Human papillomavirus, viral load and proliferation rate in recurrent respiratory papillomatosis in response to alpha interferon treatment

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The aim of this study was to identify recurrent respiratory papillomatosis patients who may benefit from interferon (IFN)-α treatment and to determine the means of IFN-α action. The presence of human papillomavirus (HPV) and viral load and proliferation rate in pre-, ongoing and post-treatment respiratory papillomatosis biopsies were examined retrospectively in 25 patients, 18 of whom were IFN-α treated and seven of whom were IFN-α non-treated. Using PCR, HPV was found to be present in 20/25 respiratory papillomatosis patients and HPV type was determined for 18/25 patients (12 HPV6 and six HPV11). Eighteen of the patients were treated with IFN-α, 14 of whom were HPV positive (eight HPV6, five HPV11 and one undefined HPV). Response to IFN-α therapy was observed in 12 patients (7/8 HPV6, 3/5 HPV11, 1/1 undefined HPV and 1/4 HPV negative), while six patients (1/8 HPV6, 2/5 HPV11 and 3/4 HPV negative) did not respond to therapy. Viral load, determined by quantitative real-time PCR (between 0.03 and 533 HPV copies per cell), and proliferation rate, determined as the percentage of Ki-67-positive cells (between 8 and 54%), were similar in IFN-α-treated and non-treated patients and were generally unaffected by IFN-α treatment. In summary, most (12/18) IFN-α-treated patients responded to therapy. Moreover, there was a tendency for patients with HPV6-positive (7/8) respiratory papillomatosis to respond more frequently to IFN-α therapy than patients with HPV11 (3/5) or HPV-negative (1/4) respiratory papillomatosis. Finally, the presence of HPV and viral load and proliferation in respiratory papillomatosis biopsies was similar in patients treated or not with IFN-α and were in general unaffected by IFN-α treatment.

INTRODUCTION

Recurrent respiratory papillomatosis (RRP) is an epithelial, exophytic, benign neoplastic growth, which rarely turns malignant (Aaltonen et al., 2002; Corbitt et al., 1988; Go et al., 2003; Pou et al., 1995; Rabah et al., 2001). It is most frequently (>90%) located on the vocal cords (Kimberlin & Malis, 2000). The most common age of onset is early childhood (juvenile RRP with age of onset below 13 years of age), but the disease is also seen in adults (adult RRP) (Abramson et al., 1987). RRP is regarded as being induced by human papillomavirus (HPV), particularly HPV type 6 (HPV6) and HPV11, and shows a variable clinical course (Abramson et al., 1987; Bonagura et al., 2004; Pou et al., 1995; Rabah et al., 2001). The primary therapeutic method is endoscopic surgery, and approximately one-tenth of patients are cured after the first surgical intervention. Spontaneous remissions are also seen, but in the majority of patients the disease is recurrent and thereby demands other treatment methods. In addition to surgery, the only well-documented treatment for laryngeal papillomatosis is interferon (IFN)-α, which has been widely used for more than 20 years and has been proposed to decrease the number of recurrences considerably (Deunas et al., 1997). Although IFN-α has been used clinically for decades as a proven anti-tumour agent in a variety of malignant and non-malignant neoplastic disorders, the underlying mechanisms of its action remain to be elucidated. Furthermore, since IFN-α treatment is burdened with side effects for most patients and the cost is considerable, markers to predict which patients may be responsive to such treatment remains a challenge for future studies. New substances for treatment of patients
with respiratory papillomatosis such as cidofovir, acyclovir and ribavirin (Kimberlin, 2004) are currently being evaluated, but their role in controlling this disease is still uncertain.

The aim of this study was to examine the presence of HPV and determine viral load and proliferation rate in respiratory papillomatosis and in response to IFN-α therapy in order to determine possible mechanisms of IFN-α action and identify patients who will respond to IFN-α treatment. Twenty-five respiratory papillomatosis patients, of whom 18 had been subjected to IFN-α treatment, were included in the study. From these patients, 54 papillomatosis biopsies, including pre-, ongoing and post-treatment biopsies, were examined.

