DNA from BK Virus and JC Virus and from KI, WU, and MC Polyomaviruses as Well as from Simian Virus 40 Is Not Detected in Non-UV-Light-Associated Primary Malignant Melanomas of Mucous Membranes

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The single most important causative factor for malignant melanomas of the skin is UV radiation. However, this is not true for melanomas on body surfaces sheltered from the sun; thus, it is important to seek new causative factors of melanoma genesis. Human papillomaviruses and gammaherpesviruses are associated with human skin cancer; for example, human papillomavirus types 5 and 8 are associated with epidermodysplasia verruciformis, and human herpesvirus 8 is associated with Kaposi's sarcoma. Recently, a newly described human polyomavirus, Merkel cell polyomavirus (MCPyV), has been associated with Merkel cell carcinoma, an unusual form of neurotropic skin cancer. Moreover, melanocytes are of neuroepithelial origin. This background impelled us to investigate if human polyomavirus DNA could play a role in the development of extracutaneous melanomas. Sixty-four extracutaneous melanomas were initially collected and dissected. Of these, 38 could be successfully used for further testing for the presence of the five human polyomaviruses known so far—BK virus (BKV), JC virus (JCV), KI polyomavirus (KIPyV), WU polyomavirus (WUPyV), and MCPyV—and of simian virus 40 (SV40). No polyomavirus DNA could be detected in any of the samples tested by use of a nested PCR detecting BKV, JCV, and SV40; a newly designed PCR detecting KIPyV and WUPyV; or a newly designed PCR for MCPyV. We conclude that since no human polyomavirus DNA was detected in primary malignant melanomas on non-sun-exposed body surfaces, these polyomaviruses presumably are not major factors for the development of extracutaneous melanomas.

The single most important factor for the development of malignant melanomas of the skin is UV radiation. In addition, numerous other risk factors or cofactors have been demonstrated, such as, e.g., hereditary/familial predisposition, a large number of nevi, the skin phenotype, and the hair and eye colors (13). However, UV radiation presumably cannot be responsible for some subgroups of cutaneous melanomas on body surfaces more or less sheltered from the sun, such as the acral lentiginous melanomas of the palms, soles, and subungual areas. Moreover, melanomas can appear on body surfaces completely sheltered from the sun, e.g., mucosal membranes of the sinonasal cavity, the anus-rectum, the vulva-vagina, and the penis. These melanomas, sometimes named extracutaneous melanomas, are rare in absolute numbers compared to the cutaneous melanomas but are similar to them in density (i.e., the average number of tumors per square unit of body surface area) is taken into account (19). This similarity in melanoma density is compelling and demonstrates the necessity of finding new causative factors of melanoma genesis. Nevertheless, there are biological differences between cutaneous and extracutaneous melanomas, and it is possible that by comparing extracutaneous melanomas with cutaneous melanomas, we may find non-UV-light-associated factors or cofactors in melanoma genesis. Viruses could be such factors. It is possible that DNA viruses are associated with melanoma genesis, even if this is extremely difficult to demonstrate. In previous reports, we have investigated whether human papillomavirus (HPV) or any of the eight viruses of the human herpesvirus group were present in more than 40 extracutaneous melanomas. No HPV DNA-positive melanoma was detected, although different general primers and several type-specific primers were used, and only two herpesvirus-positive melanomas were detected, indicating that neither HPV nor viruses of the herpesvirus family are major etiological agents for extracutaneous melanomas (7, 15).

In the present study, we were particularly interested in exploring the possible presence in extracutaneous melanomas of the five presently known members of the human papillomavirus family and simian virus 40 (SV40), since some of these viruses are potentially neurotropic and melanocytes have a neuroepithelial origin. Since their discovery in 1971, BK virus (BKV) and JC virus (JCV) (10, 18) had been the only known human papillomaviruses; last year, however, two new human viruses, KI polyomavirus (KIPyV) and WU polyomavirus (WUPyV), were identified (2, 11). In addition, more recently, a fifth human polyomavirus has been identified in several cases of Merkel cell carcinoma, a rare malignancy of the skin; this virus was therefore named Merkel cell polyomavirus (MCPyV) (9). While KIPyV and WUPyV have not yet been linked to any...
disease, MCPyV has been suggested to be a contributing factor in the pathogenesis of Merkel cell carcinoma (9). Polyomaviruses are generally species specific, and as mentioned above, five have been described as human polyomaviruses (23). However, in addition to these five, a sixth polyomavirus, SV40, from the rhesus monkey, has, to a variable but generally rare extent, been detected in humans (3). Both BKV and JCV establish latency after primary infection and can be reactivated under certain circumstances (21). Seroprevalence (in Sweden) ranges from 70 to 80% for both BKV and JCV but is not known for the newly detected human polyomaviruses. However, several studies have found KIPyV, as well as WUPyV, in 1 to 3% of nasopharyngeal aspirates from children (2, 4, 11, 17). Moreover, in one report, as many as 6% of nasopharyngeal aspirates from children (2, 4, 11, 17). However, in addition to these five, a sixth polyomavirus, SV40, from the rhesus monkey, has, to a variable but generally rare extent, been detected in humans (3). Both BKV and JCV establish latency after primary infection and can be reactivated under certain circumstances (21). Seroprevalence (in Sweden) ranges from 70 to 80% for both BKV and JCV but is not known for the newly detected human polyomaviruses. However, several studies have found KIPyV, as well as WUPyV, in 1 to 3% of nasopharyngeal aspirates from children (2, 4, 11, 17). More- ever, in one report, as many as 6% of nasopharyngeal aspirates from healthy children were positive for WUPyV (1).

