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SIMIAN VIRUS 40 DETECTION IN HUMAN MESOTHELIOMA: RELIABILITY AND SIGNIFICANCE OF THE AVAILABLE MOLECULAR EVIDENCE

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TABLE OF CONTENTS

1. ABSTRACT

Simian virus 40 was discovered as a contaminant of early poliovirus vaccines that were inadvertently administered to millions of people in Europe and the United States between 1955 and 1963. Although SV40 was proven to be oncogenic in rodents and capable of transforming human and animal cells *in vitro*, its role in human cancer could not be proven epidemiologically. The matter was forgotten until 1993 when SV40 was accidentally found to cause mesotheliomas in hamsters injected intra-cardially. Subsequently, DNA sequences associated with its powerful oncogenic principal, the large T antigen, were found with high frequency in human pleural mesothelioma using the polymerase chain reaction (PCR). Since then many laboratories have confirmed the human findings. However, a few laboratories have failed to reproduce these data and the authors of the studies have claimed that the detection of SV40 DNA may simply represent PCR contamination artefacts. The controversy raised by this viewpoint is reviewed in this article together with a critical appraisal of the reliability of the molecular techniques used to detect SV40 DNA, in order to evaluate the potential aetiopathogenic role of SV40 in human mesothelioma.

2. INTRODUCTION

2.1. Early description of malignant mesothelioma

The first recognition of large diffuse malignant tumors arising from mesothelium of the pleura and peritoneum came from Germany in 1870 (1). A review of several autopsy series from North America and Europe between 1910 and 1949 gave a prevalence rate of 0.07% for pleural tumors, in a series of 69,042 autopsies (2).

However, up until the second half of this century there was a great reluctance to make a histopathological diagnosis of malignant mesothelioma, most authorities believing that pleural tumors originated from primary adenocarcinomas of the lung or the breast. It was not until the paper by Wagner *et al*., published in 1960 (3) of mesothelioma arising in association with asbestos that pathologists began to diagnose malignant mesothelioma of the pleura in significant numbers. In their retrospective analysis of all autopsies from lung cancer patients performed at the Massachusetts General Hospital in Boston, Mark and Yokoi (4) found the first mesothelioma with consistent history and immunocytochemical criteria to have occurred in 1947. This they found was followed by an incidence of only one or two cases per year until 1955

when the numbers suddenly started to progressively increase year after year. Based on these results Mark and Yokoi (4) proposed that "mesothelioma is a new disease" although it appears likely that occasional cases had already been described in the literature.

2.2. Discovery of link between asbestos exposure and malignant mesothelioma

In 1960 Wagner *et al*., (3) published a highly significant paper which described 33 cases of pleural mesothelioma, an extremely rare tumor at the time, in subjects from the Blue Hills region of the Northwest Cape territory of South Africa. They found a strong association with the mining of crocidolite (blue) asbestos and asbestos bodies were observed in the lung tissues. Another significant observation was that the tumors not only occurred in the miners of crocidolite asbestos but also among non-occupationally exposed persons living near the mines. Since that time many cases of mesothelioma have been reported in subjects with exposure to commercial amphibole forms of asbestos - amosite (brown) and crocidolite and the incidence of tumor has increased in most developed countries (5,6). The rare cases of mesothelioma associated with chrysotile exposure appear to have been caused by tremolite, an amphibole form of asbestos, which contaminated the chrysotile ore (7).

Although most attention has been concentrated on the link between amphibole asbestos and mesothelioma, it has been realised for many years that some cases of mesothelioma have occurred without apparent asbestos exposure and other causes have been proposed. These have included exposure to non-asbestos mineral fibre - erionite (8), radiation therapy (9), chronic inflammation and metals (10).

2.3. Viral infection and malignant mesothelioma

Interestingly, viruses had been considered as a potential cause of mesothelioma once before, since injection of the strain MC29 avian leukosis virus into the peritoneal, pericardial and air sacs of the chicken was found to result in a high incidence of mesotheliomas (11). However, this hypothesis was not developed, and the concept has remained dormant until the recent discovery of the association of SV40 with hamster and human mesothelioma and other types of tumors as discussed in this review.

3. DETECTION OF SIMIAN VIRUS 40 (SV40) IN HUMAN TUMOURS

3.1. SV40 and its accidental inoculation into the human population

Simian virus 40 (SV40) was discovered by Sweet and Hilleman (12,13) as one amongst 40 or more viruses infecting Macacus rhesus as well as Macacus cynomolgus monkey kidney cells, used for preparation of polio and other virus vaccines. The importance of SV40 to human disease was first realised when Eddy *et al*., (14,15) demonstrated the capacity of SV40, present within extracts of Macacus monkey kidney cells, to induce subcutaneous sarcomas in newborn hamsters at the site of inoculation. Shein and Enders (16) subsequently showed that SV40 also had the capacity to infect as well as transform human cells grown *in vitro*. By the time the potentially serious implications of these findings had been realised, poliovirus and adenovirus vaccines prepared in Macacus kidney cells had been in use for nearly 8 years (1955-1962). By then nearly 100 million people in the United States had received one or more doses of potentially contaminated vaccine preparations (Poliomyelitis Surveillance Report No 248, Communicable Disease Centre, Atlanta, Georgia, 1962).