METHODS

Patients. Twenty-five RRP patients treated from 1977 to 1994 at the Karolinska University Hospital (Stockholm, Sweden) or from 1989 to 2002 at the Helsinki University Hospital (Helsinki, Finland) were included in this study, which was conducted in accordance with local ethical committees. During the selected time period, 18 of the 25 patients were subjected to IFN-α treatment. A total of 25 pre-treatment, 17 ongoing treatment and 12 post-treatment samples were obtained. The diagnosis of respiratory papillomatosis was confirmed by histopathology on haematoxylin/eosin-stained sections. The specimens were coded and all analyses were performed in a double blind set-up.

Therapy and response evaluation. The clinical charts of all patients were carefully reviewed. All patients were examined by indirect and direct laryngoscopy with lesion extirpation and pathological/anatomical confirmation of the laryngeal papillomatosis diagnosis. In one patient treated primarily with IFN-α, a response was defined as the absence of recurrence. All other patients were treated after recurrence and were considered as responders if no or only one relapse occurred during a 1-year period during or after the IFN-α treatment period.

Extraction of DNA. DNA was extracted from 5 × 5 mm formalin-fixed and paraffin-embedded archival tumour sections taken between 1 and 24 years prior to DNA extraction. Before and after each section, an additional section was taken for tumour tissue verification by haematoxylin/eosin staining. To check for HPV contamination between the tissue blocks, an empty block was cut, and the empty sections collected between every tumour block were subsequently treated as for the tissue sections. The paraffin was removed by xylene treatment, followed by ethanol washing. The pellet was air dried and incubated with proteinase K in 1× PCR buffer II (Applied Biosystems) at 60 °C, followed by inactivation of proteinase K at 98 °C for 8 min.

For 21 of the samples, the DNA was instead extracted using the High Pure RNA Paraffin kit (Roche Diagnostics) according to the manufacturer’s instructions, but with exclusion of DNase treatment.

Before running real-time quantitative PCR (see below), all samples that had not been extracted with the High Pure method were cleaned from cellular debris using DNA binding columns from the High Pure RNA Paraffin kit (Roche Diagnostics).

Verification of amplifiable DNA by HLA and/or S14 PCR. All tumour samples were run in an HLA and/or S14 PCR for verification of amplifiable DNA as described previously (Dahlgren et al., 2003; Mellin et al., 2000). Water was used as a negative control and DNA extracted from human fibroblasts was used as a positive control. PCR products were run on a 2.5 % agarose gel stained with ethidium bromide and visualized under UV light. Samples showing a band of 250 bp for HLA and 127 bp for S14 were considered to have amplifiable DNA. Only HLA- or S14-positive samples were used in further assays in this study.

HPV detection by PCR. The presence of HPV was analysed using the general primers GP5+/6+ (de Roda Husman et al., 1995). As positive controls, cloned plasmids of either HPV6 or -16 were used, with water as a negative control. The products were detected as above and samples with a band of 130–150 bp were considered to be positive and were run in an HPV type-specific PCR and/or sequenced as previously described (Mellin et al., 2002).

Samples negative in the GP5+/6+ PCR were run in another general PCR with the CPI/CPIG primer pair (Tieben et al., 1993) to exclude false negatives as a result of disrupted L1. The products were detected as above and samples with a band of 187 bp were considered to be HPV positive and were run in an HPV type-specific PCR and/or sequenced as previously described (Mellin et al., 2002).

HPV type-specific PCR. Samples considered to be HPV positive after the general HPV PCR were run in an HPV type-specific PCR with primers specific for HPV6, -11, -16, -18 and -33 (Karlsen et al., 1996) as described previously (Mellin et al., 2002). The positive controls consisted of plasmids containing cloned HPV6, -16, -18 or -33. The products were detected as above and samples with a band of 154 bp for HPV6, 89 bp for HPV11, 120 bp for HPV16, 172 bp for HPV18 and 211 bp for HPV33 were considered to be positive.

HPV quantification with real-time PCR specific for HPV6 and HPV11. Three nanograms of the HPV6-positive samples were run in triplicate in a type-specific quantitative PCR with a SyBrGreen protocol in an iCycler iQ (iCycler iQ Real-time PCR Detection System; Bio-Rad) with the HPV6 type-specific primers used above. The reaction mixture, in a total volume of 25 μl, consisted of 12.5 μl iQ SYBR Green Supermix (Bio-Rad) and 1.25 μl (10 pmol μl⁻¹) each of the HPV6 primers. The programme consisted of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and elongation at 74 °C for 30 s. Finally, a melting curve, starting at 40 °C and increasing by 0.5 °C every 10 s until 120 °C was reached, was run in order to verify the specificity of the obtained amplicons. Cloned HPV6 was diluted to contain 1 × 10⁵–10⁶ copies of HPV6 per test tube and used in triplicate as a standard series. For estimation of the number of viral copies per cell, 1 ng DNA was considered to be approximately equivalent to 200 cells.

