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Visions & Reflections (Minireview)

Merkel cell carcinoma

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Abstract. Merkel cell carcinoma (MCC) is a highly aggressive neuroendocrine carcinoma of the skin. More than one-third of MCC patients will die from this cancer, making it twice as lethal as malignant melanoma. Despite the fact that MCC is still a very rare tumor, its incidence is rapidly increasing; the American Cancer Society estimates for 2008 almost 1 500 new cases in the USA. These clinical observations are especially disturbing as the pathogenesis of MCC is not yet fully understood; however, a number of recent reports contribute to a better understanding of its pathogenesis. Here we describe findings regarding the role of Wnt, MAPK and Akt signaling as well as possible aberrations in the p14ARF/p53/RB tumor suppressor network in MCC. Most important, and possibly with high impact on future therapeutic approaches is the demonstration that a polyomavirus has frequently integrated in the genome of the MCC cells prior to tumor development.

Keywords. Neuroendocrine carcinoma of the skin, epidemiology, carcinogenesis, signal transduction, polyomavirus.

Merkel cell carcinoma (MCC) is an uncommon and aggressive neuroendocrine carcinoma of the skin. In 1972, Toker described five patients with unusual skin tumors where histologically anastomosing trabeculae and cell nests in the dermis dominated, so that he used the name "trabecular carcinoma of the skin" [1]. Subsequently, the discovery of electron-dense neurosecretory granules in the tumor cells allowed for classification among the neuroendocrine carcinomas. Since Merkel cells, which function as slowly adapting mechanoreceptors and belong to the amine precursor uptake and decarboxylation (APUD) system, are the only cutaneous cells which form such granules, it was postulated that these carcinomas are derived from these cells [2].

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Epidemiology

In 2008, the American Cancer Society predicted 1 500 new cases of MCC in the USA alone; the incidence of MCC would thus exceed that of cutaneous T-cell lymphomas (http://www.cancer.org). Similar data has been reported for Australia and Europe [3, 4]. Within a 15-year time period from 1986–2001 the age-adapted incidence of MCC has risen with a statistically significant annual increase of 8 %. This rise is more dramatic than the increased incidence of cutaneous melanoma [5]. Furthermore, the mortality rate of MCC with about 33 % is higher than that of melanoma. MCC is a carcinoma of the elderly; the mean age of patients at the time of initial diagnosis is about 70 years. Several lines of evidence suggest a strong link between MCC and ultraviolet light exposure. For example, the incidence of MCC is higher at more

equatorial latitudes, 81 % of primary tumors occur on sun-exposed skin, and Caucasians have the greatest risk [5, 6]. Accordingly, there is a high degree of association of MCC with squamous cell carcinoma, basal cell carcinoma and Bowen's disease.

Importantly, there is a striking epidemiologic association between immunosuppression and MCC [7–9]. Chronically immunosuppressed individuals are more than 15 times more likely to develop MCC than are age-matched controls. For example, MCC occurs much more frequently in patients with organ transplants and HIV infections (12/100,000/year) and at a significantly younger age (about 50 % < 50 years). Similarly, other forms of T-lymphocyte immune suppression such as chronic lymphocytic leukemia are linked with MCC. MCC is also more lethal in immunosuppressed patients, with a reported disease-specific mortality of up to 56 %. Interestingly, there are several case reports of MCC regression following restoration of immune function [10–12].

Clinical features and histology

MCC characteristically develops rapidly over weeks to months on chronic sun-damaged skin as a firm-elastic red to livid hemispherical tumor with a smooth, shiny surface [6]. The clinical features of MCC can be explained by the fact that the tumor usually grows in a hemispherical fashion to the outside and in an iceberg-like fashion into the deep, so that the intact epidermis is stretched. Ulcerations are very rare and are observed only in very advanced tumors.

Due to the relatively uncharacteristic features of MCC, 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 [13]. 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 between the three types are very frequent. In most cases, immunohistochemistry is required for definitive diagnosis. This is especially necessary to differentiate histologic differential diagnoses such as small cell lung cancer, small B-cell lymphomas or anaplastic small cell melanomas. In general the immunohistochemical identification of cytokeratin (CK) 20 is performed. CK20 is found in the tumor cells in a remarkable paranuclear plaque (dot-like pattern) as well as to a significantly less extent along the cytoskeleton. Expression of the inhibitor of apoptosis (IAP) survivin and the member of the p53 family p63 appears to be associated with a poorer prognosis [14, 15].

