



Identification and validation of a novel mature microRNA encoded by the Merkel cell polyomavirus in human Merkel cell carcinomas

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ABSTRACT

Background: Merkel cell polyomavirus (MCPyV) is present in approximately 80% of human Merkel cell carcinomas (MCCs). A previous *in silico* prediction suggested MCPyV encodes a microRNA (miRNA) that may regulate cellular and viral genes.

Objectives: To determine the presence and prevalence of a putative MCPyV-encoded miRNA in human MCC tumors.

Study design: Over 30 million small RNAs from 7 cryopreserved MCC tumors and 1 perilesional sample were sequenced. 45 additional MCC tumors were examined for expression of an MCPyV-encoded mature miRNA by reverse transcription real-time PCR.

Results: An MCPyV-encoded mature miRNA, “MCV-miR-M1-5p”, was detected by direct sequencing in 2 of 3 MCPyV-positive MCC tumors. Although a precursor miRNA, MCV-miR-M1, had been predicted *in silico* and studied *in vitro* by Seo et al., no MCPyV-encoded miRNAs have been directly detected in human tissues. Importantly, the mature sequence of MCV-miR-M1 found *in vivo* was identical in all 79 reads obtained but differed from the *in silico* predicted mature miRNA by a 2-nucleotide shift, resulting in a distinct seed region and a different set of predicted target genes. This mature miRNA was detected by real-time PCR in 50% of MCPyV-positive MCCs ($n = 38$) and in 0% of MCPyV-negative MCCs ($n = 13$).

Conclusions: MCV-miR-M1-5p is expressed at low levels in 50% of MCPyV-positive MCCs. This virus-encoded miRNA is predicted to target genes that may play a role in promoting immune evasion and regulating viral DNA replication.

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1. Background

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine skin cancer. In 2008, the Merkel cell polyomavirus (MCPyV) was identified in MCC tumors and appears to contribute to the causation of most MCCs through persistent expression of an oncoprotein formed from truncated viral large T-antigen.^{1–3} Other polyomaviruses, including JC virus, BK virus, and Simian virus 40, encode microRNAs (miRNAs) that are thought to be important for

their pathogenesis.^{4–8} These are expressed late in infection and suppress viral T-antigen expression, presumably to help the virus escape immune surveillance.⁹

Recently, Seo et al. took an *in silico* and *in vitro* approach to study miRNAs encoded by MCPyV, and identified a probable miRNA based on *in silico* predictions.⁹ This miRNA was shown to decrease MCPyV large T-antigen (LT) *in vitro*. In this study, we used high-throughput sequencing to directly quantify expression of small RNAs in seven MCC tumors, and subsequently validated the expression of an MCPyV-encoded miRNA.

2. Objectives

To determine whether the MCPyV encodes a miRNA that is expressed in MCC tumors.

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3. Study design

3.1. Small RNA sequencing and MCPyV genome alignment

All materials were acquired and used in conformity with the Institutional Review Board-approved protocols at the University of Washington and the Fred Hutchinson Cancer Research Center. Total RNA was extracted from 7 cryopreserved MCC tumors and 1 perilesional skin sample using mirVana miRNA Isolation Kit (Ambion). Total RNA quantity and quality were evaluated using a Nanodrop ND-1000 (Thermo Scientific). Using methods reported by Murchison et al.,¹⁰ isolated cloned small RNAs from each sample were sequenced separately using Illumina sequencers. 28.3 million acquired sequences from 5 tumors and 1 perilesional skin specimen were initially available; these were compared with the MCPyV genome [EU375803](1, 13) by the software MAQ¹¹ with up to 2 bp mismatches allowed. A single sequence matched; this was then tested with folding criteria as described by Bar et al.¹² Next, approximately 10 million sequences from 2 additional tumors were interrogated for presence of the miRNA of interest. TargetScanHuman 5.1 Custom was used to predict target genes on 12/02/10.¹³

3.2. PCR determination of virus status

Patients with available MCC tumor DNA ($n = 52$) were tested for viral load using real-time PCR according to published protocols.¹⁴ Number of copies of MCPyV was calculated by the $\Delta\Delta C_T$ method.¹⁵ The lower limit of detection was approximately 1 copy per 1000 cells.

3.3. Reverse transcription real-time PCR (qrtRT-PCR) validation of MCV-miR-M1-5p expression

A custom TaqMan[®] miRNA assay with a proprietary stem-loop primer design (Applied Biosystems) was utilized to detect levels of the mature MCV-miR-M1-5p sequence (5'-UCUGGAAGAAUUCUAGGUACA-3') in total RNA extracted from FFPE (formalin fixed paraffin embedded) and fresh MCC tumors. Assays were performed following the manufacturer's recommended protocol for Taqman qrtRT-PCR assay. This assay was successfully validated by quantitative detection of a synthetic RNA oligo of the same sequence and length as the mature miRNA (data not shown).

