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Simian virus 40 transformation, malignant mesothelioma and brain tumors

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Abstract

Simian virus 40 (SV40) is a DNA virus isolated in 1960 from contaminated polio vaccines, that induces mesotheliomas, lymphomas, brain and bone tumors, and sarcomas, including osteosarcomas, in hamsters. These same tumor types have been found to contain SV40 DNA and proteins in humans. Mesotheliomas and brain tumors are the two tumor types that have been most consistently associated with SV40, and the range of positivity has varied about from 6 to 60%, although a few reported 100% of positivity and a few reported 0%. It appears unlikely that SV40 infection alone is sufficient to cause human malignancy, as we did not observe an epidemic of cancers following the administration of SV40-contaminated vaccines. However, it seems possible that SV40 may act as a cofactor in the pathogenesis of some tumors. *In vitro* and animal experiments showing cocarcinogenicity between SV40 and asbestos support this hypothesis.

Keywords

brain tumor; malignant mesothelioma; SV40; Tag; tag; transformation

Simian virus 40

Simian virus 40 (SV40) was first isolated in 1960 from cultures of Rhesus monkey kidney cells used to produce poliovirus vaccines [1] and was assigned to the family of Polyomaviridae, Polyomavirus genus, closely related to human polyomaviruses (BK, JC, KI and WU), based on genomic organization and sequence similarity [2].

Simian virus 40 does not induce disease in its natural host, the Rhesus monkey, or in other monkey species. SV40 transmission among monkeys occurs via urine, feces and bites, and vertical transmission has been documented in monkeys and hamsters [3].

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SV40 genomic organization

Simian virus 40 is an unenveloped icosahedral virion containing a small, closed circular double-strand DNA genome. The virion is 45–50 nm in diameter [4] and 1.34–1.35 g/cm³ in density. Genome length is approximately 5 kb, depending on various strains. Different regions were identified in the SV40 genome: regulatory region (bidirectional promoter and replication origin); early genes; and late genes (Figure 1A). The terms ‘early’ and ‘late’ reflect the sequential gene transcription and translation events in the host cells. Early and late genes extend in opposite directions. Early genes encode for the large T antigen (Tag), the 17KT and the small t antigen (tag) (Figure 1B). Late genes are transcribed into two classes of late mRNAs: 16S, coding for the major capsid protein VP1; and 19S coding for the VP2, VP3, VP4 and Agnoprotein. To the best of our knowledge, all SV40 strains produce these seven viral proteins. Tag is a nuclear phospho-protein of approximately 94 kDa. Tag induces SV40 DNA replication and, together with tag, promotes cell cycle progression, S-phase entry and DNA synthesis in host cells. VP1, VP2, VP3 and VP4 enable the packaging of replicated viral DNA into transmissible infectious virions, encapsulated by 360 VP1 protein molecules, tightly bound in 72 pentamers. VP2 and VP3 are less abundant, although they also play an essential role in the SV40 packaging process, both *in vivo* and *in vitro* [5]. The Agnoprotein controls the perinuclear localization of the viral genome during virion assembly and may contribute to viral late gene transcription and translation, viral release and propagation [6]. VP4, a recently discovered late protein, triggers the lytic release of the virus [7]. Like VP3, the 15-kDa VP4 protein is synthesized starting from the third in-frame AUG codon within the VP2 transcript, and shares the same stop codon with VP3 and VP2. VP4 is expressed in the infected cells 12–24 h later than VP1, VP2 and VP3, and is not incorporated into viral particles. Mutated virus lacking VP4 expression displayed delayed lysis and reduced particle release [7].

Several different SV40 strains, described below, have been isolated from monkey and human tissues [8]. Strain SV40–776 (wild-type) is the prototype and reference strain. It was isolated from a stock of a contaminated adenovirus vaccine preparation. SV40-B2, the so called ‘Baylor strain’, was isolated from SV40-contaminated polio vaccines (Sabin, attenuated virus). VA45-54 was discovered in a normal cell culture of grivet monkey kidney cells, from which several temperature-sensitive mutants were obtained. SVPML-1 was isolated from progressive multifocal leukoencephalopathy human cells, while SVMEN was isolated from a human meningioma biopsy, an important finding because this virus comes directly from a human tumor biopsy, not from cells in culture. SVCPC, first characterized as an additional strain from a human choroid plexus tumor biopsy, has been shown to be identical to SVMEN. The DNA sequences of SVPML-1, SVMEN/SVCPC are more similar to Baylor than to 776 [9]. It should be noted, however, that these strain differences are very minimal, suggesting that mutations are not tolerated in the small SV40 genome. Most of the differences of SV40 variants were detected in the sequence encoding the carboxyl-terminal variable region of Tag, within the amino acid residues 622 and 708; in addition, some sequence variability was detected in the early transcript intron and the sequences encoding tag, VP1 and Agnoprotein, were affected [10]. Thus, no mutations have been found so far in the critical portions of the SV40 Tag, such as those responsible for binding p53, retinoblastoma protein (pRb) and so on.

SV40 strains are classified as ‘archetypal’ (harboring one copy of the 72-bp enhancer element for early and late viral genes) and ‘nonarchetypal’ SV40 (carrying two copies of this 72-bp enhancer element). VA45-54 and Baylor carry only one 72-bp enhancer element in fresh isolates, but duplication of the 72-bp sequence has occurred during replication in CV-1 monkey infected cells. No other modifications were detected in the remaining sequences, including Tag carboxyl-terminal variable region [8]. This duplication is compatible either with the hypothesis of SV40 adaptation in cell culture, by selection of a

rare variant pre-existing in the original sample, or with the generation *de novo* of the rare duplication of the regulatory region in infected cells [8,10]. The efficiency of virus production *in vitro* by archetypal SV40 776 was improved by the duplication of the naturally occurring 72-bp enhancer element [8]. Nonarchetypal SV40 strain 776 (commonly known as 'wild-type' SV40) and the Baylor strain displayed higher transformation efficiency than archetypal SV40 [11].

SV40 life cycle

Cell infection starts with the binding of SV40 virions (mainly through VP1) to specific receptors on the plasma membrane: GM1 ganglioside [12] and Class I MHC molecules [13]. SV40 enters cells by caveolae-mediated endocytosis. SV40 bypasses the Golgi complex *en route* to the endoplasmic reticulum, where calcium ions, bridging VP1 capsid proteins, are removed by endoplasmic reticulum absorption, resulting in capsid disassembly [5], which requires low cytosolic calcium concentrations [14]. Afterwards, SV40 genome is translocated to the nuclear compartment via mechanism mediated by the importin α_2/β heterodimer and by VP3 [15]. The early promoter of SV40 genome is recognized by host RNA polymerase II leading to early gene transcription soon after nuclear translocation. The primary transcripts are alternatively spliced into two mRNAs encoding Tag and tag, whose ratio varies in different cell types [16]. During the early phase of infection, the late genes transcription does not take place, because of the action of late gene transcriptional repressors [17]. What happens next varies in permissive and nonpermissive cells.