Pathogenesis

Despite a substantial research effort, the understanding of the molecular basis of MCC is still limited. Overexpression of the anti-apoptotic molecule bcl-2 was observed in three-fourths of MCC tumors in two independent studies [16, 17]. Inhibition of bcl-2 expression *in vivo* by antisense oligonucleotides in a SCID mouse/human tumor xenograft model resulted in tumor shrinkage [18]. The expression of this anti-apoptosis protein is a common finding in many cancers and suggests one of its mechanisms to avoid cell death; however, the same antisense oligonucleotides when tested in a phase II trial, demonstrated only a very limited if any efficacy in patients with MCC [19]. Moreover, bcl-2 overexpression does not illuminate the promitotic pathways that drive MCC.

Secreted Wingless type (Wnt) ligands have been shown to activate signal transduction pathways and trigger changes in gene expression, cell behavior, adhesion and polarity [20]. The role of Wnt signaling in cancer was first suggested 20 years ago with the discovery of Wnt-1 as an integration site for mouse mammary tumor virus in mouse mammary carcinoma. The Wnt pathway and its role in MCC have been evaluated by determination of nuclear accumulation of β-catenin and mutations in β-catenin and three other related genes. Liu et al. observed elevated β-catenin accumulation in only one out of 12 tumors and no mutations in any tumors; thus, they concluded that the Wnt pathway is not implicated in MCC [21].

The classical mitogen-activated protein (MAP) kinase signaling pathway plays a key role in many processes such as proliferation, suppression of apoptosis, migration and differentiation [22]. The relevance of the Raf/MEK/ERK cascade for tumorigenesis has been evident for a long time [23]. Thus, MAP kinase signaling, a common feature of many epithelial cancers, is the most studied oncogenic pathway in MCC. Expression of the receptor tyrosine kinase c-kit, a potential activator of the MAPK pathway, has been detected in MCC in several studies [24–27]. Recently, Swick et al. determined that although eight of nine MCC tumors were positive for c-kit by immunohistochemical staining, no activating mutations were present in the four exons commonly found to have mutations in this gene [28]. Similarily, the analysis of MCC samples did not reveal presence of any activating B-Raf mutations [29, 30]. Moreover, immunohistochemical

studies revealed that despite high proliferation indices and existing expression of ERK, the ERK protein generally occurs in the non-phosphorylated form and is thus inactive [29]. The lack of ERK phosphorylation was always accompanied by a strong expression of the Raf kinase inhibitor protein (RKIP), a negative regulator of the MAPK cascade. However, silencing of RKIP did not lead to an induction of ERK phosphorylation [31].

The general inactivity of the "mitogenic" cascade distinguishes MCC from many other tumors such as melanoma and raises the question if active MAP kinase signaling is perhaps even a negative selection factor for MCC cells. To test this notion, we utilized UISO cells, an MCC cell line that has been established from a primary MCC of a 46-year-old woman and which has conserved *in vitro* the *in situ* observed inactivity of the MAPK pathway [32]. We developed UISO cells that express a Raf construct in which the natural regulatory domain is replaced by an artificial hydroxy tamoxifen (OHT)-sensitive regulatory domain (Raf-ER) derived from the estrogen receptor [33]. OHT stimulation results in MEK and ERK activation in these cells. This activation of the MAPK pathway leads to a collapse of the cytoskeleton and subsequent death of the MCC cells. The induced cell death was by apoptosis and this OHT-induced apoptosis could be prevented by specific MEK inhibition indicating that apoptosis is indeed an effect mediated by the MAPK cascade.

