T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus

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Merkel cell polyomavirus (MCV) is a virus discovered in our laboratory at the University of Pittsburgh that is monoclonsal integrated into the genome of ~80% of human Merkel cell carcinomas (MCCs). Transcript mapping was performed to show that MCV expresses transcripts in MCCs similar to large T (LT), small T (ST), and 17kT transcripts of SV40. Nine MCC tumor-derived LT genomic sequences have been examined, and all were found to harbor mutations prematurely truncating the MCV LT helicase. In contrast, four presumed epistomal viruses from nonmalignant sources did not possess this T antigen signature mutation. Using common precipitation and origin replication assays, we show that tumor-derived virus mutations do not affect retinoblastoma tumor suppressor protein (Rb) binding by LT but do eliminate viral DNA replication capacity. Identification of an MCC cell line (MKL-1) having monoclonal MCV integration and the signature LT mutation allowed us to functionally test both tumor-derived and wild-type (WT) LT antigens. Only WT LT expression activates replication of integrated MCV DNA in MKL-1 cells. Our findings suggest that MCV-positive MCC tumors undergo selection for LT mutations to prevent autonomous viral genome replication. Failure to truncate the viral LT antigen may lead to DNA damage responses or immune recognition that hinders nascent tumor cell survival.

Results

Characterization of MCV T Antigen Transcripts. To characterize the MCV T antigen locus, we performed transcript mapping studies to identify the different spliced mRNAs expressed from this gene. We performed 3′ and 5′ RACE from four MCC tumors (MCC339, MCC347, MCC348, and MCC349) containing clonally integrated MCV genome (3). Transcripts corresponding to polyomavirus LT (T1) and small T (ST; T2) mRNAs are detected in all tumors, indicating that T antigen genes are commonly expressed in tumors (Fig. 1A). This transcription pattern was confirmed by detection of 3.0- and 3.5-kb transcripts by Northern blotting RNA from 293 cells expressing three MCV T antigen locus gene cassettes (Fig. 1B). TAg339 and TAg350 from MCC tumors 339 and 350, respectively and TAg206.wt from asymptatically infected appendix tissue. In addition to T1 and T2, mapping studies by RACE and Northern blotting of 293 cells transfected with the T antigen gene cassette revealed two additional mRNAs, T3 and T4 (Fig. 1B). This splicing pattern can be detected in MCC tumors by PCR with primers flanking the splicing junction and may represent transcripts for alternatively spliced analogues to SV40 17kT transcript (14). No transcript

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession nos. Appendix206, F173812; Appendix332, F173811; Bow1366, F173809; PBMC85, F173804; MCC350, F173805; MCC339, F173806; MCC344, F173807; MCC345, F173808; MCC347, F173810; MCC348, F173809; MCC349, F173813; MCC352, F173814; MCC351, F173815.)

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were aligned without gaps to known cellular genes in the RefSeq RNA database (3). Only 130 polymorphic nucleotides were found (99.93% concordance), suggesting that generally high mutation rates are not present in this tumor and that these mutations arose through selection in the tumors.

To determine whether these unexpected T antigen sequence changes are present in circulating WT virus, we next examined virus from four control tissues (two appendixes, bowel, and peripheral blood mononuclear cells) sample) from patients without MCC who were previously found to be positive at low copy number for MCV (3). Full-length LT coding sequences were obtained, and all had complete ORFs without the truncating mutations found in tumor-derived viruses (Fig. 2A). Although synonymous polymorphisms are present in both tumor-derived and nontumor-derived strains, nonsynonymous mutations leading to amino acid changes were more common in tumor-derived strains, particularly in the OBD.

Sequence analysis of N-terminal portions of both tumor- and control tissue-derived MCV LT antigens show well conserved domains (Fig. 2A and B). MCV LT encodes a penta-amino acid LFCDE (LXXCXe motif) Rb-interaction motif encoded by exon 2. To determine whether tumor-derived LT gene mutations affect the ability of LT to sequester Rb, coimmunoprecipitation assays were performed (Fig. 2C). Both WT and tumor-derived LT interact with Rb through this motif, which was confirmed by site-directed mutagenesis of the LFCDE motif to LFCDK. Thus, Rb targeting, which has been shown to contribute to tumors in animal polyomavirus models, is intact in both WT and tumor-derived MCV T antigens.