For quantification of HPV11, the GP5+/6+ protocol was adjusted to the SyBrGreen system described above and 3 ng of each sample was run in triplicates. The reaction mixture, in a total volume of 25 μl, consisted of 12.5 μl iQ SYBR Green Supermix (Bio-Rad) and 1.25 μl (10 pmol μl⁻¹) each of the GP5+ and GP6+ primers. The program consisted of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s and elongation at 71 °C for 30 s. Melting curve analysis was carried out as for HPV6 above. Cloned HPV11 was diluted to contain 10⁻¹–10³ copies of HPV11 per test tube and used in triplicate as a standard series. For estimation of the number of viral copies per cell, 1 ng DNA was considered to be approximately equivalent to 200 cells.

For quantitative HPV6 and HPV11 PCR assays, only sample values that were concordant in triplicate or duplicate were accepted. All other samples were rerun until acceptable concordant triplicate or duplicate values were obtained.
When following individual patient samples over time for variation in viral load, a 0–5 log difference was considered as a significant quantitative increase or decrease (Yun et al., 2003).

**Immunohistochemistry.** Immunostaining was performed according to the standard avidin–biotin–peroxidase complex (ABC) technique (Elite Standard ABC kit; Vector Laboratories). Paraffin sections were deparaffinized, rehydrated and pre-treated with citrate buffer at pH 6 in a microwave oven for 20 min (700 W). After rinsing, the endogenous peroxidase activity was blocked by 0·5% hydrogen peroxide for 30 min. The sections were rinsed and incubated with blocking serum (1% BSA) for 20 min and subsequently incubated overnight in a moist chamber at 8°C with the primary antibody MIB-1 (diluted 1:160; Dakopatts AB) to detect the Ki-67 antigen specific to proliferating cells. A secondary biotinylated antimouse IgG antibody (Dakopatts AB) was added to the slides and after 30 min incubation, followed by rinsing, the ABC complex was added for 30 min. The peroxidase reaction was developed using 3,3-diaminobenzidine for 6 min. Nuclear counterstaining was performed with Mayer’s haematoxylin. Tris-buffered saline (pH 7·4) was used for rinsing between each step. Staining was checked with positive controls (intestinal mucosa and a lymph node). The Ki-67 labelling index was determined by light microscopy with a ×40 objective by randomly counting cells with positive nuclear staining and expressing the results as the number of immunoreactive cells among the 200 nuclei of respiratory papilloma cells.

**Statistical analysis.** A $\chi^2$ exact test was used for all statistical analysis. A 95% confidence interval was used and $P=0·05$ was considered significant. Non-categorical variables were tested with Student’s two-tailed t-test after performing Levene’s test for normality and $P=0·05$ was considered significant.

**RESULTS**

**Clinical outcome**

Detailed information on the age and gender of the patients, as well as available biopsies from before, during and after treatment, is shown in Table 1. Most patients were males and presented an equal distribution of juvenile RRP and adult RRP, while most of the few female patients had juvenile RRP. The dominance of male patients was maintained in the IFN-α-treated group (Table 1).

Twenty-five patients, 18 of whom were IFN-α treated and seven of whom were not IFN-α treated, were included in the study, so that the study could be carried out blind, as well as allowing us to analyse whether the two groups were similar or not (for details, see Table 1). Of the 18 IFN-α-treated patients, 16 started their first IFN-α therapy after three recurrences in a 1-year period prior to IFN-α treatment and one patient after two recurrences in a 1-year period, while the final patient was introduced to IFN-α treatment within 1 month of diagnosis. For one of the patients (RP-14), biopsies were only available after a second round of IFN-α treatment. Furthermore, one of the patients (RP-2) received IFN-α treatment in two separate periods and samples were available from both these IFN-α treatment periods. The duration of IFN-α treatment ranged from 1·5 months to 10 years. Twelve of the treated patients (aged 0·5–40 years) showed a clinical response as defined above, i.e. absence of recurrence after primary IFN-α treatment or no or only one relapse occurring after 1 year of treatment, with the exception of RP-3; see response definition in the footnote of Table 1). The remaining six were judged to be non-responders (Table 1). Of the seven patients not subjected to IFN-α treatment, three were cured after the first surgical intervention, while four had recurrent disease (Table 1, and data not shown).

