DNA from Human Polyomaviruses, TSPyV, MWPyV, HPyV6, 7 and 9 Was Not Detected in Primary Mucosal Melanomas

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Abstract. *Background/Aim: Mucosal melanomas arise in non UV-light exposed areas and causative factors are yet unknown. Human polyomaviruses (HPyVs) are rapidly increasing in numbers and are potentially oncogenic, as has been established for MCPyV in Merkel cell carcinoma, an unusual skin cancer type. The aim of the present study was to investigate the association between TSPyV, MWPyV, HPyV6, 7 and 9 and mucosal melanoma. Materials and Methods: Fifty-five mucosal melanomas, were analyzed by a Luminex assay, for the presence of 10 HPyVs (BKPyV, JCPyV, KIPyV, WUPyV, TSPyV, MWPyV, HPyV6, 7 and 9) and two primate viruses (SV40 and LPyV). Results: In 37 samples the DNA quality was satisfactory for analysis. However, none of the samples analyzed were positive for any of the examined viruses. Conclusion: None of the above-analyzed HPyVs were detected in mucosal melanoma samples, and they are for this reason unlikely to play a major role in the development of this tumor type.*

Mucosal melanoma arises in non UV-light exposed areas, such as in the sino-nasal cavity, the anus-rectum or the vulva-vagina area. It constitutes another disease entity and differs in several aspects from cutaneous malignant melanoma and the patients are usually older when diagnosed with a median age of 70 years. Furthermore, while the incidence of cutaneous malignant melanomas is increasing, mainly due to UV-light exposure, the incidence of mucosal melanomas is fairly constant (1-2). However, the incidence of sinonasal melanomas in Sweden has significantly increased from 1960 to 2000 (3). In addition, whereas several risk factors have been established for cutaneous malignant melanoma besides UV-light, *e.g.* hereditary factors, no definitive risk factors have been established for mucosal melanomas.

Viruses are causal for a variety of human malignancies, the main being cervical cancer caused by human papilloma viruses and liver cancer caused by Hepatitis B and C viruses (4). However, viruses are also involved in less common malignancies, or subsets of these, *e.g.* gastric carcinoma by EBV; primary effusion lymphoma by HHV8; T-cell leukemia by HTLV-1; and Merkel cell carcinoma (MCC) by MCPyV. The latter being a member of the polyomavirus (PyV) family, with members with well-established oncogenic potential. It is likely that other rare malignancies or subsets of them, are caused by viruses and this could be the case for mucosal melanomas, especially given the lack of other known risk factors.

The first human polyomaviruses (HPyVs), BKPyV and JCPyV, were discovered in 1971, but it was not until 2007, that two more HPyVs: KIPyV and WUPyV were discovered in nasal aspirates (5, 6) followed by MCPyV, isolated from MCC in 2008 (7). Since then, seven new HPyVs have been characterized from samples derived from the skin (HPyV6, 7 and TSPyV) (8, 9) blood (HPyV9) (10, 11), faeces (MWPyV and STLPyV) (12-14) and from the gastrointestinal tract (HPyV12) (15). Like most HPyVs they are also present in a large part of the general population, and their seroprevalence range from 20% to >90%, with HPyV9 being the less common among them (16-18). So far only BKPyV, JCPyV, MCPyV and TSPyV, have, been associated with disease (19-20). In immunocompromised individuals, reactivation of BKPyV is associated with haemorrhagic cystisis, BKV nephropathy, and ureteral stenosis, while JCPyV is associated with progressive multifocal leukoencephalopathy and TSPyV associated with Trichodysplasia spinulosa, a rare skin disease (20). As noted above, MCPyV is a frequent cause of MCC, a skin malignancy, mainly in immunosuppressed or older individuals (7). However, despite that only MCPyV has been associated with cancer, most other HPyVs express proteins that may potentially contribute to cancer development, and all except HPyV12 have putative binding sites for the retinoblastoma protein and most also for p53 (9-10, 12, 15).

