare that they have no conflict of interest.

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