The inactivity of the MAP kinase pathway together with the report that inhibition of Ras farnesylation by apoptosis induction suppresses the growth of MCC xenografts in the naked mouse model suggest that another Ras-dependent signaling pathway may be of special relevance in MCC [34]. The most important Ras-regulated signal pathway next to the Raf/MEK/ERK cascade involves class 1 phosphoinositide 3 kinase (PI3K) and the Akt kinase [35]. PI3K phosphorylates phosphatidylinositol 4,5-biphphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3), an important second messenger lipid. The antagonist of PI3K that dephosphorylates PIP3 is the phosphate and tensin homologue deleted in chromosome 10 (PTEN) that is frequently inactivated in human tumors. Accumulation of PIP3 after PI3K activation leads to a translocation of Akt to the plasma membrane where it is phosphorylated by two PIP3-dependent kinases and thus activated. Akt develops its anti-apoptotic and cell cycle-regulating effects via a series of target molecules (over 30 have already been described) such as *e.g.,* the pro-apoptotic protein Bad, pro-caspase 9, forkhead transcription factors or the mammalian target of rapamycin (mTOR) [36]. Surprisingly mTOR functions, on the one hand, in a rapamycin-sensitive complex with raptor as an effector of Akt, on the other hand, it belongs in a rapamycin-insensitive complex with rictor to the Akt-activating kinases. Interestingly, an Akt/mTOR-regulated suppression of p53 protein expression that functions via induction of the translation of the p53-specific ubiquitin ligase mdm2 has been described. Furthermore, Akt can directly activate mdm2 and thus induce p53 protein degradation [35]. As the apoptosis induced by Ras inhibition in the MCC xenografts is accompanied by p53 induction, an involvement of the PI3K/Akt signal pathway in this process as well as in the molecular pathogenesis appears very likely [34].

The frequent heterozygous loss of chromosome 10 or the long arm of chromosome 10 in MCC suggests that the tumor suppressor PTEN encoded there plays a relevant role [37]. A recent study using MCC tissue arrays to measure the expression of various proteins (especially matrix metalloproteinases) revealed that PTEN could hardly be identified in the samples examined, which could indicate inactivation of the second allele, *e.g.,* by epigenetic mechanisms [38].

The epigenetic silencing of another tumor suppressor gene in MCC had been recently reported [39]. Lassacher et al. observed that promoter hypermethylation of the INK4a-ARF locus is common in MCC. Their analysis revealed the presence of methylated DNA at the p14ARF promoter in eight of 19 MCC samples; in contrast, the p16INK4a promoter was only methylated in one case. This observation sheds important light on the pathogenesis of MCC because DNA methylation in promoter regions helps to regulate the gene expression. In particular, hypermethylation of CpG islands located in the promoter regions of tumor-suppressor genes such as p16INK4a, BRCA1, and hMLH is now established as an important mechanism of gene inactivation in cancer [40]. The INK4a-ARF locus encodes the two protein products (p16INK4a and p14ARF) whose respective mRNAs are generated from separate promoters (Fig. 1). p16INK4a and p14ARF are integral parts of the two cell-cycle control pathways targeted most frequently during tumorigenesis, *i.e.,* the RB and p53 pathway [41]. In the case of MCC, alterations in the p53 pathway appear to be most crucial, as suggested by the high frequency of p14ARF promoter methylation and low frequency of p16INK4a promoter methylation.

A multitude of functions have been described for the tumor suppressor p53 [41]. The anti-proliferative activity of p53 plays a significant role in the avoidance of tumor development, but also requires effective mechanisms to control p53 in normal proliferating cells. The probably most important mechanism involves the control of

