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一般的名称	①乾燥抗 HBs 人免疫グロブリン ②ポリエチレングリコール処理抗 HBs 人免疫グロブリン		研究報告の 公表状況	Science 2008; 319: 1096-1100	公表国 アメリカ	使用上の注意記載状況・ その他参考事項等
販売名 (企業名)	①ヘプスリン (ベネシス) ②静注用ヘプスリン-IH (ベネシス)					
研究報告の概要	<p>メルケル細胞癌 (Merkel cell carcinoma: MCC) は、稀ではあるが進行の早いヒトの皮膚がんであり、主に高齢者や免疫抑制者にみられる。われわれは今回、digital transcriptome subtraction (DTS) 法を用いて MCC 検体について調べ、新種のポリオーマウイルスを同定し、メルケル細胞ポリオーマウイルス (MCV または MCPyV) と命名した。このウイルスは、MCC 腫瘍の 10 検体のうち 8 検体 (80%) で検出されたが、体内のさまざまな部位から採取した対照組織では 59 検体中 5 検体 (8%)、対照皮膚組織では 25 検体中 4 検体 (16%) でしか検出されなかった。MCV 陽性であった 8 MCC 検体のうち 6 検体では、ウイルス DNA は腫瘍ゲノム内に組み込まれており、そのパターンより、MCV の感染と組み込みにより腫瘍細胞のクローン増殖となったことが示唆された。したがって、MCV は MCC の発生に寄与する因子であると考えられる。</p>					<p>代表として静注用ヘプスリン-IH の記載を示す。</p> <p>2. 重要な基本的注意 (1) 本剤の原材料となる血液については、HBs 抗原、抗 HCV 抗体、抗 HIV-1 抗体、抗 HIV-2 抗体陰性で、かつ ALT (GPT) 値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV 及び HCV について核酸増幅検査 (NAT) を実施し、適合した血漿を本剤の製造に使用しているが、当該 NAT の検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した高力価の抗 HBs 抗体を含有する血漿を原料として、Cohn の低温エタノール分画で得た画分からポリエチレングリコール 4000 処理、DEAE セファデックス処理等により抗 HBs 人免疫グロブリンを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において 60℃、10 時間の液状加熱処理及び過膜処理 (ナノフィルトレーション) を施しているが、投与に際しては、次の点に十分注意すること。</p>
	報告企業の意見				今後の対応	
<p>メルケル細胞癌の発生に寄与すると考えられる新規のポリオーマウイルスを同定したとの報告である。ポリオーマウイルスは、直径 40nm のエンベロープを有しない DNA ウイルスである。静注用ヘプスリン-IH については、万一本剤の原料血漿にポリオーマウイルスが混入したとしても、EMC 及び CPV をモデルウイルスとしたウイルスバリデーション試験成績から、本剤の製造工程において十分に不活化・除去されると考えている。</p> <p>ヘプスリンについては、EMC 及び CPV をモデルウイルスとしたウイルスバリデーション試験成績からは、製造工程において不活化・除去が十分であるとは説明困難であるため、ポリオーマウイルスの原血漿への混入が判明した場合にはその事実を総合機構及び厚生労働省に報告する。</p>				<p>ポリオーマウイルスに関連する情報については、今後とも注視することとする。</p>		

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ains of TRF1 and TRF2, these structural variations emphasize that the TRFH domain is a versatile framework for interactions with different proteins.

The crystal structure of the TRF2_{TRFH}-Apollo_{TBM} complex is corroborated by mutagenesis. Mutations of the conserved hydrophobic residues of Apollo (F504, L506, and P508) or TRF2 (F120) completely abolished the interaction both in vitro and in vivo (Fig. 4, F and G). We further assayed the cellular localization of wild-type and mutant Apollo by expressing hemagglutinin (HA)-tagged proteins in human telomerase reverse transcriptase (hTERT)-immortalized human BJ fibroblasts. Although wild-type Apollo showed the expected telomere localization, the L506E/P508A double mutant was distributed throughout the nucleoplasm with no obvious accumulation at telomeres (Fig. 4H). This result confirms the structural information and indicates that the binding of Apollo to the TRFH domain of TRF2 is required for the telomeric localization of Apollo.

