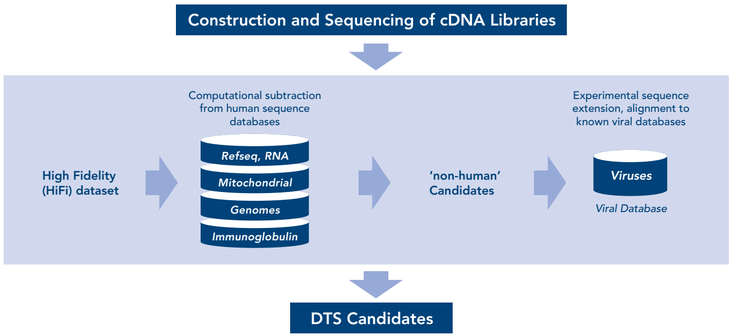

Figure 1: Digital transcriptome subtraction (DTS) *relies on alignment of high throughput sequencing of cDNA from tumors to the human genome databases. Sequences that do not align are candidate "nonhuman" sequences that can be examined using molecular techniques to determine if they belong to a foreign virus.*

passenger virus that infects the tumor after the tumor has emerged. The mechanistic requirement for two independent mutation events to occur during tumorigenesis (virus integration and T antigen truncation) may explain why MCC is rare. Ongoing studies are attempting to ascertain whether the viral mutations result from pyrimidine dimer substitutions that could arise from ultraviolet irradiation from sun exposure.

Direct in situ evidence for MCV infection in MCC tumor cells also points to the virus playing a direct role in carcinogenesis. (**See Figure 2.**)[8] Several MCC cell lines have been found to harbor the viral genome stably, allowing functional studies and examination of virus gene expression.[14] A monoclonal antibody raised against a T antigen shows uniform staining for MCV cells in a nuclear pattern. Quantitative PCR (qPCR) studies also show that MCV is generally present at > 1 copy per cell in MCC tumors positive for the virus, whereas uninvolved tissues can harbor virus but at 2-3 logs lower levels.[8,15] Thus, there is a biological gradient for detecting the virus in MCC that is consistent with the virus causing the tumor.

While most studies thus far show MCV associated only with MCC, individual reports of infection using PCR have described MCV in small cell lung cancers[16] and various skin cancers.[12,17] These results are not replicated elsewhere,[7,18,19] so further studies

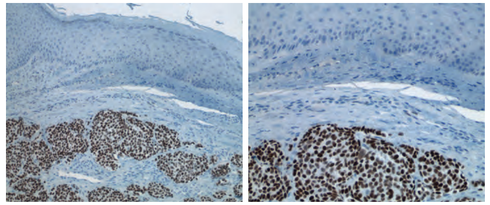

Figure 2: *Merkel cell carcinoma infiltrating the skin stained for MCV T antigen using the CM2B4 antibody. Note on high power view (right) that viral T antigen is localized to nuclei of tumor cells and is not found in surrounding non-neoplastic tissues.*

are needed to determine whether technical errors account for these results or whether subpopulations of these tumors harbor the virus. Low-level MCV infection can be found in non-MCC tissues, such as some hematolymphoid malignancies and skin,[1,8] which is consistent with coincidental infection.

Is MCV a Common Human Infection?

These findings raise the question whether MCV is a common infection of humans that rarely causes MCC, or a rare infection that commonly causes MCC. Serologic testing suggests the former. Kean, et al developed an antibody assay based on recombinant MCV VP1 protein, finding that 42 percent of adults have evidence for past MCV exposure.[20] A substantial fraction of children under age 15 have MCV antibodies, indicating that infection can be acquired early in life. This is consistent with several studies that have found MCV in the upper aerodigestive tract, digestive system, and respiratory secretions, indicating that respiratory and/or gastrointestinal transmission may be a common route of infection.[21,22,15]

We confirmed this using a serologic assay made from virus-like particles (VLP) generated by expression of the MCV VP1 and VP2 proteins.[23] No cross-reactivity was present for MCV VLP with VLP from other polyomaviruses, suggesting it is a suitable antigen for a blood test.[23] All MCC patients whose tumors are known to be positive for the virus have significantly higher IgG (but not IgM) antibodies against MCV. In a small set of MCC patients with MCV-negative tumors, approximately half show evidence of past MCV exposure — a rate nearly identical to that of various control populations without MCC. This is consistent with the view that MCC tumors without MCV infection have a separate etiology from MCV-positive tumors, rather than simply having lost the virus as the tumor progresses. Evidence for MCV infection was found among children under age 5, and MCV seropositivity rates increased with age, reaching 80 percent by age 50.[23]