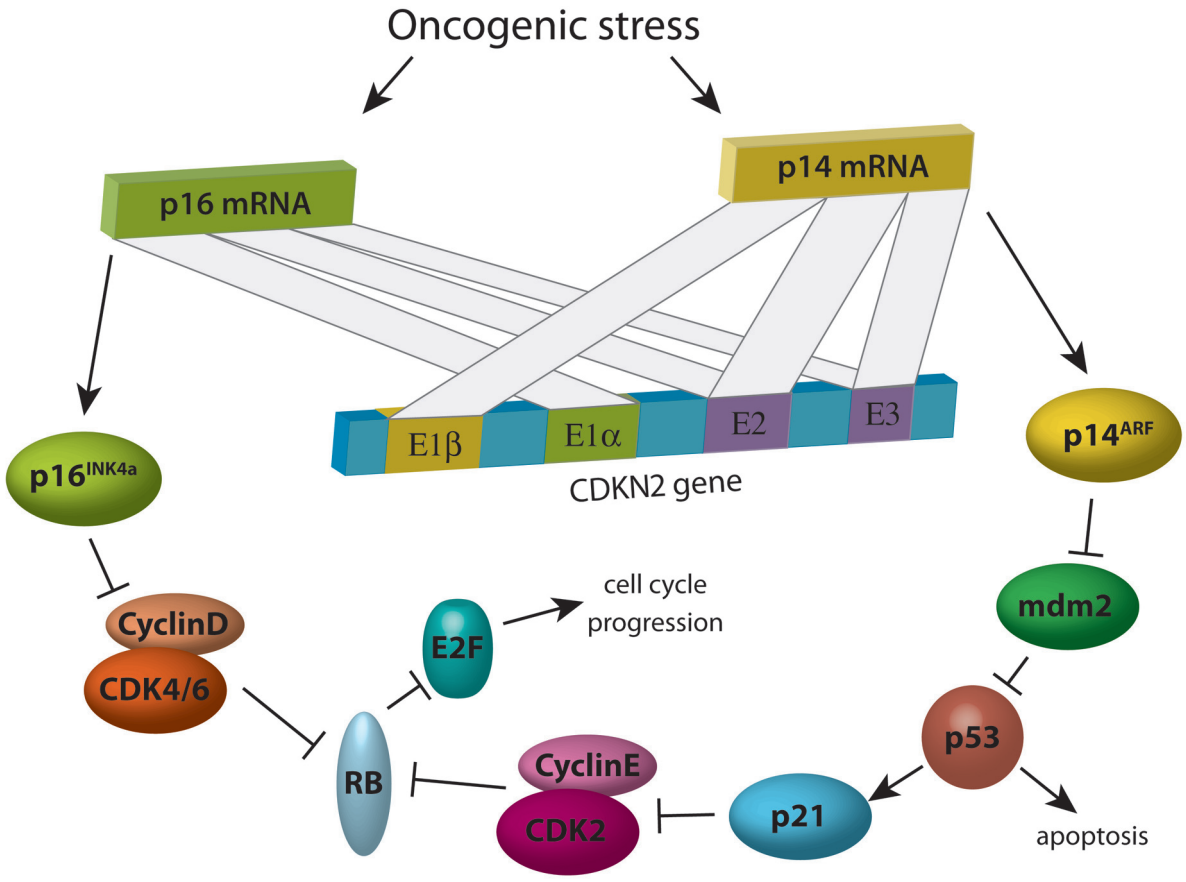

Figure 1. The control of the cell cycle progression by Rb1, p53 and CDK inhibitors.

protein stability with ubiquitination by the p53-specific ubiquitin ligase mdm2 and subsequent proteasomal degradation playing a central role. Oncogenic stress results in the induction of the tumor suppressor protein p14ARF, which binds to mdm2, inactivates it and thus prevents degradation of p53 (Fig. 1) [42].

p53 is the protein that is most frequently present in a mutated form in tumors. It is found altered in about 50 % of human tumors, and in tumors with wild-type p53, its activation is often disturbed. p53 mutations are occasionally found in MCC. In three of 15 tumors examined and in two of six MCC cell lines, p53 mutations were present [43, 44]. On the other hand, the previously mentioned apoptosis induction associated with p53 derepression in MCC xenografts suggests that the regulation of p53 expression or stability may be disturbed in MCC [34]. For another member of the p53 family, p63, it has been shown that its expression in tumor cells correlates to an exceptionally high degree with the aggressiveness of the disease [15]. p63 is a transcription factor essential for proliferation of stem cells and for stratification in epithelia, mutated in human hereditary syndromes characterized by ectodermal dysplasia.

