HLA-DQ Alleles and Human Papillomavirus DNA in Adult-Onset Laryngeal Papillomatosis

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Sixty-two patients with histologically confirmed adult-onset laryngeal papilloma were clinically examined; their HLA class II DQA1 and DQB1 alleles and the presence and type of human papillomavirus (HPV) in their laryngeal papilloma biopsies were determined by polymerase chain reaction–based methods. No differences in the DQA1 or DQB1 frequencies appeared between the patients as a group and the reference population. When the patients were divided into groups according to number of laryngeal procedures performed, no HLA association was noticed with any group, nor did the presence of HPV-6 or HPV-11 DNA in the laryngeal specimen correlate with HLA type. A suggestive association was found between the DQB1*0501 allele and the 16 patients whose laryngeal biopsy was HPV-negative, but because of the small series, additional patients need to be studied. Earlier, the DQB1*0501 allele was reported to be protective against cervical cancer, another HPV-associated disease.

Patients and Methods

Patients and samples. This study was based on the adult-onset (age at diagnosis, ≥17 years) laryngeal papilloma patient population without malignant transformation, treated at Department of Otorhinolaryngology, Helsinki University Central Hospital, during 1975–1994. Of the 113 patients, 43 were excluded: 5 had died, 17 were >80 years old, and 21 did not live in the hospital district. Of the 70 patients invited to join the study, only 8 did not participate.

Thus, 62 patients filled in a questionnaire, were examined by an otolaryngologist, and had their larynxes video-recorded. A blood sample was taken for HLA assay. HPV DNA from the most recent formalin-fixed paraffin-embedded laryngeal biopsies was detected by PCR and Southern blotting. DQA1 and DQB1 allele frequencies obtained from 93 cadaver kidney donors served as a reference population [6].

PCR-based HLA class II typing. The DQA1 alleles were determined as described by Ota et al. [7]. The assay distinguishes 7 different DQA1 allele-groups: *0101/2/4, *0103, *02, *03, *04, *05, and *06. The DQB1 alleles were determined with a DQB1 reverse dot blot kit (Inno-Lipa; Innogenetics, Zwijndrecht, Belgium), which detects 25 of the 26 currently known DQB1 alleles.

DNA extraction for HPV DNA detection. Paraffin-embedded laryngeal papilloma blocks from 61 patients (one block was lost) were cut into sections in a series of 10 blocks, every 7th block containing only paraffin. Papilloma tumor histology was confirmed by examination of van Gieson–stained sections of the specimens. After deparaffinization, DNA was purified by the phenol-chloroform extraction.

PCR. PCR was performed both with β-globin primers [8] and with type-specific primers for HPV-6, -11, and -16 [9] on all specimens. Specimens negative with type-specific primers were subjected to PCR with general primers GPS+/6+/10]. Because of the small quantity of DNA, PCR was performed in two phases: first 20 cycles and then 35 cycles, in which 5 μL of the former PCR product served as a template. The reaction mixtures contained 1.5 mM
Table 1. HPV-type of 61 laryngeal papilloma biopsies from 61 adult-onset laryngeal papilloma patients as detected by polymerase chain reaction (PCR) and Southern blotting.

<table>
<thead>
<tr>
<th>HPV-type</th>
<th>No. of biopsies (%), n = 61</th>
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<tbody>
<tr>
<td>6</td>
<td>20 (33)</td>
</tr>
<tr>
<td>11</td>
<td>8 (13)</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Some other HPV type</td>
<td>2 (3)</td>
</tr>
<tr>
<td>No HPV DNA detected</td>
<td>16 (26)</td>
</tr>
<tr>
<td>Samples not amplifiable in PCR</td>
<td>16 (26)</td>
</tr>
</tbody>
</table>

NOTE. One patient had both HPV-6 and HPV-11 DNA in her biopsy.

MgCl₂, each primer at 0.4 μM, each dNTP at 0.2 mM, and 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ). The PCR reaction was initiated with a “hot start” (10 min at 95°C) followed by 1 min of denaturation at 95°C, 2 min of annealing, 1.5 min of chain elongation at 75°C, and another 4 min of chain elongation at 72°C after the last cycle. The annealing temperature was 40°C for GP5+/6+, 58°C for β-globin, HPV-6, and HPV-11 primers; and 62°C for HPV-16 primers. All reactions were performed with an automated PCR machine (PTC-100; MJ Research, Watertown, MA).

