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review

Presence and role of Simian Virus 40 (SV40) in malignant pleural mesothelioma

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Background. *Evidence of a possible role of viruses in cancer first emerged in the early 1900s and was confirmed after the discovery of Epstein-Barr virus (EBV) in Burkitt's lymphoma cells. Thereafter, several oncogenic viruses and retroviruses were characterised. It is estimated that 15% of human malignancies are of viral aetiology. Oncogenic viruses use different proteins to interfere dramatically with the cellular cell cycle and affect many signalling pathways and checkpoints, causing genomic instability, immunoresistance and immortality.*

Conclusions. *Simian virus 40 (SV40) is a small DNA virus from the genus polyomavirus, closely related to human polyomaviruses John Cunningham virus (JCV) and BK virus (BKV) and is highly oncogenic for rodents. The virus accidentally entered the human population through contaminated early batches of polio vaccine in the 1960s. After the discovery of SV40-like DNA sequences in mesothelioma samples in 1994, a new wave of research started, focusing on the role of SV40 in malignant pleural mesothelioma and human cancer in general. Although the virus is not considered a cancer causing agent for humans, it is thought to have a (not yet defined) role in the development of the malignancy. Further research to better understand the interactions between the virus and the mesothelial cell is still ongoing.*

Key words: viral carcinogenesis; simian virus 40; mesothelioma; T antigen

Introduction

Evidence of a possible role of viruses in cancer first emerged in the early 1900s, but because malignancies were considered to be non-contagious, the findings were received with scepticism. The debate was finalised in 1964, when Epstein-Barr virus (EBV) was discovered in Burkitt lymphoma malignant cells. Further studies identified several other cancer–inducing human viruses and, in 1990, it was estimated that viruses and other infecting agents are associated with about 15% of all human cancer cases worldwide.[1] In addition to EBV, the most prominent human oncogenic DNA viruses are hepatitis B virus (HBV), human papilloma virus (HPV) and Kaposi sarcoma herpes virus (KSHV). Oncogenic retroviruses exist, too, and their role in human cancers has been discussed elsewhere.[3]

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Oncogenic viruses dramatically interfere with the cell cycle and affect many signalling pathways and checkpoints.[2] Different viruses use specific products to induce a progressive transition to the malignant phenotype. They may cause genetic instability of the infected cell, resistance to the immune system and cellular immortality.

Oncogenic DNA viruses adopt several different mechanisms to trigger host cell malignant transformation; a common feature of all oncogenic viruses is the ability to use multiple strategies. HPV oncoproteins, for example, induce genomic instability by inducing centrosome duplication errors and formation of abnormal centrioles, leading to a dysfunctional mitotic spindle and chromosomal imbalancies in daughter cells and, at the same time, prevent apoptosis. KSHV and EBV oncoproteins induce cell immortalization by modulating telomerase activity and promoting the alternative lengthening of telomeres (ALT) pathway. These mechanisms help cells bypass crisis and grant a dramatically prolonged lifespan. Other viral proteins destabilize p53, allowing cells to ignore cell cycle checkpoints and undergo undisturbed proliferation. Viral proteins interfere with the cellular microenvironment and impair communication with adjacent cells, ensuring a stable malignant phenotype and invasiveness of the host cell.[1]

Other DNA viruses can cause malignant transformations *in vitro*, but their definite role in human cancer is not determined. This group includes several adenoviruses and polyomaviruses. Very recently, a new polyomavirus has been identified and isolated from human Merkel cell carcinoma and could be the main causative agent for this skin malignancy.[3] Another polyomavirus, Simian virus 40 (SV40), is being extensively studied for its possible role as a human carcinogen, since it is highly oncogenic for rodents. The virus can also transform human cells *in vitro* and the first successful attempt to convert normal human cells to a stable malignant phenotype with a defined set of genetic elements actually used a combination of SV40, hRAS and hTERT sequences.[4]