The proteins whose expression is induced by p53 include p21 (Fig. 1). Together with p27 and the proteins of the Ink4 family p21 belongs to the inhibitors of the cyclin-dependent kinases (CDK). These CDK inhibitors prevent the transition from the G1 to the S phase of the cell cycle [45]. Rb1 (product of the retinoblastoma susceptibility gene) is a key molecule in gene expression promoting the G1/S transition. In its hypo-phosphorylated state this tumor suppressor protein prevents entry into the S phase by forming an inhibiting complex with transcription factors of the E2F family [46]. After multiple phosphorylations of Rb1 by cyclin D/CDK4/6 and cyclin E/CDK2 complexes, Rb1 is released and degraded, the inhibition of E2F is suspended. The RB/E2F signaling pathway is of such central significance for the control of cell proliferation that it is assumed that its regulation is disturbed in practically all tumors. Gene loss or inactivating mutations of Rb1, viral Rb1-inactivating oncoproteins (*e.g.,* human papilloma virus E7), overexpression and activating mutations of CDK 4/6, amplification and overexpression of CDK2, amplification or translocation of cyclin D genes as well as inactivating mutations and loss of

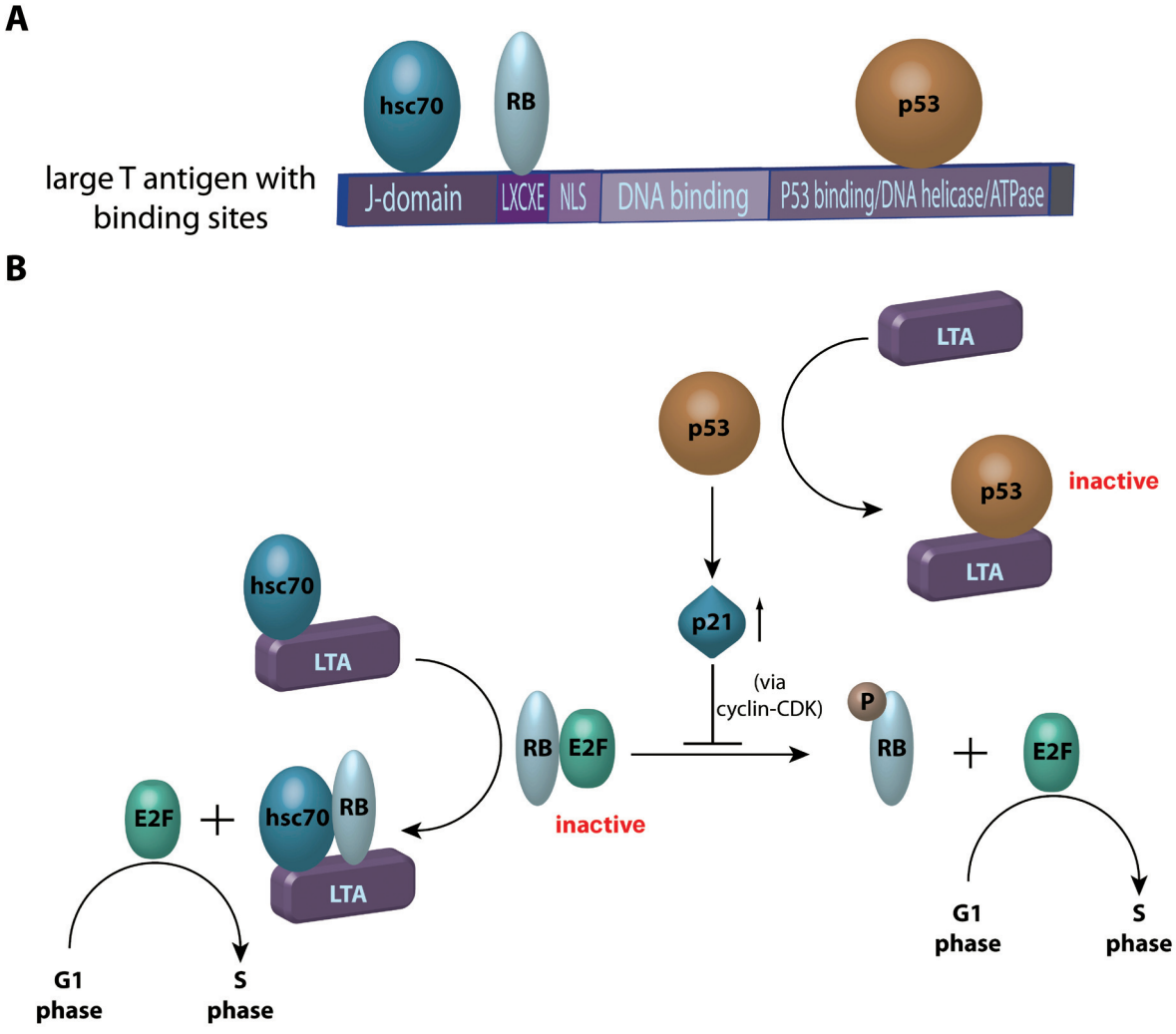

Figure 2. Binding sites of the large T antigen for the tumor suppressor gene p53 and the members of the Rb gene family (*A*) and its impact on these pathways.

expression of p16Ink4a and p15Ink4b have been described. For MCC, little data on the regulation of the cell cycle by Rb1 exists and studies on a possible loss of Rb1 are contradictory. Leonard and Hayard found loss of heterozygosity for the marker D13S233 (13q14.3), which is located close to the Rb1 gene, in 18 of 