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Laryngeal papillomatosis: molecular, histopathological, and clinical evaluation

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Abstract Molecular, histopathological, and clinical studies were carried out on a series of 79 laryngeal papillomas (LP) from 36 patients in order to investigate the hypothesis that juvenile and adult LP may represent a biological entity causally related to Human papilloma virus (HPV) infection. Using in situ hybridization with biotin-labelled probes and polymerase chain reaction, we detected human papilloma virus (HPV) 6/11 in 28 of 29 juvenile LP, in 26 of 30 adult multiple, and in 17 of 20 adult solitary LP. None of LP was found to harbour HPV types 16, 18, 31, 33, and 51. There were no clear-cut histological differences between juvenile and adult LP, the presence of koilocytosis was equally observed in both, and there was no prevalent type of epithelial hyperplasia in either form, except that all three cases of atypical hyperplasias (precancerous lesions) were found among adult patients. During a 14 year follow-up, no carcinomatous transformation of LP was observed. All juvenile LP in our study had frequent recurrences of the disease, however, numerous surgical procedures were also required in 16 of 27 adult patients. Our study supports Lindeberg's hypothesis of a similar pathogenesis for all forms of LP caused by the HPV types 6/11.

Key words Laryngeal papillomatosis · Aetiology
Molecular pathology techniques · Histopathology
Follow-up

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Introduction

Application of molecular hybridization techniques has increased our knowledge of Human papilloma virus (HPV) and associated tissue lesions. HPV infections are found to be prevalent in the uterine cervical squamous epithelium and associated with proliferative lesions [4, 21]. In addition to the cervical epithelium, HPV expresses a significant epitheliotropism to the skin and mucous membranes of the urogenital and upper aerodigestive tracts, producing hyperplastic, usually papillomatous lesions [5].

A significant number of studies have confirmed the opinion that laryngeal papilloma (LP), the most frequent benign neoplasia of the upper respiratory tract, has a viral aetiology [5]. The papilloma appears as an exophytic, branching, pedunculated or sessile mass, single or multiple, most frequently located on the true vocal cords. Other parts of the laryngeal mucosa may also be affected. Histologically, the tumour consists of a central fibrovascular core covered by squamous epithelium. LP is classified into four groups: juvenile multiple, juvenile solitary, adult multiple, and adult solitary. This classification is based on age at the onset of the disease, the number of lesions revealed at first presentation, and a difference in sex distribution [16]. Strong evidence has supported a viral aetiology in juvenile groups, caused most probably by HPV 6 and 11 [20, 24, 25]. In contrast, some authors found a markedly lower frequency of HPV infections in adult papillomas. A possible difference in aetiology for these lesions, particularly solitary adult LP, has thus been suggested [23, 25, 27]. Using two methods of molecular pathology, in situ hybridization (ISH) and polymerase chain reaction (PCR), this retrospective study was performed to prove or disprove Lindeberg's hypothesis [13] that all forms of LP might represent a biological entity caused by the same HPV types 6/11. To substantiate this hypothesis further, a comparison of the histopathological features and clinical behaviour of juvenile and adult groups of LP has been investigated.

Materials and methods

Thirty-six patients with a diagnosis of laryngeal squamous cell papillomas confirmed by traditional light microscopy, were selected. The juvenile group included 3 males and 6 females, the adult 17 males and 10 females. The youngest patient was 10-months-old and the oldest 71 years; the mean age was 37 years. The study spanned a period of 14 years between 1980–1993.

All 79 biopsy specimens obtained from the patients were fixed in 10% neutral buffered formalin, embedded in semi-synthetic paraplast wax, and routinely processed. The covering epithelium of the LP was classified into simple (squamous cell hyperplasia), abnormal (mild dysplasia), and atypical (moderate and severe dysplasia) hyperplasia following Kambič's classification [3, 8, 9, 10, 17], which is essentially comparable with the current WHO classification, although the terminology is somewhat different [23]. The presence of koilocytosis, the only visible sign of HPV infection by traditional light microscopy, limited to the superficial layers of the squamous epithelium, was also evaluated.

Additional 4 μm sections from the paraffin blocks were cut, from all 79 specimens for ISH, and from 32 for PCR. ISH analysis was performed on all tissue specimens with HPV 6/11, 16/18, and 31/33/51 biotin-labelled probes from an ENZO PathoGene in situ human papillomavirus tissue hybridisation kit (New York, USA) according to the manufacturer's directions. For negative controls, autopsy material was obtained from 5 normal vocal cord specimens. As positive controls, 5 condylomata accuminata containing HPV 6/11 and 3 cervical squamous cell carcinomas containing HPV 16/18, were chosen.

