Human Herpes Virus DNA is Rarely Detected in Non-UV Light-associated Primary Malignant Melanomas of Mucous Membranes

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Abstract. Background: UV-radiation is the most important causative factor for malignant melanomas of the skin. However, this is not the case for melanomas on sun-sheltered body surfaces. The aim of this study was to investigate if human herpes virus DNA could be found in malignant melanomas in sun-sheltered body areas and if these viruses play a role in the development of extracutaneous melanomas. Materials and Methods: Forty-one extracutaneous melanomas were dissected and used for further analysis. Quantitative PCR methods were used for detection of the eight human herpes viruses in melanoma samples. Results: Human herpes virus DNA was absent in 37/41 melanomas, however, cytomegalovirus DNA was detected in two samples, and one sample each exhibited presence of Epstein-Barr virus and Human Herpes virus-6 DNA respectively. Conclusion: Human herpes virus DNA is rarely detected in primary malignant melanomas in non-sun exposed body surfaces and is not a major factor for the development of extracutaneous melanomas.

UV radiation is the single most important factor for the appearance of malignant melanomas of the skin. However, several other risk factors or co-factors, such as hereditary/familial predisposition, a large number of nevi, the skin phenotype, and the hair and eye colour have been identified (1). Moreover, exposure to UV-radiation cannot account for the development of some subgroups of cutaneous melanomas, e.g., acral lentiginous melanomas of the palms, soles and subungually and possibly melanomas of the nodular type (2). Furthermore, melanomas can emerge on sun-sheltered body surfaces, i.e., nasal cavity, anus, rectum, vulva and penis. These melanomas, sometimes named extracutaneous melanomas, are usually found in mucous membranes, and, while few in absolute numbers when compared to the cutaneous melanomas, their local density (i.e., the number per square unit of body surface) is on average comparable with melanomas on the whole body surface (3, 4). The similarity in melanoma density between sun-exposed and sun-shielded body surfaces is fascinating and compelling for seeking new causative factors of melanoma genesis. There are also many biological differences between the cutaneous and extracutaneous melanomas (4, 5), and it is possible that studying extracutaneous melanomas and comparing them with cutaneous melanomas can be instrumental in finding non-UV light-associated factors or co-factors in the melanoma genesis, such as viruses. One hypothesis is that DNA viruses are associated with melanoma genesis, even if it is extremely difficult to demonstrate such a connection. In a previous report, the presence of human papilloma virus (HPV)-DNA was investigated in 40 extracutaneous melanomas using a highly sensitive PCR protocol, but HPV-DNA positive melanoma was rarely detected, indicating that HPV is not a major etiological agent for extracutaneous melanomas (6).

In the present study, possible presence of members of the herpes virus family in extracutaneous melanomas was of special interest, since some of these viruses are neurotropic and the melanocytes have a neuroepithelial origin. The herpes virus family has approximately 100 members, which are widely distributed in nature. Herpes viruses are species-specific and so far eight herpes viruses have been

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found to infect humans (7). All herpes viruses establish latency after primary infection and can be reactivated under certain circumstances. Human herpes viruses are grouped into three subfamilies according to their biological properties: the alpha-herpesvirinae are: the varicella zoster virus (VZV) and the herpes simplex virus 1 and 2 (HSV-1 and -2); the beta-herpesvirinae are: cytomegalovirus (CMV) and the human herpes virus 6 and 7; (HHV-6 and -7) and the gamma-herpesvirinae are: Epstein Barr virus (EBV), and Kaposi sarcoma-associated herpes virus (KSHV), also known as human herpes virus 8 (HHV-8) (7). The alpha-herpes viruses have mucocutaneous cells as their primary targets and establish latency in neurones. The beta-herpes viruses can infect a broad range of cells, such as macrophages, peripheral blood leukocytes, dendritic cells, fibroblasts, epithelial and endothelial cells (8-10). CMV has, for example, been shown to establish latency in monocytes, bone marrow progenitor populations and endothelial cells (8, 10). Monocytes and the epithelia of the salivary and bronchial glands have been suggested as sites of HHV-6 latency (11). The gamma-herpes viruses, such as EBV, have B-lymphocytes and epithelial cells in the oropharynx as their primary target cells and establish latency in B-lymphocytes, while HHV-8 may infect lymphoblastoid cells (12, 13). All herpes viruses are distributed throughout the world and are common among all populations. The sero-prevalence in Sweden ranges from 20% (HHV-8) to 99% (EBV) in the adult population (14-17).

In the present study, we aimed to investigate if human herpes viruses could be detected in melanomas from sun-sheltered body areas and possibly be considered as causative agents for melanoma genesis. For this reason, 64 formalin-fixed melanomas were collected and 41 of these were examined for the presence of human herpes virus DNA.