In the present study, we aimed to investigate whether human polyomaviruses could be detected in melanomas from non-sun-exposed body areas and whether they could possibly be considered causative agents in the genesis of some melanomas. For this purpose, more than 30 formalin-fixed extracutaneous melanoma samples were examined for the presence of human polyomavirus DNA.

**MATERIALS AND METHODS**

**Patients’ tumor material.** Thirty-eight mucosal melanoma samples with confirmed PCR-amplifiable DNA tested in two previous studies (7, 15) were selected from samples from 64 patients who were diagnosed from 1985 to 2003 and reported to the (compulsory) Swedish Cancer Registry. The present study was conducted with the permission of the Swedish Data Inspection Authority and the Human Ethical Committee at the Karolinska Institutet and was in compliance with their rules. All histological slides were reviewed, and only primary melanomas, not metastases, were used for further analysis.

**Preparation of samples.** One 4-μm-thick and two 20-μm-thick consecutive sections were cut from each paraffin block of formalin-fixed tissue. The 4-μm sections were stained with hematoxylin and eosin to guide the dissection of tumor tissue to be used for DNA analysis. Tumor tissues were dissected with a scalpel under light microscopy in each case in order to avoid potential contamination with virus from surrounding tissues and from heavily inflammatory cell infiltrates. This procedure resulted in approximately 90% pure tumor samples for further analysis. Similarly, micrometer knives and scalpels were washed in 70% ethanol between each preparation, and each section was stretched on a drop of distilled water placed on each slide, instead of using a regular water bath, in order to avoid contamination. In seven cases, the whole biopsy specimen contained tumor tissue and further dissection was not necessary.

**DNA extraction.** DNA was extracted using the High Pure RNA extraction kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s protocol, but without DNase treatment. Briefly, the paraffin was removed by treatment with xylene and ethanol. The tissue pellet was disrupted by overnight incubation at 55°C in tissue lysis buffer with the addition of sodium dodecyl sulfate and proteinase K. On day 2, the DNA was bound to a membrane in a fresh collection tube, washed, and eluted, and finally the DNA amount and purity were measured by NanoDrop technology (NanoDrop Technology Inc., Wilmington, DE). To avoid and check for virus carryover between the melanoma samples, tubes with a slice from an empty paraffin block were placed between the samples and treated in the same way as the melanoma samples throughout the experimental procedures.

**Verification of amplifiable DNA.** To avoid false-negative results due to DNA unsuitable for PCR analysis, a control PCR with β2-microglobulin primers was run in 100 to 120 ng DNA for all samples in a previous study (7).

**Nested PCR for the detection of BKV, JCV, and SV40.** To detect the presence of BKV, JCV, or SV40, a nested PCR was performed as described previously (5, 12). In addition, each reaction tube contained 0.4 μg/μl purified bovine serum albumin (BSA) to avoid possible inhibition of the PCR by a high concentration of melan in the DNA extract. A urine sample positive for BKV, used as a positive control, was kindly provided by Anna-Lena Hammarin (SMI, Karolinska Institutet, Stockholm, Sweden).

**PCR assay for the detection of KIPyV and WUPyV.** To simultaneously detect the presence of KIPyV and WUPyV, a standard PCR was performed with a first described by Arthur et al., 1989 (2a).

## TABLE 1. Sequences of primers used in PCRs for detection of BKV, JCV, and SV40, KIPyV and WUPyV, and MCPyV

<table>
<thead>
<tr>
<th>Nested outer primers&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P-3</th>
<th>GTATACACAGCAAAAGGAAAC</th>
<th>BKV</th>
<th>LT</th>
<th>2475–2494</th>
<th>V01109 (strain MM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-4</td>
<td>GCTCATCACGCCTGATTHTGTG</td>
<td>JCV</td>
<td>LT</td>
<td>4179–4198</td>
<td>NC_001699 (strain Mad-1)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SV40</td>
<td>LT</td>
<td>4327–4346</td>
<td>AF316139 (strain 77b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BKV</td>
<td>LT</td>
<td>2827–2846</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>JCV</td>
<td>LT</td>
<td>4790–4809</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SV40</td>
<td>LT</td>
<td>4940–4959</td>
<td></td>
</tr>
</tbody>
</table>