The vast majority of these had received subcutaneous dose(s) of the formaldehyde inactivated Salk type polio vaccine (IPV). This preparation, though thought in earlier trials to be free of live virus, was proved subsequently by more sensitive tests to contain significant amounts (i.e. 102 to 107 p.f.u. /ml) of live infectious SV40 resistant to formaldehyde inactivation (13,17). This finding forced an immediate abandonment of the use of the Macacus monkey kidney cells, and a switch to the use of African Green Monkey kidney cells tested to be virtually free of indigenous viruses. In addition, each batch of the vaccine from then onwards was prepared with SV40 antiserum-treated virus stocks. The lack of SV40 DNA sequence contamination of the presently used oral poliovaccines (OPV) as well as those archived since 1971 at National Institute for Biological Standards, UK, has been recently confirmed by Sanger *et al*., (18) using molecular analysis. Nevertheless, by 1961, before the safety measures became effectively implemented, an estimated 62% (92 million) of U.S. residents (19) and approximately a similar proportion of the British population had received potentially SV40-contaminated poliovirus vaccines.

It has been suggested that during the period 1955-1962, the vaccinated individuals probably received (a) SV40-free vaccine, (b) vaccine containing inactivated SV40, or (c) vaccine containing a small amount of live, potentially infectious SV40, in addition to inactivated SV40 (19). According to prospective serological studies conducted on children given the subcutaneous doses of IPV, it seems about 1 in 5 of the vaccinees may have received live SV40 containing vaccine dose(s). This is suggested by the persistently high titre of SV40 specific antibody response observed in approximately 20% of the inoculated children (20).

3.2. Lack of pathogenic effects of inoculated SV40

Despite evidence suggestive of persistent immunogenic and/or infective presence of SV40 in a significant proportion of the vaccinated individuals, a review of the epidemiological data by Shah and Nathanson (17) revealed no evidence of 'frequent', 'immediate' or 'unusual' consequences of SV40 exposure. Nevertheless low frequency and long term complications such as induction of neoplastic, or chronic neurological disease could not be ruled out by these limited studies. A subsequent specific survey of brain tumor incidence, because of possible similarity of SV40 to principally neurotropic BK and JC human homologue polyoma viruses, also failed to show any obvious SV40 associated increased incidence of any expected forms of central

nervous system cancers (21). Overall therefore, the inoculation SV40 as a contaminant of polio vaccines into a substantial proportion of human subjects was considered to be free from any serious consequences over 5 week to 22 year follow-up periods (22).

3.3. Discovery of SV40-like DNA in human mesothelioma and other types of tumors

Whilst analysing the unusual capacity of small t deletion mutants of SV40 to induce true histiocytic lymphomas in hamsters, Carbone and colleagues (23,24) were surprised to find wild type SV40 (injected intrapericardially as a negative control) caused pericardial/pleural mesotheliomas with an overall 60% incidence. Subsequently, when SV40 was deliberately injected intrapleurally it was found to induce pleural mesotheliomas in all of the injected hamsters (25). Virtually all these animals died of the mesothelioma at 4-6 months following inoculation. The overall findings were particularly unexpected in that SV40 had apparently never been previously associated with mesothelioma in mammals (see reviews in (26,27)). In addition, mesothelioma induced following inhalation or injection of various forms of asbestos affected only 0-65% of animals (hamsters, mice and rats) with a latent period of > 1 year (see review by Carbone (28)). Thus compared to asbestos, SV40 appeared to be a considerably more potent carcinogen for induction of mesothelioma in animals.

These observations taken together with the facts that a) asbestos exposure alone is unable to fully explain the aetiopathogenesis of human pleural mesothelioma, since 20% of cases with mesotheliomas lack a history of asbestos exposure; and b) the prevalence of mesotheliomas in individuals with known prolonged heavy exposure to asbestos is only 2-10%, prompted Carbone and colleagues to investigate the possible aetiopathogenic role of SV40 in human mesothelioma.