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We have previously investigated the possible presence of HPV, eight herpes viruses, and five HPyVs, in around 40 mucosal melanomas of a material of originally more than 60 mucosal melanomas obtained in 1985-2003 (21-23). None of the tested melanomas were positive for HPV, or the five at the time known HPyVs: BKPyV, JCPyV, KIPyV, WUPyV and MCPyV, while four were positive for herpes viruses (two for CMV and one each for EBV and HHV-6) but with viral loads below 1 viral genome/10,000 cells (24-26).

In the present study, the presence of five recently discovered HPyVs; HPyV6, 7, 9, TSPyV and MWPyV have been analyzed in 37 mucosal melanomas, of the originally obtained mucosal melanomas above. In addition the assay also included the analysis of BKPyV, JCPyV, KIPyV, WUPyV and MCPyV as well as the primate PyVs, SV40 and LPyV.

Materials and Methods

Patients' tumor material. Fifty-five mucosal melanoma samples, from patients diagnosed between 1985-2003 and reported to the Swedish Cancer registry, included in three previous studies (21-23), were also included in the present study. Some of these had been excluded from the evaluation in the earlier studies due to lack of amplifiable DNA. However, since the method utilized in the present study was, in general, more sensitive than in the former studies, all 55 samples were re-analyzed regardless of earlier results. A representative example of mucosal melanoma of the vulva is shown in Figure I. The study was performed according to permissions obtained from the Stockholm Regional Ethical Committee and in accordance with the rules of the Swedish Data Inspection Authority. As noted in (23) all histological slides were reviewed and only primary melanomas, and no metastases, were included in the study.

Sample preparation. Samples were prepared as described by Giraud *et al*. (23). Briefly, consecutive sections were cut from each block of formalin-fixed paraffin-embedded tissues. One section was haematoxylin/eosin stained to guide for the dissection of tumour tissue for DNA extraction. Tumour tissue was micro-dissected by scalpel under light microscopy, in order to avoid potential contamination of viral DNA from adjacent tissue. In addition, areas with abundant infiltration of inflammatory cells were avoided. To further avoid contamination, microtome knives and scalpels were washed in 70% ethanol between each sample processing and the sections were stretched on a drop of distilled water placed on a slide, instead of using a water bath. As noted above the same tumors have been included in earlier studies of our group. However, for the majority of samples, new sections were also cut and analyzed.

DNA extraction. DNA was extracted using the "High Pure RNA extraction kit" (Roche, Diagnostics GmbH, Mannheim, Germany) in accordance with the instructions from the manufacturer, but with exclusion of DNase treatment. Briefly, cuts were treated with xylene and ethanol to remove paraffin. The dried tissue pellet was then dissolved by incubation overnight at 55°C in 100 μl tissue lysis buffer with 16 μl 10% SDS and 40 μl Proteinase K before the mix was added to the collection tubes. The DNA was the washed repeatedly before elution. After the final elution step, the amount and purity of DNA was evaluated on a NanoDrop instrument (NanoDrop Technology Inc, Wilmington, DE). To monitor for possible contamination or for transfer of viral DNA between melanoma samples the following procedure was performed: After each melanom sample an additional slice was cut from an empty paraffin block. These paraffin slices were then included in the DNA extraction procedure and evaluated for presence of PyV DNA together with the tumour samples.

Table I. *Origin of mucosal melanomas included in the study.*

Tumour site	N
Nasal cavity	9
Tongue	2
Oral cavity	1
Cervix/vagina/vulva	12
Penis	2
Anus/Rectum	11
Total	37

PCR for detection of PyVs and β-globin. For the simultaneous detection of 10 different human PyVs; BKPyV, JCPyV, KIPyV, WUPyV, MCPyV, TSPyV, HPyV6 ,7, 9 and MWPyV (HPyV10) and 2 primate PyV; SV40 and LPyV, and β-globin, a newly-developed PCR assay, was utilized (24). The details of this assay, as well as the primers included, are described by Gustafsson *et al*. (24). In this assay, 42 primers, targeting the small T-antigen (ST) and the viral protein 1 (VP1) region of all the 12 viruses, were included. The β-globin primers utilized in Gustafsson *et al*. (24), were prolonged to increase their melting point and were in the present study; β-globin1168.F, 5'-GTACACATATTGACCAAATCAGGGTAA-3' and β-globin1293.R 5'-GCCCTGAAAGAAAGAGATTAGGGAAAG-3.