Materials and Methods

Patients and tumour characterisation. Sixty-four patients diagnosed with mucosal melanomas 1985-2003 and reported to the (compulsory) Swedish Cancer Registry were included in this study. With permission and in compliance with the rules of the Swedish Data Inspection Authority and the Human Ethical Committee at the Karolinska Institute, clinical records, pathology reports and histopathological specimens from all 64 patients were collected from hospitals and pathology laboratories throughout Sweden. All histological slides were reviewed and only primary melanomas, not metastases, were used for further analysis. For technical reasons, mainly because of a shortage in tumour tissues in the paraffin blocks, only 41 cases could be analysed for the presence of DNA from EBV, CMV, HHV-6, HHV-7, HHV-8, VZV, HSV-1, or HSV-2. The sites of these 41 melanomas were: anus 13, rectum 5, nasal cavity (8), vulva (7), vagina-cervix (4), tongue (1), penis (1), subungual (1) and skin (1).

Preparation of the samples and DNA extraction. One 4 µm- and two 20 µm-thick consecutive sections were cut from each paraffin block of formalin-fixed tissue. Cryostat knives and scalpels were washed in 70% ethanol between each preparation and each cryostat-cut section was stretched on a drop of distilled water placed on each slide instead of using a regular water bath. The 4 µm sections were stained with haematoxylin and eosin to guide the dissection of tumour tissue to be used for DNA analysis. A dissection of tumour tissues, resulting in around 90% pure tumour samples for further analysis, according to histology, was performed in each case in order to avoid potential contamination with virus from surrounding tissues and from heavy inflammatory cell infiltrates. In seven cases, the whole biopsy contained only tumour tissue and further dissection was not necessary.

DNA was extracted using the "High Pure RNA extraction kit" (Roche, Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol, but with exclusion of DNase treatment. In short, 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 addition of SDS and Proteinase K. On day two, the DNA was bound to a membrane in collection tubes, washed and eluted by an elution buffer. The Proteinase K treatment was repeated once (incubation for 1 h at 55°C) for removal of trace proteins. The DNA was bound to the membrane in a fresh collection tube, washed, eluted and finally the DNA amount and purity was measured using NanoDrop technology (NanoDrop Technology Inc., Wilmington, DE, USA). To avoid and check for virus carry-over 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.

Real-time quantitative PCR. Real-time PCR (TaqMan) was used for detection and quantification of EBV, CMV, HHV-6, HHV-7, HHV-8, VZV, HSV-1 and HSV-2. The PCR reaction was carried out using 12.5 µl TaqMan Universal PCR Mastermix (Applied Biosystems, Stockholm, Sweden), and various amounts and concentrations of probes, primers, Mg and water, depending on the specific type of virus (Table I (18-21)). All samples were analysed in 96-well plates, with a total of 25 µl in each well, including 5 µl sample (corresponding to DNA from 20,000 cells). The PCR conditions during the run consisted of 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 sec at 95°C and 1 min at 60°C; the analysis was performed on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Stockholm, Sweden). The patient samples were analysed in duplicate in all eight virus assays except one where the samples were analysed in triplicate due to the addition of an inhibition control. A positive control containing 50 EBV genomes was added to the third well of each sample in order to rule out a false negative result due to inhibition. An external EBV DNA standard was used to calculate the number of genomes in each sample (18). Discordant results in the duplicate analyses were reanalysed and thereafter designated as either positive (≥50% positive of total number of analyses) or negative.

Results

A total of 37 out of 41 patient samples that had PCR-amplifiable DNA gave a negative result in the PCR analysis. However, one patient with a subungual melanoma tested positive for EBV (4 genomes/20,000 cells) (Table II). Two patients were positive for CMV in anal (3 genomes/20,000
cells) and nasal cavity melanomas (0.3 genomes/20,000 cells), respectively. Finally, one patient, also displaying nasal cavity melanoma, was positive for HHV-6 (7 genomes/20,000 cells) (Table II).

Discussion

In this study, 37 out of 41 patients tested negative for human herpes virus DNA and only 4 out of 41 (10%) patient samples tested positive for any one of the eight tested human herpes viruses. Furthermore, the number of viral genomes/cell was low suggesting that the known human herpes viruses cannot be regarded as major causative agents of non-UV light-associated melanoma. Furthermore, the presence of virus DNA in four of the samples should be considered with caution. These three relevant viruses (EBV, HHV-6, and CMV) are common in the adult population with a seroprevalence reaching 100% for EBV and HHV-6 and 70% for CMV and they all induce latent infection in blood leukocytes (14-16). In inflammatory diseases, leukocytes migrate to the site of inflammation resulting in the clustering of cells that may contain a possible latent viral infection. It is feasible in this investigation that inflammatory cells in and around the tumour tissue harboured the detected virus, although the use of heavily inflamed tumour tissue was avoided by micro dissection.

Table I. PCR conditions, primer sequences, probe sequences, and amplicon length of amplified EBV, CMV, HHV-6, HHV-7, HHV-8, VZV, HSV-1, and HSV-2 fragments in the quantitative PCR assay. Number of base pairs and final concentrations of primers, probes, and Mg in parentheses and references of PCR assays in brackets.