Infected cells can be permissive, nonpermissive and semi-permissive to SV40. African green monkey and Rhesus kidney cells are permissive. In these cells, Tag binds as a double hexamer to the viral replication origin (*ori*), promoting DNA replication. With time, the cellular repressor proteins are titrated-off from the late promoter, while the transcription from the early promoter is repressed. Tag also unwinds the DNA double helix, promoting the recruitment of host cellular DNA polymerase and protein A, which starts transcription. In addition, Tag modulates intracellular signaling by recruiting several cellular proteins involved in cell progression and apoptosis pathways, and Tag transactivates several cellular genes including *IGF-1* [18,19], *cdc2* [20], hepatocyte growth factor receptor (*Met*) [21] and *Notch-1* [22], thus promoting G2 phase progression (see SV40-mediated human primary mesothelial cells [HM] and astrocyte transformation). These events trigger the transition from early to late phase of SV40 infection. When most of the viral genome is replicated, the capsid proteins are synthesized. The activation of checkpoint kinase Chk1 by ataxia telangiectasia mutated-Rad3-related-dependent phosphorylation blocks the mitosis of the target cells, at the same time late viral proteins accumulate inside the cell [23]. Only the SV40 genome, but not the host's genome, contains six tandem GC boxes, which represent the viral packaging signal for capsid assembly and viral DNA packaging. The transcriptional factor SP1 binds to the GC box, recruits VP2 and VP3, which in turn bind to VP1 pentamers and start viral assembly [24]. This is followed by the attachment at low affinity of multiple capsomers surrounding the minichromosome. The formation of this immature complex increases the capsomer local concentration and accelerates the assembly in a cooperative manner. The final icosahedron arises either from the progressive addition of single pentamers to the growing shell or from an organized pentamer clustering [25]. The process is followed by VP4-mediated activation of poly (ADP-ribose) polymerase, which leads to cell necrosis. Large amounts of viral particles are released, new cells are infected and a new infection cycle begins. The nuclear factor of activated T-cells (NFATs), specifically NFAT3 and NFAT4, is an important transcriptional factor controlling SV40 infection [26]. In human cells, SV40 Tag increases NFATs activity and NFATs provide a positive feedback loop, transactivating the SV40 promoters.

The tag, inactivates serine/threonine protein phosphatase 2A, thus altering the phosphorylation of several cellular proteins promoting S phase entry. The tag cooperates with Tag supporting virus production in permissive cells [27].

Nonpermissive rodent cells do not support viral DNA replication – or at least support limited viral DNA replication [28]. However, Tag and tag are expressed and promote cell division. Because virus production does not take place in these cells, the cell progeny do not contain SV40 and cells stop growing, a process known as ‘abortive transformation’. Occasionally, the SV40 DNA can integrate into the host chromosomal DNA. When the virus sequences that code Tag and tag are not altered by the integration process, they may be expressed and may cause malignant transformation (a rare event occurring in approximately one in 10^7 – 10^9 infected cells). SV40 integration occurs randomly, often near the nuclear matrix attachment region in tumor cells [29]. It has been suggested that the disruption of human chromosomal interval at 1q21.1, caused by SV40 integration, is an essential step in the process of cellular immortalization [30].

Human cells are called semi-permissive in that they support SV40 replication, although less efficiently than monkey cells. SV40 lyses human fibroblasts and very rarely (less than $1/10^8$ cells) these cells may become transformed, if the viral DNA integrates into the host cell genome [31]. Shein discovered in 1967 that human astrocytes were susceptible to SV40 infection and that, following infection, these cells could become transformed [32]. SV40 transformed astrocytes released infectious SV40 [32], while transformed fibroblasts did not [33]. Whether SV40 enters a true latent state remains to be fully addressed. We discovered that following SV40 infection, $1/10^3$ HM [34] and one in seven human primary astrocytes [11] are transformed and can be established as continuous cell lines (i.e., they do not undergo ‘crisis’). At the same time, in similar experiments conducted on many different fibroblast batches in our laboratory during the past 26 years, we have never developed a single SV40 transformed human fibroblast line. Instead, in parallel experiments we developed several dozen HM and astrocytic cell lines. Some of these HM and astrocytic cell lines release variable amounts of infectious SV40: in a few, the amount of virus released significantly decreased during cell culture, an effect we linked to the production of an antisense mRNA originating in the early region of the SV40 genome that leads to the degradation of late genes mRNAs [11,35] [Carbone M, Unpublished Observations].

SV40-mediated immortalization & transformation

To study SV40 infection of human cells it is necessary to prevent SV40 cell lysis, a normal outcome of SV40 infection of human cells. For this purpose, DNA replication-defective SV40 mutants and SV40 oncogene constructs under the control of a heterologous promoter are often used. The expression of both Tag and tag causes higher transformation efficiency [27]. Following SV40 infection, fibroblasts delay senescence from 40–50 to 60–70 generations, then undergo cell crisis and apoptosis. It has been reported by others that $1/10^7$ – $1/10^9$ fibroblasts may escape senescence and become immortalized cell lines [36], a phenomenon we have never observed in our laboratory. The expression of Tag and tag following SV40 infection correlates with the induction of telomerase activity in infected HM [34]. Transfection of SV40 constructs does not induce telomerase in fibroblasts unless SV40 is placed under a strong heterologous promoter, such as human cytomegalovirus [34].

It has been proposed that active telomerase mitogenic stimuli (i.e., H-ras pathway) and SV40 early genes, by altering p53, pRb and PP2A activities, are the indispensable genetic requirements to achieve fully malignant transformation of human cells [36]. However, the functions of Tag and tag broadly extend to a number of other host cellular signaling pathways [16].

Some human cell types are more susceptible than others to SV40-mediated transformation. In HM and brain cells, Tag binds and inactivates the tumor suppressor activities of cellular p53, pRb, p130 [37], p300 and p400. At the same time the Tag-p53-multiprotein complex acquires its own oncogenic activity and binds and activates the promoter, which in turn causes IGF-1 secretion and tumor cell growth [18].

Human primary mesothelial cells are an example of human cells that can be transformed solely by SV40 exposure [35]. Approximately one in 5000 of the infected HM develops foci and these foci can be easily propagated in tissue culture. Stable cell lines can be established from about 95% of foci and tumors develop when these cells are injected into immunodeficient mice [38]. In SV40-transformed HM abundant viral DNA persists in episomal form, and a low amount of infectious viral particles are produced and released during passage in tissue culture [35].