Unanswered Questions

We are just beginning to investigate this important new human pathogen. Fortunately, MCV's close relatives, SV40 and mouse polyomavirus, have been studied for over 50 years and form part of the bedrock of modern molecular and cancer biology. Applying knowledge gained from these viruses to MCV will rapidly accelerate basic research on this new human polyomavirus. Since direct evidence for MCV causing cell transformation has not been established, investigation into the mechanisms used by this virus to initiate carcinogenesis has become critical to MCC pathology. Although MCV appears to be relatively specific for MCC tumors, the search for MCV in other cancers is far from over. The conflicting results on the presence of MCV in non-MCC cancers needs to be resolved. Moreover, the natural history of MCV infection is largely unknown, and efforts to determine whether MCV is linked to non-neoplastic diseases have only just begun.

It is important to answer whether or not MCV infection affects the clinical course of MCC and its treatment. One retrospective study with long-term follow-up showed that the presence of MCPyV DNA in MCC tumor samples predicted better overall survival.[6] This raises the possibility that virally mediated MCC may be more immunogenic and less genetically complex than MCV-free MCC. Future therapies targeting the MCV T antigen or augmenting large T antigen-specific immune response may prove beneficial for these patients. The availability of MCV-specific monoclonal antibodies provides an immediately available clinical benefit in differentiating MCC from other round cell tumors of the skin.[18]

Finally, a substantial minority of MCC tumors are not infected with MCV. How do these tumors arise, what is their clinical course, and how should they be treated? The answers to these questions will be particularly important for Merkel cell carcinoma, but may shed light on other cancers as well.

Conclusions

Does MCV cause MCC? Evidence already firmly indicates that MCV is the infectious cause of a portion of MCCs. Current data suggest that MCC actually includes two different diseases, one caused by MCV and another having an unknown etiology. There is remarkable consistency among many studies performed in different settings showing that virus is present at high levels in most but not all MCC tumors. This is supported by detection of viral DNA using a variety of techniques, by serologic studies, by monoclonal antibody-staining, and by in situ hybridization. Among tumors that are MCV-positive, the virus is clonally integrated and present at high copy number. Further viruses isolated from tumors contain signature mutations and can no longer independently replicate, whereas virus isolated from normal tissues retains the wild-type genetic sequence. While MCV is a common human infection, most studies agree that MCV infection in tumors is largely specific to Merkel cell carcinoma. Taken together, there is already strong, convincing evidence that MCV is the cause of most but not all MCC tumors.

Despite the good news that a new virus is the probable fundamental cause of most Merkel cell carcinomas, it is sobering to remember another virus that causes an important skin cancer, Kaposi's sarcoma (KS). KS is the most commonly reported cancer in sub-Saharan Africa and remains the most common malignancy among AIDS patients in the US. In the 15 years since Kaposi's sarcoma herpesvirus (KSHV) was first described, a wealth of basic, translational and clinical research on KSHV has accumulated, but thus far none of this data has been used to improve KS clinical care. One hopes that enthusiasm for investigating MCV and Merkel cell carcinoma will take a different path, with the recent basic science findings being actively applied to new methods to diagnose, treat, and prevent this dismal cancer.

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THE MELANOMA LETTER

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PERRY ROBINS, MD, President MARY STINE, Executive Director

Merkel Cell Carcinoma

Jürgen C. Becker, MD, PhD
Professor, Department of Dermatology, Venereology, and Allergy
University of Würzburg, Germany

Merkel cell carcinoma (MCC) is an uncommon, aggressive neuroendocrine carcinoma of the skin, with high rates of recurrence, metastatic spread, and mortality. In 1972, Toker[1] described five patients with unusual skin tumors where histologically anastomosing trabeculae (pathologically connecting bands of neoplastic tissue two or more cells wide) and cell nests in the dermis dominated; he used the name "trabecular carcinomas of the skin" to describe them.