We next asked whether other shelterin-associated proteins might contain the F/Y-X-L-X-P motif suggestive of an interaction with the TRFH domain of TRF1 or TRF2. We identified this motif in PinX1, originally identified as a TRF1-interacting protein in a yeast two-hybrid screen (6). An 11-residue fragment of PinX1 (R287-D-F-T-L-K-E-K-K-R-R297), referred to as PinX1_{TBM}, closely resembles TIN2_{TBM} (fig. S12A), suggesting that it may bind to TRF1_{TRFH} in the same fashion as does TIN2_{TBM}. ITC data confirmed the TRF1_{TRFH}-PinX1_{TBM} interaction, whereas no measurable interaction was observed between TRF2_{TRFH} and PinX1_{TBM} (fig. S12B). Mutagenesis studies

showed that PinX1-L291 and TRF1-F142 are critical for the interaction, whereas PinX1-P293 is not (fig. S12C). These results are consistent with those of the TRF1_{TRFH}-TIN2_{TBM} interaction (Fig. 2D) and indicate that PinX1, like TIN2, binds the TRFH domain of TRF1 but not TRF2. Protein sequence database searches showed many instances of telomere-associated proteins containing the F/Y-X-L-X-P motif (fig. S13). Future studies are needed to address whether this motif mediates the TRF1/TRF2 binding of these telomere-associated proteins in vivo.

Our results indicate that binding to the TRFH docking site involves the sequence F/Y-X-L-X-P in shelterin-associated proteins, which contacts the same molecular recognition surface of the TRFH domains of TRF1 and TRF2 with distinct specificities. Because TRF1 and TRF2 play different roles in telomere length homeostasis and telomere protection (1), we propose that the TRFH domains of TRF1 and TRF2 function as telomeric protein docking sites that recruit different shelterin-associated factors with distinct functions to the chromosome ends.

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17. Materials and methods are available as supporting material on Science Online.
18. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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20. Coordinates and structure factor amplitudes have been deposited in the Protein Data Bank with access numbers 3BQ0 (TRF1_{TRFH}-TIN2_{TBM}), 3BU8 (TRF2_{TRFH}-TIN2_{TBM}), and 3BUA (TRF2_{TRFH}-Apollo_{TBM}). We thank F. Wang and K. Wan for assistance. Work was supported by an NIH grant (to T.de L.) and an American Cancer Society Research Scholar grant and a Sidney Kimmel Scholar award (to M.L.). Use of Life Sciences Collaborative Access Team Sector 21 was supported by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor (grant 085P1000817). Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under contract no. DE-AC02-06CH11357.

Supporting Online Material

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Materials and Methods
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Clonal Integration of a Polyomavirus in Human Merkel Cell Carcinoma

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Merkel cell carcinoma (MCC) is a rare but aggressive human skin cancer that typically affects elderly and immunosuppressed individuals, a feature suggestive of an infectious origin. We studied MCC samples by digital transcriptome subtraction and detected a fusion transcript between a previously undescribed virus T antigen and a human receptor tyrosine phosphatase. Further investigation led to identification and sequence analysis of the 5387-base-pair genome of a previously unknown polyomavirus that we call Merkel cell polyomavirus (MCV or MCPyV). MCV sequences were detected in 8 of 10 (80%) MCC tumors but only 5 of 59 (8%) control tissues from various body sites and 4 of 25 (16%) control skin tissues. In six of eight MCV-positive MCCs, viral DNA was integrated within the tumor genome in a clonal pattern, suggesting that MCV infection and integration preceded clonal expansion of the tumor cells. Thus, MCV may be a contributing factor in the pathogenesis of MCC.