Identification of a Naturally MCV-Infected Cell Line, MKL-1. To investigate the effects of tumor-derived T antigen mutations, we sought to identify an MCC cell line that stably propagates the virus. MCC has been divided into classic and variant types depending on whether tumor-derived cell lines form suspension cultures (classic) or adherent (variant) cell cultures (Fig. 3A) (18). These cell culture characteristics reflect differences in gene expression profiles, suggesting that MCC may have multiple different causes (19). We tested one classic (MKL-1) (20, 21) and three variant (UBSO, MCC13, MCC26) cell lines (21–23), and several common laboratory cell lines, for the presence of MCV infection [supporting information (SI) Table S1]. Only the classic cell line MKL-1 harbors MCV infection. Southern blotting of EcoRI-digested MKL-1 DNA with MCV probes generates 5.1- and 3.9-kb monoclinal integration bands consistent with a head-to-tail tandem array pattern (Fig. 3B). Sequencing reveals full-length MCV genome integrated in MKL-1 cells (GenBank accession no. FJ173815). Both LT (T1) and ST (T2) mRNAs are expressed in MKL-1 cells, as seen in MCC tumors (Fig. 3C). Deletions at a putative splice-donor junction (nucleotides 1612–1658) eliminate splicing of T-3 and T-4 transcripts, which is confirmed by Northern blotting (Fig. 3C), and the cell line does not express the analog to the SV40 17K protein. As expected for a tumor-derived virus, the MKL-1 LT has a truncating mutation (stop codon at nucleotide 1452) that prevents endogenous expression of both the OBD and helicase domain (Fig. 2A).

Tumor-Derived Mutations Prevent LT-Dependent Origin Replication. Expression of full-length WT T antigen in an MCV-integrated cell line could be expected to initiate replication from the viral origin that might lead to collisions with cellular replication forks. To examine T antigen helicase activity, virus replication assay was developed by using pMCV-Ori, a plasmid containing the MCV replication origin (5074–270 nt), which is transfected together with LT expression vectors. After 48 h, low molecular weight DNA is extracted, digested with methylation-sensitive DpnI and BamHI, and probed by Southern blotting. Nontumor WT LT (TAG206) from appendix tissue initiates MCV origin...
replication in both UIOS (22), a Merkel cell line not infected with MCV (Table S1) and 293 cells, whereas no origin replication occurs during expression of two different T antigens derived from tumors (TAg339 or TAg350) (Fig. 4A). MCV origin replication was compared with SV40 origin replication in a similar assay (Fig. 4B). LT expression was confirmed in all transfections by immunoblotting for a V5 tag fused to the carboxyl terminus of the LT constructs (Fig. S1). Despite similarities in sequence between replication origins for MCV and SV40 (24), neither MCV nor SV40 LT initiates replication from each others’ viral origin (Fig. 4A and B).

To determine whether tumor-derived and WT T antigens can activate endogenous viral replication from MLK-1 cells, Southern blotting was performed. Endogenous replication of MCV was not detected with an MCV VP1 gene probe, nor did transfection of tumor-derived LT in MLK-1 cells activate MCV replication (Fig. 4C). However, when WT LT from nontumor virus was transfected into MLK-1 cells, robust replication of the integrated virus was readily detected (Fig. 4C). These results lead to one possible model in which active MCV replication after integration leads to replication fork collisions (Fig. 4D). Under these conditions, strong selection pressure would exist for viral
clones possessing T antigen mutations that truncate OBD–helicase domains while sparing growth promoting tumor suppressor interaction domains. We were unable to detect increased helicase domains while sparing growth promoting tumor-suppressing clones possessing T antigen mutations that truncate OBD–helicase domains while sparing growth promoting tumor suppressor interaction domains. We were unable to detect increased γH2AX foci or apoptotic cell death in short-term expression assays of fully transformed MLK-1 cells expressing WT T antigen. It is possible that DNA damage response pathways have already been altered in these aneuploid cells or that LT overexpression itself abrogates these pathways.