<table>
<thead>
<tr>
<th>PCR assay (gene) [Ref #]</th>
<th>MgCl (mM)</th>
<th>Forward primer Reverse primer</th>
<th>Probe sequence</th>
<th>Amplicon length (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV (LMP1 ) [17]</td>
<td>1.5</td>
<td>5′-AAGGTCAAAGAACA AGGCCAAG-3′ (0.9)</td>
<td>5′-GCATCGGAAGTC GGTGGG-3′ (0.9)</td>
<td>5′-FAM-AGGAGCTGTGCC CGTGAGG-TAMRA-3′ (0.2) 65 bp</td>
</tr>
<tr>
<td>CMV (UL65) [18]</td>
<td>3.5</td>
<td>5′-AAAAACGTGCTCA CCCACT-3′ (0.3)</td>
<td>5′-ACGACACTTATTT GCGGTC-3′ (0.1)</td>
<td>5′-FAM-ACACCATCTTTCCG GAGTGCGG-TAMRA-3′ (0.15) 74 bp</td>
</tr>
<tr>
<td>HHV-6 (U67) *</td>
<td>-</td>
<td>5′-ATGCTGACCTGACAAAGAGC -3′ (0.9)</td>
<td>5′-AAATGCAAGYG CAYGAG-3′ (0.9)</td>
<td>5′-FAM-CAGCCATATTTCGCGG ATAGCCTATCGAATGCT-3′ (0.15) 76 bp</td>
</tr>
<tr>
<td>HHV-7 (U100) [19]</td>
<td>-</td>
<td>5′-ATCTACAAATACG CTCCACTTGG-3′ (0.5)</td>
<td>5′-AGAGCTGCGTGTG GTGATGTT-3′ (0.9)</td>
<td>5′-6FAM-AGCACGCGACGGAAT AACCTGAGAGATATAATCG-3′ (0.2) 100 bp</td>
</tr>
<tr>
<td>HHV-8 (ORF26) [17]</td>
<td>-</td>
<td>5′-GCTCGAGTCAAGAC GGATTTG-3′ (0.3)</td>
<td>5′-AAATAGGCTGCC CAGTGGC-3′ (0.9)</td>
<td>5′-FAM-TTTCCCGATGGGT CGTGCAGC-TAMRA-3′ (0.2) 67 bp</td>
</tr>
<tr>
<td>VZV *</td>
<td>-</td>
<td>5′-TGACGCGCGGAA CTTTTTA-3′ (0.3)</td>
<td>5′-GCTTCCAGTTCC AACCAC-3′ (0.9)</td>
<td>5′-TCAGCCTCA TTTAA-3′ (0.175) 56 bp</td>
</tr>
<tr>
<td>HSV-1 (HE1CG) [20]</td>
<td>-</td>
<td>5′-GGCCTGGTCTCC GGAGA-3′ (0.3)</td>
<td>5′-GGGCAGAGCAC ATCAGCA-3′ (0.3)</td>
<td>5′-FAM-CAGCAGCAGACTTG GCCTCTTG-GT-Dark Quencher-3′ (0.175) 62 bp</td>
</tr>
<tr>
<td>HSV-2 (US4) [20]</td>
<td>-</td>
<td>5′-AGATATCTTCTTAT CATCAGCAAC-3′ (0.3)</td>
<td>5′-TTTGTGCTGCCA AGGCCA-3′ (0.3)</td>
<td>5′-HEX-CAGACAAGAAGCC CCGCG-Dark Quencher-3′ (0.175) 72 bp</td>
</tr>
</tbody>
</table>

# The sensitivity of the assays are 1-5 copies per assay and have for CMV, HSV1, HSV2, VZV and HHV-6 been correlated to electron microscope counted particles, as well as extracted viral genome quantified by spectrophotometry. For more details, see specific references.

* Unpublished data.
herpes viruses can be reactivated for various reasons and EBV and HHV-6 are, for example, frequently shed in the saliva of healthy persons, whilst CMV can be excreted asymptomatically in urine (22, 23). These circumstances can give misleading information and should be taken into consideration when analysing the results.

It is well established that members of the human herpes virus family have the potential to cause certain malignant tumours. EBV is associated with Burkitts, Hodgkin’s, B-, T- and NK-lymphomas, nasopharyngeal and gastric carcinoma, while HHV-8 is associated with Kaposi’s sarcoma (12, 13).

Previously published studies on herpes viruses associated with human melanoma are sparse. Metastatic spread of cutaneous melanomas mimicking herpes zoster has however been reported (24-26). Only one report on herpes viruses and extracutaneous melanoma has previously been published and the results were at variance with this report. Out of nine melanomas in the nasal cavity area tested for EBV, by means of EBER-1 in situ hybridization combined with immunostaining for the EBV-related protein LMP-1, three (33,3%) and two (22%), respectively, turned out positive (27). The reason for this discrepancy is unknown.

The present investigation indicates that the human herpes viruses EBV, CMV, HHV-6, HHV-7, HHV-8, VZV, HSV-1 and HSV-2, do not play a major role in the development of extracutaneous melanomas.

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