The preparation of DNA from formalin-fixed, paraffin-embedded tissue sections for the PCR has been described previously [22]. All known precautions to avoid PCR-product carryover and sample-to-sample contamination were taken rigorously [11]. The prepared specimens were used immediately for PCR.

PCR amplifications that target a portion of the HPV L1 region (approximately 450 bp) were performed using HPV L1 consensus primers MY09 (5′-CGTCCMARRGGAWACTGATC; M=A+C, R=A+G, W=A+T) and MY11 (5′-GCMCAGGGWCATAAYAATGG; Y=C+T) (Perkin-Elmer, Norwalk, USA) as previously described [18, 19, 26]. All samples were tested initially using the beta-globin gene specific primers GH20 (5′-GAAGAGCCAAGGACAGGTAC) and PC04 (5′-CAACTTCATCCACGTTCACC) (Perkin-Elmer). Successful amplification of the beta-globin fragment (268 bp) indicated that the sample was adequate for PCR analysis and that no PCR inhibitors were present. To further reduce the possibility of false negative results, the quality of DNA preparation was checked in HPV PCR negative samples by amplification of a longer segment (720 bp) of the human bcl-2 gene using 5′-ATGGCGCACGCTGGGAGAACAGGGTA and 5′-TGGGTGCCTATCTGGGCCACAAGTGA primers.

For typing of HPVs after PCR, restriction fragment analysis of the HPV L1 amplified products was performed, according to the original method developed by Manos and Wheeler of the Department of Infectious Diseases, Cetus Corp, Emeryville, Calif., USA [18, 19, 26]. All the enzymes used: *Bam*H I, *Dde* I, *Hae* III, *Hinf* I, *Pst* I, *Rsa* I, and *Sau*3A I, were from Gibco-BRL Life Technologies, Inc. (Bethesda, M.D., USA). The PCR product (5 μl) was mixed with 5–10 U of restriction enzyme in the optimal buffer system provided for each enzyme by the manufacturer, in a total volume of 15 μl. The reactions were performed in a 37° C water bath for 1 h and stopped by adding 2 μl of DNA gel loading buffer (15% Ficoll 400, 0.35% Brom Phenol Blue). Digested bands were separated by electrophoresis in 3% agarose gel prepared with 0.5×TRIS-borate-EDTA buffer, followed by staining with ethidium bromide.

Results

Thirty-six patients with 79 LP were selected in a random manner and the present group does not represent our general population with LP. Twenty-four (67%) of these patients had multiple surgical procedures, among them all patients with juvenile papillomas. Only one surgical removal of LP was performed in 12 (33%) patients.

With regard to the covering epithelium, simple hyperplasia (squamous cell hyperplasia) was found in 9 of 12 patients who had had one surgical procedure; the epithelium was mainly thickened because of the augmented cells in the spinous layer, while the basal layer was normal. The remaining three patients showed abnormal hyperplasia (mild dysplasia): the epithelium was thickened because of "basalification". The basaloid cells extended to the midportion of the epithelium. Rare dyskeratotic cells were present; no distinct atypias or pathological mitoses were observed. In 5 out of 24 patients with multiple recurrences, repeated LP showed simple, and in 4 abnormal epithelial hyperplasia. In 12 patients, a deterioration of epithelial changes was found, from simple to abnormal hyperplasia, and in 3 patients from abnormal to atypical hyperplasia (moderate and severe dysplasia). In this type of lesion, the cells of almost the entire epithelium consisted of immature basaloid cells with moderate nuclear and cytoplasmic atypia; rare mitosis and numer-

Table 1 Results of in situ hybridization performed on 79 LP

LP type	HPV 6/11	HPV 16/18	HPV 31/33/51
Juvenile multiple LP	25/29[a]	0/29	0/29
Adult multiple LP	23/30	0/30	0/30
Adult solitary LP	13/20	0/20	0/20