**Detection of HPV by PCR with general HPV primers in respiratory papillomatosis and in response to IFN-α therapy**

The presence of HPV DNA was found by PCR in 20/25 (80%) patients and it was possible to establish HPV type for 18 of these 20 patients (Table 1). Furthermore, the frequency of HPV determined by PCR was similar between patients subjected (14/18, 77·8%) and not subjected (6/7, 85·7%) to IFN-α treatment (Table 1).

The number of HPV-positive respiratory papillomas from IFN-α responders was in general higher than respiratory papillomas from IFN-α non-responders, i.e. 11/14 and 1/4, respectively (Table 1), but this difference was not statistically significant ($P=0·083$, $\chi^2$ exact test).

Of the available pre-treatment respiratory papillomas, 9/12 (75%) of IFN-α-treated responders were HPV positive, while only 2/4 (50%) of the available pre-treatment papillomas of IFN-α non-responders were HPV positive (Table 2). Furthermore, 8/8 (100%) of available ongoing and 5/5 (100%) of available post-treatment IFN-α-responsive respiratory papillomatosis were HPV positive, while only 1/3 (33%) of available ongoing and 2/3 (67%) of post-treatment IFN-α non-responsive papillomas were HPV positive (Table 2). However, none of these differences was statistically significant.

**Determination of HPV type by type-specific PCR and sequencing in respiratory papillomatosis and in response to IFN-α therapy**

HPV type-specific PCR and sequencing showed that 12 patients harboured HPV6 and six patients harboured HPV11 in their respiratory papillomas, while for two patients HPV type could not be determined due to limited material (Table 1).

The frequency of HPV6 was similar in IFN-α-treated (8/18, 44%) and non-treated (4/7, 57%), while the presence of HPV11 was higher in IFN-α-treated (5/18, 28%) than in non-treated (1/7, 14%) patients (Table 1).

However, while 7/8 (87·5%) patients with HPV6-positive papillomas responded to IFN-α therapy, only 3/5 (60%) patients with HPV11 responded, but this difference was not statistically significant ($P=0·5105$, $\chi^2$ exact test). There was also no significant statistical difference between the 7/8 patients with HPV6-positive papillomas or the 3/5 patients with HPV11-positive papillomas compared with the only patient (1/4) with an HPV-negative papilloma who
responded to IFN-α therapy (\(P=0.067\) and \(P=0.524\), respectively, \(\chi^2\) exact test).

**Table 1.** Respiratory papillomatosis patients, age at onset, gender, HPV status, treatment duration, response to IFN-α treatment and number of available biopsies