Polyomaviruses have been suspected as potential etiologic agents in human cancer since the discovery of murine polyoma virus (MuPyV) by Gross in 1953 (1). However,

although polyomavirus infections can produce tumors in animal models, there is no conclusive evidence that they play a role in human cancers (2). These small double-stranded DNA viruses

[~5200 base pairs (bp)] encode a variably spliced oncoprotein, the tumor (T) antigen (3, 4), and are divided into three genetically distinct groups: (i) avian polyomaviruses, (ii) mammalian viruses related to MuPyV, and (iii) mammalian polyomaviruses related to simian virus 40 (SV40) (5). All four known human polyomaviruses [BK virus (BKV), JC virus (JCV), K1 virus (K1V), and WU virus (WUV) (6, 7)] belong to the SV40 subgroup. In animals, integration of polyomavirus DNA into the host genome often precedes tumor formation (8).

Merkel cell carcinoma (MCC) is a neuroectodermal tumor arising from mechanoreceptor Merkel cells (Fig. 1A). MCC is rare, but its incidence has tripled over the past 2 decades in the United States to 1500 cases per year (9). It is one of the most aggressive forms of skin cancer; about 50% of advanced MCC patients

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live 9 months or less. Gene expression profiling studies indicate that MCC may comprise two or more clinically similar diseases with distinct etiologies (10). Like Kaposi's sarcoma (KS), MCC occurs more frequently than expected among immunosuppressed transplant and AIDS patients (11). These similarities to KS, an immune-related tumor caused by KS-associated herpesvirus (12), raise the possibility that MCC may also have an infectious origin.

To search for viral sequences in MCC, we used digital transcriptome subtraction (DTS), a methodology we developed that can identify foreign transcripts by using human high-throughput cDNA sequencing data (13). We generated two cDNA libraries from a total of four anonymized MCC tumors. One library was prepared with the use of mRNA from a single tumor (MCC347), and the other was prepared with mRNA pooled from three tumors (MCC337, 343, and 346) to increase the likelihood of detecting rare viral sequences (table S1).

From these two libraries, we respectively pyrosequenced 216,599 and 179,135 cDNA sequences (~150 to 200 bp). These 395,734 cDNA sequences were trimmed with LUCY stringency equivalent to PHRED scores of 20 or higher (14). Copolymers of adenine or thymidine [poly(A) and poly(T), respectively], dust (low-complexity), human repeat, and primer adaptor sequences were then removed, leaving 382,747 sequences to form a high-fidelity (HiFi) data set. Of these, 380,352 (99.4%) aligned to human RefSeq RNA,

mitochondrial, assembled chromosomes, or immunoglobulin sequences in National Center for Biotechnology Information (NCBI) databases. Of the remaining 2395 HiFi candidate sequences, one transcript (DTS1) from MCC347 cDNA aligned with high homology to African green monkey (AGM) lymphotropic polyomavirus (LPyV) and to human BK polyomavirus T antigen sequences. A second DTS transcript (DTS2) had no homology to deposited polyomavirus sequences but was subsequently identified by aligning HiFi candidates to the full-length viral genome (see below). These two sequences define a previously unknown human polyomavirus that we call Merkel cell polyomavirus (MCV or MCPyV) because of its close association with MCC.

