SV40 associated miRNAs are not detectable in mesotheliomas

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BACKGROUND: Simian virus-40 (SV40) is a DNA tumour virus that was introduced into the human population with contaminated poliovirus vaccine, and its role in mesothelioma is widely debated. PCR based testing has been called into question, as false positives can be because of cross-reactivity with related viruses, or to laboratory contamination. The Institute of Medicine has recommended the development of more sensitive and specific tests to resolve this controversy.

METHODS: We have characterized highly sensitive RT–PCR based assays that are specific for SV40-encoded microRNAs (miRNAs), as an alternative to current testing methods.

RESULTS: Using this sensitive and specific detection method, we were unable to identify SV40 miRNA expression in human malignant pleural mesothelioma (MM) samples.

CONCLUSION: Our work indicates that SV40 miRNAs are not likely to contribute to mesothelioma tumourogenesis, but highlights the value of this approach when compared with the relatively unspecific current testing methods.

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Simian virus-40 (SV40) is a monkey polyomavirus that was first introduced into the human population in the 1950s with contaminated poliovirus vaccine, and continued until at least the early 1970s (Sweet and Hilleman, 1960). SV40 has been shown to cause tumours in hamsters and sporadically produce small quantities of infectious virus (Eddy et al, 1961; Sabin and Koch, 1963). The transforming roles of the early viral proteins, large T antigen and small t antigen, have been clearly established in vitro (Frisque et al, 2006; Khalili et al, 2008). The fact that SV40 can have cytopathic effects and can transform human cells has led to the debate as to whether this virus can cause cancer in humans. Notably, SV40 has been implicated as a co-carcinogen in MM.

Published reports have detected SV40 DNA in up to 50% of MM patients (Carbone et al, 1994). In 2002, a study was published by the Immunization Safety Review Committee of the United States Institute of Medicine, which explored the correlation between SV40 exposure and human cancers. The conclusion was that the evidence was inadequate to determine a causal relationship but did recommend the development of sensitive and specific serological tests for the virus (Stratton et al, 2002).

A recent report has demonstrated that SV40-encoded microRNAs (miRNAs) are expressed in infected cells (Sullivan et al, 2005). The five known SV40 miRNAs are different length splice variants produced from the 5′ and 3′ arms of a precursor miRNA, derived from the late transcript, a portion generally not included in laboratory plasmids. SV40 miRNAs are distinguishable from the miRNAs encoded by the closely related human polyomaviruses, with the 5′ and 3′ mature sequences having only 50 and 77% identity to the JCV and BKV sequences (See et al, 2008). For these reasons, we concentrated on the SV40-encoded miRNAs to develop a more sensitive and reliable SV40 detection technique.

METHODS

Viruses and cell lines

We used SVG-A cells, a subclone of SVG cells (ATCC no. CRL-8621), an SV40-transformed human astroglial cell line and SV-T2 cells (ATCC no. CCL-163.1, Manassas, VA, USA), a mouse fibroblast cell line transformed with SV40. We also used SV40-negative human embryonic kidney cells (HEK)-293 cells (ATCC no. CRL-1573), a human kidney cell line transformed with adenovirus 5 DNA. All cell lines were grown in ATCC recommended media supplemented with foetal bovine serum under standard conditions.

Tissue ascertainment

Fresh frozen tissues were obtained from the CREST Biorepository of the National Cancer Research Institute, Genova, Italy, the Masonic Cancer Center’s Tissue Procurement Facility at the University of Minnesota, the National Mesothelioma Virtual Bank,
either one or two mismatches to the minimal 5′ and 3′ sequences (Figure 1D). Neither the 5′ assay nor the 3′ assay could detect differences between the minimal miRNA and the longer splice variants, but they were specific for the sequences themselves. Each assay discriminated the mismatched oligonucleotides, although the position of the mismatches influenced the sensitivity of the assays. The single mismatch U→G had less cross-reactivity in both cases than the combined U→A and U→C mismatches. Owing to the ability of the minimal 5′ and 3′ assays to detect the longer respective miRNA splice variants, the minimal assays were used in all future experiments (Figures 1A and 1B). Dilutions of the minimal synthetic 5′ and 3′ RNA oligos were used to measure the sensitivity of the assays, which are linear over multiple orders of magnitude (Figure 1C).