Later, the discovery of electron-dense neurosecretory granules in the tumor cells allowed them to be classified among the neuroendocrine carcinomas. Since Merkel cells, which function as slowly adapting mechanoreceptors, are the only cutaneous cells that form such granules, it was postulated that these carcinomas are derived from them. Thus, this tumor was named "Merkel cell carcinoma."

Epidemiology

Within a 15-year time period from 1986 to 2001, the age-adapted incidence of MCC rose, with a statistically significant annual increase of 8 percent. This rise is even more dramatic than the increased incidence of cutaneous melanoma.[2] Furthermore, the mortality rate for MCC is about 33 percent, also higher than that of melanoma. The American Cancer Society predicted 1,500 new cases of MCC in the US for 2008.

MCC is typically a carcinoma of the elderly; the mean age of patients at time of initial diagnosis is about 70 years. Several lines of evidence suggest a strong link between MCC and ultraviolet (UV) light exposure. Accordingly, MCC is highly associated with squamous cell carcinoma, basal cell carcinoma, and Bowen's disease.

There is also a striking epidemiologic association between immunosuppression and MCC.[3] Chronically immunosuppressed individuals are more than 15 times

Continued on page 2

SPECIAL REPORT:

From the Editors

"Skin cancer" is actually a broad term that encompasses a host of malignancies, each developing from distinct cellular compartments of normal skin: keratinocytes, melanocytes, lymphocytes, and fibroblasts, to name a few. Since the vast majority of skin cancers are basal cell carcinomas, squamous cell carcinomas, and melanomas, they deservedly receive the most attention in the medical literature and in the media. However, this does not mean that other cancers of the skin are insignificant.

This issue of *The Melanoma Letter* is dedicated to one of the less common skin cancers, Merkel cell carcinoma (MCC). This potentially lethal cancer – more frequently fatal than melanoma – was recently featured in headline news worldwide due to the landmark discovery that viral oncogenesis may be responsible for triggering its development. Evidence is mounting that, much the way human papillomavirus induces cervical cancer and herpesvirus induces Kaposi's sarcoma, a mutated polyomavirus may induce the majority of Merkel cell carcinomas.

In our lead story, Dr. Jürgen Becker presents an excellent review of the epidemiology, pathogenesis, staging and current treatment options for Merkel cell carcinoma. In the accompanying report, the evidence linking a polyomavirus to Merkel cell carcinoma and the fascinating process by which this evidence was uncovered are described in detail by Drs. Moschos, Chang and Moore, integral members of the group that discovered the role of this polyomavirus in MCC. It is the shared hope of researchers and clinicians that their discovery will lead to a better understanding of MCC and successful therapies.

Allan C. Halpern, MD
Editor-in-Chief

Ashfaq A. Marghoob, MD
Associate Editor

Merkel Cell Carcinoma, *from page 1*

likelier to develop MCC than are age-matched controls. For example, MCC occurs much more frequently (12/100,000 per year) and at a significantly younger age in patients with organ transplants and HIV infections. (About 50 percent of tumors in immunosuppressed patients occur before the age of 50.)

Clinical Features and Histology

MCC characteristically develops rapidly over weeks or months on chronically sun-damaged skin, appearing as a firm-elastic, red-to-livid hemispherical tumor with a smooth, shiny surface.[4] Ulcerations are very rare, observed only in very advanced tumors.

Due to the relatively uncharacteristic features of MCC and the difficulty differentiating it clinically from other lesions, the diagnosis in most cases is first made on the basis of histopathology. Histologically, MCC appears as an asymmetric dermal tumor with irregular margins composed of tumor cells arranged in strands or nests.[5] The tumor spreads into the reticular dermis and subcutis; the papillary dermis, epidermis, and adnexa are usually spared. The mitotic index is very high, and many atypical mitoses are seen.

According to the arrangement and appearance of the tumor cells, three histologic patterns are differentiated: the trabecular, the intermediate, and the small cell type. Mixed and transitional forms among the three types are very frequent. In most cases, immunohistochemistry is required for definitive diagnosis. This is especially necessary to exclude histologic differential diagnoses such as small cell lung cancer, small B-cell lymphomas, or anaplastic small cell melanomas. In general, immunohistochemical identification of cytokeratin (CK) 20 and neuron-specific enolase is sought. CK20 is found in the tumor cells in a remarkable paranuclear plaque (dot-like pattern) as well as to a significantly lower extent along the cytoskeleton.