SV40 oncogenicity in animals

Hamsters injected with SV40 developed osteosarcomas, sarcomas, ependymomas, mesotheliomas, choroid plexus tumors and true histiocytic lymphomas [16]. The types of tumors induced are influenced by the route of SV40 inoculation and by cell susceptibility, for instance, most cell types are infected, but few can be transformed by SV40 [39]. Over 50% of hamsters injected with SV40 intracardiacally or intraperitoneally, and 100% of those injected intrapleurally, developed mesothelioma [40].

Simian virus 40 transgenic animals have provided excellent models to study the development of various cancers, and to search for possible therapeutic targets and approaches. To date, several transgenic models have been established. The first SV40 transgenic model was established in 1984 by integrating the SV40 early region genes and their own promoter–enhancer into a mouse germ line genome. High percentages of the transgenic mice population developed brain tumors within 3–5 months [41]. Moreover, in a similar model, by using Tag and the SV40 72-bp enhancer element as transgenes, the same group demonstrated that Tag expression is sufficient for tumor induction and that the enhancer region influences tissue tropism, as these animals developed only choroid plexus tumors [42]. Expression of Tag under the control of the glucagon gene 5' flanking sequences in transgenic mice led to the development of large bowel carcinoma [43]. Tag transgenic mice can produce heritable eye tumors, including ocular tumors and lens tumors [44].

Several different transgenic models have been developed by driving SV40 genes expression under the control of different tissue-specific promoters [45–65]. Table 1 lists a panel of SV40-based transgenic models commonly used to study breast, prostate and other tumors. These transgenic models indicate that the expression of SV40 Tag can cause cancer in any organ, making SV40 Tag the most potent oncogene known to date. The types of cancer induced are determined by the promoter used, which in turn determines the tissue in which Tag is expressed. When Tag is under the control of its own promoter, it causes primarily brain and bone tumors, mesotheliomas and a type of lymphomas known as true histiocytic lymphomas.

These animal models provide convenient and accurate tools to monitor and observe the events underlying tumor initiation and development, and provide models to test novel preventive and therapeutic approaches.

Malignant mesothelioma

Malignant mesothelioma (MM) is an aggressive cancer, resistant to conventional therapies; about 90% of those diagnosed with this disease die within 2 years [66]. The incidence of

MM is increasing in the Western world; in the USA approximately 3000 cases are diagnosed annually, compared with approximately none until 1950 [67]. MM develops from the transformation of the HM of the pleural, pericardial and peritoneal tissues that line the body cavities; about 70% of MM is of pleural origin. Exposure to the mineral fibers asbestos and erionite is the main cause of MM; SV40 infection and radiation are potential cofactors [68]. SV40 is the only agent known to cause malignant transformation of HM in tissue culture. Asbestos does not transform HM in tissue culture; however, when HM are exposed to both SV40 and asbestos, the rate of transformation increases significantly. Thus, SV40 and asbestos are cocarcinogens, and act synergistically in causing malignant transformation of HM *in vitro* [38,69].

SV40-mediated HM & astrocyte transformation

For a long time, we were puzzled by the observation that SV40 remained episomal in human tumor cells. For example, the isolation of infectious SV40 from brain tumors [8] clearly indicated the presence of episomal virus, yet integrated virus was not detected in the same tumors. How could episomal SV40 persist in human cells when, as discussed previously, episomal SV40 should replicate, produce VP4 and cause cell lysis? We discovered that in HM and in human astrocytes, during SV40 late phase infection, late gene expression is suppressed by antisense RNAs that are produced as a result of extension of the early transcripts beyond the early polyadenylation signal into the late region (Figure 2) [35]. The double-stranded RNA molecules, generated by this peculiar mechanism, may either be degraded by Dicer-mediated processing, or they may not be exported into cytoplasm. Experiments are in progress to address this mechanism. Viral late gene silencing allows HM to survive SV40 infection, as viral particles and VP4 are not produced, thus these cells are not lysed. At the same time the continuous expression of Tag causes malignant cell transformation [35]. These findings provided a mechanistic rationale for the unusual susceptibility of HM to SV40-mediated malignant transformation. Consistently, SV40-associated microRNAs were not detected in human MM samples by a highly sensitive reverse transcription PCR survey [70]. Such microRNA could not be produced because of the presence of the antisense RNA discussed previously.

Figure 3 summarizes the molecular events driven by the expression of SV40 Tag in HM that leads to HM transformation and malignant growth. Tag expression also induces a HGF autocrine circuit in Rb-dependent manner in HM, and possibly in other cell types. The resulting activation of HGF receptor Met causes epithelial–mesenchymal transition of HM and S-phase entry, cell cycle progression, virus particle assembling and infection of adjacent cells [21]. VEGF is also expressed and secreted upon SV40 infection of HM, by an autocrine mechanism [71]. VEGF is an important growth factor for MM growth and is a potent angiogenic factor promoting vascularization of MM [72]. Tag and tag are able to upregulate Notch-1 at the transcriptional level, to induce ERK pathway and to induce telomerase activity [22]. Interestingly, Notch-1 and Met are highly expressed in HM and astrocytes transformed by nonarchetypal SV40, while the archetypal SV40 variant can only transform astrocytes [11]. SV40 tag binds and inactivates the PP2A leading to an increase of ERK pathway activity and tumorigenesis [27].

SV40-asbestos: cocarcinogenesis

Asbestos is a mineral fiber that when deposited in sufficient amounts in the lungs can cause progressive fibrosis (i.e., asbestosis), lung cancer and MM. When HM in tissue culture are exposed to both SV40 and asbestos, the rate of malignant transformation increases synergistically [73]. By using the tag-defective SV40 (strain dl883), which does not induce MM *in vivo*, we demonstrated that pleural and peritoneal injection of crocidolite asbestos, combined with intraventricular administration of dl883 SV40, induced mesothelioma in

approximately 90% of hamsters with a shorter tumor latency compared with 20% mesothelioma induction in animals injected with asbestos alone [73]. Therefore, two very different carcinogens, SV40 and asbestos, are cocarcinogens in causing malignant transformation of HM *in vitro* and in causing MM in animals. This finding was independently reproduced in different laboratories [61,74]. The exact mechanisms underlying cocarcinogenesis are unknown.

Asbestos deposition in tissues causes a chronic inflammatory process. When asbestos fibers are ingested by macrophages and HM, these cells undergo programmed cell necrosis that is a process that leads to the release of high molecular weight binding protein 1 (HMGB1) into extracellular space. Extracellular HMGB1 promotes the secretion of TNF- α by surrounding macrophages and HM cells that reduce asbestos-induced cytotoxicity by activating the nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) [75–77]. The release of HMGB1 starts and propagates a chronic inflammatory process characterized by the release of reactive oxygen/nitrogen species that cause DNA damage [68]. It appears possible that this inflammatory environment may favor the growth of HM that have accumulated DNA damage owing to the reactive oxygen/nitrogen species released by cells exposed to asbestos, or owing to SV40 Tag expression, or both. Moreover, Tag expression protects HM from asbestos cytotoxicity by activating the PI3K/Akt pathway, thus increasing the percentage of cells that survive asbestos exposure and can therefore undergo malignant transformation [69]. Tag also upregulates calretinin expression [78].