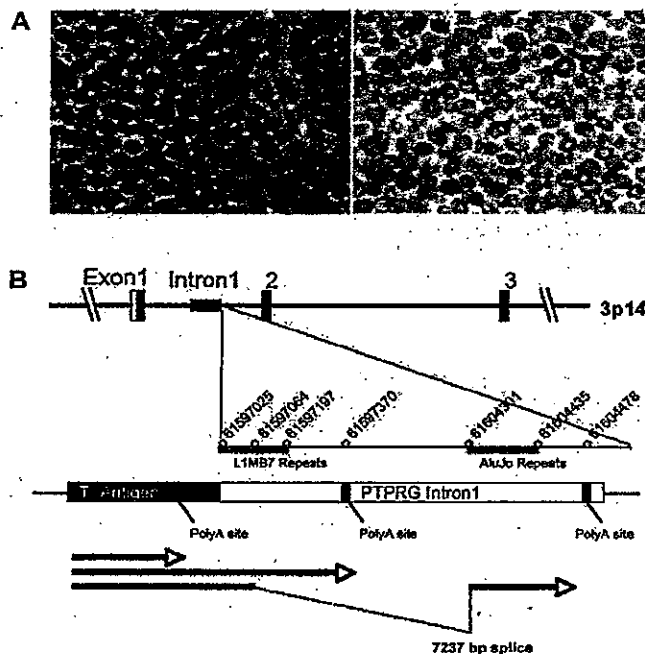
Rapid amplification of cDNA ends (3'-RACE) extended DTS1 to three different cDNAs (Fig. 1B): One transcript terminated at a poly(A) site in the T antigen sequence, and two cDNAs read through this weak poly(A) site to form different length fusions with intron 1 of the human receptor tyrosine phosphatase type G gene (*PTPRG*) (GenBank:18860897) on chromosome 3p14.2. Viral integration at this site was confirmed by sequencing DNA polymerase chain reaction (PCR) products with the use of a viral primer and a human *PTPRG* primer. The same three RACE products were independently cloned from MCC348, a lymph node metastasis from the MCC347 primary tumor, indicating that this tumor was seeded from a single tumor cell already positive for the T antigen-*PTPRG* fusion transcript.

By viral genome walking, we sequenced the complete closed circular genome of MCV (5387 bp, prototype) from tumor MCC350. A second genome, MCV339 (5201 bp), was then sequenced by using MCV-specific primers. The sequences of MCV350 and MCV339 have GenBank accession numbers EU375803 and EU375804, respectively. Both viruses encode sequences with high homology to polyomavirus T antigen, VP1, VP2/3, and replication origin sequences (Fig. 2A). MCV has an early gene expression region [196 to 3080 nucleotides (nt)] containing the T antigen locus with large T and small T open reading frames and a late gene region containing VP1 and VP2/3 open reading frames between 3156 and 5118 nt. The T antigen locus has features conserved with other polyomavirus T antigens, including *cr1*, DnaJ, pRB1-binding Leu-X-Cys-X-Glu (LXCXE) motif, origin-binding, and helicase/adenosine triphosphatase (ATPase) domains. Mutations in the C terminus of MCV350 and 339 large T open reading frames are predicted to truncate large T protein but are unlikely to affect small T antigen protein expression. The replication origin is highly conserved with that of other polyomaviruses and includes features such as a poly(T) tract and conserved T antigen binding boxes (fig. S1). MCV has highest homology to viruses belonging to the MuPyV subgroup and is most closely related to AGM LPyV (Fig. 2B) (15). It is more distantly related to known human polyomaviruses and SV40. The principal differences between MCV350 and MCV339 are a 191-bp (1994 to 2184 nt) deletion in the MCV339 T antigen gene and a 5-bp (5216 to 5220 nt) insertion in the MCV339 late promoter. Excluding these sites, only 41 (0.8%) nucleotides differ between MCV350 and 339.

To investigate the association between MCV infection and MCC, we compared tumors from 10 MCC patients to two tissue control groups. The first control group was composed of unselected tissues from various body sites (including nine skin samples) from 59 patients without MCC (table S2). These samples were taken consecutively on a single surgical day and tested for MCV positivity with two PCR primer sets in the T antigen locus (LT1 and LT3) and one in the VP1 gene (VP1). These primers do not amplify cloned human BKV or JCV genomic DNA or SV40 genome from COS-7 cells. A second control group composed of skin and skin tumor samples from 25 immunocompetent and immunosuppressed patients without MCC were tested with LT1 and VP1 primers (table S2). Samples were randomized and tested in a blinded fashion. Southern blotting of PCR products was performed to increase sensitivity (fig. S2).