[a] No. positive/total No. biopsies

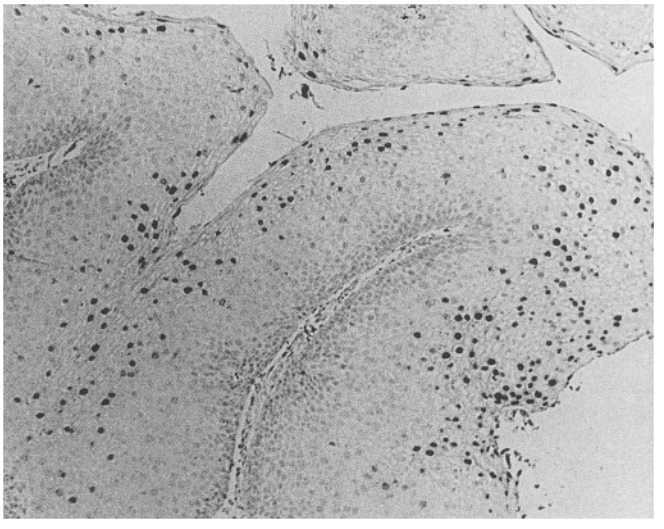

Fig. 1 Positive in situ hybridization (ISH) signal for human papilloma virus (HPV) type 6 in a juvenile laryngeal papilloma (LP). ×82

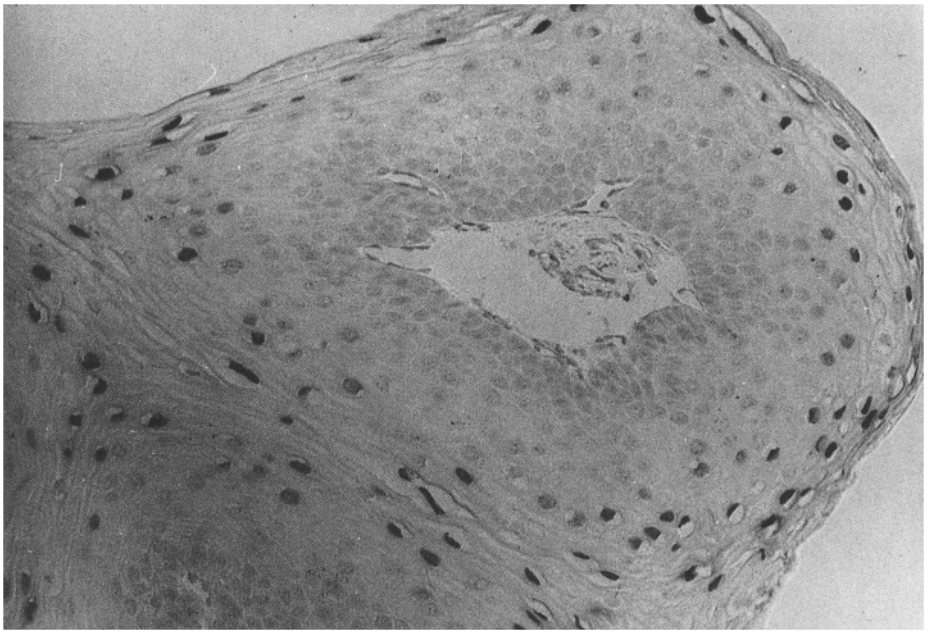

Fig. 2 Positive ISH signal for HPV type 6 in a adult LP. ×309

Table 2 Results of polymerase chain reaction

Tissue sample	No. positive/ No. biopsies	% positive
ISH positive LP	10/10[a]	100
ISH negative LP	10/18	55
Normal vocal cord	0/5	0

[a] No. positive/total No. biopsies

ous dyskeratotic cells were seen throughout the epithelium. Koilocytes showing nuclear enlargement, angularity or pyknotic forms, and cytoplasmic vacuolization, were present in 71/79 (90%) specimens. In relation to the type of epithelial hyperplasia and the occurrence of koilocytosis, no particular differences were found between juvenile and adult LP.

The results of ISH analysis performed on all 79 papillomas are shown in Table 1. The hybridization signal (red or reddish-brown stain) was always confined to the nuclei, involving mostly numerous cells in the superficial squamous layers, and a positive correlation was found with koilocytosis (Figs. 1, 2). Using ISH, we discovered the presence of HPV 6/11 in 25 (86%) of 29 LP of juvenile onset, in 23 (77%) of 30 with adult multiple and in 13 (65%) of 20 with adult solitary papillomas. None of the LP was found to harbour HPV types 16, 18, 31, 33, and 51. The five autopsy specimens of normal vocal cords were also found to be HPV negative.