Using a polymerase chain reaction (PCR) method pioneered by Bergsagel *et al*., (29) for detecting SV40 specific DNA in human ependymomas and choroid plexus tumors, Carbone *et al*., (30) were able to find SV40-like DNA sequences in 29 out of 48 (60%) of frozen tissue DNA extracts of mesotheliomas, compared to 1 of 28 of normal appearing background lung tissue obtained from the same series of cases. In addition, none of the DNA extracts obtained from frozen samples of 23 non-mesothelioma lung tumors, 10 non-lung tumors, and 22 tumor and non-tumor associated lung specimens, were found to be SV40 DNA positive. A similarly selective association of SV40 DNA with human pleural mesothelioma was corroborated by studies of two independent groups (in Italy and Wales, respectively) on DNA extracts obtained from formalin-fixed paraffin-embedded archival tissue specimens (31,32).

In a subsequent study, Carbone *et al*., (33) found the presence of SV40-related DNA to be selectively restricted to bone tumors amongst a wide range of other tumours examined. From this it became apparent that the range of SV40 DNA positive types of human neoplasms was similar to the range of tumors the virus was capable of inducing in hamsters. The studies also suggested that the virus was endemically prevalent in the human population, since some of the SV40 DNA positive bone tumors belonged to children born of mothers themselves born well after the contaminated vaccine period.

The nature and weight of the available molecular evidence at this stage was sufficient to prompt the NIH and the FDA in the USA to organise a special International Workshop in January 1997 to evaluate the significance of SV40-like DNA detection in human tumors. Both scientists and members of public attended the meeting, and while there was a general acceptance of SV40 as a possible endemic human polyoma virus, a consensus on its role as a potential human carcinogen could not be reached. This was largely due to a) the lack of SV40 DNA in human mesothelioma reported at the meeting by Shah (34); b) the reports of SV40 DNA detection in normal tissues and body fluids (35) and in non-malignant tissue (36); and c) the inadequacy of epidemiological support, as presented by Olin and Giesecke (37) and Strickler and Goedert (38) - (see the summary of the meeting conclusions by Lewis, (39)).

Since the meeting in 1997, new epidemiological and molecular evidence has nevertheless emerged to revive the interest in the potential oncogenic role of SV40 in mesothelioma and other types of human tumors. The rest of this review is therefore devoted to examination of the old and new information, with the particular aim of evaluating the potential pathogenic significance of SV40 DNA detected in human pleural mesothelioma.

3.4. Epidemiological evidence

Large sample based cohort studies offer probably the most reliable approach for analysing the possible influence on cancer incidence or mortality rates resulting from exposure to a single a potential carcinogen such as SV40 contaminated poliovirus vaccine.

The earliest relevant cohort study was conducted by Fraumeni *et al*., (40) which compared 6 million 6-8 year old American children selected from regions exposed to batches of IPV containing either high or low titres of SV40, or no SV40. The study showed no significant differences in cancer mortality directly attributable to immunisation with SV40-contaminated vaccine. Though the study compared large sample sizes derived from the same birth cohorts, the subjects selected were not immunized in infancy; the age of highest susceptibility to SV40 induced tumour formation according to the animal studies (41). Also, the follow-up period was too short for the detection of cancers with more than 4-year latent periods. However, a subsequent study on neonates exposed to SV40 contaminated OPV and IPV, also showed no cancer related deaths after a 17-19 year follow-up period. The study was however conducted on only a small sample of 1073 children, and the follow-up period was still not long enough to accommodate the particularly lengthy latent period associated with mesothelioma.

A 22 year follow-up study in Germany by Geissler (21), examined cancer incidence in 0.9 million

infants, 86% of whom were immunised with SV40 contaminated OPV between 1959-1961, and compared it with that observed amongst a similar number of infants born in 1962-1964, given SV40-free vaccines. No significant difference in overall cancer incidence was observed. However, 25% of brain tumours observed in the SV40-contaminated vaccine exposed group were found to show the presence of SV40 DNA and large T antigen expression in approximately 25% (14/53 and 18/68, respectively) (42). This finding was corroborated by the detection of large T antigen expression in 2/39 brain tumors by Tabuchi *et al*., (43) as well as that of SV40 messenger RNA in 11/32 brain tumors by Ibelgaufts and Jones (44).

A cohort-based study by Heinonen *et al*., (45) examined 50,000 children born of mothers reportedly either exposed or not exposed to SV40-contaminated IPV at the time of pregnancy. The data showed a significant increase in the rate of malignancy in the exposed group (14/18,342 vs. 10/32,555; $p<0.05$), with a particular bias towards tumours of neural origin.