Of the 10 MCC tumors from different patients, 8 (80%) were positive for MCV sequences by PCR (Table 1 and table S1). Seven tumors showed robust amplification, and one tumor was positive only after PCR-Southern hybridization. MCC348 (metastasis from MCC347) and

Fig. 1. (A) MCC is an aggressive skin cancer derived from Merkel mechanoreceptor cells that expresses neuroendocrine and perinuclear cytokeratin 20 markers, distinguishing it from other small round cell tumors (MCC349, left, hematoxylin and eosin; right, cytokeratin 20 staining, 40 \times . Scale bar represents 10 μ m). **(B)** Discovery of Merkel cell polyomavirus transcripts in (MCC). 3'-RACE mapping of an MCC fusion transcript between the MCV T antigen and human *PTPRG*. A cDNA corresponding to a polyomavirus-like T antigen transcript was found by DTS analysis of MCC. This T antigen cDNA was extended by 3'-RACE to map three mRNA sequences (arrows), one of which terminates at a viral polyadenylation site and two of which extend into flanking human sequence and terminate in intron 1 of the human *PTPRG* gene on chromosome 3p14, indicative of viral DNA integration into the tumor cell genome. The two viral-human chimeric transcripts were generated by read-through of a weak polyadenylation signal in the viral T antigen gene. Identical RACE products were also sequenced from a lymph node metastasis of this primary tumor.



MCC338 (infiltrating tumor from MCC339) were also positive. Two tumors, MCC343 and 346, remained negative after testing with 13 PCR primer pairs spanning the MCV genome. None of the 59 control tissues, including nine skin samples, was positive by PCR alone, but five gastrointestinal tract tissues tested weakly positive after PCR–Southern hybridization (8%, $P < 0.0001$, table S2). Viral T antigen sequences were recovered from three of these samples, confirming low copy number infection. Similarly, only 4 of 25 (16%, $P = 0.0007$, table S2) additional skin and

non-MCC skin tumor samples from immunocompetent and immunosuppressed patients tested positive for MCV sequences (Table 2 and table S2).

To determine whether MCV DNA was integrated into the tumor genome, we examined MCC samples by direct Southern blotting without PCR amplification. When MCV DNA in MCC tumor is digested by single-cutter restriction endonucleases, such as EcoRI or BamHI, and probed with viral sequence, four possible patterns are predicted to occur: (i) if the viral DNA exists as freely replicating circular epi-

some, then a ~5.4 kilobase (kb) band will be present (integrated-concatenated virus will also generate a ~5.4 kb band); (ii) if MCV DNA integrates polyclonally, as might occur during secondary infection of the tumor if MCV is a passenger virus, then diffuse hybridization from different band sizes is expected; (iii) if MCV DNA integrates at one or a few chromosomal sites, then the tumors will have identical or near-identical non-5.4-kb banding patterns; or (iv) if MCV DNA integrates at different chromosomal sites before clonal expansion of the tumor cells, then distinct bands of different sizes will be present (monoclonal viral integration).

Eight of 11 MCC DNA samples (including MCC348 metastasis from MCC347) digested with either BamHI or EcoRI showed robust MCV hybridization, and these corresponded to the same tumors positive by PCR analysis with multiple primers (Fig. 3A and fig. S3). Monoclonal viral integration (pattern iv) was evident with one or both enzymes in six tumors: MCC339, 345, 347, 348, 349, and 352 (solid arrowheads). EcoRI digestion of MCC339, for example, produced two distinct 7.5- and 12.2-kb bands that would arise only if MCV is integrated at a single site in the majority of tumor cells. MCC344 and 350 bands have episomal or integrated-concatemeric bands (open arrowhead, pattern i). MCC352 has a monoclonal integration pattern (solid arrowheads, pattern iv) on BamHI digestion as well as an intense 5.4-kb band (open arrowhead), consistent with an integrated concatemer. All three tumors negative by PCR with ethidium bromide staining (MCC337, 343, and 346) were also negative by direct Southern blotting.

