Specific Types of Human Papillomavirus Found in Benign Proliferations and Carcinomas of the Skin in Immunosuppressed Patients

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Abstract

A total of 118 biopsies from skin lesions of 46 renal allograft patients was analyzed for human papillomavirus (HPV) DNA by polymerase chain reaction with degenerate primers and also partially by subsequent sequencing of the amplified fragment. Sixty-two % of the benign proliferations (31 of 50) contained DNA of known HPV types as well as HPV sequences related to a group of human papillomavirus (HPV)-associated HPV types. HPV DNA sequences were found in 14 (56%) of 25 biopsies from squamous cell and basal cell carcinomas. One squamous cell carcinoma contained HPV 41 DNA. A novel 640-base pair fragment sharing homology with HPV 29 (82.7%) was found in 15% (3 of 20) of sequences related to a number of epidermodysplasia verruciformis-associated HPV types. HPV DNA sequences were found in 14 (56%) of 25 biopsies from squamous cell and basal cell carcinomas. One squamous cell carcinoma contained HPV 41 DNA. A novel 640-base pair fragment sharing homology with HPV 29 (82.7%) was found in 15% (3 of 20) of squamous cell carcinomas, in 9.4% (3 of 32) of dysplastic warts and in 8.5% (4 of 47) common warts. The remaining positive carcinoma biopsies contained HPV-related DNA in such a low copy number that additional analysis is required. The identification of new HPV types in skin cancers of immunosuppressed patients (other than epidermodysplasia verruciformis patients) further expands the spectrum of HPV-linked human malignancies and permits new approaches to study the pathogenesis of skin cancers.

Introduction

Multiple skin disorders are commonly seen in transplant patients. These disorders include warts, hyperkeratoses, keratoacanthomas, and skin malignancies, the latter usually developing on sun-exposed areas (1). An increase of squamous cell carcinomas (relative risk, 27.6) is noted when compared to the population at large (2). An infection with the main types of cutaneous HPV (e.g., HPV 1, 2, 3, and 4, has frequently been demonstrated in the benign lesions (3-5). Patients with EV have a genetic predisposition to infections with a specific group of HPV types, some of which have an oncogenic potential. These EV-specific HPV types have not been detected in the normal population, except in immunosuppressed patients (5, 6). Interestingly, these EV-specific HPV infections were frequently detected with HPV 3 or HPV 3-related types, a phenomenon which seems to be associated with severe defects of the cell-mediated immunity. Such lesions do not, however, display the morphological and histological pattern characteristic for the same HPV infections in EV patients (5, 6).

Published data on the prevalence of HPV DNA in benign and malignant lesions taken from immunosuppressed patients were based mainly on the method of hybridization using available HPV types as probes. These experiments were usually restricted by the limited number of HPV types used as probes on the small amount of material available. In our study we applied a degenerate PCR method to evaluate the spectrum and distribution of any known and potentially unknown HPV types in a variety of lesions from immunosuppressed patients.

Materials and Methods

Patients and Materials. All 291 patients with a functioning renal allograft attending the Royal London Hospital were examined for the presence of viral warts and nonmelanoma skin cancer. Of the 291 patients, 172 (59%) were found to have cutaneous warts and 64 (21.9%) had nonmelanoma skin cancer, increasing to 40% of patients who had been transplanted for more than 9 years. Fifteen patients were at particularly high risk, with extensive dysplastic change and frequent development of malignant lesions (7). Because many of the warty keratoses were difficult to diagnose clinically, all suspicious lesions were biopsied. The specimens were bisected; one-half of the specimens was placed in formalin for routine histopathology and one-half was placed in liquid nitrogen and then stored at -70°C. All formalin-fixed biopsies were assessed by a histopathologist and classified as benign warts, dysplastic warty keratoses, carcinoma in situ, squamous cell carcinoma, or basal cell carcinoma. No features distinguishing squamousproliferative lesions from these patients from a similar range of lesions from immunocompetent patients could be found when masked sections were assessed by two histopathologists (8). The frozen blocks from 118 lesions from 46 patients were examined for the presence of HPV by DNA analysis. These 118 lesions included samples from 14 of the high risk patients with multiple skin cancers.