The largest and the longest cohort study conducted to date relates to that published by Strickler *et al*., (46). They examined the data available from the Surveillance, Epidemiology, and End Results (SEER) programme (1973-1993), Connecticut Tumour Registry (1950-1969) and national mortality statistics (1947-1973), in order to measure the relative risk (RR) of cancer types closely related to SV40 associated animal tumours (i.e. ependymomas, osteosarcoma, and mesothelioma). For this, cohorts of SV40 "exposed" children born between 1956-1962 versus "unexposed" children born between either 1947-1952 or 1964-1969 were examined. The results of this more than 30-year retrospective follow-up study showed no increase in RR for ependymomas or osteosarcoma. However, they did show a 3.3-fold increase in RR for mesothelioma (RR 8.0 versus 2.45, respectively), amongst the exposed birth cohort despite the fact that they have not yet reached the age at which most mesotheliomas occur.

Fisher *et al*., (47) have since challenged the negative conclusions drawn by Strickler *et al*., (46) from the SEER data, mainly on the basis that their comparison of cancer incidence in the SV40 contaminated vaccine exposed and non-exposed populations, did not use age-matched cohorts. Re-analysing the same data matched for age, Fisher *et al*., (47) have found increased age-adjusted incidence rates of ependymomas (37%), osteogenic sarcoma (26%), other bone tumors (34%) and mesothelioma (90%) among 2 million potentially exposed to SV40 contaminated poliovaccines as compared to 2 million unaffected age-matched cohort related individuals. Fisher *et al*., (47) have however, emphasised that the small number of mesotheliomas occurring in young individuals has prevented studies of significance and that the apparently major difference observed in their incidence in the exposed cohort may simply reflect the very limited number of mesotheliomas occurring in this age population.

Fisher *et al*., (47) have argued that the available epidemiological data do provide a sufficient basis to pursue SV40 as a potential risk factor in the development of mesothelioma and certain forms of brain and bone tumours. They have proposed the need for multidisciplinary investigations involving descriptive epidemiological studies based on larger, age-matched samples and analytic case-control or cohort design based approach, to more accurately define the power of epidemiological association of SV40 with human cancer.

Strickler and Goedert (38) have equally emphasised that their largely negative findings should not be interpreted as strong evidence against a possible role for SV40 as a human pathogen. They have argued that the relatively low titres of live SV40 associated with IPV, and the observed lack of SV40 viraemia associated with contaminated OPV (48), may not have amounted to a significant increase in exposure to the virus above its endemically circulating levels in the community. They have therefore highlighted the need for examining the incidence and mode of transmission of SV40 in the general community.

Investigation of SV40 DNA presence in blood and tissue samples obtainable from healthy and tumour affected individuals with or without history of SV40 exposure, offers a possible way for obtaining the epidemiological information espoused by Fisher *et al*., (47) and Strickler and Goedert (38). However, the reproducibility as well as reliability of the methodology presently available for SV40 DNA analysis has been questioned by Strickler *et al*., (49) Griffiths *et al*., (50), Volter *et al*., (51) and Mulaterro *et al*., (52). It is therefore important to re-evaluate these in the light of the overall information available.

3.5. Reproducibility and reliability of SV40 DNA detection

The reproducibility of SV40 DNA detection by the PCR technique has been challenged by Strickler *et al*., (49) and Mulatero *et al*., (52), on the basis of their failure to detect SV40 DNA in 50/50 and 12/12 specimens of human pleural mesothelioma, respectively. The molecular studies by both these groups were conducted on paraffin wax embedded sections. Both have contended however, that their failure has not been due to either the poor quality of extracted DNA or a lack of adequate sensitivity of the PCR assays used. Strickler *et al*., (49) have emphasised that the lack of SV40 DNA detection was matched by an equal lack of SV40-specific neutralising antibodies in sera obtained from both mesothelioma or osteogenic sarcoma, as well as cancer-free controls. Their data therefore contradict those of Carbone *et al*., (30) who showed the presence of SV40-like DNA in 29/48 (60%) of mesothelioma tumor samples as well as large T antigen reactive antibodies (detected by immunoprecipitation and Western blot studies and not by neutralisation of plaque forming activity of the SV40 virus) in 13/16 (82%) serum samples from the same case series.

Since 1996, however more than 30 different laboratories have independently detected the presence of SV40 DNA in mesotheliomas, osteosarcoma, bone tumors and a variety of brain tumors (see reviews (28,53-55)). As

discussed next, it seems that methodological differences may account for the contradictory results obtained.

3.6. Sensitivity of SV40 DNA detection

The PCR technique of Bergsagel *et al*., (29) used originally for the detection of SV40-like DNA sequences in ependymoma and choroid plexus tumors, has become the principal method used by the majority of the groups to study the molecular presence of SV40 in human pleural mesothelioma (see Table 1). The method essentially involves extraction of the DNA from the tumor sample, and PCR amplification of the extracted DNA with primer sets designed to first check the quality of the extracted DNA and then the presence in it of any SV40 DNA-like sequence(s).