Suggestions of asbestos/SV40 cocarcinogenesis in human MM were reported in some areas in Northeastern Italy [79] and Egypt [80], both with high levels of asbestos exposure and MM incidence. The Italian town of Casale Monferrato (Northwestern Italy) was extensively exposed to asbestos [81]. In this town, 16% of blood specimens from healthy individuals tested positive for SV40 Tag DNA sequences [82]. The unusually high incidence of SV40 in specimens from Italy can be attributed to the inadvertent use of SV40 contaminated vaccines in Italy as recently as 1999, when following a note from the British National Institute for Biological Standards and Control (UK), the Italians finally switched to the SV40-free WHO seed to produce polio vaccines [11] (to the best of our knowledge Italy is the only country in the Western world where SV40 contaminated vaccines have been used until few years ago). Several SV40 strains have been isolated from Italian patients [83].

SV40 association with human cancers

Rhesus monkey cells in culture were extensively used in the preparation of polio vaccines, by using either killed (Salk vaccines) or attenuated virus (Sabin vaccines). In these cultures, indigenous SV40 was not completely killed by the formaldehyde inactivation process used for polio vaccines. Poliovirus vaccines potentially contaminated with SV40 were inadvertently administered to millions of individuals between 1953 and 1963 in the USA and in several other countries [16]. Whether the increased incidence of mesothelioma observed since then in the USA and throughout the world was influenced by these contaminated vaccines continues to be debated. Infectious SV40 particles were also detected in adenovirus vaccines produced between 1957 and 1960, although the distribution of contaminated adenovirus vaccines was much less widespread and largely restricted to military personnel [8]. A seed of polio vaccines from a major eastern European manufacturer that contained infectious SV40 particles was used from 1965 to 1978 throughout the world [84]. In human populations, individuals exposed to SV40 may develop a transient infection, approximately 60% of the male volunteers (ages 21–35 years) developed antibodies after intranasal exposure to SV40 [85]. Whether these people were/are at higher risk of developing cancer remains to be elucidated.

SV40 possible transmission route among human populations

Humans have been exposed to both archetypal and nonarchetypal SV40. SV40 virus with an archetypal configuration of the regulatory region, are the most common viruses present in monkeys, therefore, these were the strains most commonly detected in polio vaccines [8,84]. Following oral administration of SV40-contaminated polio vaccine, SV40 was detected in the feces for several weeks, indicating that the virus was replicating in some of the cells of the GI tract [86]. SV40 was detected in the urine of immunocompromised patients and patients with collapsing focal segmental glomerulosclerosis [87], and in the tonsils of immunocompetent children [88]. Polyomaviruses (BK, JC or SV40) were detected in 46% of stool samples from hospitalized children [89] and 20 out of 110 stool specimens from healthy adults [90]. Among the 20 positive samples, one of the stool samples contained both SV40 and JC virus [90]. These findings indicate that SV40 infects and replicates in humans, and that the GI tract may be a site of polyomavirus persistence. It appears possible that human to human SV40 infection occurs as observed for other viruses. More research is needed to address this question.

SV40 & MM

The association of SV40 and human MM has been extensively explored. Table 2 summarizes most of the reports published since 2002; previous work has been well summarized by Klein *et al.* [91]. The presence of SV40 in human MM was first reported in approximately 60% of US specimens in 1994, by using PCR, western blotting, immunoprecipitation and immunohistochemical (IHC) staining [92]. It was confirmed by a multi-institutional study [93], and by several other research teams [79,80,94–98]. Microdissection experiments confirmed IHC findings, indicating that SV40 was present in tumor cells but not in the surrounding stroma [95]. However, some research groups did not detect SV40 in MM biopsies [99–102].

SV40 & brain tumors

Tag expression was first detected in a meningioma in 1975 [103], and infectious SV40 was isolated from specimens of this tumor [104]. Between 1975 and 2002, 13 studies reported an overall prevalence of about 18% of brain tumors containing SV40 sequences (reviewed in [105]). High frequencies of SV40 DNA sequence were found in choroid plexus tumors, ependymomas, low-grade astrocytomas, anaplastic astrocytomas and secondary glioblastomas, and lower frequencies were detected in gemistocytic astrocytomas, oligodendrogliomas, glioma and medulloblastoma (Table 3) [106–108]. Some research teams did not detect SV40 in brain tumors (Table 3) [109–111].

At least three reports showed the presence of SV40 in Li-Fraumeni Syndrome (LFS) [106,112,113]. A total of 18 out of 151 LFS patient specimens were SV40 positive, and 11 of these were osteosarcoma biopsies from LFS patients [106]. SV40 DNA sequences and Tag protein were detected in the choroid plexus carcinoma (CPC) sample from one LFS patient. This patient also had an embryonal rhabdomyosarcoma; SV40 was detected only in the CPC. In a second LFS patient that had developed CPC, osteosarcoma and renal cell carcinoma, SV40 DNA and Tag were detected in both CPC and renal cell carcinoma specimens [113].

SV40 & other tumors

SV40 has been detected in several other tumor types, including lymphomas [114–117], osteosarcomas [118,119], breast carcinomas [120] and, more recently, colon cancer [121]. The significance of these findings remains to be established.

Geographic differences in SV40-positive tumors

It has been suggested that SV40 may be transmitted in humans by both horizontal and vertical infection [16]. In the USA, about 90% of children and 60% of adults were immunized with polio vaccines between 1955 and 1961, are, therefore, potentially infected [16]. In the former USSR, and in countries under its influence, polio vaccines containing infectious SV40 were administered until 1978 [84], and possibly later. Moreover, at least one polio seed virus from which vaccines were prepared in Italy until 1999 was SV40 contaminated [122] [Phil Minor, Pers. Comm.]. Some other countries, such as China, still produce polio vaccines using monkey cells, raising the possibility that their vaccines remain contaminated with SV40 [84].

Simian virus 40-positive tumors were found in the USA, Canada, China, Japan, Europe and New Zealand [123], but not in Finland, Turkey, Yugoslavia and Austria, countries that did not use SV40-contaminated vaccines [16]. Human exposure to SV40 through contaminated polio vaccines has been extensive, and has been influenced by geographical differences associated with the use of contaminated or noncontaminated vaccines [16]. The reasons that some countries did not use contaminated vaccines vary, some of them started polio vaccination in the 1970s, when the vaccines had been cleaned from infections SV40 (Turkey); others made their own vaccines and they made them using SV40-free substrates (Yugoslavia).

The incidence of SV40 positive MM shows dramatic geographical differences, whether these differences are related to the use of contaminated vaccines remains a controversial issue because of potential liability and litigation. For example, tumor tissue samples from the USA and Europe were found positive in 20–83% of cases [92,93,95–98], however, we and others did not detect SV40 sequences in samples from Turkey [96].