In the PCR reaction mix, concentrations for primers were; 0.2 μM for each PyV primer and 0.1 μM for the β-globin primers. The PCR was performed on 10 or 50 ng sample DNA with the Multiplex PCR Master Mix (Qiagen) in a total volume of 25 μl. For blank samples, prepared together with the melanoma samples, a corresponding sample volume was used. The PCR was initiated with 15 min denaturation at 94˚C followed by 40 cycles, each consisting of 20 sec at 94˚C, 90 sec at 48˚C and 80 sec at 72˚C, and terminated with an additional elongation step for 4 min at 71˚C. As a positive general control for the PCR, cellular DNA from the Siha cell line was included in an amount corresponding to 5, 50 and 500 cell genomes, were include in each assay.

Detection of PCR amplicons on a MagPix instrument. To detect for PyV amplicons, a bead-based multiplex assay on a Magpix instrument (Luminex, Houston, TX, US) was utilized, as detailed in Gustafsson *et al*. (24). Briefly, 5 μl from the PCR reaction was incubated together with a mixture of 25 different bead types, coupled with probes for ST or VP1, for each of the 12 different PyV, and for b β-globin. The output was given as median fluorescent index (MFI). A MFI value over 2x background + 20 was considered as a positive value. The assay was validated for sensitivity and specificity through use of plasmids containing the corresponding