F, Female; M, male; R, response; NR, no response.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at diagnosis (years)</th>
<th>Sex</th>
<th>Final HPV DNA*</th>
<th>IFN-α treatment duration</th>
<th>Response to IFN-α</th>
<th>No. available biopsies</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RP-1</td>
<td>5</td>
<td>M</td>
<td>6</td>
<td>2-5 years</td>
<td>R</td>
<td>Pre-treatment During Post-treatment</td>
</tr>
<tr>
<td>RP-2</td>
<td>1</td>
<td>M</td>
<td>6</td>
<td>1 year +3 years†</td>
<td>R</td>
<td>1 1 1</td>
</tr>
<tr>
<td>RP-3‡</td>
<td>2</td>
<td>M</td>
<td>6</td>
<td>4 months</td>
<td>R</td>
<td>2 1 1</td>
</tr>
<tr>
<td>RP-4</td>
<td>2</td>
<td>F</td>
<td>6</td>
<td>4 years</td>
<td>R</td>
<td>1 2 0</td>
</tr>
<tr>
<td>RP-5</td>
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<td>M</td>
<td>6</td>
<td>2 months</td>
<td>R</td>
<td>1 0 0</td>
</tr>
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<td>6</td>
<td>3 years</td>
<td>R</td>
<td>1 3 0</td>
</tr>
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<td>M</td>
<td>6</td>
<td>1 year</td>
<td>R</td>
<td>1 1 0</td>
</tr>
<tr>
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<td>38</td>
<td>M</td>
<td>6</td>
<td>1-5 months</td>
<td>NR</td>
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<td>RP-9</td>
<td>4</td>
<td>M</td>
<td>11</td>
<td>3 years</td>
<td>R</td>
<td>1 2 1</td>
</tr>
<tr>
<td>RP-10</td>
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<td>M</td>
<td>11</td>
<td>6 months</td>
<td>R</td>
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</tr>
<tr>
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<tr>
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<td>26</td>
<td>M</td>
<td>11</td>
<td>3 months</td>
<td>NR</td>
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</tr>
<tr>
<td>RP-13</td>
<td>5</td>
<td>M</td>
<td>11</td>
<td>4 years</td>
<td>NR</td>
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</tr>
<tr>
<td>RP-14</td>
<td>9</td>
<td>M</td>
<td>+</td>
<td>5 years</td>
<td>R</td>
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</tr>
<tr>
<td>RP-15</td>
<td>2</td>
<td>M</td>
<td>–</td>
<td>1-5 years</td>
<td>R</td>
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<tr>
<td>RP-16</td>
<td>5</td>
<td>M</td>
<td>–</td>
<td>10 years§</td>
<td>NR</td>
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<tr>
<td>RP-17</td>
<td>62</td>
<td>M</td>
<td>–</td>
<td>3 months</td>
<td>NR</td>
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<td>–</td>
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<tr>
<td>Total: 18 patients</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>14/18 17 17 12</td>
</tr>
<tr>
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<td></td>
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<td>8</td>
<td>F</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>RP-20</td>
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<td>M</td>
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<td>–</td>
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<tr>
<td>RP-21</td>
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<td>M</td>
<td>6</td>
<td>–</td>
<td>–</td>
<td>1 0 0</td>
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<tr>
<td>RP-22</td>
<td></td>
<td></td>
<td>59</td>
<td>M</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>RP-23</td>
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<td></td>
<td>3</td>
<td>F</td>
<td>11</td>
<td>–</td>
</tr>
<tr>
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<td></td>
<td>18</td>
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<td></td>
<td></td>
<td></td>
<td>6/7 8 0 0</td>
</tr>
<tr>
<td>Overall total: 25 patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>25 17 12</td>
</tr>
</tbody>
</table>

*For patients with multiple samples available, this indicates the presence of HPV DNA at any time (in any samples) during the study.
†This patient received IFN-α treatment at two separate time intervals.
‡This patient no longer required a tracheotomy, had a 50% reduction in the number of papillomas and had a normalization of voice quality.
§This patient was a non-responder according to our strict criteria. However, treatment was continued for 10 years due to subjective clinical benefits.
||These patients could have been considered for IFN-α treatment due to recurrent papillomas.

Viral load estimation with real-time quantitative PCR for HPV6 and HPV11 in respiratory papillomatosis and in response to IFN-α therapy

The viral load in HPV6- and HPV11-positive samples was estimated by a real-time quantitative PCR and ranged from 0-25 to 533 copies per cell for HPV6 and from 0-03 to 87 copies per cell for HPV11 (Table 2).

Patients with different pre-treatment viral loads (6-2–126 HPV6 copies per cell and 0-07–0-1 HPV11 copies per cell) responded to IFN-α therapy, and major changes in viral load in biopsies from individual patients with HPV-positive tumours were generally not observed during IFN-α treatment (Table 2). Furthermore, in the three patients who had HPV-positive respiratory papillomas and who still did not respond to IFN-α treatment, the viral load did not differ significantly from those patients that responded to therapy (Table 2).

HPV6 viral load could be compared before and during treatment for four IFN-α-responding patients (Table 2).
The viral load was somewhat lower in all patients during treatment, but this decrease was significant (>0.5 log) only for one patient (RP-2, showing a drop from 6.2 to 0.65 HPV6 copies per cell) and not for the remaining three patients (RP-3, RP-6 and RP-7; Table 2). HPV6 viral load was available for only two patients post-treatment and in both cases a clear increase in viral load was observed (Table 2).

HPV11 viral load could be compared before and during treatment for two IFN-α-responding patients and one non-responding patient (Table 2). The viral load was somewhat higher in all patients during treatment, but this increase was not significant (<0.5 log) for two IFN-α-responsive patients (RP-9 and RP-11; Table 2). For the remaining IFN-α non-responsive patient RP-12, a significant increase in viral load was seen initially during treatment and then it dropped again (from 0.55 to 0.04 to 0.08 HPV11 copies per cell) and the viral load increased to 8.76 HPV11 copies per cell after termination of IFN-α treatment (Table 2).