Geographical differences were reported for the SV40 incidence in bone tumors as well. In 143 out of 277 (42%) osteosarcoma tissue samples from Germany and Hungary, SV40 DNA sequences were detected by quantitative real-time PCR. However, differences in the distribution of SV40 were observed between the two countries. Approximately 74% of Hungarian osteosarcoma samples harbored high copy numbers of SV40 genomic DNA (>100 copies), whereas 24% of the German osteosarcoma samples contained low copy numbers of SV40 genomic DNA (about ten copies) [119]. On the other hand, no SV40 sequences were detected in ten osteosarcomas and 14 giant cell tumors from Austrian patients [99]. These results support geographical differences associated with the detection of SV40 in human specimens [16].

Expert commentary

The presence of SV40 sequences in several human cancers, including MM, bone and brain tumors, and lymphomas has raised concerns about the possible pathogenic role of SV40 in humans. Unfortunately, the controversy over the percent of tumor specimens containing SV40 DNA and proteins has paralyzed this research field. This controversy was magnified by the legal implications of associating the production and distribution of contaminated poliovaccines to the development of human mesotheliomas and brain tumors. Study sections reviewers have been unwilling to support SV40 research citing the need to first address the ‘controversy’, yet without funding it is impossible to conduct studies to address controversial findings.

Regardless of its pathogenic role, the presence of SV40 in some tumors provides a potential target for therapy. However, until the existing controversy over the presence of SV40 in human tumors is addressed and funding becomes available, it appears unlikely that progress

will be made to develop therapies aimed at tumor cells carrying SV40 and or expressing SV40 antigens. So, the reader may ask, is SV40 really present in some human biopsies?

Over 50 laboratories, using different techniques, have detected SV40 in human tumors (mostly in MM and brain tumors). The techniques used to detect SV40 included PCR, quantitative PCR, Southern blotting, IHC staining for Tag, DNA sequencing, *in situ* hybridization *et cetera*. It is unlikely that so many researchers using different technical approaches would consistently be wrong. A critical review of the papers reporting SV40 in human specimens show that some were carefully planned and executed, yet others lacked appropriate controls, and were of poor quality. Therefore, it is possible that some of these 'positive' studies were flawed and overestimated the presence of SV40 in human tumors. At the same time, many other papers, for example those from the laboratories of J Butel, J Lednický, A Gazdar, R Garcea and J Melnick, to cite a few, were carefully controlled and appear to have been well executed. On the other hand, several reports were negative, or better the titles claimed negative results, but the data contained in those papers reported a low incidence, 5–6% positivity of SV40 in the specimens tested, and it is the low percentage of positivity that was interpreted as insignificant by the authors (Table 2) [124,125]. This is a critical point of the controversy: most 'negative' papers were actually not negative at all. These papers instead reported a low incidence of SV40 in human specimens. In addition, some authors detected SV40 DNA but did not detect SV40 proteins, raising the question of whether finding SV40 DNA was of any pathogenic significance (Table 2) [126–128]. These are valid questions (i.e., if SV40 proteins are not expressed, is the presence of SV40 DNA alone of any pathogenic significance?) that should be carefully studied, as suggested by a review of the Institute of Medicine in 2002 [129]. The problem is that analyzing the so-called 'hit and run mechanism' or 'indirect carcinogenesis' is not an easy task, as the investigator is faced with the outcome of the carcinogenic process and must reconstruct the events that led to cancer. There is documented evidence in the literature for a 'hit and run' carcinogenesis by SV40 in causing tumors in transgenic animals [130], but we do not know how to study this phenomenon in humans.

It is of course impossible to provide a comprehensive satisfactory explanation for controversial findings, as multiple factors may have been at play. At times, technical reasons may have caused some of the discrepancies in the literature. We experienced this problem in our own laboratory, when Maurizio Bocchetta told one of the authors of this review (Carbone) that there was no Tag in MM biopsies [131]. Previously, Carbone had performed the experiments using ¹²⁵I-labeled antibodies [132], but as lab techniques evolved, Bocchetta was using enhanced chemiluminescence (ECL). Eventually, after much discussion and repeated attempts, we discovered that the results were influenced by the type of ECL kit used for detection, the least sensitive one gave 0% positivity. By using an intermediate sensitivity method, which allowed us to test different exposure times, at least seven out of 20 MM samples tested Tag positive. Instead, the most sensitive ECL detected only two out of 20 positive samples, because the reaction was so rapid that we had to interrupt it after 1–2 sec before the whole membrane became black, and we could not test different exposure times [131]. If we had not previously done these same experiments, most likely we would not have taken the time to repeat multiple times the experiments to address the discrepancy between present and past results, and we would have erroneously published 'negative' results.

The discrepancies in the results may have also been influenced by geographical differences linked to the distribution of contaminated poliovaccines [84]. For example, the high incidence of SV40 reported in Italian specimens correlates with the finding that Italy is the only country in the Western world that used SV40 contaminated vaccines until 1999 [84].

Some authors reported that positive results were due to contamination by laboratory plasmids containing SV40 sequences [124]. Lopez-Rios *et al.* found that the high incidence of SV40 in human MM they tested (52–62%) was obtained with primers annealing to sequences present in common laboratory plasmids used in their own laboratory [124]. When the results were repeated with different primers, only 6% of the MM biopsies were confirmed as SV40 positive. It is possible that the high incidence of SV40 reported by some studies may have suffered from similar technical pitfalls.

The presence of SV40 in some human specimens is supported by serological studies showing widespread human exposure to SV40. Recent studies using ELISA assays that reliably distinguish among SV40, and the human polioviruses JCV and BKV revealed that 1% of those tested had anti-SV40 antibodies, suggesting widespread human exposure to a virus (SV40) that should be confined to monkeys [133].

In summary, the data suggest that at least 5% of human mesotheliomas and brain tumors – according to the so-called negative papers, or more according to the ‘positive papers’ – contain SV40 DNA, the possible pathogenic role of SV40 in these tumors should be further investigated to address causality issues.

It is unfortunate that the controversy about the presence of SV40 in human tumors has been too heated to allow an unbiased analysis of the results. As noted by a panel of the Institute of Medicine that reviewed this issue, this area of investigation deserves further attention [134]. At the same time, the conflicting arguments and lack of funding caused many researchers to leave this field. Progress in the near future remains unclear because of lack of funding. We are pleased to note, however, that regardless of the ‘controversy’ over the role of SV40 in human carcinogenesis, this research has led to important discoveries about mechanisms of SV40 infection, carcinogenesis and cocarcinogenesis that are important to the whole field of cancer. In addition, this research has uncovered the extent of human exposure to SV40, and has and will continue to influence future regulatory decisions related to vaccine production and safety.