24 examined MCC samples [47]. Further, in all nine MCC cell lines they analyzed, no Rb1 could be detected in the Western blot. In contrast, Popp et al. reported no loss of the 13q region in 10 MCCs analyzed by comparative genome hybridization [43]. Our own as yet unpublished experiments suggest that loss of Rb1 is not a frequent occurrence in MCC. However, as described below, the frequent association of MCC with a new polyomavirus which encodes an Rb-binding protein may explain a disturbed regulation of this pathway [48].

The role of UV light in the development of MCC may be rather immunosuppressive than mutagenic. Pathogenetically, in addition to disturbed antigen presentation, the induction of immunosuppressive cytokines such as IL-10 and TNF-α, the isomerization of trans-urocanic acid to cis-urocanic acid and the formation of reactive oxygen species are blamed [49, 50]. This notion goes in parallel with the striking epidemiologic association between immunosuppression and MCC [51]. The observation that MCC occurs more frequently than expected among immunosuppressed transplant and AIDS patients is very similar to Kaposi's sarcoma. This similarity to Kaposi's sarcoma, an immune-related tumor caused by Kaposi's sarcoma-associated herpes virus, raised the possibility that MCC may also have an infectious origin. Indeed, Feng and coworkers recently were able to provide evidence for a possible viral oncogenesis [52]. They studied

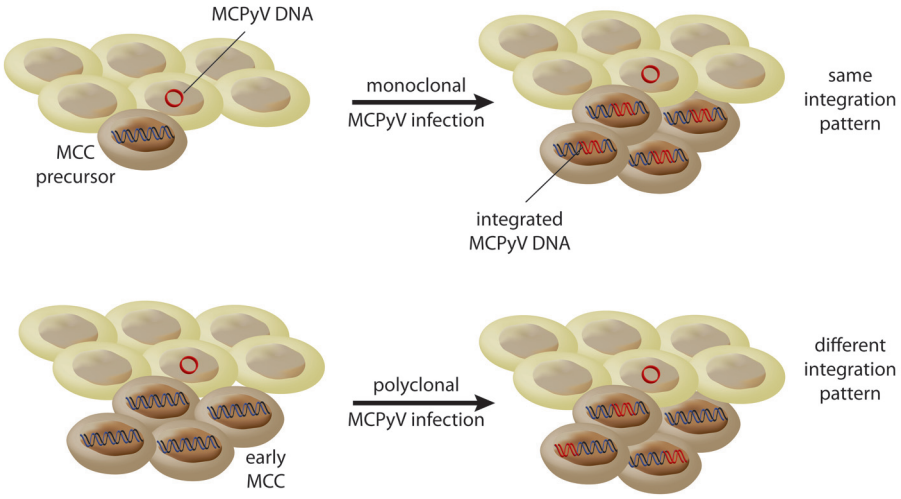

Figure 3. Monoclonal versus polyclonal MCPyV infection.

MCC samples using digital transcriptome subtraction. These studies resulted in the discovery of a genome encompassing 5 387 base pairs of a new polyomavirus, the Merkel cell polyomavirus (MCPyV).