Pathogenesis

Certain genetic and epigenetic changes cause cells to evade apoptosis, become self-sufficient in growth signals and insensitive to growth-inhibitory signals, develop limitless replicative potential, boost angiogenesis, escape immune surveillance, and allow tissue invasion and metastasis. These changes, considered the main reasons that a normal cell turns into a cancer cell, are mediated by oncogenes that are activated and tumor suppressor genes that are inactivated. Recent evidence has revealed that different cancer types have different characteristic patterns of aberrations. Nevertheless, scientists have identified a distinct set of proteins and pathways that are repeatedly involved in the carcinogenesis of many different tumor types. Several of the genes involved have been analyzed for their relevance in the molecular pathogenesis of MCC and their potential impact on the clinical course of this aggressive disease.

The classic **m**itogen-**a**ctivated **p**rotein (MAP) kinase signaling pathway plays a key role in the oncogenesis of several cancers. Expression of c-Kit in tumor samples has suggested the impact of dysregulated c-Kit

The mortality rate for MCC is about 33 percent — higher than that of melanoma.

(the gene that encodes for the KIT protein, which serves as a stem cell factor receptor) as oncogenic tyrosine kinase for autocrine stimulation of MAP kinase signaling in MCC.[6] However, to date no activating c-Kit mutations have been detected in MCC. Similarly, analysis of MCC samples has not revealed the presence of any activating B-Raf mutations. Moreover, immunohistochemical studies have revealed that despite high proliferation indices and existing expression of **e**xtracellular signal-**r**egulated **k**inases (ERK), the ERK protein generally occurs in the non-phosphorylated form in MCC and is thus inactive.

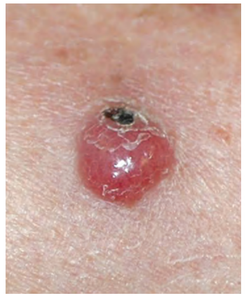
The role of UV light in the development of MCC appears to be immunosuppressive (e.g., by induction of immunosuppressive cytokines such as interleukin-10) rather than mutagenic; there is a striking epidemiologic association between immunosuppression and MCC.[7] The observation that MCC occurs more frequently than expected among immunosuppressed patients is quite comparable to what is observed in Kaposi's sarcoma (KS). This similarity to KS, an immune-related tumor caused by Kaposi's sarcoma-associated herpesvirus, originally raised the possibility that MCC may also have an infectious origin. Indeed, Feng, et al recently provided evidence of a possible viral oncogenesis.[8] By means of a technique called digital transcriptome subtraction, they discovered a genome encompassing 5,387 base pairs of a new polyomavirus, the Merkel cell polyomavirus (MCPyV). The presence of MCPyV in the majority of MCC samples has been confirmed by three independent groups. [See *"Merkel Cell Polyomavirus: The Seventh Human Cancer Virus?"* in this issue.]

This observation is particularly interesting, since polyomaviruses express genes, including large and small T antigens, and bind to host proteins to force the cell into S phase (the cell-cycle phase when the DNA is replicated). Notably, the large T antigen regulates the life cycle of the virus as well as the cell cycle of the host cell. The latter occurs via interaction with the tumor suppressor gene p53 and the members of the retinoblastoma protein (Rb) gene family.

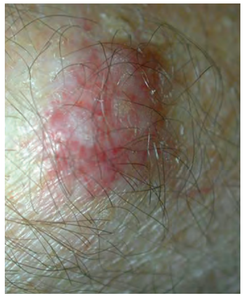
Staging, Prognosis and Follow-up

The staging of Merkel cell carcinoma is not uniformly defined. Frequently the stage classification shown in **Table 1** is employed. The five-year survival rate of Merkel cell carcinoma patients is 75 percent, 59 percent, and 25 percent, respectively, for primary tumors, lymph node metastases (and/or local recurrences), and distant metastases. Most recurrences occur within 2 years after diagnosis of the primary tumor. Due to the high frequency of lymphatic metastases, sentinel lymph node biopsy (SLNB) is frequently performed, revealing micrometastatic involvement in about 30 percent of cases. The presence of micrometastases in the sentinel lymph nodes denotes poorer prognosis. Very likely, consideration of lymph node status in the future will lead to improved prognostic appraisal and an altered stage classification system.