Five-year view

Simian virus 40 remains an invaluable tool to study mechanisms of viral carcinogenesis and regardless of the controversial issues related to the possible pathogenic role of SV40 in humans, SV40 will continue to be studied to address basic mechanisms of carcinogenesis. The precise mechanisms by which SV40 either lyses or transforms different human cell types are becoming more evident. Recent work has indicated that an antisense mechanism that appears unique to HM and brain cells suppresses late viral gene expression, allowing these cell types to survive infection, maintain SV40 as episomal with expression of Tag and tag but not of the VP1–4 proteins and become transformed. It is hoped that these mechanisms will be fully elucidated in the next 5 years, and that the information may help us understand the process and possible biological significance of SV40 infection in humans, and more generally advance our understanding of viral carcinogenesis. Future study will have to elucidate the cellular factors that appear unique to HM and brain cells, and that regulate the expression of this antisense RNA. Researchers are starting to address the puzzle of why archetypal SV40 DNA sequences are detectable in human brain tumors, while the SV40 DNA sequence in MM samples are most often nonarchetypal. A recent paper showed that these findings reflect the tissue tropism of SV40 [11] and that host cell signaling contributes to the transformation process [135].

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Papers of special note have been highlighted as:

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Key issues

- Simian virus 40 (SV40) is a small DNA tumor virus, transmitted from monkeys to humans through contaminated polio vaccines. It has been postulated that SV40 is transmitted among humans both vertically and horizontally.
- The early gene product SV40 large T antigen promotes viral DNA replication and is the main SV40 oncogene. SV40 Tag interacts with and transactivates multiple intracellular signaling proteins. Tag-p53 complex recruits retinoblastoma protein, p300, p400 and CREB-binding protein to induce specific gene transcription, resulting in the activation of IGF-1, Met and Notch-1 pathways.
- Recent studies using ELISA assays that reliably distinguish among SV40, JC and BK viruses revealed that 1% of those tested had anti-SV40 antibodies.
- SV40 and asbestos are cocarcinogens in causing mesothelioma in hamster and mice, and in causing malignant transformation of human primary mesothelial cells in tissue culture.
- Many laboratories reported the presence of SV40 DNA, RNA or proteins in human tumor biopsies, other laboratories reported negative results, or more often reported a low incidence of SV40 in these specimens (6%). These conflicting findings have generated controversy that has paralyzed this research field.
- The conflicting results can be at least in part attributed to technical discrepancies, laboratory contamination with SV40 plasmids, inadequate technical approaches and geographical differences in the prevalence of SV40 linked to the distribution of contaminated poliovaccines.
- The high incidence of SV40 sequences in Italian specimens, for example, is probably linked to the fact that Italy is the only country in the Western world that used contaminated vaccines as late as 1999.

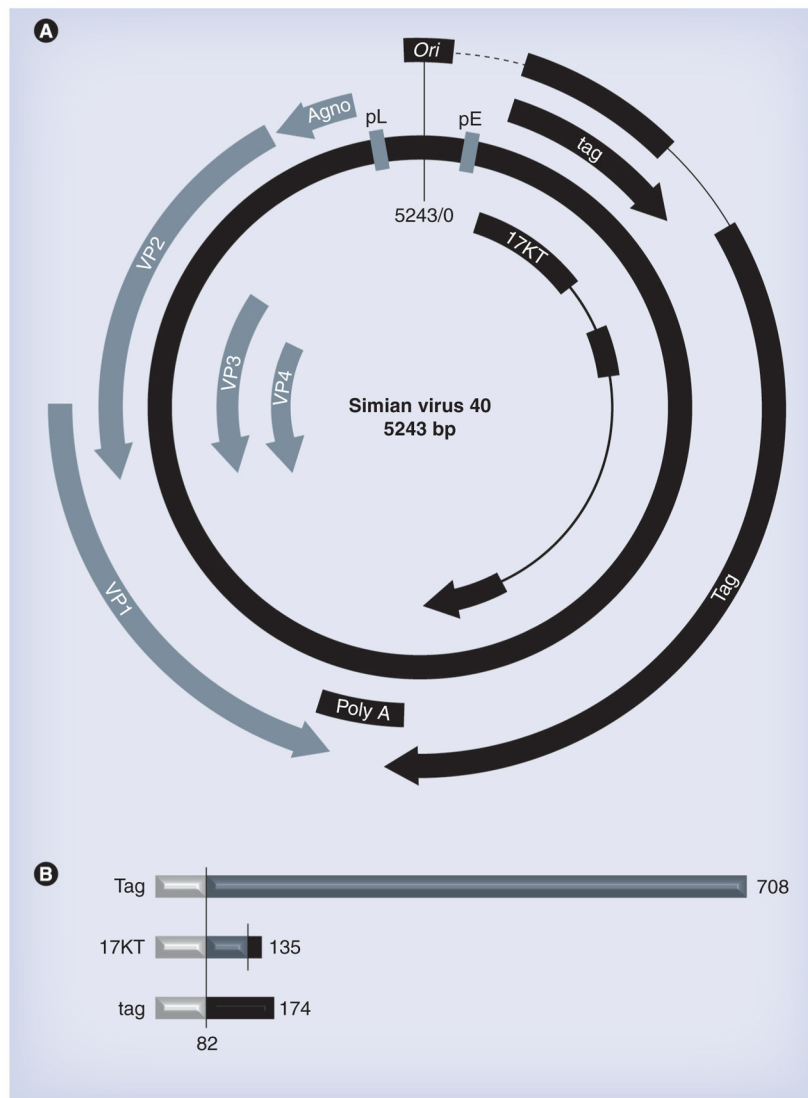


Figure 1. Structure and function of simian virus 40

(A) Physical and functional map of the SV40 genome. The circular DNA genome is 5243 bp. Ori (black). Tag, tag and 17KT (black): 'Early gene' mRNAs, controlled by the pE (blue). pL (blue) shares the same sequence with pE, but is oriented in the opposite direction. VP1, VP2, VP3, VP4 and Agno (blue): transcripts of 'late genes', are under control of pL and encode capsid proteins VP1, VP2, VP3, and VP4 and Agnoprotein assembly promoting proteins. VP2-4 share the same stop codon. **(B)** SV40 early proteins: Tag, 17KT and tag share the first 82 amino acid residues at the N-terminal (gray). Tag extends up to 708 residues in length (blue), but skips the tag encoding region (black), owing to differential splicing. 17KT encodes a 135 amino acids protein, sharing the first 131 residues (gray and blue) with Tag, followed by four amino acids encoded by the third intron of the Tag gene, retained by an alternative splicing. SV40 tag is a 174 residue protein. Ori: Origin of viral DNA replication; pE: Early promoter; pL: Late promoter; SV40: Simian virus 40; Tag: Large T antigen; tag: Small t antigen.