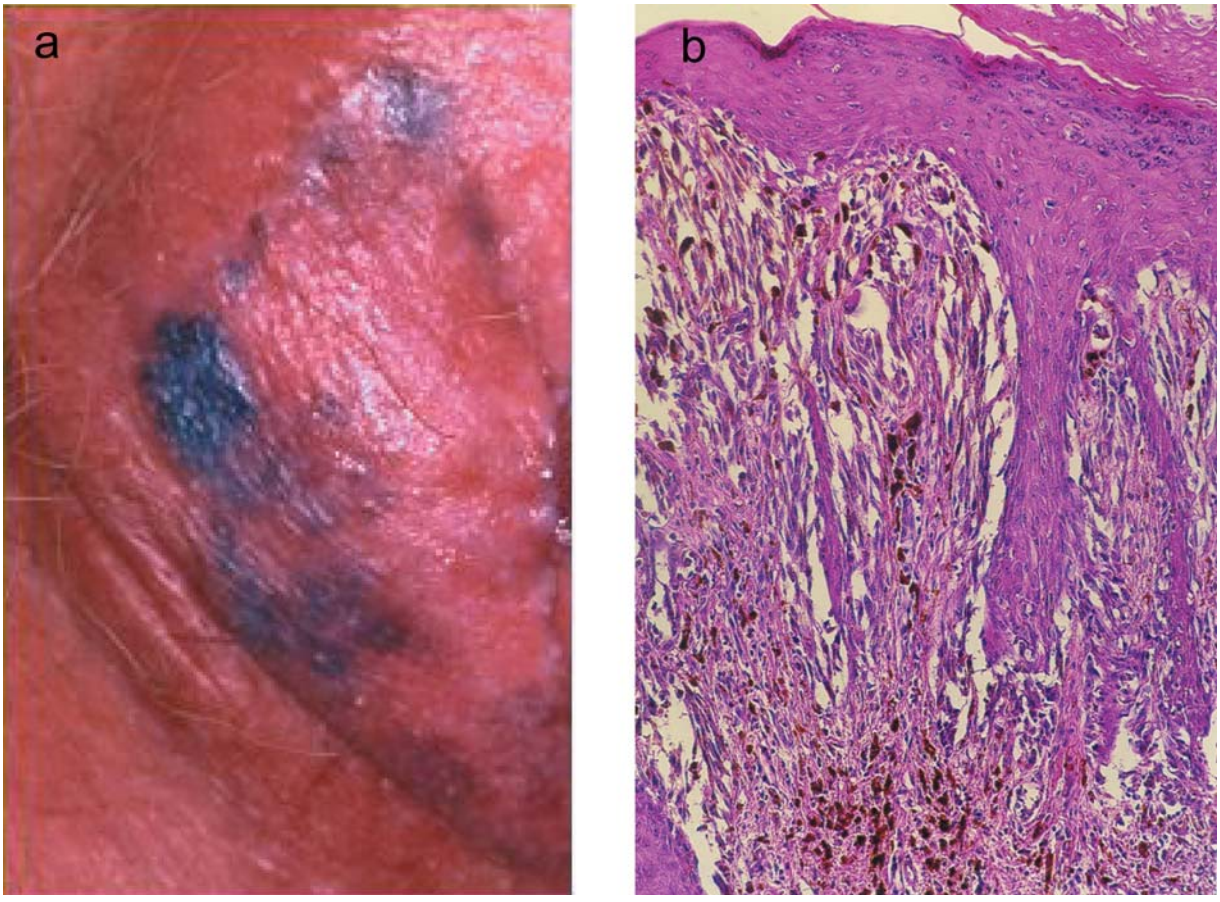

Figure 1. *Representative photos of: a) Malignant melanoma of the labium minus of the vulva. b) Hematoxylin-eosin stained section of a mucosal lentiginous type of malignant melanoma of the vulva.*

genomes, or in one case (WUPyV) with a virus-positive sample as, described in Gustafsson *et al*. (24). For all viruses with the exception of KIPyV, where it was <50 copies, the sensitivity was <5 copies. No cross-reactions between the different viruses were observed. Samples with a MFI value for β-globin <30 was considered not to have amplifiable DNA and were excluded from the study. All samples were included in at least two assays.

Results

In total, 55 mucosal melanoma samples were analyzed using a multiplex bead-based PyV assay on a MagPix instrument. Out of these 37 had amplifiable DNA, as assessed by successful amplification of a part of the β-globin gene. The locations of these 37 melanomas are presented in Table I and as shown, the majority were from the anogenital area or the nasal cavity.

When assayed for the presence of 12 different PyVs, (BKPyV, JCPyV, KIPyV, WUPyV, TSPyV, HPyV6, 7, 9, MWPyV, SV40 and LPyV) none of the 37 mucosal melanomas were positive for any of these viruses.

Discussion

In the present study 37 mucosal melanoma samples, collected between 1985-2003, were analyzed for the presence of 12 different PyV species, including the recently-discovered HPyV6, 7, 9, TSPyV and MWPyV. However, none of them were positive for any of the viruses analyzed for. HPyV6, 7, 9, TSPyV and MWPyV are all in general common in the population and sequence data suggests they all are potentially oncogenic, *i.e*. by binding to Rb and p53. Thus, the possibility that one or more of these virus is causative for a subset of tumours is a plausible hypothesis.

The tropism of these viruses is not well-understood. TSPyV, HPyV6 and 7 are, similarly to MCPyV, common on skin (8-9, 25). HPyV9 is mainly, although rarely, found in the blood (10), but has also been isolated from skin (11). MWPyV, isolated from faeces, has been detected in faecal samples from different regions with prevalence rates of 2-14% (12, 14).

In spite of the high serological prevalence of the newly-discovered HPyVs, as noted in the introduction of the article, the prevalence with regard to detection of viral DNA in most sample types is low. In a recent study, the occurrence of HPyV6, 7, 9, TSPyV and MWPyV in over 2,000 samples from faeces, urine, blood, CSF and respiratory swabs from both healthy volunteers and patients, was investigated (26). Only a minority of respiratory and faecal samples were positive for HPV6 and 7, TSPyV and MWPyV. The only virus with prevalence rates >2% was MWPyV, with a prevalence of 9.2 and 12.8% in respiratory and faecal samples respectively, of healthy subjects.

Out of these five HPyVs, so far only TSPyV is known to be disease-associated; Trichodysplasia spinulosa, a rare skin disease in immunocompromised patients (9). So far none of HPyV6, 7, 9, TSPyV and MWPyV have been found to be associated with cancer development (19). However, since tumour viruses are often associated with subsets of specific tumour types, further extensive studies remain to be performed before the involvement of these viruses in tumour development can be excluded.

Since risk factors for the development of mucosal melanomas are mainly unknown, a viral factor remains a possibility. Nevertheless, the results of the present study indicate that none of the PyVs tested for were involved in the origin of these tumours.

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