### Nested inner primers<sup>a</sup>

<table>
<thead>
<tr>
<th>P-1</th>
<th>AGTCATTAAGGGCTCTCTACC</th>
<th>BKV</th>
<th>LT</th>
<th>2551–2570</th>
<th>V01109 (strain MM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>JCV</td>
<td>LT</td>
<td>4255–4274</td>
<td>NC_001699 (strain Mad-1)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SV40</td>
<td>LT</td>
<td>4406–4425</td>
<td>AF316139 (strain 77b)</td>
</tr>
<tr>
<td>P-2</td>
<td>GGTCACCTACCTGGAACAG</td>
<td>BKV</td>
<td>LT</td>
<td>2707–2726</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>JCV</td>
<td>LT</td>
<td>4408–4427</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SV40</td>
<td>LT</td>
<td>4453–4527</td>
<td></td>
</tr>
</tbody>
</table>

### MCPyV primers<sup>a</sup>

| MCPyVT1709.F | CAGGCGATGCGCTGGGAATTAGGATG | MCPyV | LT | 1709–1732 | EU375803 |
| MCPyVT1846.R | CAGGCGACTTTTATTCACCTCC | MCPyV | LT | 1846–1827 |

<sup>a</sup> First described by Bogdanovic et al., 1994 (5).

<sup>b</sup> From reference 9a.

<sup>c</sup> Site of mismatch with the WUPyV sequence is boldfaced.

<sup>d</sup> From reference 9a.
single primer pair detecting part of the VP1 region of both viruses (Table 1). The reverse primer KIPyV2404.R had a length of 27 bases to compensate for one mismatch with the WUPyV sequence at base 20. The 50-µl reaction mixture consisted of 50 mM NaCl, 10 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, 200 µM of each deoxynucleoside triphosphate, 1 U of Taq DNA polymerase, 0.3 µM of each primer, and 500 to 1,000 ng of amplifiable DNA, corresponding to 1 × 10⁵ to 2 × 10⁶ cells per reaction. In addition, each reaction mixture contained 0.4 µg/µl purified BSA to prevent possible inhibition of the PCR by a high concentration of melanin in the DNA extracts. An initial 1-min denaturation at 94°C was followed by 40 cycles of 30 s at 94°C, 30 s at 53°C, and 45 s at 72°C, which, in turn, were followed by a final step of 5 min at 72°C. To produce a positive control, KIPyV bp 1395 to 2159 was cloned into pBSKS.

PCR assay for the detection of MCPyV. To detect the presence of MCPyV, a standard PCR was performed with a primer pair detecting part of the large T (LT) region of MCPyV (Table 1). The 50-µl reaction mixture consisted of 50 mM NaCl, 10 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, 200 µM of each deoxynucleoside triphosphate, 1 U of Taq DNA polymerase, 0.3 µM of each primer, and 500 to 1,000 ng of amplifiable DNA, corresponding to 1 × 10⁵ to 2 × 10⁶ cells per reaction. In addition, each reaction tube contained 0.4 µg/µl purified BSA to prevent possible inhibition of the PCR by a high concentration of melanin in the DNA extracts. An initial 1-min denaturation at 94°C was followed by 40 cycles of 30 s at 94°C, 30 s at 53°C, and 45 s at 72°C, with a final step for 5 min at 72°C. Plasmid pUC57MC1 containing MCPyV bp 1681 to 1870 (constructed upon request by GenScript Corporation), was used as a positive control. To define the detection levels of the two newly designed MCPyV PCR assays, the assays were performed with increasing concentrations of the respective plasmids, from 5 copies to 1 billion.

RESULTS AND DISCUSSION

To simultaneously assay for the presence of the two related polyomaviruses, KIPyV and WUPyV, a PCR assay utilizing primers directed at identical or nearly identical sequences in the two viruses was established (Table 1). The resulting amplicon had a size of 142 bp, and as few as 10 copies of a plasmid containing the KIPyV VP1 gene could be detected consistently and reliably. The detection limit remained unchanged when genomic DNA was added to the assay (data not shown). In addition, to assay for the presence in melanomas of the early region of MCPyV, a PCR assay utilizing primers detecting the central part of the LT antigen open reading frame was established (Table 1). This assay generated an amplicon of 138 bp, and 1,000 copies of plasmid pUC57MC1, containing this part of LT, could consistently be detected. The detection level was unaffected when pUC57MC1 was mixed with genomic DNA (data not shown). The two new methods for the detection of KIPyV, WUPyV, and MCPyC were highly sensitive—comparable in sensitivity to the nested PCR for BKV, JCV, and SV40—at levels of 10⁵ to 10⁶ copies per reaction. In additional, each reaction mixture contained 0.4 µg/µl purified BSA to prevent possible inhibition of the PCR by a high concentration of melanin in the DNA extracts. An initial 1-min denaturation at 94°C was followed by 40 cycles of 30 s at 94°C, 30 s at 53°C, and 45 s at 72°C, with a final step for 5 min at 72°C.

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REFERENCES


AUTHOR’S CORRECTION

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Volume 46, no. 11, p. 3595–3598, 2008. Page 3597, column 1, line 12: “KIPyV bp 1395 to 2159 was cloned into pBSKS” should read “KIPyV bp 1493 to 2653 was cloned into pcDNA3.”