DNA Analysis. Total DNA was extracted from all the biopsies and subjected to Southern blot hybridization as described previously (9). The radio-labeled probes included the complete genomes of HPV 1, 2, 3, 5, 7, 10, 37, and 41.

Samples of total cellular DNA were amplified by PCR using degenerate primers (10). A forward primer HPV2 with the sequence (5’-3’) GGTGAGTCGTC-GTGGGGTGGGGGGGGGGA was used. The lower case letters indicate the BamHI linker, whereas the capital letters indicate the following degenerate papillomavirus-specific sequence: N as A, G, T, or C; M as A or C; and Y as A or T. The primer corresponds to the same region of the HPV 4 genome, respectively, resulting in a PCR product of 573 base pairs. These experiments were therefore marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. 7 The abbreviations used are: HPV, human papillomavirus; EV, epidermodysplasia verruciformis; PCR, polymerase chain reaction; EtiBr, ethidium bromide; SCC, squamous cell carcinoma; BCC, basal cell carcinoma.

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2 To whom requests for reprints should be addressed.

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were determined by means of cycle sequencing (Cycle Sequencing Kit; New England Biolabs) using 32P-labeled primer HVP2 or primer mixture C as sequencing primers. In a number of samples the virus-specific PCR bands were detected after hybridization although they had not been visible after EtBr staining. In such cases the PCR DNA was reamplified as follows. The DNA was subjected to gel electrophoresis in 1.5% agarose. The area corresponding to 600–700 base pairs was cut out and the DNA was purified from the gel. An aliquot of the purified DNA (5–10%) was amplified again using the same primers as described above and the products were analyzed in an 1.5% agarose gel. After EtBr staining, visible PCR bands of the expected size (approximately 650 base pairs) were purified from the gel. No virus-specific bands could be detected in human placenta DNA, which was used throughout as a negative control. The purified amplified DNA was digested with BamHI and cloned into the pBluescript II KS plasmid (Stratagene). The nucleotide sequence of the cloned insert was determined by dideoxy sequencing using the Sequenase 2.0 DNA Sequencing Kit (United States Biochemical Corp.). All sequences obtained were compared to the available HPV sequences in the EMBL Databank and Genbank, as well as unpublished sequences provided by Dr Hajo Delius.

Results

A total of 118 biopsies of skin lesions derived from renal transplant patients was tested for HPV DNA. The series included 20 SCCs from 12 patients, as well as 5 BCCs from 4 patients. The analysis of the extracted DNA from these lesions was performed by Southern blot analyses and PCR.

All samples were hybridized by Southern blot to at least 2 different HPV types and in most cases to 4 of the following HPV types: HPV 1, 2, 3, 5, 7, 10, 37, and 41. This resulted in clear positive signals in 17 samples (14.4%) hybridizing to HPV 2 and HPV 3/10. The copy number varied, although the viral DNA bands were already visible after ethidium bromide staining in the majority of cases, including the SCC sample which contained HPV 41 DNA.

All samples were subsequently analyzed by PCR. The yield of amplified DNA at the expected size of approximately 600–700 base pairs varied significantly. Occasionally a double band was observed in this region, suggesting the presence of more than one HPV fragment. The copy number of the amplified HPV product was either too low in a number of samples or the primers were too degenerate (resulting in low sensitivity and specificity) to obtain sufficient quantities of the product to determine the sequence even after consecutive rounds of amplification. The DNA of the other samples were reamplified, after which the products were cloned and sequenced. As a control, the cloned probes were used for hybridization with the original cellular DNA. Amplified products of high copy number were either sequenced directly by cycle sequencing, or in cases where a mixture of sequences resulted in two fragments with a total size of approximately 8 kilobases. The initial screening with the degenerate primers revealed the presence of the HPV 29-related sequence in 7 biopsies, among them 3 SCCs. Specific primers derived from the HPV 29-related sequence were designed (forward 5'TGACATATAGTCCTGACTCCG3', backward 5'ACCATAGCCACTAGGTTGG3'; product 484 base pairs) and all the samples were retested. This procedure permitted the detection of 3 additional positive biopsies the identity of which to the HPV 29-related sequences was confirmed by (direct) cycle sequencing of the purified PCR products. A total of 9 (7 identified and 2 not characterized) of the 10 samples containing the HPV 29-related sequences were positive in the initial screening, pointing to the relatively high sensitivity of this method despite the degeneracy of the primers.