SV40 miRNAs are not expressed in transformed cells

We next used SV40-transformed mouse (SV-T2) and human (SVG-A) cell lines to test the sensitivity of all five assays compared with the species-specific small RNAs, snorNA202 (mouse) and RNU48 (human). These cells contain at least one copy of the entire genome but do not express the late transcript and therefore should not express the mature miRNAs (Botchan et al., 1976; Major and Matsumura, 1984). We compared the background detection of each assay in small RNA-enriched lysates of both cell types and found that both the 5′ and 3′ assays detected <1% of the relative control (Figure 2). The minimal assays had the least cross-reactivity in transformed cell lines with the 3′ and 5′ assays being specific to 100× and 300×, respectively.

SV40 miRNA expression in infected human cells

We chose the minimal 5′ and 3′ assays to detect miRNA in actively infected cells following SV40 infection of human embryonic kidney cells HEK and measured the expression over time. We found that the 3′ miRNA was first detected 48 h after infection. The level continued to increase over 96 h of the initial round of infection in accordance with the known timeline of late transcription (Figure 3). In these cells, the 5′ transcript was undetectable during the entire 96-h time course (data not shown).

SV40 miRNA not detected in human mesothelioma biopsies

We used the minimal 5′ and 3′ assays to detect the expression of SV40-encoded miRNAs in 94 human malignant mesothelioma tumour biopsies as well as in 20 lung adenocarcinoma cancers and 28 non-malignant control biopsies (Figure 4). Despite the high sensitivity of these assays, we were unable to find any evidence of SV40 miRNA expression in human tissues. To ensure that the lack of SV40 miRNA expression in mesotheliomas was not because of technical problems isolating miRNA from these tissue samples, or to a general miRNA processing defect of this tumour type, we used quantitative PCR to detect three separate host-encoded miRNAs in mesotheliomas compared with non-malignant lung tissue (Supplementary Figure 1).

CONCLUSION

We characterized sensitive and specific quantitative PCR assays that are based on the expression of the mature SV40-encoded miRNAs. We showed that, although the assays cannot distinguish the minimal 5′ and 3′ miRNAs from the longer splice variants, the assays are highly sensitive to sequence. Single mismatches in the detected sequence result in decreased amplification. We used synthetic oligonucleotides to make standard curves and show that both minimal 5′ and 3′ assays are sensitive over many orders of magnitude.
magnitude. We also used SV40-transformed and non-SV40-transformed cell lines to characterize the levels and timeline of miRNA expression following infection with the virus.

Our results indicated that these assays were both sensitive and specific enough to meet the standard for serological SV40 tests recommended by the Institute of Medicine. We have tested these
assays on 94 different human MM samples from multiple tissue banks. Using the 5’ and 3’ assays, we were unable to detect SV40 miRNAs in any human tissue samples. The initial paper describing these miRNAs suggested that they may function to downregulate SV40 early protein expression and allow the virus to escape immune clearance (Sullivan et al., 2005). This led us to postulate that these miRNAs may be detectable in mesotheliomas and could provide a useful target to develop a more sensitive test for the virus. From our work, we have concluded that mature SV40 miRNA are not detected in human tissue samples, indicating that they are not likely to contribute to mesothelioma tumorigenesis. This can imply that the virus itself does not contribute to the development of mesothelioma. Another interpretation is that, because only the early SV40 genes are necessary to transform cells, only these genes are expected to be expressed in mesotheliomas (Carbone et al., 2008). The lack of false-positive results in an array of cancerous and non-cancerous human tissue shows that these assays are less vulnerable to laboratory contamination and cross-reactivity. The consistent lack of detection of SV40 miRNAs in human malignant mesotheliomas is indicative of a lack of association of miRNA-producing infectious SV40 with this tumour type.

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We cannot, however, completely exclude the association of human mesothelioma with a transforming form of SV40.

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