Retrospective studies reveal the following unfavorable prognostic factors: advanced tumor stage, male gender, location of the primary tumor in the head-and-neck region or on the trunk, and the presence of immunosuppression. Prognostic significance is also assigned to the histological type: The trabecular type of tumor is the best differentiated, while the small cell type is least differentiated.



Merkel cell carcinoma, local recurrence, forehead.



Merkel cell carcinoma, primary tumor, lower arm.

STAGE	PRIMARY TUMOR
Ia	Diameter < 2cm
Ib	Diameter > 2cm
II	Locoregional metastases
III	Distant metastases

Table 1: *Clinical Staging of MCC*

Measuring tumor thickness also appears to enhance prognostic classification. In addition, a higher mitotic index, the presence of tumor-infiltrating lymphocytes, and overexpression of p63 or survivin all are associated with worse prognosis.

Therapy

For primary tumors without indications of organ metastases, complete surgical excision is the basic therapy. Due to the high rate of local metastases that can be attributed to subclinical satellite metastases, a safety margin should be observed, if possible. MCCs are radiosensitive; thus, the local recurrence rate after surgery can be reduced significantly by locoregional adjuvant radiation therapy (to the surgical scar with a 3 cm safety margin, as well as to the regional lymph node basin). The required total dose is considered to be 50 Gy with a single dose of 2 Gy five times weekly.[9] Due to the high rate of subclinical micrometastases in the draining lymph node, a sentinel lymph node biopsy should be considered. When micrometastases are present in the sentinel lymph node, this should be followed by complete lymphadenectomy or adjuvant radiation of the respective lymph node region.

Even though Merkel cell carcinoma is considered chemosensitive, evidence-based, standardized chemotherapy does not yet exist. Due to morphological similarities in the past, schemes that are established for small cell lung cancer have often been chosen; these include, among others, anthracyclines, anti-metabolites, bleomycin, cyclophosphamide, etoposide, and platinum derivatives singly or in combination. With administration of these potentially highly toxic regimens, relatively high remission rates of up to 70 percent are achieved, but due to generally short remissions, this does not lead to a significant increase in survival time. Furthermore, no obvious correlation between intensity of therapy and response exists. Therefore, systemic chemotherapy is indicated as a palliative measure when distant metastases are present, but especially due to the highly toxic effects of most chemotherapeutic agents on elderly patients (reduced hepatic and renal function as well as reduced hematopoiesis), it must be adapted to the individual case. Well-tolerated monotherapies include etoposide or anthracyclines.[10]

Now that Feng, et al's description of Merkel cell carcinoma as a polyomavirus has made a viral oncogenesis a possibility, new therapeutic options have opened, such as the use of interferons for their antiviral effects or the development of immunotherapeutic strategies. For the latter purpose, antigens may include not only viral proteins but also proteins induced by polyomaviruses, such as survivin, the regulator of apoptosis. To date, only sporadic case reports exist where immunotherapy agents have been used in MCC. Anecdotal case reports exist showing the successful use of interferon-α and anti-CD56 antibodies or vaccines, but controlled clinical trials will be needed. We still lack prospective clinical trials for any strategy used to treat patients suffering from MCC.

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Mark Teich, Editor *(mteich@skincancer.org); Paul Melia,* Managing Editor; *Michelle Marcano,* Production Coordinator.

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SPECIAL REPORT:

Merkel Cell Polyomavirus:
The Seventh Human Cancer Virus?

Stergios Moschos, MD
Yuan Chang, MD
Patrick S. Moore, MD, MPH
University of Pittsburgh Cancer Institute
Pittsburgh, PA

The recent discovery of a new human polyomavirus in Merkel cell carcinoma (MCC) has markedly changed the directions and opportunities for research on this enigmatic cancer.[1] MCC has several epidemiologic features that suggest it might have an infectious etiology, the most important of which is the strong association between MCC and immunosuppression.

Feng, et al developed a direct sequencing approach called *digital transcriptome subtraction* (DTS) to discover new viral infectious agents in human cancers.[2] This technique is based on creating complementary DNA (cDNA) from RNA extracted from fresh tumor tissue through reverse transcription and exhaustively sequencing the cDNA library. All sequences matching known human sequences are then computationally subtracted. The remaining nonmatching — presumably non-human — sequences are candidates that may represent pathogen cDNAs (**Figure 1**).