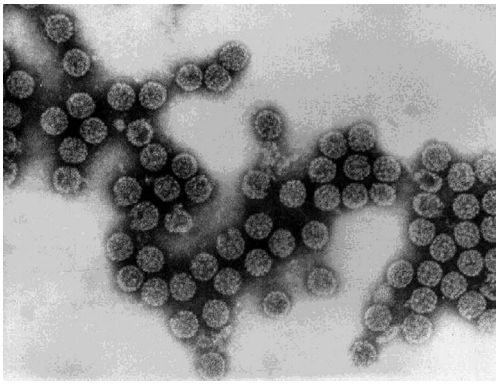

Figure 1. Transmission electron micrograph of polyomavirus SV40 (photo by Dr. E. Palmer, Center for Disease Control (CDC), GA, USA; no copyright restrictions under Public Domain – Property of the United States federal government (PD-USGov)).

SV40 in cancer

SV40, also known as Simian vacuolating virus 40, is a small (approximately 40 nm in diameter) icosahedral non-enveloped DNA virus from the genus polyomavirus (Figure 1). It is closely related to, and shares approximately 70% sequence similarity with, the human polyomaviruses John Cunningham virus (JCV) and BK virus (BKV; named after the initials B.K. of a renal transplant patient; viral particles were found in his urine). Its genome consists of a single circular double stranded DNA molecule and can be divided into three distinct regions – early, late and regulatory. The early region is expressed soon after entrance into the host cell, while the late region is expressed efficiently only after successful viral DNA replication has begun and it encodes for the capsid proteins (Figure 2).[5]

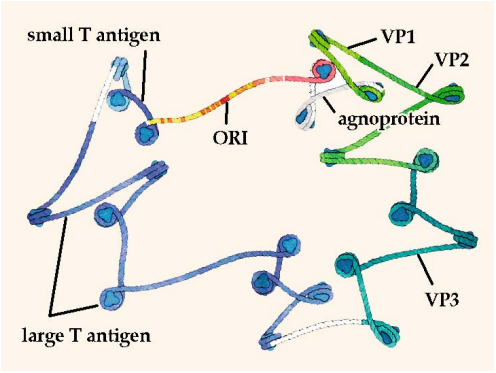

Figure 2. Structural view of the 5243 nucleotide SV40 genome with its characteristic nucleosomes. Blue highlights the early region, while the late region is green. Yellow and red denote the regulatory region of the viral genome (modified from D.S. Goodsell. Simian Virus 40 – November 2003 Molecule of the Month. Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank; no copyright restrictions under PD-USGov).

Its primary hosts are rhesus macaque (*Macaca mulatta*) monkeys. The virus is capable of causing tumours when injected into rodents.[6] Low antibody titters in human sera show that, compared to monkeys, replication of SV40 in humans is inefficient and that the virus is poorly adapted to the alternative host.[5]

Since the accidental contamination of large early batches of polio vaccine in the 1950s and 1960s, the previously unknown virus has spread in the human population and has become seen as an emerging human pathogen.[7] Both oral and intravenous vaccines were initially produced using rhesus monkey primary cell lines, some of which were naturally contaminated with SV40. The virus was discovered several years after the start of the mass polio immunization programme.[8] An unknown proportion of the vaccine was contaminated, presumably with small amounts of SV40.[9] The number of patients given the contaminated vaccine is also unknown, but estimates exist of more than 98 million people exposed to the contaminated vaccine in the United States alone.[10] Batches of intravenous polio vaccine (IPV – intravenous inactivated (Salk's) polio vaccine) produced after 1963 were SV40-free, while the virus remained present in the oral vaccine (OPV – oral live attenuated (Sabin's) polio vaccine) until the 1970s. Contaminated OPV and IPV were inadvertently used in many countries other than the United States, including the former Soviet Union, Japan, the United Kingdom, Italy, Mexico and several Central American and European countries.[5] No official records are available for Slovenia, but it is thought that no contaminated vaccine was used. It is presumed that SV40 has a tumour inducing or promoting role in humans.[10] Possible effects on human health, including the development of cancer, have been extensively researched in the past fifty years and many studies are still ongoing.