Table 1. PCR for MCV DNA in MCC tissues. A plus symbol indicates that the sample was strongly positive by ethidium bromide staining only with one or more primers. A minus symbol indicates that the tissue was negative for all primers. Entries with both plus and minus symbols, indicate that the sample was negative by ethidium bromide staining but positive after Southern hybridization of PCR products.

MCC cases (n = 10)		
Patient	Tissue ID	MCV positivity
1	MCC337	-/+
2†	MCC338	+
2	MCC339	+
3	MCC343	-
4	MCC344	+
5	MCC345	+
6	MCC346	-
7	MCC347	+
7‡	MCC348	+
8	MCC349	+
9	MCC350	+
10	MCC352	+
Total (%)		8/10 (80)

†MCC338 was from an infiltrating tumor in skin tissue adjacent to MCC339 tumor. ‡MCC348 taken from a metastatic lymph node from MCC347.

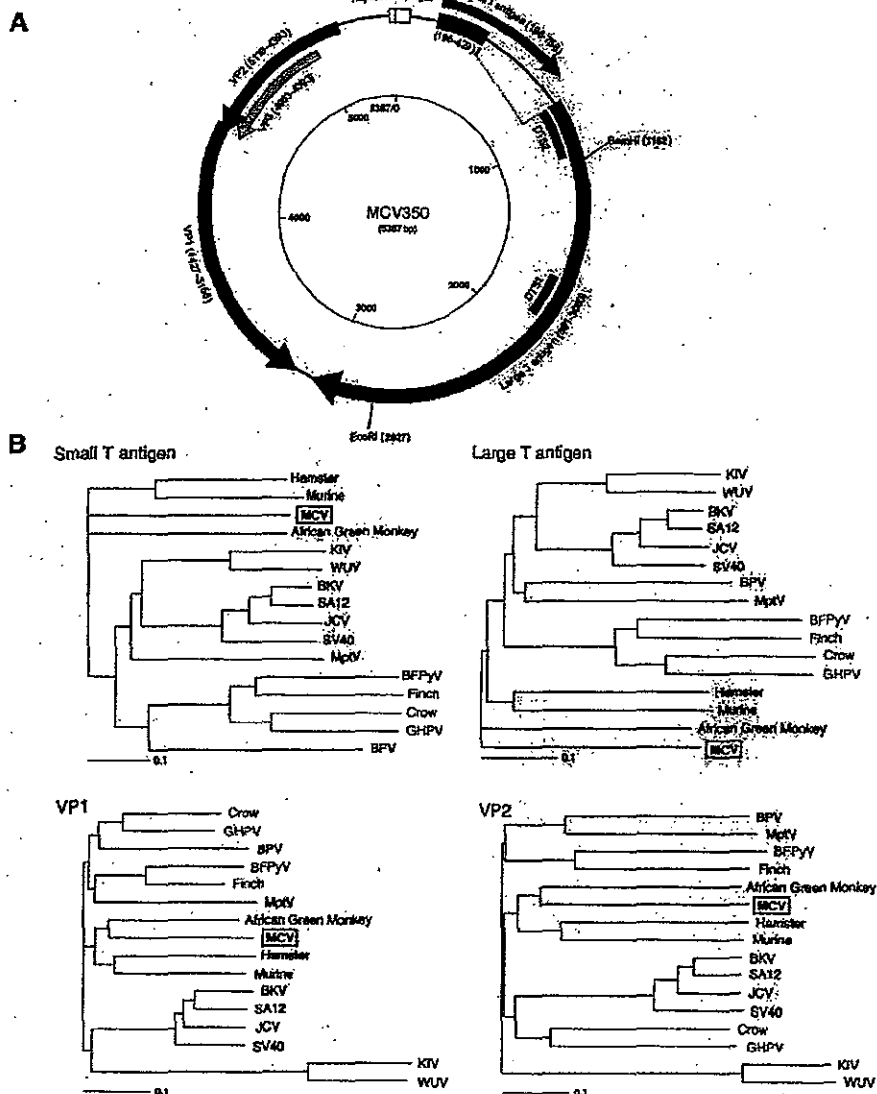


Fig. 2. (A) Schematic of MCV genome. Genome walking was used to clone the full MCV genome from tumor MCC350. The genome encodes typical features of a polyomavirus, including large T (purple) and small T (blue) open reading frames. Also shown are predicted VP1 (green) and overlapping VP2 (orange) and VP3 (yellow) genes. DTS1 and DTS2 (red) represent cDNA fragments originally identified by DTS screening. The former was used to identify MCV, and the latter is a spliced transcript with no homology to known polyomavirus sequences. (B) Neighbor-joining trees for putative MCV large T, small T, VP1, and VP2 proteins. The four known human polyomaviruses (BKV, JCV, KIV, and WUV) cluster together in the SV40 subgroup (blue), whereas MCV is most closely related to MuPyV subgroup viruses (red). Both subgroups are distinct from the avian polyomavirus subgroup (orange). Scale bars indicate an evolutionary distance of 0.1 amino acid substitutions per position in the sequence.

The Southern blot banding patterns (Fig. 3A) were identical for MCC347 and its metastasis, MCC348, in line with 3'-RACE results (Fig. 1B) and confirming that MCC348 arose as a metastatic clone of MCC347. Because the genomic integration site (the *PTPRG* locus on chromosome 3p14) is mapped for these tumors, we performed Southern blotting with flanking human sequence probes to examine cellular monoclonal integration. *NheI*-*SacI* digestion of MCC347