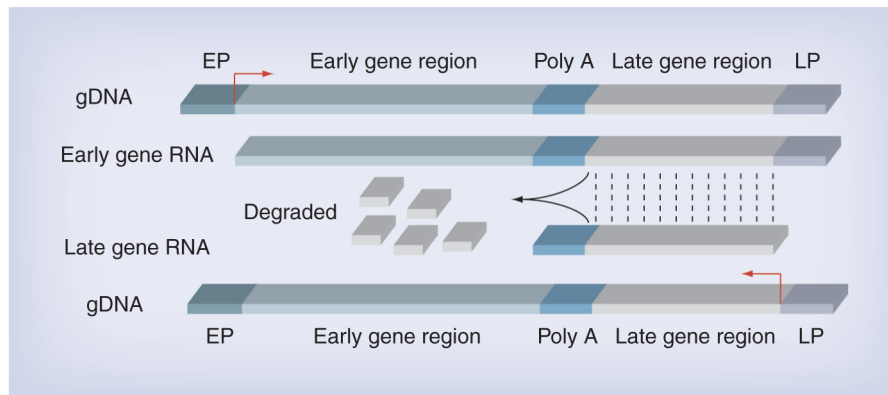


Figure 2. Silencing mechanism of simian virus 40 late gene transcription

Early genes transcripts extend beyond poly A signal and overlap the late gene region (light gray), leading to late gene antisense RNA. The resulting double-strand RNA is degraded, impairing late gene expression.

EP: Early promoter; gDNA: Genomic DNA; LP: Late promoter.

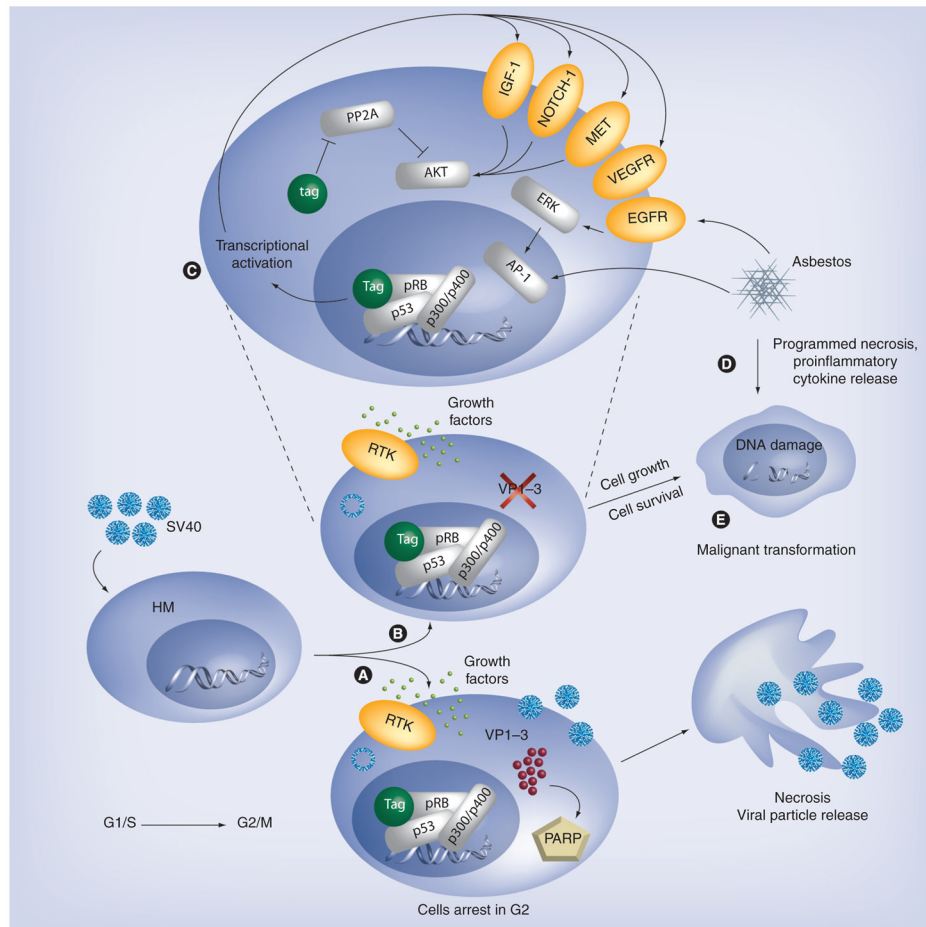


Figure 3. Mechanisms of simian virus 40 infection and cell transformation

Upon SV40 entry, Tag is expressed and accumulates in the host cells, where it binds to p53, pRb, p300, p400 and possibly other proteins, to form a multiprotein complex that activates transcription of IGF-1. Tag also induces, possibly with similar mechanisms, the expression of other growth factors. (A) In the majority of infected HM, late gene transcription leads to VP1-3 viral capsid protein expression and virion assembly. Host cells undergo necrotic lysis, owing to massive viral release and a PARP-mediated mechanism. (B) In a fraction of infected HM, VP1-3 expression is blocked by the antisense mechanism [35], virions are neither assembled nor released and host cells survive. (C) In these cells, the signaling induced by the autocrine IGF-1/IGF-1R loop (and by other ligand/receptor autocrine circuits) eventually leads to G1/S progression and cell proliferation. Also tag contributes to cell survival inducing Akt activity as a consequence of PP2A inhibition. (D) Exposure to asbestos activates EGFR signaling, leading to ERK activity and AP-1 transcription, and induces programmed cell necrosis with consequent release of HMGB1 and other proinflammatory cytokines [77], leading to chronic inflammation. (E) The signaling induced by asbestos and SV40 infection, either independently or in a cooperative manner, can promote HM transformation and mesothelioma development.

EGFR: EGF receptor; HM: Human primary mesothelial cell; HMGB1: High molecular group binding protein 1; PARP: Poly (ADP-ribose) polymerase; pRb: Retinoblastoma protein; SV40: Simian virus 40; Tag: Large T antigen; tag: Small t antigen; VEGFR: VEGF receptor.

Table 1

SV40 transgenic animal models.

