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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.

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 Hutchison 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 MAQ[11] 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′-UCUGGAAGAAUUUCUAGGUACA-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 (MCCL17) 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

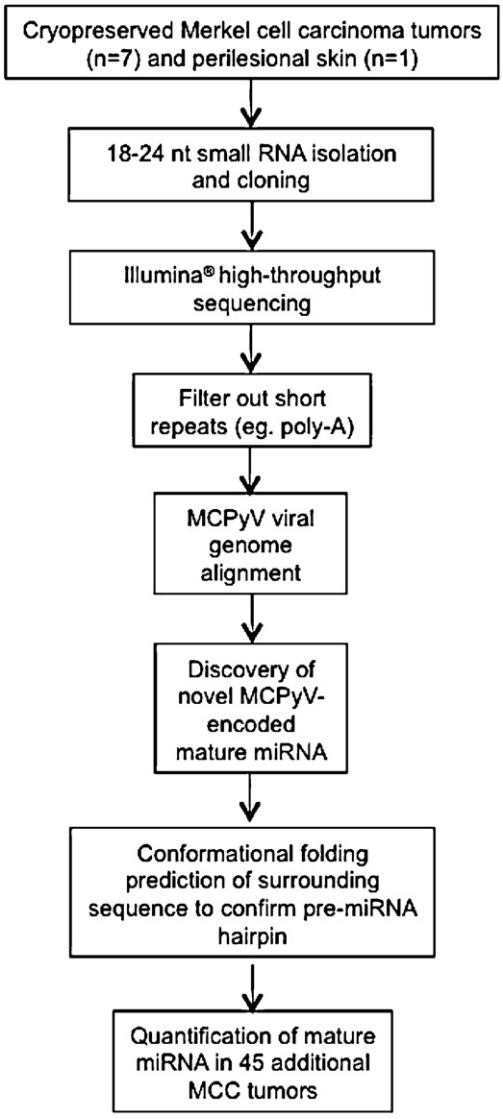

Fig. 1. Overview of high-throughput sequencing of small human RNAs in Merkel cell carcinoma and data analysis pipeline.

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,