and 348 is predicted to generate a 3.1-kb fragment from the wild-type allele and a 3.9-kb fragment from the allele containing the integrated MCV DNA. Hybridization with a flanking human *PTPRG* sequence probe revealed that the 3.9-kb allele was present in MCC347 and 348 DNA but not in control tissue DNA (Fig. 3B). As predicted, the same fragment hybridized to a MCV T antigen sequence probe, consistent with both cellular and viral monoclonality in this tu-

mor. These results provide evidence that MCV infection and genome integration occurred in this tumor before clonal expansion of tumor cells. MCV in MCC may have some parallels to high-risk human papillomavirus (HPV), which causes cervical cancer mainly after viral episome disruption and integration into the cervical epithelial cell genome (16).

If MCV plays a causal role in tumorigenesis, it could conceivably do so by several mechanisms, including T antigen expression, insertional mutagenesis, or both. Our DTS results show tumor expression of MCV T antigen, which has conserved DnaJ (4), pocket protein-binding LXCXE (17), and pp2A-binding (18, 19) domains previously shown to play roles in polyomavirus-induced cell transformation. Mutational disruption of the *PTPRG* gene, which is suspected to be a tumor suppressor (20), could also play a role in MCC, although our Southern blot data suggest that MCV integration occurs at various genomic sites in different MCC tumors.

Our study validates the utility of DTS for the discovery of cryptic human viruses, but it has also revealed some limitations of the approach. Of the four tumors we sampled, only one (MCC347) was infected at high copy number. MCV transcripts in this tumor were present at 10 transcripts per million or about 5 transcripts per tumor cell. In future searches for other directly transforming tumor viruses (21), DTS should be used on multiple highly uniform samples sequenced to a depth of 200,000 transcripts or greater. Because DTS is quantitative, it is less likely to be useful in its current form for discovery of low-abundance viruses in autoimmune disorders or other chronic infectious diseases. Discovery of MCV by DTS nonetheless shows that DTS and related approaches (22) are promising methods to identify previously unknown human tumor viruses.