### Proliferation before, during and after IFN-α treatment

Ki-67 staining was mainly observed in the basal layer of the respiratory tract epithelium, although occasional staining was also detected in more superficial layers of the epithelium.

A high proliferation rate was generally seen in all samples, with Ki-67 indexes ranging from 8 to 54 % (median 35 %; Table 2). There were no major differences in proliferation...
rate between pre-treatment samples from IFN-\(\alpha\) responders (8–50\%, median 35\%) and non-responders (22–35\%, median 35\%), or during (10–54\%, median 28\%) and after (19–47\%, median 37.5\%) IFN-\(\alpha\) treatment (Table 2). More specifically, in the IFN-\(\alpha\)-treated group, in available samples from eight responding patients, the Ki-67 index increased in half and decreased in half of the patients, during or after treatment (Table 2). In available samples from the 3/4 IFN-\(\alpha\) non-responding patients that could be analysed, the Ki-67 index increased in one and decreased in the remaining two patients during or after treatment (Table 2).

**DISCUSSION**

With its clinical characteristics of early onset, frequent recurrences and therapeutic resistance, respiratory papillomatosis constitutes a challenge for clinicians not only to improve therapeutic results, but also to decrease the impairment of life quality due to disease-related symptoms and the side effects of therapy. Since surgery alone is commonly not sufficient for disease control of RRP, there is a need for adjuvant treatment. IFN-\(\alpha\) has, for several decades, been the drug of choice (Kimberlin & Malis, 2000). To achieve remission, long-term treatment, in many cases associated with severe side effects, is necessary. In order to prevent unnecessary treatment for those patients resistant to IFN-\(\alpha\) therapy and those with a low risk of relapse after surgery, an extended investigation of potentially predictive factors is desirable.

In this study, biopsies from 25 respiratory papillomatosis patients, 18 of whom had been subjected to IFN-\(\alpha\) treatment, were examined for the presence of HPV and to determine viral load and proliferation rate in order to find a possible means of IFN-\(\alpha\) action and to identify patients who might benefit from therapy.

Of the respiratory papillomatosis biopsies investigated, the majority were determined to be HPV positive, which was similar to previous observations (Lie et al., 1996; Rabah et al., 2001). Furthermore, also similar to previous studies, HPV6 and HPV11 were found to be the predominant HPV types (Kimberlin & Malis, 2000; Rabah et al., 2001). We also noted a dominance of HPV6 among the Swedish cases, while HPV11 dominated among the Finnish cases (data not shown), showing a variation that has been described previously in other populations (Rabah et al., 2001).

Of the patients who were subjected to IFN-\(\alpha\) therapy, the majority (67\%) responded successfully, which is similar to previously published results (Deunas et al., 1997). There was no evident difference in age or in the presence of HPV in patients who were or were not subjected to IFN-\(\alpha\) treatment or who responded. However, males were dominant as a whole and more so in the IFN-\(\alpha\)-treated group (Table 1).

Biopsies taken prior to IFN-\(\alpha\) treatment were available for the majority of the treated patients and presence of HPV was demonstrated by PCR in 11/16 cases and 73\% of the HPV-positive cases responded to therapy. It is remarkable that HPV could not be detected in some biopsies before treatment, but this could be due in part to a low viral load. In fact, among the five pre-treatment respiratory papillomatosis cases that were originally determined as HPV negative, two biopsies taken later during IFN-\(\alpha\) treatment were HPV6 positive and these two patients also responded to IFN-\(\alpha\) treatment.

Summarizing the data on HPV and HPV type, a tendency was observed for HPV6 cases to respond more readily to therapy than HPV11 and HPV-negative cases, but this difference was not statistically significant, although it was close to the borderline (\(P=0.067\)) between the HPV6 and HPV-negative groups. We have no explanation for the tendency of HPV6-positive papillomas to respond more readily to IFN-\(\alpha\) treatment than the HPV11-positive cases; however, it has been suggested HPV11-associated papillomatosis is more aggressive than HPV6-associated disease (Rabah et al., 2001). Furthermore, a correlation between virus genotype and response to IFN treatment has been noted for other viruses such as hepatitis C virus (Tan & Katze, 2001).