HPV-6 and -11 plasmid DNA (5 ng/reaction) or HPV-6– and -11–positive laryngeal specimens served as positive controls. Genomic DNA (5 ng/reaction) isolated from SiHa cells (HTB35; American Type Culture Collection, Rockville, MD) served as a positive control in HPV-16 PCR, and DNA (5 ng/reaction) isolated from an HPV-16-containing cervical carcinoma biopsy, for GP5+/6+ PCR. Our negative controls were HPV-6–, -11–, and -16–negative laryngeal specimens, and for GP5+/6+, DNA extracted from laryngeal fibroblasts of a patient with no known history of HPV-related disease. For paraffin and water controls, glycogen was added to serve as a carrier for possible DNA contamination. The PCR products (5 μL each) were run in 2% agarose gel, which was visualized with ethidium bromide, and the products of HPV type-specific PCR reactions were transferred to nylon membranes for detection. The test detected 100 ag of HPV plasmid DNA corresponding to ~12 copies of HPV DNA.

Southern blot hybridization. The PCR products were transferred to nylon membranes (Hybond N+; Amersham Life Science, Amersham, UK). The membranes were prehybridized and hybridized with digoxigenin-labeled oligonucleotide probes. The hybrids were immunologically detected with alkaline phosphatase–conjugated anti-digoxigenin Fab fragments (Boehringer Mannheim, Mannheim, Germany), followed by a chromogenic reaction (4-nitro blue tetrazolium chloride and x-phosphate/5-bromo-4-chloro-3-indoly-phosphate; Boehringer Mannheim).

Statistical analysis. Pearson’s χ² test was used for statistical analysis to determine HLA allele frequencies and the Kruskal-Wallis test was used when clinical data between 2 patient groups was compared.

Results

Patient description. The male-to-female ratio was 3:1, comprising 47 male patients (mean age, 53 years; range, 26–77) and 15 female patients (mean age, 48 years; range, 27–71). The mean follow-up time was 10 years (range, 1–35). No hereditary component was discovered for the disease, and in otolaryngologic examination, no HPV-associated lesions were detected outside the larynx. A laryngeal tumor relapse occurred in 14 of the 62 patients (2 female, 12 male); in 2 of these men, their relapses proved in subsequent histopathology of the biopsies to be carcinomas.

HPV DNA detection. Formalin-fixed paraffin-embedded biopsies were available from 61 of the 62 patients. The results of PCR and Southern blot hybridization are seen in table 1. Only 1 patient, female, had a mixed HPV-6/11 infection. Of all 61 specimens, 45 (74%) were amplifiable in our PCR, and 16 (26%) were negative to all primers used. Of the 45 amplifiable samples, 29 (64%) were HPV-positive. HPV-positive biopsies were taken on average 9 years before the serum samples for HLA assay, and HPV-negative samples, as well as those negative to all primers (including β-globin), were taken, on average, 7 years before.

Relation between HLA status, HPV type of laryngeal biopsy, and clinical features. No differences in the DQA1 or DQB1 frequencies were detected between patients as a group and the reference population. Moreover, when the patients were divided into groups according to the number of laryngeal procedures performed (1 or 2, 3–6, >6), no HLA association was noticed with any group. Neither did the presence of HPV-6 or -11 DNA in the laryngeal specimen correlate with HLA type. However, patients whose laryngeal biopsy showed a typical histology of HPV infection but was HPV-negative had increased frequency of the DQB1*0501 allele compared with those whose laryngeal biopsy was HPV-positive (uncorrected P = .0053, Pearson’s χ² test; Bonferroni corrected, P = .053) or compared with the reference population (uncorrected P < .001, Pearson’s χ² test; Bonferroni corrected, P = .002). The DQB1 alleles determined in HPV-positive and -negative patients and the reference group are seen in table 2. Patients without detectable HPV DNA in their latest laryngeal biopsy had also undergone fewer laryngoscopies (mean, 2.8; range, 1–17) than those whose bi-
oposy contained HPV DNA (mean, 5.7; range, 1–70) \( (P = .0031\), Kruskal-Wallis test). The former patient group was also older than the latter at diagnosis \( (P < .001\), Kruskal-Wallis test). Those with an HPV-6- or -11-positive laryngeal biopsy did not differ from each other in regard to age, sex, number of laryngeal procedures performed, or number of relapses found in the clinical examination.