The primers (SV.for3/SV.rev) most frequently used for the identification of SV40-like DNA, have been designed to amplify the sequence that codes for Rb, p107 and Rb2/p130 binding domain of the large T antigen, which is central to the oncogenic action of SV40. The pertinent details of the conditions of DNA extraction and the application of the PCR technique used by various groups for SV40 DNA detection in human pleural mesothelioma specimens are summarised in Table 1.

3.7. Incidence of SV40 DNA Detection in human tumors

To date at least 23 independent groups have published data on the PCR based detectability of SV40 DNA specifically in human pleural mesothelioma samples. The incidence rates of SV40 detection recorded by each of these groups using the most commonly employed SV.for3/SV.rev primer pair as well as other sets of primers are summarised and compared in Table 1. The incidence rates are seen to range from 0% to 100%.

The wide variability in the detection rates may be partly explained by the relatively small number of cases analysed in each study. However, it may be due to a combination of differences in the quality of the tissue (e.g. unfixed frozen versus formalin-fixed paraffin-embedded specimens), the respective efficiency of the DNA extraction, PCR amplification and product detection methods, as well as demographic differences of the cases, used by the various groups.

Strickler *et al*., (49) have claimed the sensitivity of their PCR technique to be sufficient to detect 10-100 copies of SV40 genome per PCR reaction mixture. This compares less well with the detection limit of 1-10 genome copies per PCR reaction claimed by Carbone *et al*., (30). Mulatero *et al*., (52) have however claimed analytical sensitivity of 1-10 genome copies, but do not state whether this is per cell or per final PCR reaction mixture. Mulatero *et al*., (52) in addition did not use the Southern blotting or filter hybridisation method used by Carbone *et al*., (30) and others (see Table 1), required for detection of the products of amplification more sensitively. Thus Griffiths *et al*., (50) have stated a dramatic increase in the sensitivity of detection with the use of Southern blotting based on probes targeted at SV40 large T antigen gene sequence. Accordingly, only 4 cases were found by them to be positive after ethidium bromide staining compared to 26/26 after filter hybridisation.

Different methods used for DNA extraction may account for the variable detection rate, since the spooling technique used for high quality DNA extraction from fresh tissue is inapplicable to formalin-fixed paraffin-embedded tissue material. Pass *et al*., (56) have nevertheless emphasised the advantage of centrifuging DNA at high speed following phenol/chloroform extraction, for more efficient isolation of small molecular weight DNA including any episomal SV40 DNA.

Inadequate sampling of the tumor specimen is yet another possibility, which could account for the lack of SV40 detection. In mesothelioma biopsies the tumor component in some cases may be the minor component, the major portion being represented by various reactive elements. Thus if the tumor component in the biopsy material is poorly represented or the total amount of biopsy material from which DNA extracted is small, then there would be an increased chance of failure of detection of the SV40 in such a sample. In addition, notwithstanding open thoracotomy biopsy samples, antemortem mesothelioma biopsies are most likely to be tru-cut or fine needle aspirate biopsies, and therefore significantly small enough to suffer from the problems of sampling errors as illustrated by the recent work of McLaren *et al*., (57). The latter situation is likely to be further compounded by any intratumoral variation in SV40 distribution of the type noted by Arrington *et al*., (58).

In the above context, it is noteworthy that Strickler *et al*. (49) had used for all their cases minute amounts of DNA extracted from individual paraffin wax sections scraped off glass slides. Similarly, Mulatero *et al*., (52) had relied upon antemortem diagnostic biopsy material from which 200 ng of extracted DNA was used per PCR reaction, instead of the more optimal amounts of 500 to 1000 ng DNA employed by laboratories producing consistently positive results. In this context Shah (59) has estimated the amount of DNA obtainable from up to 5000 cells contained within a standard 5 mm thick paraffin wax section taken from a tissue block biopsy. Assuming an average of 6 pg of genomic DNA/diploid cell, around 50 ng of DNA is expected from a standard 5 micron paraffin wax section. Hence, for 500-1000 ng of DNA required for reproducible PCR detection of SV40 DNA (cf (60)), 10-20 x 5 micron paraffin sections are likely to be needed.

Finally, the Finnish and Turkish mesothelioma studies of Hirvonen *et al*., (61) and Emri *et al*., (62), respectively have helped to demonstrate the possible marked effect of demographic differences on the rate of SV40 DNA detection (see Table 1).