**Discussion**

Our results demonstrate that MCC-derived T antigens acquire mutations ablatting full-length LT expression and MCV replication capacity. Similar results have been seen in vitro and in vivo for transformation studies of animal polyomaviruses. Gluzman and colleagues (25–27) reported enhanced transforming activity for defective SV40 with mutations disrupting viral replication and helicase activity. In vitro MuPyV-transformed cell lines lack LT protein expression required for viral replication but express MT and ST oncoproteins that do not encode the T antigen helicase domain (28). Hamster cell lines established from tumors induced by MuPyV also lose full-length LT protein expression as a result of a T antigen carboxyl-terminal deletion (29). Our study confirms these results and suggests that loss of full-length LT in tumors is not an experimental artifact but is a general feature of polyomavirus-mediated carcinogenesis. Mutations in the MCV T antigen gene may serve as a useful genetic marker to distinguish tumorigenic from nontumorigenic MCV strains in humans.

MCV LT derived from tumors is functionally incapable of replicating virus. This is evidence, independent from monoclonal insertion (3), that these replication-incompetent viruses are not capable of secondarily infecting a preexisting tumor. This finding is also consistent with studies suggesting that polyoma viral transformation does not require free, episomal viral replication (25, 28, 30). We cannot exclude the possibility that intact helper WT MCV are present that allow packaging and transmission of mutant MCV but we repeatedly failed to recover WT LT sequences from human tumors and thus far have found no evidence for such a mechanism. Both T antigen mutations and virus monoclonal insertions in tumors make it unlikely that MCV is a passenger virus of MCC tumors.

The risk of MCC is increased by exposure to UV and possibly ionizing radiation exposure (31, 32). We did not find CC → TT signature mutations that can be induced by UVA but a high rate of pyrimidine dimer substitution among the LT mutations is consistent with UV irradiation playing a role in MCV mutagenesis. We propose a model for MCC evolution in which integration sustains MCV in the tumor cell but secondary mutations must arise that eliminate viral replication functions while sparing Rb targeting (Fig. 4D). Consistent with this model, WT T antigen expression can initiate viral DNA replication from integrated viral DNA within an MCC cell, which is expected to lead to replication fork collisions and DNA damage. Similarly, when the papillomavirus E1 helicase protein gene, which is usually lost after integration, is expressed in cervical cancer cells, these cells undergo a DNA damage response (33). Loss of replication capacity thus may be a generalized phenomenon among integrated DNA tumor viruses.

We do not know whether MCV integration or T antigen mutation arises first during MCC tumorigenesis. T antigen mutations will cause loss of virus maintenance and so mutant viruses would be rapidly lost unless rescued by an integration event. In contrast, if integration occurs first, this could generate a pool of clonal descendants that undergo secondary selection for T antigen mutations. Genomic integration also increases single-nucleotide mutation rates at the integrated element (34), which may increase the likelihood for secondary mutations.
MCV integration into host chromosomes can be expected to lead to autonomous viral origin DNA replication when WT T antigen is expressed. Newly replicated DNA replication (arrow) after transfection. MCC26 was used as negative control for this replication assay. EtBr-stained agarose gel is shown as a loading control. (Empty) were cotransfected with MCV replication origin plasmid (pMCV-Ori) in UISO and 293 cells, and replication was detected by Southern blotting. The positions of replicated DNA (DpnI-resistant) and unreplicated DNA (DpnI-sensitive) are indicated by solid and open arrows, respectively. (Empty) were cotransfected with MCV replication origin plasmid (pMCV-Ori) in UISO and 293 cells, and replication was detected by Southern blotting. The positions of replicated DNA (DpnI-resistant) and unreplicated DNA (DpnI-sensitive) are indicated by solid and open arrows, respectively. (Empty) were cotransfected with MCV replication origin plasmid (pMCV-Ori) in UISO and 293 cells, and replication was detected by Southern blotting. The positions of replicated DNA (DpnI-resistant) and unreplicated DNA (DpnI-sensitive) are indicated by solid and open arrows, respectively.

Although our data point toward loss of the MCV replication machinery as an essential event in tumor cell development, it is also possible that immune surveillance against vestigial T antigen domains contributes to positive selection for LT truncations. We found that the MKL-1 MCC cell line harbors MCV as an integrated virus possessing the tumor-specific LT mutation signature, which demonstrates the cell culture propagation of MCV. MCC tumors have been divided into tumors that generate cell lines with a classic (rounded-up, suspension) or variant (flattened, adherent) cell morphology (18). MKL-1 is the only classic MCC cell line that we have tested and so additional studies are needed to determine whether differences in cell culture morphology reflect differences in MCV infection among parental tumors. If only classic cell lines harbor MCV infection, then cell culture studies based on variant cell lines may not reflect the biological properties of MCV-infected MCC tumors.