Among 33 tissue specimens analysed by PCR, 18 biopsies were included with negative results for ISH, 10 cases with proven viral infection by ISH, and 5 autopsy samples of normal vocal cord mucosa. The results are presented in Table 2. An additional 10 HPV DNA positive cases of LP were found by PCR, including 3 of juvenile LP, 3 of adult multiple, and 4 of adult single LP.

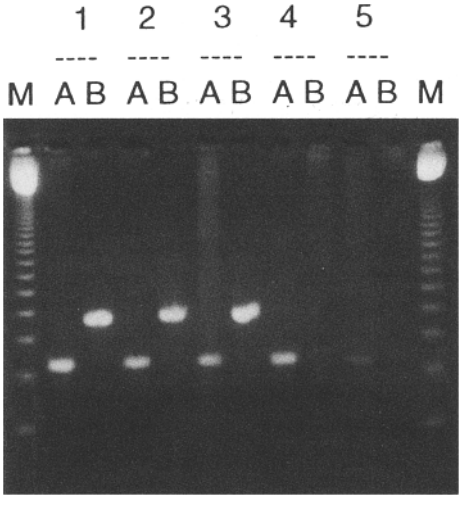

Fig. 3 Detection of HPV DNA present in LP samples by polymerase chain reaction (PCR). Agarose gel analysis of beta-globin gene (lanes labelled with A; 268 bp) and HPV L1 (lanes labelled with B; 449 bp) specific PCR products. Lanes M, 123 bp DNA ladder (Gibco-BRL); lanes 1–5, amplification products of DNA extracted from five ISH HPV-negative cases of LP

Three of these positive cases are shown in Fig. 3, lanes 1, 2 and 3. Using both methods, there were altogether 71 (90%) positive LP of 79, including 28 (97%) of 29 juvenile LP, 26 (87%) of 30 multiple adult, and 17 (85%) of 20 solitary adult LP. To determine the HPV type, the PCR products of all ISH negative-PCR positive cases were digested with 7 restriction endonucleases, and analysed by agarose gel electrophoresis. The restriction patterns were found to be unique for HPV type 6 in all ISH negative-PCR positive cases (one of them is seen on Fig. 4). All other HPV ISH-negative cases of LP and the 5 autopsy specimens of vocal cords were successfully am-

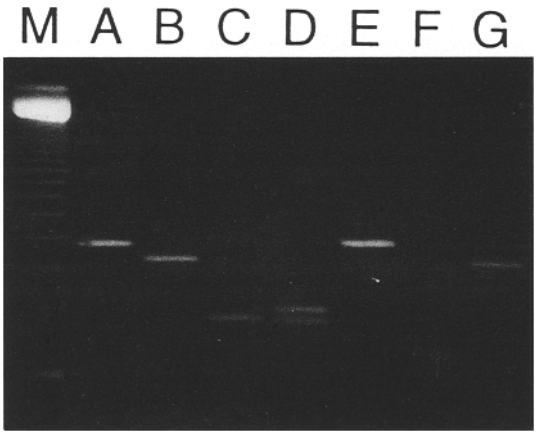

Fig. 4 Determination of HPV type by restriction fragment analyses of the HPV L1 PCR products. Electrophoresis patterns of PCR product of DNA extracted from a case of ISH negative LP, cleaved with *Bam*H I (lane A; 449 bp), *Dde* I (lane B; 382, 67 bp), *Hae* III (lane C; 217, 124, 108 bp), *Hinf* I (lane D, 234, 215 bp), *Pst* I (lane E; 449 bp), *Rsa* I (lane F; 161, 149, 72, 67 bp), and *Sau*3A I (lane G; 366, 63, 20 bp). Lane M, 123 bp DNA ladder (Gibco BRL)

plified only with internal control human beta-globin primers (two of them are shown on Fig. 3, lanes 4 and 5) and human *bcl*-2 primers.

Discussion

A more precise detection of viruses in tissue specimens was enabled by the techniques of molecular pathology in the early 1980's. This advanced methodology has been also performed in studies of HPV infection in LP. The accurate location of viruses within the tissue can only be revealed by ISH used on fresh or fixed material, applying both radioactive [15, 27] and nonradioactive probes [20, 21, 24, 25]. Recently, a highly sensitive PCR has been utilized for detection of HPV DNA in LP [2, 7, 12].