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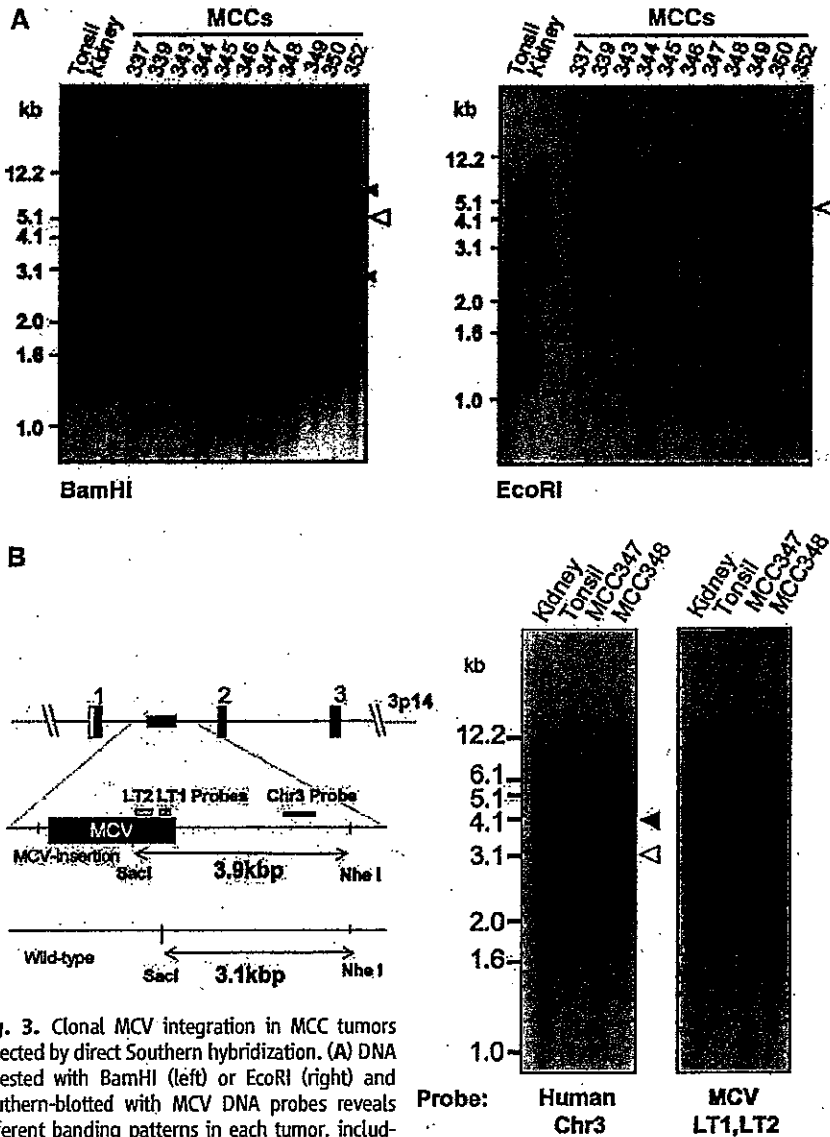


Fig. 3. Clonal MCV integration in MCC tumors detected by direct Southern hybridization. (A) DNA digested with BamHI (left) or EcoRI (right) and Southern-blotted with MCV DNA probes reveals different banding patterns in each tumor, including >5.4-kb bands. Open arrowhead shows the expected position for MCV episomal or concatenated-integrated genome (5.4 kb) with corresponding bands present in tumors MCC344 and 350. Tumors MCC339, 345, 347, 348, and 349 have different band sizes and doublet bands (solid arrowheads), consistent with genomic monoclonal integration. MCC352 has a prominent 5.4-kb band as well as higher and lower molecular weight monoclonal integration bands (BamHI), consistent with an integrated concatemer. Tumors MCC337, 343, and 346 have no MCV DNA detected by Southern blotting [bands at 1.5 kb (kidney) and 1.2 kb (MCC346) are artifacts]. (B) Viral and cellular monoclonality in MCC347 and 348. Tumor MCC347 and its metastasis MCC348 were digested with *SacI* and *NheI* and Southern-blotted with unique human flanking sequence probe [Chr3 (red), left] or viral probes [LT1 and LT2 (yellow), right]. The wild-type human allele is present in all samples at 3.1 kb (left). The MCC tumors, however, have an additional 3.9-kb allelic band formed by MCV DNA insertion into chromosome 3p14. Hybridization with probes for MCV T antigen sequence (yellow, right) generates an identical band.