Expression of MCV-miR-M1-5p in 6 of 7 sequenced and 45 additional MCC tumors were validated. One sequenced tumor (MCC17) was omitted due to insufficient miRNA. RNU6B, a small, non-coding RNA (Applied Biosystems, product number 4373381), was used as an RNA loading control and was found to be positive in all samples except water. Cycle 34 was used as a cutoff for detection of expression since non-specific products were detected at 36 cycles or greater among tumors known by DNA and protein studies to be virus-negative.

4. Results

Direct sequencing of small RNAs was used to profile the entire MCC microRNA-ome (miR-ome) of 7 MCC tumor samples and 1 perilesional skin sample (Fig. 1). Alignment of MCC miR-ome sequences against the published MCPyV genome identified a 22-nucleotide sequence (5'-UC UGG AAG AAU UUC UAG GUA CA-3') with perfect homology to the MCPyV large T-antigen nucleotides 1217–1238 (Fig. 2a). This sequence has no match within the human genome (best MAQ homology: 17/22 nucleotides in human genome build 36). Furthermore, folding of the flanking viral sequences using an established computer algorithm produced a hairpin structure

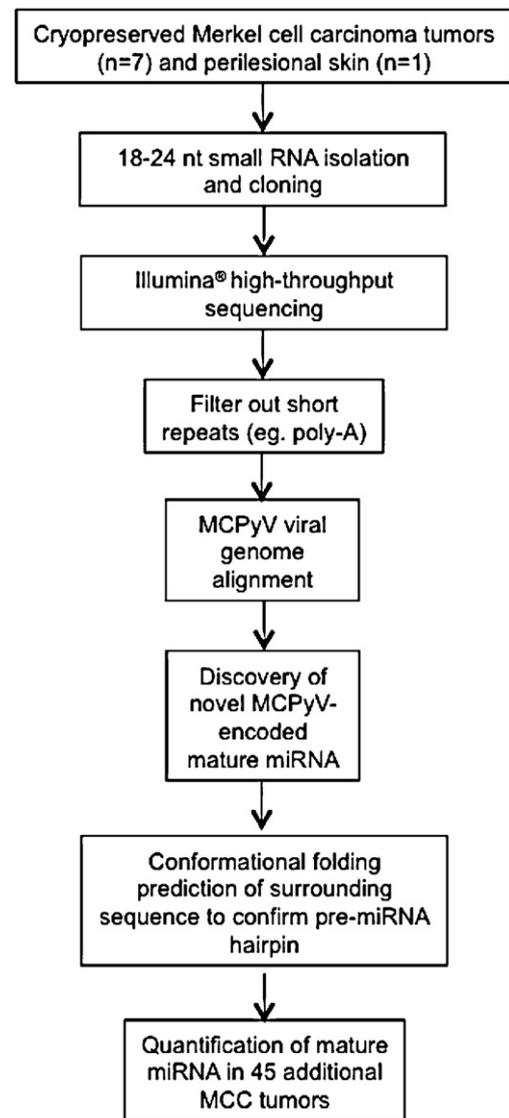


Fig. 1. Overview of high-throughput sequencing of small human RNAs in Merkel cell carcinoma and data analysis pipeline.

consistent with a pre-miRNA (free energy 31.10, shape probability 0.99890, base pairing 77%, and p -value 0.001) (Fig. 2b).¹²

Among 7 sequenced tumors, 3 were positive for MCPyV DNA and 4 had undetectable MCPyV DNA. The sequenced mature miRNA was detected at low levels in 2 of 3 MCPyV DNA-positive tumors. In tumor MCCw160, 78 reads of the mature miRNA among 5.6 million total reads were detected (3.1 million of these reads corresponded to known human miRNAs). In tumor MCCw200, only 1 read of the mature miRNA was detected. In contrast, MCV-miR-M1-5p was not detected in any of the 4 MCPyV-negative tumors or in the perilesional skin sample. All 79 sequences had an identical 5' end. No sequences were detected corresponding to MCV-miR-M1 star strand.