3.8. Specificity of SV40 DNA detection

The question of specificity of SV40 detection has been challenged on two grounds. The failure of Strickler *et al*., (49) to detect SV40 in their samples has been used as evidence to contend that the laboratories which have scored positive reactions may have done so on the basis of intra-

Table 1. Methodological details of PCR based analysis of human mesothelioma tissue used by different laboratories

Primer pair	Amplicon size	Position within the genome	Type and amount of tumour sample used for DNA extraction	PCR conditions	Band visualization method	Detection rate	Ref
SVfor3 (4476-4453) Svrev (4399-4372)	105bp	4372-4476	F tissue	40,52ºC	FH	29/48 (60.4%)	30
SVfor3 (4476-4453) Svrev (4399-4372)	105bp	4372-4476	PE tissue	40,52ºC	FH	8/18 (44.4%)	81
SVfor3 (4476-4453) Svrev (4399-4372)	105bp	4372-4476	F and PE tisuue, 100ng/reaction	HS, 45, 63ºC	EB	13/28 (46%)	82
SVfor3 (4476-4453) Svrev (4399-4372)	105bp	4372-4476	PE tissue, 300-500ng DNA/reaction	40, 52ºC	FH	26/26 (100%)	50
SVfor3 (4476-4453) Svrev (4399-4372)	105bp	4372-4476	PE tissue	45, 53°C	EB	5/11 (45.4%)	83
SVfor3 (4476-4453) Svrev (4399-4372)	105bp	4372-4476	F tissue 500ng/reaction	40, 52ºC	EB SEQ	9/25 (36%)	84
SVfor3 (4476-4453) Svrev (4399-4372)	105bp	4372-4476	F tissue	45, 52ºC	FH	38/42 (90%)	56
SVfor3 (4476-4453) Svrev (4399-4372)	105bp	4372-4476	PE tissue	45, 52ºC	EB	4/9 (44.4%)	32
SVfor3 (4476-4453) Svrev (4399-4372)	105bp	4372-4476	F and PE tissue	40,52ºC	EB	57/118 (48%)	77
SVfor3 (4476-4453) Svrev (4399-4372)	105bp	4372-4476	PE tissue scraped from slides	40, 52ºC	FH	0/50 (0%)	49
SVfor3 (4389-4412) sic Svrev (4308-4334) sic	105bp	4308-4412 sic	Cell lines and biopsies	45, 52ºC	EB	5/5 (100%) cell lines 7/7 (100%) biopsies	57
PYV.for (4574-4552) Pyv.rev (4425-4402)	172bp	4402-4574	F tissue	40,52ºC	FH SEQ	29/48 (60.4%)	30
PYV.for (4574-4552) Pyv.rev (4425-4402)	172bp	4402-4574	F tissue	40, 52ºC	FH	10/21 (47.6%)	36
PYV.for (4574-4552) Pyv.rev (4425-4402)	172bp	4402-4574	PE tissue	40,52ºC	FH SEQ	11/26 (42.3%)	50
PYV.for (4574-4552) Pyv.rev (4425-4402)	172bp	4402-4574	PE tissue 200ng DNA/reaction	40, 52ºC	EB	0/12 (0%)	52
PYV.for (4574-4552) Pyv.rev (4425-4402)	172bp	4402-4574	PE tissue	45, 52ºC	EB	4/9 (44.4%)	32
PYV.for (4574-4552) Pyv.rev (4425-4402)	172bp	4402-4574	PE tissue	HS, 45,52ºC	FH SEQ	32/62 (53%) E MM 17/21 (81%) MM	85
PYV.for (4574-4552) Pyv.rev (4425-4402)	172bp	4402-4574	PE tissue, pleural effusions	HS,44, 60ºC	FH	9/23 (39.5%) tissue 5/12 (42%) pleural effusions	86
PYV.for (4574-4552) Pyv.rev (4425-4402)	172bp	4402-4574	F tissue	45, 52ºC	FH SEQ	29/48 (60.4%)	60
PYV.for (4574-4552) Svrev (4399-4372)	202bp	4372-4574	PE tissue scraped from slides	40,52ºC	FH	0/50 (0%)	49
Svfor2 (4945-4970) Svrev (4399-4372)	574bp	4945-4372	F tissue 1µg DNA/ reaction	40, 62ºC	EB	0/49 (0%)	61
Svfor2 (4945-4970) Svrev (4399-4372)	574bp	4945-4372	F tissue	45, 55ºC	FH	10/42 (25%)	56
Svfor2 (4945-4970) Svrev (4399-4372)	574bp	4945-4372	PE tissue, pleural effusions	HS,44, 60ºC	FH	9/23 (39.5%) tissue 5/12 (42%) pleural effusions	86
Svfor2 (4945-4970) Svrev (4399-4372)	574bp	4945-4372	F tissue	45, 55ºC	FH SEQ	29/48 (60.4%)	60
SVfor2 (4857-4881) sic Svrev (4308-4334) sic	574bp	4308-4881 sic	Cell lines and biopsies	45, 55ºC	EB	0/5 (0%) cell lines 0/7 (0%) biopsies	57
SV8 (2821-2838) SV9 (2548-2565)	289bp	2548-2821	Cell lines and biopsies	45, 62ºC	EB	5/5 (100%) cell lines 7/7 (100%) biopsies	57
SV5 (4551-4570) SV6 (4402-4425)	169bp	4402-4570	F tissue	45, 59ºC	FH SEQ	10/12 (83%)	63
SV1 (not specified) SV2 (not specified)	574bp	(not specified)	PE tissue (Turkey, Italy)	not specified	EB	0/29 (0%) Turkey 1/1 (100%) Italy	62
RA3 (358-336) RA4 (5119-5142)	413 (485)bp	358-5119	PEtissue	2x35, 52ºC	EB, SEQ	1/1 (100%)	58
RA3 (358-336) RA4 (5119-5142)	482bp	5142-336	PE tissue	40, 64ºC	FH	10/18 (55.5%)	81
RA1 (266-245) RA2 (5195-5218)	242 (315)bp	266-5195	PE tissue	2x30, 52ºC	EB, SEQ	1/1 (100%)	58
R1 (266-245) R2 (5195-5218)	315bp	266-5195	F tissue	40, 60ºC	EB	22/42 (52.3%)	56
Not specified	739bp	4400-5138	Cell lines	35, 58ºC	EB	5/5 (100%)	78
TA1 (3070-3049) TA2 (2630-2652)	441bp	2630-3070	PE tissue	2x35, 52ºC	EB, SEQ	1/1 (100%)	58
T3 (not specified) T4 (not specified)	338bp	(not specified)	PE tissue (Turkey, Italy)	not specified	EB	0/29 (0%) Turkey 1/1 (100%) Italy	62
C-terminus of Tag	329bp	2573-2902	F tissue	45, 59ºC	FH SEQ	10/12 (83%)	63