The results of this study provide insights into epidemiologic and clinical characteristics of MCC. The requirement for at least two distinct mutagenic steps (integration and LT truncation) to occur in MCV-positive MCC tumors helps to explain why a relatively common viral infection leads to cancer only under rare circumstances. Exposures to environmental skin mutagens (e.g., UVA) and MCV infection are likely to increase the risk for MCC. These findings also guide choices for future experimental therapies against MCV-associated MCC: helicase inhibitors are predicted to be ineffective for MCC but drugs that target MCV-tumor suppressor interactions may provide specific and effective means for treating MCC-positive tumors.

Materials and Methods

Cells and Clinical Specimens. MCC cell lines (UISO, MCC13, and MCC26; kindly provided by Mireille Van Gele, Ghent University Hospital, Ghent, Belgium) and MKL-1 were maintained in RPMI medium 1640 supplemented with 10% FBS, glutamine, penicillin and streptomycin (pen/strep) (20, 21). Human embryonic kidney 293 cells were maintained in DMEM supplemented with 10% FBS and pen/strep. MCC tumor specimens (MCC330, MCC339, MCC344, MCC345, MCC347, MCC348, MCC349, and MCC352) and MCV positive controls (appendix206, appendix332, and bowl366) have been described (3).

DNA Sequencing of T Antigen Loci. PCR direct sequencing was used for mutation screening of MCV T antigen genes to obtain dominant viral sequences in clinical samples. MCC.P1–3 primers were used for control cases (Table S2). MCC.contig1–8 primers, as described (3), were used for MCC cases and MKL-1 cells. All PCR was performed by using Platinum High Fidelity Taq polymerase (Invitrogen).

Plasmids. For genomic constructs used in transcript mapping of MCV T antigens, the full-length T antigen gene locus was PCR-amplified from control tissue (app206) and MCC tissues (MCC350 and MCC339) with a common primer set: MCV.EcoRV(S)–MCV.XhoI(AS). For replication and Rb-binding assays, tumor-derived, truncated forms of LT protein were C-terminally tagged with a VS epitope immediately before premature stop codons. Constructs of Tag339 and Tag350 coding frames were amplified from full-length genomic T antigen constructs by using two different primer sets, MCV.EcoRV(S)–MCV.XhoI(AS) and MCV350.EcoRV(MCV350.XhoI(AS), respectively, and cloned in-frame with VS epitope tag into pcDNA6/V5-HisB (Fig. S1). We generated LXCMVXO206.wtv.LXCMXXK, and Tag350.LXCMXXK by using a QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) with the MCV.T.E216K(S)–MCV.T.E216K(AS) primer set according to the manufacturer’s instructions. MCV replication origin was amplified from MCC339 genomic DNA with the Ori.P074.XhoI(S)–Ori.270.EcoRV(AS) primer set and cloned in pCR 2.1 (Invitrogen) to produce pMCV-Ori. Primer sequences are listed in Table S2.

RACE Analysis. We conducted both 5’ and 3’ RACE analysis with a GeneRacer Kit (Invitrogen) using total RNA extracted from MCC tissues (MCC339, MCC347, MCC348, and MCC349) or 293 cells transfected with vector expressing the full genomic region of T antigen transcripts. T antigens of different lengths were amplified with the primers shown in Table S2. PCR fragments were separated in agarose gels and extracted with a QIAEX II Gel Extraction Kit (Qiagen) and cloned into pCR 2.1 vector (Invitrogen) for DNA sequencing.