LP have been traditionally divided into two entities, juvenile and adult, in view of the biphasic age distribution, the difference in sex incidence, and the clinical course of the disease [6, 16]. Not least, a significantly lower incidence of HPV detection in adult solitary papillomas described in some studies has suggested a possible different aetiology for the two lesions [23, 25, 27]. In contrast, Lindeberg's hypothesis that all laryngeal papillomas may form a biological entity, was based on the demonstration of HPV 6/11 in 19 of 20 adult solitary LP obtained in 16 patients by ISH with sulphonated DNA probes [15].

Our results on a large series of cases are almost entirely comparable with the Lindeberg's and Johansen's analysis [15]. Using both methods of molecular pathology, ISH with biotin-labelled probes and PCR, we detected the presence of HPV 6/11 in 28 of 29 juvenile LP, in 26 of 30 adult multiple and in 17 of 20 adult solitary LP.

The results of traditional light microscopical studies did not provide significant differences between juvenile and adult LP. Abramson et al. [1] demonstrated in their cases of LP abnormal squamous maturation with parakeratosis, retardation of superficial cell maturation, and basal hyperplasia of the papillary branches epithelium. Their analysis did not show any correlation of these histopathological findings, either with the age of onset or the natural course of LP. Furthermore, Lindeberg [13] performed a histomorphometric analysis in 162 LP, divided according to clinical parameters into juvenile multiple, adult multiple and adult solitary forms; the volume fractions of basal cells, koilocytes and nuclei, as well as the mean nuclear volume, were determined in each group. His analysis disclosed no significant differences between them.

As with the above mentioned findings, our study established no clear-cut histological differences between juvenile and adult LP. The presence of koilocytosis, observed in the upper part of the spinous layer in 90% of cases, was not proved to be a discriminating feature between the two forms of LP. The most significant histopathologic feature of all 79 LP was epithelial hyperplasia. In 33 LP of 14 patients, we classified the thickened epithelium of the papillary branches as simple hyperplasia (squamous cell hyperplasia), as abnormal hyperplasia (mild dysplasia) in 43 LP of 19 patients, and as typical hyperplasia (moderate and severe dysplasia) or risky epithelium in 3 LP of 3 patients. There was no prevalent type of epithelial hyperplasia in either form, except that all three cases of atypical hyperplasias were found among adult patients. These three patients developed risky epithelium in the first, fourth, and seventh year after the first removal of LP, and underwent multiple surgical procedures. None of our patients with LP, including these three with risky epithelium, were found to harbour HPV types 16, 18, 31, 33, and 51, which have been demonstrated to be associated with squamous precancerous lesions and carcinomas in the anogenital region, skin and in the upper aerodigestive tract [5].

Biphasic age distribution is thought to be the most significant parameter in distinguishing the two forms of LP. It is generally accepted that HPV transmission to neonates occurs during fetal passage through the birth canal [5, 21]. The mode of adult infection remains unclear. The reactivation of a latent infection connatally acquired, or a post-partum infection, are suggested as two possibilities [5]. We favour the second possibility. It seems more likely that a solitary adult HPV 6/11 positive LP diagnosed in our female patient at the age of 71 years for the first time, is causally related to a viral infection acquired during life rather than to an endogenous reactivation of a virus obtained intrapartum. Rimell et al. [21] also believe that the theory of birth canal transmission in LP is unproven because, of 38 patients studied, at least some should have been infected by HPV 16/18, the prevalent HPV types in the uterine cervix. Lindeberg and Elbrond [14] think that if latent infections originating from birth are responsible for adult papillomas, they could be expected to have a more uniform sex distribution, but in these patients, male preponderance, confirmed also in

our study, is evident. The clinical course of the disease in children is usually more serious and dramatic than in adults. This could be one of the main reasons why the disease is divided on the basis of the patient's age. However, we have to be aware that the significantly smaller diameter of the airways in children, rather than differences in the biological behaviour of LP, may best explain the dangerous or even fatal clinical outcome occuring occasionally in patients with LP of this age group.

Frequent recurrences of the disease are more characteristic of juvenile LP. In this study, all children underwent from 4 to 15 repeated surgical removals. Nevertheless, 16 of 27 adult patients had also had frequent operative procedures (from 2 to 9). It might speculatively be suggested that a lower number of viral copies in adult LP compared to juvenile [5], is the cause of the observed differences in the recurrence rate between LP of the two age groups.

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