The virus triggers malignant transformations of both animal and human cells *in vitro* and is oncogenic for rodents when injected intraperitoneally or pericardially. Malignant transformation occurs if SV40 DNA becomes integrated in the host cell genome or remains in stable episomal form in the cytosol.[5] In mice, latent infection with SV40 can cause malignancy, mediated by two early viral proteins: large T antigen (Tag) and small t antigen (tag). Labelled one of the most potent oncogenic proteins, Tag has multiple functions in the host cell. It mainly acts as a transcriptional suppressor of tumour suppressor genes, while tag inhibits protein phosphatase 2A and thus serves as a modulator of Tag. Other important roles of Tag are direct binding and inactivation of expressed p53 and Rb and induction of insuline-like growth factor-1 (I-LGF 1) and its receptor[11] and, as recently discovered, p53-Tag complexes act as transcription factors acting as an inductor of I-LGF 1 promoter.[12]

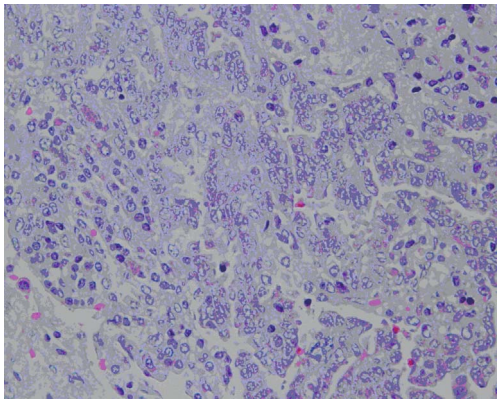

Figure 3. Classic HE stain of malignant pleural mesothelioma.

Large T antigen alone can initiate and maintain malignancy in hamster cells, but it is presumed that other events on the cellular level are required for *in vitro* malignant transformation of human cells.[13] Experimental data has shown that co-expression of SV40 early genes (Tag and tag genes), telomerase activity and oncogenic hRAS are sufficient for stable malignant transformation of human cells *in vitro*. This is mediated by perturbation of several intracellular signalling and metabolic pathways.[5]

The possible role of SV40 in human malignancies has been intensively debated. It is presumed that SV40 has either a tumour inducing or promoting role in humans.[10] Some human tumours (bone sarcomas, non-Hodgkin's lymphomas, brain tumours and mesothelioma in particular) frequently contain proviral DNA, usually in episomal form.

The strongest association between SV40 and human malignancies has been shown for malignant pleural mesothelioma.[14]

SV40 in malignant mesothelioma

Malignant pleural mesothelioma (MPM) is a rare but highly invasive tumour that is very rarely curable.[15,16] It arises from the malignant transformation of mesothelial cells, a uniform layer of non-differentiated serosal epithelial cells lining the pleura, the peritoneum and the pericardium (Figure 3).[11,15]

Among mesothelial malignancies, MPM is the most common, accounting for about 90% of all mesothelioma cases. It causes on average one death per million inhabitants worldwide, with very significant geographical variability,[16,17] since the incidence in Europe and Australia is considerably higher than in the Americas.[18]

There is no doubt that the vast majority of MPM are caused by asbestos fibres, since only 20% of mesothelioma cases occur without previous exposure to asbestos.[14,16] The data of patients with mesothelioma who had not been exposed to asbestos has varied.[15,19]

However, since only a small fraction of subjects exposed to asbestos fibres develop MPM, it has been suggested that additional factors may be involved in mesothelial malignant transformations. Other causes, in addition to asbestos, including non-asbestos fibres (erionite, in Cappadocia, Turkey), therapeutic radiation and intense pleural scarring (caused by prior plombage therapy for tuberculosis) have been reported to cause mesothelioma in rare cases.[18] Detection of Simian virus 40 DNA in several MPM tissue samples suggests that the virus itself could be the factor that renders some asbestos exposed individuals more susceptible to MPM.[20]