MCV-miR-M1-5p expression levels were validated by qrtRT-PCR in 6 of 7 sequenced tumors with sufficient miRNA availability. Confirming our sequencing results, MCCw160 and MCCw200 tumors had evidence of very low-level expression of MCV-miR-M1-5p (PCR amplification observed between cycles 30 and 32). 45 additional MCC tumors were tested – 10 DNA virus-negative and 35 virus-positive detected by PCR – giving a total of 51 MCC tumor samples (Fig. 2c). Most tumors express very low levels of MCV-miR-M1-5p (PCR amplification observed after cycle 30). Overall,

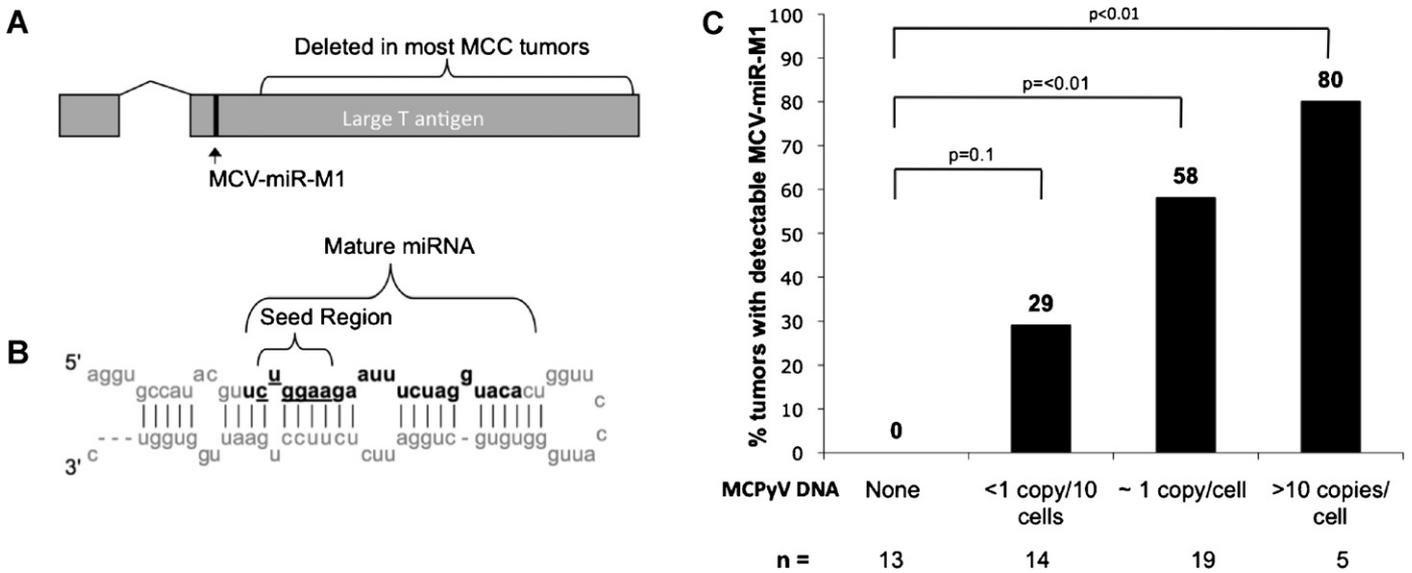


Fig. 2. Merkel cell polyomavirus encodes a microRNA expressed in Merkel cell carcinoma tumors. (A) MCV-miR-M1 binding site. The 22-nt sequence is complementary to a portion of the large T antigen transcript (nt 1217–1238) that is upstream of the reported deletions and truncations in MCCs.¹⁵ (B) MCV-miR-M1 hairpin structure. The pre-miRNA structure is shown, with the mature sequence, MCV-miR-M1-5p, bolded and seed region (nt 2–7) underlined. Mature sequence was determined by sequencing of 22-nt RNAs from MCC tumors and (C) MCV-miR-M1 mature miRNA expression in MCC tumors with different levels of detectable MCPyV DNA. No MCV-miR-M1 was detected in tumors without detectable MCPyV DNA. MCV-miR-M1 was detected in 28.6% of weakly MCPyV positive tumors (4/14). MCV-miR-M1 was detected in 57.9% of moderately MCPyV positive tumors (11/19). MCV-miR-M1 was detected in 80% of strongly MCPyV positive tumors (4/5). A significant positive trend was observed (total $n = 51$, including 6 sequenced tumors with 45 additional tumors).

19 of 38 MCC tumors with detectable MCPyV DNA had MCV-miR-M1-5p expression (50%). As expected, 0 of 13 MCC tumors without detectable MCPyV had MCV-miR-M1-5p. The amount of viral DNA positively correlated with copies of MCV-miR-M1-5p – samples having more viral DNA express more copies of MCV-miR-M1-5p.