Since the discovery of the mouse polyomavirus by Gross in 1953, polyomaviruses have been suspected as possible causes of cancer in humans [53, 54]. Even though polyomaviruses can induce tumors in animal models, there has not yet been any definitive proof that they play a relevant role in human carcinogenesis. These small (40–50 nanometer in diameter) double-stranded DNA viruses code for several proteins, among them large T(umor) antigen, in their circular genome. Polyomaviruses, as the most extensively studied Simian Virus 40 (SV-40), express genes in two waves: early and late. The early-expressed genes, including large and small T antigens, bind to host proteins to force the cell into S phase (the cell-cycle phase when the DNA is replicated) and facilitate viral replication [55, 56]. The late genes encode components of the viral coat and enable lysis.

The SV-40 large T antigen regulates the life cycle of the virus as well as the cell cycle of the host cell. The last occurs via interaction with the tumor suppressor gene p53 and the members of the Rb gene family (Fig. 2). This viral stimulation of the cell cycle is the main driving force of the oncogenic potential of polyomaviruses [55, 56]. Importantly, the predicted MCPyV large T antigen contains many of the features common to oncogenic polyomaviruses, including an LxCxE motif that may directly bind pRb [52]. Polyomaviruses often induce latent infections without manifest disease, but can, for example in an immunosuppressed host, induce tumors [57]. In animal models, tumor development is usually preceded by the integration of the polyomavirus DNA into the host genome [53]. In this respect, it is important to note that when screening ten MCC tumors for MCPyV and found that seven were strongly positive for MCPyV DNA by PCR and an additional tumor was weakly positive. The strength of the PCR signals suggested that the viral DNA was integrated in a clonal fashion (Fig. 3). To test this notion, genomic DNA from MCC tumors was digested with two restriction enzymes and analyzed by Southern hybridization using a MCPyV DNA probe. The resulting banding pattern in five of the eight MCPyV-positive MCC tumors indicated a monoclonal integration of the virus; in one tumor, a monoclonally integrated viral concatemer was observed and in the remaining two other tumors, an indeterminate integration pattern was observed [52]. Moreover, for one tumor with a monoclonal integration pattern, both primary and metastatic tumor tissue was available and both specimens showed an identical viral integration pattern, demonstrating that integration of MCPyV preceded metastasis formation. Furthermore, the distinct integration patterns between tumors imply that the virus integrated at different locations within the human genome in the different tumors.

The presence of MCPyV in the majority of MCC samples as been confirmed by three independent groups [58–60]. Interestingly, our own data suggest a poorer prognosis for MCC patients with the identification of MCPyV in tumor tissue. In all published MCPyV genome sequences premature stop codons are predicted within the second exon of the large T antigen. The predicted truncated proteins potentially preserve some of the cell-cycle progression activities of the amino terminus of large T but prevent cell-lethal genomic instability related to the replicative functions of the carboxyl terminus [55, 56]. If the large T antigen were intact, replication initiated at an integrated origin would lead to genomic instability

and cell death [48]. Indeed, in permissive cells transformed by SV-40, the large T antigen is often mutated such that the viral replication functions are inactive but cell-cycle progression functions are preserved. The presence of MCPyV DNA in Merkel tumors does not prove a causal involvement of this virus in Merkel carcinomas. The clonal pattern of integration in tumor tissue as well as in its metastasis, however, could favor this interpretation. The preferential occurrence of this cancer in immunosuppressed patients might also support a viral etiology. Merkel cell carcinoma seems to represent the first human malignancy with a relatively consistent presence of integrated sequences of a specific type of polyomavirus.

Conclusion

The gradual improvement of our comprehension of the pathogenesis of Merkel cell carcinoma will hopefully translate into an improved management of patients suffering from this highly aggressive cancer, which is currently dismal. Due to similarities in immunohistochemistry and morphology with small cell lung cancer, metastatic disease is treated with various combination chemotherapy regimens of cisplatin and etoposide, or vincristine, cyclophosphamide, and doxorubicin. These regimens have response rates of 60% to 75%; however, median survival is measured in months. The identification of epigenetic aberrations, particularities in signal transduction pathways and most recently the Merkel cell polyomavirus are exciting discoveries and will provide opportunities to explore new targeted therapies for this disease.

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