Fig. 4. Initiation of MCV replication by WT but not tumor-derived LT. (A) T antigen expression vectors (Tag339, Tag350, and Tag206.wt) or pcDNA vector (Empty) were cotransfected with MCV replication origin plasmid (pMCV-Ori) in UISO and 293 cells, and replication was detected by Southern blotting. The positions of replicated DNA (DpnI-resistant) and unreplicated DNA (DpnI-sensitive) are indicated by solid and open arrows, respectively. (B) SV40 LT expression vector and SV40 origin plasmid were used as a positive control for the replication assay. Neither MCV nor SV40 LT initiates replication from each others’ viral origin. (C) Southern blot of MKL-1 cells for MCV VP1 gene shows WT 208LT, but not tumor-derived 339LT or 350LT, induces endogenous integrated MCV DNA replication (arrow) after transfection. MCC26 was used as negative control for this replication assay. EtBr-stained agarose gel is shown as a loading control. (2) MCV integration into host chromosomes can be expected to lead to autonomous viral origin DNA replication when WT T antigen is expressed. Newly replicated virus DNA strands may collide with cellular replication forks unless secondary mutations eliminate viral LT antigen helicase activity.
Northern Blotting. Total RNA from MKL-1 and 293 cells transfected with Tag339, Tag350, and Tag206-wt was extracted by using TRizol (Invitrogen). Fifteen micrograms of MKL-1 or 5 micrograms of 293 RNA were electrophoresed in 1.2% formaldehyde-agarose gels and transferred onto nitrocellulose membranes (Amersham) with 10× SSC. MCV fragments (t–v) spanning all exons and introns of T antigen were amplified by PCR using the primers listed in Table S2. PCR products were used to generate α³²P dCTP-labeled probes by the Rediprime II Random Prime Labeling System (Amersham). Hybridization was performed at 42°C in 5× SSC, 50% formamide, 5× Denhardt’s solution, 2% SDS, 10% dextran sulfate, and 100 μg/ml of denatured salmon sperm DNA (Stratagene). Blots were rinsed in 2× SSC/0.1% SDS at 60°C for 30 min.

Immunoprecipitation and Immunoblotting. The 293 cells were cotransfected with 5 μg of HA-Rb (35) and various T antigen expression vectors with LKXCE Rb binding site or its mutant LCKXX (Tag206-wt, Tag350, and SV40 TAg) by using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 h after transfection, suspended in lysis buffer (50 mM Tris-HCl, pH 7.4) supplemented with protease inhibitors. Lysates were precleared for 1 h with Protein A-Sepharose beads (Amersham) and immunoprecipitated with monoclonal anti-SV40 antibody, PAb108, or monoclonal anti-V5 antibody (Bethyl) overnight at 4°C. Protein A Sepharose beads were incubated with the lysates for 3 h at 4°C with gentle shaking. Beads were collected by centrifugation, washed four times with lysis buffer, and resuspended in SDS loading buffer. Proteins were separated by SDS/PAGE. Immunoblotting was performed with anti-HA antibody (Covance).

Genomic DNA Southern Blotting. Genomic DNA was extracted by standard phenol-chloroform technique. Fifteen micrograms of genomic DNA was digested overnight with EcoRI and separated by electrophoresis in 0.7% agarose gels. Completion of digestion was checked with ethidium bromide staining. Genomic DNA was transferred onto nitrocellulose membrane (Amersham) with 10× SSC and hybridized overnight with probes (2.7 × 10⁶ dpm/ml) at 42°C in hybridization buffer used in Northern blotting. Membranes were rinsed in 0.2× SSC/0.5% SDS at 60°C. The MCV DNA fragments (Contig1, Contig3, Contig6, Contig9, and Contig12) amplified by PCR were used to generate probes (3).

MCV Origin Replication Assay. MISO and 293 cells (1.5 × 10⁶ cells) were transfected with T antigen expression vector (2.5 μg), pMCV-ori (2.5 μg), or pSV40-ori (36) (2.5 μg) by Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were lysed with TE-SDS [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.6% SDS] for 10 min at room temperature. One-third of lysate was used for immunoblotting to examine LT expression. NaCl was added to two-thirds of lysate at a final concentration of 1 M. Lysate was kept at 4°C overnight to salt-potentiate chromatin DNA and centrifuged at 20,000 × g for 30 min at 4°C to collect supernatant containing episomal DNA fraction. After phenol-chloroform extraction, one-fifth of the collected DNA was double-digested overnight with 40 units each of BamHI and DpnII to digest nonreplicated DNA. Digested DNA were then separated in 1% agarose gel, transferred on nitrocellulose membrane, and subjected to Southern hybridization. DNA fragments containing MCV origin (Contig1) (3) or SV40 ori digested from pSV40-Ori were used to generate probes. To analyze origin replication of integrated MCV in MKL-1 cells, T antigen expression vectors were transfected with Nucleofector by using Kit V (Amaxa). Genomic DNA extracted from MKL-1 cells was digested with EcoRI and BamHI and analyzed by Southern hybridization with probes generated from PCR fragments of VP regions (Contig 9, Contig11, and Contig13).

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