**Discussion**

We found no differences in the HLA-DQA1 and -DQB1 frequencies between the patients as a group and the reference population. Moreover, there was no HLA association with the number of laryngeal procedures performed or with the presence of HPV-6 or -11 DNA in the laryngeal specimen.

Of our 61 samples, 16 were negative to all primers used: \( \beta \)-globin, GP5+/6+, and HPV type-specific primers. In 5 of these 16, there was probably not enough DNA for PCR. The rest of the negative samples might have contained some inhibitors preventing the PCR reaction. However, in our experience this kind of archival material is suitable for HPV PCR; in fact, the HPV-positive blocks were even older than the negative ones.

No HPV-16-positive laryngeal biopsies existed, whereas HPV-6 was the most commonly found HPV type. After 10 years of follow-up, 19% of the patients had a papilloma relapse in their larynx, and 2 of these appeared to be malignant. Of interest, both of the patients with malignant transformation had the HLA-DQBI\( ^*0501\) allele and an HPV-negative laryngeal biopsy. Whether the observed apparent association between HPV-negativity and malignancy is merely accidental remains to be determined by further studies.

Compared with the reference population, the adult-onset laryngeal papilloma patients as a group did not exhibit a different HLA-DQA1 or -DQB1 profile. Neither could we confirm the result of Bonagura et al. \[5\] about the enriched DQw3 phenotype. Notably, the DQB1\( ^*0501\) allele was more common among adult-onset patients whose laryngeal biopsy was HPV DNA-negative compared with HPV-positive patients or with the reference population. After the Bonferroni correction (multiplying \( P \) values by the number of alleles tested, \( 10 \times P \)), the corresponding \( P \) values increased to .053 and .002, respectively. The latter is still statistically significant, but the former is only marginally significant. However, clinical data also support the protective role of DQB1\( ^*0501\). The patients with increased frequency of the DQB1\( ^*0501\) allele did not have HPV DNA in their laryngeal biopsies; they also had undergone significantly fewer laryngomicroscopies, which indicated less frequent relapses compared with relapses of those whose laryngeal biopsies were HPV-positive. Moreover, in previous studies, the DQB1\( ^*0501\) allele has been associated with protective effect against cervical carcinoma, another HPV-associated disease \[11, 12\].

HPV-negative patients with increased frequency of the DQB1\( ^*0501\) allele were, at time of diagnosis, significantly older than HPV-positive patients. An association of HLA with a patient’s age at disease onset is also found in many other diseases, including rheumatoid arthritis: Expression of two DR4 alleles is associated with earlier disease onset \[13\].

Histology of the HPV-negative specimens showed typical features of HPV infection with koilocytes, parakeratosis, and dyskeratotic cells. Because koilocytes are considered characteristic of HPV infection, these patients obviously have had a previous HPV infection in the larynx. It is possible that there is a bimodal age distribution of adult-onset laryngeal papillomatosis, with elder patients more likely to be HPV-negative than HPV-positive, as has been shown for some HPV-related cancers. This is in line with our previous findings with adult-onset laryngeal papilloma patients: Young patients at diagnosis are at the greatest risk for frequent relapses \[14\]. The DQB1\( ^*0501\) allele could help patients eradicate the HPV genome and in this way protect them from frequent relapses. However, because of the small number of patients, a study of another patient population is clearly warranted. Comparable data are available on the clearance of hepatitis B virus; the HLA class II allele DQBI\( ^*1302\) is associated with protection against persistent hepatitis B virus infection \[15\]. Further studies will be aimed at elucidating the role of the HLA-DQBI\( ^*0501\) allele in the cellular immune response against HPV and in laryngeal carcinogenesis.

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**References**