The number of biopsies taken from each patient varied. Different HPV types were detected in the multiple biopsies, ranging from benign to malignant proliferations taken from certain patients. There was no concordance in the HPV types found. Four biopsies yielded clones with sequence homology to more than one HPV type, indicating double or triple infections. The subsequent analysis showed that they contained HPV 10 + HPV 27, HPV 17-related + HPV 29-related, HPV 6 + HPV 12-related, or HPV 10 + HPV 25-related + HPV 27, respectively. From most samples an average of approximately 300 base pairs of the total of 600–700 base pairs were determined. Homologies above 90% to known HPV prototypes were considered as sufficiently similar to exclude them from further analysis (13). Homologies below 90% led to complete sequence analysis of the PCR product, particularly when an identical DNA was isolated from different samples (Table 2). The percentage of homology of the partial sequences of several products was confirmed by subsequently sequencing both DNA strands of the clone.

Interestingly, the HPV 29-related sequence present in the 3 SCC samples (from 3 different patients) was found in 3 of 32 dysplastic warts, as well as in 4 of 47 common warts. One additional SCC contained HPV 41 DNA and in 7 additional tumors, virus-specific

<table>
<thead>
<tr>
<th>Type of lesion</th>
<th>No. tested</th>
<th>Total positive (%)</th>
<th>Uncharacterised</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warts</td>
<td>47</td>
<td>28 (60)</td>
<td>12</td>
</tr>
<tr>
<td>EV-like lesions</td>
<td>3</td>
<td>3 (100)</td>
<td>2</td>
</tr>
<tr>
<td>Dysplastic warts/verrucous keratoses</td>
<td>32</td>
<td>16 (53)</td>
<td>6</td>
</tr>
<tr>
<td>SCC in situ/Bowen’s</td>
<td>8</td>
<td>6 (25)</td>
<td>1</td>
</tr>
<tr>
<td>KA/SCC</td>
<td>3</td>
<td>2 (67)</td>
<td>1</td>
</tr>
<tr>
<td>SCC</td>
<td>20</td>
<td>12 (55)</td>
<td>7</td>
</tr>
<tr>
<td>BCC</td>
<td>5</td>
<td>4 (60)</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>118</td>
<td>66 (56)</td>
<td>32</td>
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</table>

<table>
<thead>
<tr>
<th>Identified to known HPV</th>
<th>Related to known HPV</th>
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<tbody>
<tr>
<td>6</td>
<td>9</td>
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<tr>
<td>10</td>
<td>20</td>
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<tr>
<td>27</td>
<td>28</td>
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<tr>
<td>41</td>
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<tr>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>29</td>
<td>49</td>
</tr>
</tbody>
</table>

a Double or triple infection.

b Two samples have the same sequence related to HPV 17.

c One wart and 1 dysplastic wart harbor the same sequence related to HPV 15.
d All samples harbor the same sequence related to HPV 29.

Table 1 Papillomavirus detected in skin lesions of renal transplant patients
DNA thus far remain uncharacterized. Similarly, we were unable to identify the HPV-related DNA in the 3 of the 5 BCCs.

Besides these malignant tumors, HPV 10 and 28 were detected in 6 of 66 (9.1%) positive samples, whereas 9 of 66 (14%) contained either HPV 27 or 57 DNA. One wart on the arm contained HPV 6b DNA. EV-specific HPV types detected in dysplastic warts and in situ SCC included HPV 9 and HPV 20 [2 of 66 (3%)]. Sequences closely related to HPV types 12, 15, 17, 25, and 49, all EV-specific types, were found in 15% (10 of 66) of the samples. The degree of homology of these sequences to the respective prototype DNA varied between 73 and 83%, still permitting a degree of cross-hybridization even under stringent conditions (EMBL Databank accession nos. X79941, X79942, X79943, X79944, X79945, X79946, X79948, and X79949). With the exception of the HPV 49-related sequences, the copy numbers of these related sequences were extremely low in the respective samples, leading to negative results in the Southern blot hybridizations, even under conditions of low stringency with the respective HPV prototypes.