The discovery of SV40-like sequences in the genome of several MPM patients by Carbone *et al*.[21] in 1994 caused much controversy. Several studies of the presence of specific anti SV40 antibodies in patients' sera were mostly negative or detected extremely low antibody levels. Engels *et al*.[22] stated that these results mean that the antibody titter declined over the years, probably due to a lack of virus replication, indicating

a latent infection. Only the discovery of viral proteins within tumour tissues has confirmed the actual presence of the virus.[23]

Traces of either viral DNA or proteins have been repeatedly found in various independent studies. It is now accepted that mesothelial cells are unusually susceptible to SV40 mediated transformation,[5] but there is no indisputable epidemiological proof that contamination with SV40 increases the risk of developing MPM or cancer mortality.[10]

In vitro testing has shown that SV40 infects only about 20% of human fibroblasts and epithelial cells and becomes integrated in the cellular genome in only a small fraction ($1/10^7$) of infected cells. This results in cell lysis and thus prevents malignant transformation. Mesothelial cells, on the other hand, behave very differently compared to fibroblasts and epithelial cells. They seem to be more permissive for SV40 infection, but about 80% of infected mesothelial cells survive infection and undergo latent, rather than lytic, infection. Infected mesothelial cells actively express Tag, but do not produce viral particles. A very high rate, approximately $1/10^3$ cells, undergo malignant transformation.[11]

One of the easiest and most sensitive techniques for SV40 DNA detection in tissue and cell culture samples is polymerase chain reaction (PCR). The vast majority of researchers who have published scientific papers on the presence of SV40 in mesothelioma have used this technique. However, wide discordance in the results, poor reproducibility and episodes of positive results in no-template negative controls, has led to a questioning of the specificity and sensitivity of PCR assays.[11] Other viral detection techniques, such as Southern blotting, DNA sequencing, mRNA *in situ* hybridization, Tag immunoprecipitation, electron microscopy, immunohistochemistry and immunofluorescence, have been used to confirm the positiveness of PCR results.[5]

It is surprising that immunostaining of Tag in tissue samples revealed that viral expression is present in MPM cells, but not in the adjacent stromal and lung tissue.[11] This observation, together with low viral DNA copy numbers and the virtual absence of anti-SV40 antibodies in patients' sera, suggests that SV40 might be the factor that initiates carcinogenesis and becomes redundant and lost at a later stage, allowing the cancerous cells to evade the immune response, survive and grow.[5] This behaviour is consistent with the »hit and run« hypothesis in viral carcinogenesis. According to this hypothesis, infected cells evade the innate immune system response in early phases of viral infection and malignant transformation. In later phases, the cells become stably malignant and some lose the viral genome before specific antibodies are formed. Malignant cells that do not express viral proteins are less immunogenic, which gives them a selective advantage compared to malignant cells with an active viral infection. There are no conclusive data on its credibility yet, but the »hit and run« hypothesis is a possible explanation for the lack of specific antibodies in the sera of patients with putative virus-associated cancers.[1]

Asbestos and SV40 co-carcinogenity

Recent studies have shown a strong co-carcinogenic effect of crocidolite asbestos fibres and SV40 on hamster and human mesothelial cells. Asbestos exposure complements SV40 infected cells in malignant transformation.[11] Moreover, the same effect has been observed and extensively studied in experimental animals.[20] Human mesothelial cells are particularly sensitive to both asbestos genotoxicity and SV40 transformation. It is assumed that the latter may have the pivotal malignancy inducing role