Given that hundreds of thousands of sequences are screened in DTS, even miniscule error rates in sequencing or analysis can lead to dozens of potential candidate sequences that do not match the human genome. Furthermore, highly polymorphic human sequences such as mitochondrial DNA and immunoglobulin hypervariable regions can also pass through computational screens to become part of the pool of candidate sequences. Thus, the bulk of the effort in DTS involves careful screening and selection of high-quality sequences prior to computational subtraction.

Discovery of Merkel Cell Polyomavirus

Feng and colleagues applied DTS to RNA extracted from four human MCC tumors. After examining 400,000 cDNA sequences, one cDNA was found with homology to a large tumor (T) antigen sequence belonging to polyomaviruses—a group of viruses known to cause cancers in experimental animals, particularly rodents. Flanking viral DNA regions were then isolated, allowing identification of the entire 5.4-kilobase genome of a new human polyomavirus, termed Merkel cell polyomavirus (MCV or MCPyV).

The MCV genome includes an early coding region encoding the large tumor (T) and small tumor (t) antigen proteins named for their ability to induce tumors and cell transformation. MCV also possesses a late coding region, which encodes the viral capsid proteins VP1 and VP2/3.[1] MCV large T antigen, like large T antigens from other polyomaviruses, is multifunctional and possesses highly conserved domains that bind known host cell tumor suppressor proteins. Other large T antigen domains are involved in viral DNA replication. The MCV genome is normally a circular double-

MCC has several epidemiologic features that suggest it might have an infectious etiology, the most important of which is the strong association between MCC and immunosuppression.

stranded DNA; T antigen binds to the viral replication origin, then a helicase domain in the T antigen carboxyl region opens the double-stranded DNA to allow the viral DNA to replicate.

Evidence of a Causative Role

Polyomaviruses are controversial candidates for causing human cancer.[3] Two of them, BK virus and JC virus, have been isolated from human cancers, but proof of an etiopathogenic role has been elusive. Similarly, simian virus 40 (SV40) induces tumors in experimental animals and has been proposed to cause human cancer, but there is no clear evidence for involvement of this virus in human disease.

In their initial report, Feng, et al found 7 of 10 MCC tumors from different patients positive for MCV by Southern (blot) hybridization, a technique that is highly specific and not prone to contamination. An additional tumor was positive for the virus but only with PCR, a less reliable but highly sensitive method. This high rate of MCV positivity has been subsequently confirmed in over a dozen studies around the world that included several hundred MCC patients.[4-6]

Extensive surveys have also been performed on other tumors, including cutaneous melanomas, basal cell carcinomas, and squamous cell carcinomas. Most,[7-10] but not all,[11-12] studies have found that MCV infection is specific for MCC. In fact, MCV is clonally integrated into the Merkel cell carcinoma genome.[1] This suggests that MCV infection preceded initial cancer development and that the virus integrated into the host genome prior to clonal tumor cell expansion, a pattern analogous to that of high-risk human papillomavirus integration into cervical carcinoma cells. Since MCV cannot "excise" itself from the human genome, the virus is in a non-transmissible, dead-end state in MCC tumor cells. Integration occurs at different sites in the genome in different individual cases;[1,13] in one case, metastatic tumor had the same monoclonal integration pattern as the primary tumor, showing that the metastasis arose from a single cancer cell already infected with the virus.[1] Although this does not rule out insertional mutagenesis playing a role in MCV-related cancer, no obvious patterns for virus integration into the human genome have been described to date.

A peculiar set of mutations are also present in tumor-derived viruses, which supports MCV having a direct etiopathogenic role in MCC.[14] These tumor-specific signature mutations truncate the helicase portion of T antigen and are not present in viruses isolated from morphologically normal tissues. In the polyomavirus, the large T antigen regulates the life cycle of the virus as well as the cell cycle of the host cell. The latter occurs via interaction with the tumor suppressor gene p53 and the members of the retinoblastoma protein (Rb) gene family. The mutated viruses retain their ability to inhibit the retinoblastoma protein but can no longer initiate replication and would be lost without integration into the cellular genome.[14] For this reason, the virus cannot be a