EB= ethidium bromide staining, FH= filter DNA hybridisation , F= snap-frozen, PE= paraffin-embedded

Primers' sequences (5'-3')			
Waheed et al.	AGTCCTCGAGTCTTTGCAGCTAATGGACCT AGTCTCTAGATCCTTTGTGGTGTAAATAGC		
SVfor3	TGAGGCTACTGCTGACTCTCAACA	SV6	GGAAAGTCCTTGGGGTCTTCTACC
SVfor2	CTTTGGAGGCTTCTGGGATGCAACT	RA1	AATGTGTGTCAGTTAGGGTGTG
SVrev	GCATGACTCAAAAAACTTAGCAATTCTG	RA2	TCCAAAAAAGCCTCCTCACTACTT
PYVfor	TAGGTGCCAACCTATGGAACAGA	RA3	GCGTGACAGCCGGCGCAGCACCA
PYVrev	GGAAAGTCTTTAGGGTCTTCTACC	RA4	GTCCATTAGCTGCAAAGATTCCTC
T3	ACCACAACTAGAATGCAGTGAAAAAA	SV5	TAGATTCCAACCTATGGAACTGAT
T4	GAAGACAGCCAGGAAGAAAATGCTGATAA	SV6	GGAAAGTCCTTGGGGTCTTCTACC
R1	AATGTGTGTCAGTTAGGGTGTG	SV8	GCCAGGAAAATGCTGATA
R2	TCCAAAAAAGCCTCCTCACTACTT	SV9	GATGCTATTGCTTTATTT
SV1	CTTGGAGGCTTCTGGGATGCAAACT	TA1	GACCTGTGGCTGAGTTTGCTCA
SV2	GCATGACTCAAAAAACTTAGCAATTCTG	TA2	GCTTTATTTGTAACCATTATAAG
SV5	TAGATTTCCAACCTATGGAACTGAT		

and/or inter-laboratory sample contamination with laboratory strain SV40 DNA. Griffiths *et al*., (50) have estimated the risk of such cross-contamination to be quite high due to the now long (20 years) and widely distributed usage of the SV40 early region base vectors and gene constructs. Testa *et al*., (63) have addressed this criticism by undertaking a study of blinded DNA samples extracted from 12 mesotheliomas, distributed to 4 independent laboratories, three of which had never before worked with SV40. Strict precautions were taken to avoid any contamination of the samples with SV40 during DNA extraction, by undertaking the extraction procedure in a single independent center not previously exposed to SV40 work. This study was able to verify the presence of SV40 in 9 out of 12 (75%) mesotheliomas with complete concordance. In addition, the positive and negative controls were consistently found to yield the expected results in all 4 laboratories. Recently, Hirvonen *et al*., (61) and an independent American laboratory, have shown high reliability of SV40 detection by consistently demonstrating SV40 positivity in 0/49 Finnish and 3/3 American mesothelioma specimens, using a single blind analysis approach.