Direct sequencing results confirmed previously published pre-miRNA hairpin structure,⁹ but revealed a distinct mature sequence and seed region that is shifted by 2 nucleotides from the prior report (5'-GGAAGA-3' – *in silico* vs. 5'-CUGGAA-3' – *in vivo*). Analysis of this distinct seed region in TargetScanHuman 5.1 Custom¹³ resulted in a

list of predicted human target genes of the experimentally observed mature MCV-miR-M1 (Table 1).

5. Discussion

We found evidence that MCPyV encodes a miRNA, MCV-miR-M1-5p, with the same pre-miRNA hairpin as previously reported *in silico* but with a different 22-nt mature sequence.⁹ It is possible that a different mature sequence is made by MCPyV depending on cellular context and that the previously reported sequence is indeed also present *in vivo* in certain settings. This miRNA is one of the

Table 1
In silico prediction of the most likely human target genes for MCV-miR-M1-5p using TargetScanHuman 5.1 Custom.¹³

Gene symbol	Number of conserved sites			Gene name	Function
	8mer	7mer-m8	7mer-1a		
AMBRA1	2	0	0	Autophagy/beclin-1 regulator 1	Regulates autophagy and nervous system development ²¹
RBM9 or FOX2	2	0	0	RNA binding motif protein 9	Regulates alternative exon splicing in the nervous system and other cell types ²⁰
MECP2	1	0	2	Methyl CpG binding protein 2	Binds to methylated DNA, represses transcription ^{18,19}
PIK3CD	1	1	0	Phosphoinositide-3-kinase, catalytic, delta polypeptide	Expressed in leukocytes, regulates immune functions – T cell activation, B cell proliferation ²²
PSME3	1	1	0	Proteasome activator subunit 3 (PA28 gamma; Ki)	Immunoproteasome activator for processing class I MHC peptides, involved in cell cycle modulation ²³
RUNX1	1	1	0	Runt-related transcription factor 1 (acute myeloid leukemia 1; AML1 oncogene)	Binds to promoters and enhancers, activates polyomavirus DNA replication ²⁴

Based on the input heptamer, CUGGAAG, TargetScanHuman generated a list of 442 target genes and a prediction of their likelihood of being downregulated by MCV-miR-M1-5p.

This table lists the six target genes that are most likely to be downregulated. 7mer-m8 site indicates a match to the miRNA seed with an additional match to nucleotide 8 of miRNA. 7mer-1a site indicates a match to miRNA seed followed by an additional adenine. The 8mer site in the 3' UTR of mRNA is typically the most reliable indication and the most stringent requirement for miRNA targeting.

few currently known virus-encoded miRNAs expressed in human cancers.^{16,17}

Consistent with prior reports,⁹ we find that MCV-miR-M1-5p is in a different viral genomic location than the known miRNAs encoded by BK and JC viruses. This further supports distinct evolutionary pathways between these viruses.¹

MCV-miR-M1 likely regulates both viral and cellular genes. Due to the perfect reverse complementarity/homology to the viral large T-antigen, it is likely that MCV-miR-M1 autoregulates expression of the large T-antigen to potentially evade immune surveillance.⁹ However, in MCC tumors, expression levels of the viral miRNA were low (0.005% of total miRNAs). Indeed, low expression of MCV-miR-M1-5p is not surprising given that T-antigen expression is required for MCC growth, and the miRNA would thus suppress a required oncoprotein.³

MCV-miR-M1 has several potentially relevant predicted cellular targets, as revealed by *in silico* analysis (Table 1).^{13,18–21} Two genes, PIK3CD and PSME3, are especially interesting because they are potentially involved in mediating the host immune response against MCPyV. Inactivation of PIK3CD in mice impaired antigen receptor signaling in B and T cells.²² PSME3, a subunit of the immunoproteasome, promotes presentation of murine cytomegalovirus peptides to cytotoxic T cells.²³ Immune evasion could thus occur via downregulation of PSME3-dependent antigen presentation by the host cell. MCV-miR-M1 may also regulate viral proliferation through another of its predicted cellular targets, RUNX1, that is involved in polyomavirus replication.²⁴ By downregulating RUNX1, MCV-miR-M1 would aid the viral life cycle transition from early to late. Functional studies will be required to investigate the biological relevance of this viral miRNA to MCC and to the viral life cycle in non-cancer host cells.

Conflict of interest

The authors report no conflicts of interest.

Acknowledgments

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Abbreviations: miR; miRNA, microRNA; miRNAs, microRNAs; miR-ome, microRNAome; MCPyV; MCV, Merkel cell polyomavirus; MCC, Merkel cell carcinoma; MCCs, Merkel cell carcinomas; PCR, Polymerase chain reaction; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2011.08.012.

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