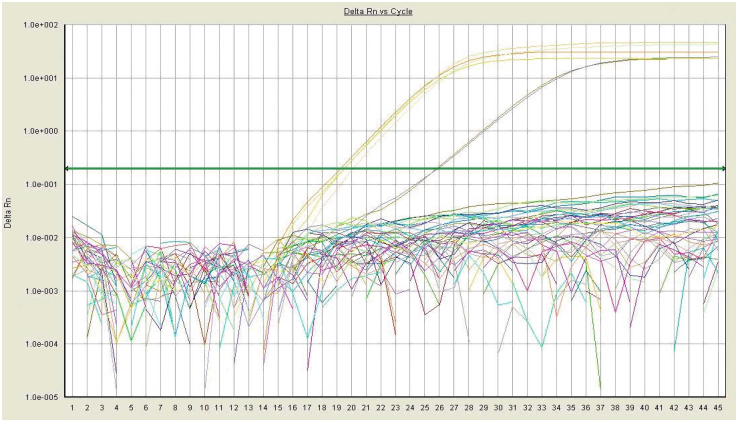

Figure 4. Amplification curve of a quantitative (real-time) polymerase chain reaction (qPCR) assay performed on DNA from mesothelioma tissue samples and DNA from SV40-transformed Wi-38 cells (ATCC acc. no. CCL-75.1) using a primer set for SV40 large T antigen (SV.for3 and SV.rev primers)[27] and two custom-made primer sets for SV40 small t antigen. Positive results were obtained only for the Wi-38 derived DNA, while mesothelioma samples were SV40 free.

in the mesothelium, especially in cells with asbestos damaged DNA.[24]

Asbestos fibres easily penetrate inside a cell, where they induce extensive DNA damage. The fibres generate genotoxic reactive oxygen and nitrogen species, cathalyse the synthesis of nitrotyrosine and induce DNA strand breaks. The presence of SV40 in asbestos exposed mesothelial cells affects the cell cycle, DNA repair and signalling pathways, especially through the interaction of some viral proteins (Tag and tag) with p53. The strong affinity of Tag for binding and inactivating p53 results in the cell avoiding key cell cycle checkpoints, thus surviving the otherwise fatal DNA damage and undergoing mitosis.[24,25] By escaping apoptosis and surviving, asbestos-damaged SV40 infected mesothelial cells pave the way to malignant transformation. It is very likely that asbestos fibres and viral Tag and tag interact and synergistically activate cellular signalling pathways, especially the ERK/AP-1 pathway, that lead to cell proliferation, malignant transformation and invasion.[20]

Conclusions and future aspects

Dilemmas and controversies about the role of SV40 as a human carcinogen are still strong, despite the ongoing extensive research. There is no powerful evidence of the exact role of SV40 in human cancers and MPM in particular. Some studies have suggested that the virus does not have a major implication in the development of MPM,[9,24] but several others claim the opposite.[20]

Researchers are still not able to find consensus, mainly because of flaws attributed to different SV40 detection techniques. The lack of appropriate, standardized approaches and quality control measures further adds to the controversy.[26] There is a high risk of false positive results, especially when using sensitive DNA detection techniques such as PCR. Some recent studies have proved that many previously published results of SV40 positive tumour specimens were not reproducible. The authors suggested contamination with laboratory plasmids and, for serological assays, cross-reactivity with antibodies to the SV40-related human polyomaviruses BKV and JCV.[9] The viral DNA sequences are common in many engineered plasmids and several immortalized cell lines, including the extensively used Wi-38 (ATCC acc. no. CCL-75.1). SV40 sequences containing DNA from these sources can easily contaminate PCR reaction mixes and laboratory equipment.

Extremely careful experiment design for every single step in the process is thus a must for reliable results, especially to avoid sample contamination and cross-contamination. Choosing multiple and appropriate primer sets for PCR can also avoid false-positive results and increase reproducibility (Figure 4).

Better understanding of viral carcinogenesis in general could provide new insights in our understanding of cancer biology and treatment.[1] Further studies, aimed not only at confirming the presence of the virus, but also at elucidating its role, will provide answers to open questions about a possible causal link.

Ongoing extensive research on the topic proves that elucidating the link between SV40 and MPM is of major interest to the scientific community. What seemed like a complex jigsaw puzzle even a few years ago is now being assembled and clarified and we will probably be able to tell the whole story very soon.

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