Some usually common cutaneous HPV types were not found in this series. This accounts for HPV types 1, 2, 3, and 4. Previous findings of HPV 3 in lesions of similar patients may originate from the homology of this type with HPV 10, 28, and 29 (11), resulting in positive signals under conditions of hybridization used. In our series, Southern blot hybridization with HPV 3 DNA resulted in positive signals in 5 biopsies. The sequences of the corresponding PCR fragments were either identical to HPV 10 and HPV 28 or related to HPV 29. The same applies for HPV 2, which has an even higher homology to HPV 27 and 57. Southern blot analyses indicated the presence of HPV 2 DNA (Fig. 2), whereas the sequences obtained after PCR amplification were identical to HPV 27 or HPV 57, respectively.

**Discussion**

HPV sequences in lesions derived from immunosuppressed patients until very recently were detected mainly by either Southern blot or in situ hybridization methods, frequently applied under conditions of reduced stringency. Even the use of specific primers in the PCR has been restricted to the limited number of HPV sequences available. Screening varying numbers of samples from skin lesions, the prevalence of different HPV types were determined in the general population (14, 15) or in immunosuppressed patients (5, 16, 17). The mentioned limitations probably led in some studies to inaccurate typing, particularly in subgroups of types relatively closely related to
each other. Whereas our study identified a sequence of an apparently novel HPV 29-related type relatively frequently in skin lesions, the closely related HPV 3 was not detected in the present analysis, which is in contrast to previous reports. Similarly, HPV 2, also noted in previous studies, was not found here, although DNA of the related types 27 and 57 was seen in a number of our biopsies.

The availability of DNA sequences from the majority of known HPV types (11) and the use of a more sensitive technique (PCR), combined with sequencing of amplified DNA, presently facilitate a more accurate analysis of HPV-related DNA. By taking advantage of these procedures, our results indicate the presence of a different spectrum and of different prevalence rates of HPV types in lesions of immunosuppressed patients than those previously reported.

Infections with HPV types 10 and 28 and with 27 and 57 were relatively frequent in warts of these patients. Sequences related to EV-specific types were also detected here, implying the wide distribution of these viruses in the general population, probably as latent infections. The EV-related sequences analyzed in this study are indicative of a series of yet unidentified HPV types. The cloning and characterization of their complete genomes is presently underway. In the present analyses we could not detect HPV DNA in 40% of the wart samples. This is probably due to the presence of yet unidentified HPV types and/or low copy numbers of HPV DNA.

Dysplastic warts in immunosuppressed patients harbored more or less the same HPV types as other types of warts in this group, with the exception of HPV 9. Another observation, frequently reported in EV patients (6), was the simultaneous infection of one lesion with more than one HPV type. Three of four multiple infections included either HPV 10 or the HPV 29-related sequence. Simultaneous infections with HPV 3 or 3-related viruses and other HPV types have previously been reported for immunosuppressed patients (5).

The most striking finding in this series is the large number of malignant tumors (SCCs and BCCs) harboring detectable HPV sequences (14 of 25, 56%), contrasting data reported for skin carcinomas in immunocompetent patients (14). The novel sequence of the HPV 29-related sequences is of particular interest since it has also been found in a number of dysplastic warts, suggesting the identification of a new high risk virus. Besides this DNA, one SCC contained HPV 41 DNA, one KA/SCC contained HPV 27, and one in situ SCC contained HPV 20. HPV 41 has previously been detected in SCCs of three patients with a history of PUVA treatment (18). In 10 additional carcinomas studied here, HPV-related sequences were detected and await further characterization.

The most frequent HPV type found in carcinomas of EV patients is HPV 5 (19). These carcinomas arise mainly in lesions at sun-exposed sites. Only single cases of HPV 5 containing malignant lesions have been reported in other immunosuppressed patients (17, 20, 21). HPV 5 DNA was not detected in the malignant tumors studied here, although they too developed in sun-exposed areas of the skin. One wart, however, contained an HPV 12-related sequence. HPV 12 is closely related to HPV 5.

The identification of new HPV types involved in the development of malignant tumors of the skin in patients with immunosuppression other than EV contributes to the substantial list of cancers already linked to this group of viruses and may provide us with clues to study the molecular pathogenesis of skin cancer.

Acknowledgments

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References

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