The viral load varied considerably among biopsies, and patients with various pre-treatment loads responded to IFN-\(\alpha\) therapy, indicating that patients with a low as well as a high viral load can respond to IFN-\(\alpha\). In addition, viral load was generally not significantly affected during IFN-\(\alpha\) treatment, although in some HPV6 cases the viral load had a tendency to decrease, while the opposite tendency was observed for the HPV11 cases. Nevertheless, a significant increase in viral load could be observed after IFN-\(\alpha\) withdrawal in four cases (Table 2), indicating a risk for rebound phenomena, observed in clinical practice as an increase in the numbers of papillomas (Kimberlin & Malis, 2000).

The absence of influence of IFN-\(\alpha\) on viral load during treatment was not anticipated and could possibly be due to the limited number of patients included in this study. However, this has been reported previously although not by quantitative PCR (Steinberg et al., 1988).

It is possible that IFN-\(\alpha\) exerts its mechanisms by other means. One possibility is enhancement of an immunological response against the HPV-infected cells, for example by increasing major histocompatibility complex (MHC) expression (Grander & Einhorn, 1998). An increase in MHC expression would consequently increase the ability of the immune system to respond to and combat HPV-positive respiratory papillomatosis, without directly influencing the viral load extensively in the remaining respiratory papillomatosis cells during treatment. This mechanism could also explain why cell proliferation was not affected during IFN-\(\alpha\) treatment as discussed below.

Another possibility could be that IFN increases the rate of apoptosis in virus-infected cells. This could also lead to a decreased amount of papilloma cells, but with maintained
levels of virus. IFN-α has been shown to act pro-apoptotically in neoplastic cells and we have recently found that expression of the HPV16 E7 oncogene may sensitize tumour cells to IFN-induced apoptosis (Thyrell et al., 2005). Whether induction of apoptosis explains the clinical effects of IFN-α in respiratory papillomatosis, however, remains to be investigated.

IFN-α is also known to exert anti-proliferative effects in many cell types and this has been suggested also to be of importance in its anti-neoplastic activities (Grander & Einhorn, 1998).

Cell proliferation was determined by the percentage of Ki-67-positive cells by immunohistochemistry and there was no significant difference between the cell proliferation observed in patients treated and not treated with IFN-α. Moreover, the Ki-67 index was not significantly affected during IFN-α treatment and did not differ significantly between patients who responded or did not respond to IFN-α therapy, which was unexpected. To our knowledge, there have been no other reports on comparison on the effect of IFN-α on the proliferation rate in respiratory papillomatosis.

HPV clearly induces perturbed regulation of the cell cycle, manifested in the relatively high percentage of cycling cells in most papillomas. IFN-α seems to act as an anti-proliferative agent through alterations impinging on the G1 cyclin-dependent kinase complexes, and the HPV-induced alteration in the cell-cycle machinery may be responsible for the lack of IFN-α-induced anti-proliferative effects (Sangfelt et al., 2000). IFN-α may also exert its anti-tumour function by other means, such as inhibition of angiogenesis, as well as induction of differentiation (Grander & Einhorn, 1998), which could play a role in the papillomatosis setting, although this needs to be studied in detail.

In conclusion, this is one of few studies investigating the putative anti-tumour effects of IFN-α in vivo during treatment, aimed at defining the mechanism of action of IFN-α, as well as defining predictive markers for IFN-α therapy. One reason for the limited number of similar studies is that IFN-α treatment is not used as a standard therapy for RRP. Nevertheless, even though the RRP patient group in this study was heterogeneous (age, clinical course, treatment strategy), most IFN-α-treated patients responded to therapy. Furthermore, there was a tendency for HPV6-positive cases to respond better than HPV-11-positive and HPV-negative cases to IFN-α therapy. Interestingly, in this highly IFN-α-responsive disease, the prominent anti-tumour effects were not reflected as IFN-α-induced changes in fundamental disease-related parameters, such as viral load and the hyperproliferative state of the HPV-affected epithelium. Finally, although this study does not conclusively pinpoint the mechanism for successful IFN-α treatment, it suggests that some of these analysed factors can be ruled out as possible markers for response prediction.

The study also indicates areas for future investigation aimed at selecting patients who may benefit from IFN-α treatment.

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REFERENCES