Study (year)	Sequence	Promoter	Host	Tumor type	Ref.
Hanahan (1985)	Tag	Rat insulin II	Mouse	Insulinoma	[45]
Wikenhaiser <i>et al.</i> (1992)	Tag	Human surfactant protein C	Mouse	Adenocarcinoma	[46]
Maroulakou <i>et al.</i> (1994)	Tag, tag	Rat prostatic steroid binding protein C3(1)	Mouse	Adenocarcinoma (only in females)	[47]
Greenberg <i>et al.</i> (1995)	Tag, tag	Rat probasin	Mouse	Invasive adenocarcinoma (only in males)	[48]
Hurwitz (2001)	Tag, tag	Rat probasin	Mouse	Invasive adenocarcinoma (only in males)	[49]
Santarelli <i>et al.</i> (1996)	Tag, tag	Mouse whey acidic milk protein	Mouse	Breast	[50]
Perez-Stable <i>et al.</i> (1996)	Tag	Human fetal IgG γ -globulin	Mouse	Prostate	[51]
Garabedian <i>et al.</i> (1997, 1998)	Tag	Mouse cryptdin-2	Mouse	Prostate	[52,53]
Asamoto <i>et al.</i> (2001)	Tag	Rat probasin	Rat	Prostate carcinoma	[54]
Masumori <i>et al.</i> (2001), Gabriel <i>et al.</i> (2002)	Tag	Rat probasin	Mouse	Adenocarcinoma	[55,56]
Gabril <i>et al.</i> (2002)	Tag	PSP94	Mouse	Adenocarcinoma	[56]
Hicks <i>et al.</i> (2003)	Tag	Clara-cell secretory protein 10	Mouse	Adenocarcinoma, lung parenchymal	[57]
Garson <i>et al.</i> (2003)	Tag	Ovarian-specific promoter 1	Mouse	Ovarian and brain tumors, abdominal and bone sarcoma	[58]
Lou <i>et al.</i> (2005)	Tag, tag	Human antithrombin III gene	Mouse	Hepatocarcinoma	[59]
Grippio <i>et al.</i> (2000)	Tag	Cytokeratin 19	Mouse	Urinary bladder tumors Mesothelioma	[60]
Robinson <i>et al.</i> (2006)	Tag	Mesothelin	Mouse	Mesothelioma	[61]
Köbber <i>et al.</i> (2008)	Tag	SM22 α	Mouse	Cardiac rhabdomyosarcoma	[62]
ter Brugge <i>et al.</i> (2009)	Tag	Immunoglobulin heavy chain, opposite orientation	Mouse	B-cell chronic lymphocytic leukemia	[63]
Stahl <i>et al.</i> (2009)	Tag inducible	Hepatocyte-specific albumin	Mouse	Hepatocellular carcinoma	[64]
Iwakura <i>et al.</i> (2009)	Tag	Human ghrelin	Mouse	Ghrelinoma	[65]

Tag: Large T antigen; tag: Small t antigen.

Table 2

Simian virus 40 incidence in malignant mesothelioma.

Study (year)	SV40 (%)	Asbestos (%)	SV40 + asbestos (%)	Source [†]	Techniques	Ref.
Carbone <i>et al.</i> (1994)	60	76	38	USA	PCR, IHC, WB, IP	[92]
Testa <i>et al.</i> (1998)	83	71	71	USA	PCR	[93]
Ramael <i>et al.</i> (1999)	60	100	60	Belgium	PRINS and IHC	[94]
Shivapurkar <i>et al.</i> (1999)	57	NA	NA	Germany, North America	PCR and DNA seq	[95]
De Rienzo <i>et al.</i> (2002)	36/0 [‡]	NA	NA	USA, Turkey	PCR and DNA seq	[96]
Pfiftakis <i>et al.</i> (2002)	10	NA	NA	Sweden	PCR and DNA seq	[97]
Cristaudo <i>et al.</i> (2005) [§]	42	68	88	Italy	PCR, DNA seq, F-Hybri	[98]
Comar <i>et al.</i> (2007) [¶]	16	90	29	Italy	PCR and DNA seq	[79]
Zekii <i>et al.</i> (2007)	50	78	58	Egypt	qRT-PCR	[80]
Gordon <i>et al.</i> (2002)	6	NA	NA	USA	qRT-PCR	[125]
Lopez-Rios <i>et al.</i> (2004)	6	NA	NA	USA	PCR and DNA seq	[124]
Jin <i>et al.</i> (2004)	44/0 [#]	NA	NA	Japan	PCR, DNA seq, IHC	[126]
Aoe <i>et al.</i> (2006)	6/0 ^{††}	NA	NA	Japan	qRT-PCR	[127]
Hmeljak <i>et al.</i> (2010) ^{‡‡}	4/0 ^{§§}	NA	NA	Slovenia	qRT-PCR and IHC	[128]
Leithner <i>et al.</i> (2002)	0	NA	NA	Austria	PCR	[99]
Hubner <i>et al.</i> (2002)	0	NA	NA	Belgium	PCR	[100]
Mayall <i>et al.</i> (2003)	0	NA	NA	New Zealand	qRT-PCR	[101]
Brousset <i>et al.</i> (2005)	0	NA	NA	France	IHC	[102]

[†] Source: country of origin of the specimens tested in the study.

[‡] 36% of US samples and 0% of Turkish samples are SV40 DNA positive.

[§] 33% SV40-positive DNA in bladder urotheliomas.

[¶] 16.7% normal liver tissues and 12.5% kidney tissues tested positive for SV40.

[#] 44% positive specimens by PCR and 0% by IHC.

^{††} 6% positive specimens by qRT-PCR and 0% by IHC.

^{††} Croatia, South Africa, UK and USA.

^{§§} 4% positive specimens by qRT-PCR, 0% by IHC.

DNA seq: DNA sequence analysis; F-Hybri: Filter hybridization; IHC: Immunohistochemistry; IP: Immunoprecipitation; NA: The presence of asbestos was not investigated in these studies; PRINS: Primed *in situ*; qRT-PCR: Quantitative real time PCR; SV40: Simian virus 40; WB: Western blotting.

Table 3

Simian virus 40 incidence in brain tumors.

Study (year)	Brain tumors (%)	Source [†]	Techniques	Ref.
Weiss <i>et al.</i> (1976)	38 ME	Germany	IHC	[104]
Wang <i>et al.</i> (1998)	50 CP; 90 EM	USA	PCR and DNA seq	[106]
Huang <i>et al.</i> (1999)	25 OL; 32 GA 59 LA, AA, SG;	France	PCR, Southern blotting	[107]
Vilchez <i>et al.</i> (2003)	21	Different [‡]	PCR, IHC	[105]
Rollison <i>et al.</i> (2005)	1.8 GL, MD	USA	PCR, Southern blotting, qRT-PCR	[108]
Kim <i>et al.</i> (2002)	0	USA	PCR	[109]
Montesions-Rongen <i>et al.</i> (2004)	0	German	PCR	[110]
Sabatier <i>et al.</i> (2005)	0 CNS	France	IHC	[111]

[†] Source: country of origin of the specimens tested in the study.

[‡] Assessment of 13 different reports on primary brain tumors from different sources.

AA: Anaplastic astrocytoma; CNS: CNS-related tumor; CP: Choroid plexus tumor; DNA seq: DNA sequence analysis; EM: Ependymoma; GA: Gemistocytic astrocytoma; GL: Glioma; IHC: Immunohistochemistry; LA: Low-grade astrocytoma; MD: Medulloblastoma; ME: Meningioma; OL: Oligodendroglioma; qRT-PCR: Quantitative real-time PCR; SG: Secondary glioblastoma.