To increase the specificity of SV40 detection, several researchers have resorted to the use of multiple primer sets to identify genomic sequences from different parts of the SV40 genome as well as sequence specific variation, as a mark of the authenticity of SV40 DNA detection (see Table 1, reviews (28,53-55), and also below for further details).

Despite this doubts have persisted about the authenticity of SV40 DNA detected in human tissues, and it has proved necessary to organise a multi-institutional study involving 9 laboratories under the auspices of FDA in the United States. The results of this study are awaited (Strickler *et al* - submitted).

4. BIOLOGICAL EFFECTS OF EXPRESSION OF SV40 GENE PRODUCTS IN HUMAN TUMORS

4.1. Biological activity and physical state of SV40 DNA detected in human mesothelioma

SV40 is an oncogenic virus and the large T antigen, Tag, is known to mediate the bulk of its oncogenicity (reviewed in (64)). The oncogenic effect of Tag is mediated through its capacity to bind and inactivate the activity of two powerful tumor suppressor gene products, p53 and retinoblastoma protein, Rb, and related proteins p107, p130. This leads to a powerful combination of loss of cell cycle regulation combined with marked genomic instability, responsible for SV40 induced cell transformation, immortalisation, and tumor formation. It is noteworthy that the 105 bp DNA segment most frequently used to detect SV40 DNA by PCR in mesotheliomas, encodes the highly conserved pocket binding domain of Tag, responsible for the binding to the Rb family of proteins.

Hence, one could argue that the detection of 105 bp SV40 Tag DNA sequence in a high proportion of tumor specimens is indicative of a possible oncogenic role of SV40 in human mesothelioma. However, in order to ascertain whether this is so, it is necessary to analyse the biological state of the SV40 DNA associated with the tumor cells. In particular, it is important to determine whether its presence is associated with active expression of oncogenically active Tag protein. It is also important to determine whether the DNA is exclusively present in the tumor cells and in what proportion of them. Furthermore it is important to analyse whether it is present in its infective (i.e. packaged within a virus particle), episomal (i.e. in a free molecular form) or integrated (i.e. linked to the host cell genomic DNA) states.

Largely due to the paucity of the tumour tissue available for analysis, only a limited amount of information has been gathered so far regarding the biological and physical state of the SV40 DNA detectable in human mesothelioma and other types of human cancer. The available information is reviewed briefly below to evaluate the putative oncogenic potential of SV40 in human mesothelioma.

4.2. Characteristics of SV40 DNA associated with human and animal tumors

In snap-frozen tumor specimens obtained from 42 consecutive cases of pleural and peritoneal mesotheliomas, Pass *et al*., (56) were able to ascertain using the PCR technique the presence of the SV40 specific 105 bp DNA segment in 90% of the cases. Using Svfor2/SVrev primers they were able to show the presence of the larger region of amino terminus Tag in 25% of cases. In addition, 38% specimens contained sequences of the carboxyl terminus important for tissue specific replication of SV40, and 52% showed amplification of the regulatory region. In 4 cases analysed further this was found to be identical to that seen in hamster mesothelioma which was derived from the laboratory-propagated virus strain 776 associated with two 72 bp enhancer elements. Duplication of the 72 bp repeat elements is apparently important in conferring a growth advantage to SV40 during its *in vitro* culture. Overall 24% of the mesotheliomas studied yielded amplification of all the three regions, suggestive of the presence of full length SV40 DNA either in its viral, episomal and/or integrated state.

The infective viral state of SV40 genome has so far been isolated from only one choroid plexus tumor (65). In 14 out of the 17 SV40 positive tumors in this series, the viral DNA appeared to be predominantly in its full-length episomal state. The same was true of the viral DNA associated with human osteosarcoma (66). In addition, the tumor associated viral DNA sequences appeared to represent authentic natural strains of SV40 with only one 72bp enhancer element, distinct from the laboratory isolates of SV40 776, SV40-Baylor and VA45-54 strains containing two-enhancer element. This is in contrast to the two-enhancer elements associated with SV40 DNA found in the mesotheliomas studied by Pass *et al*., (56), and the thyroid papillary carcinomas described by Pacini *et al*., (67). Interestingly, in osteosarcoma both one and two-enhancer elements have been found to be involved, as described by both Lednicky *et al*., (66) and Carbone *et al*., (33).

In the brain tumor study (65), each tumor appeared to be associated with a single homogenous viral DNA species defined by its unique sequence related to the variable domain (i.e. the last 86 amino acids - 622-708) of the C-terminus of large T antigen. This is in contrast to the mixture of genetically heterogeneous SV40 DNA isolated from the brain tissue of immunocompromised monkeys exhibiting disseminated viral infection (68).

In most of the above studies, the possible integrated state of the virus could not be confirmed or excluded mainly due to insufficient tumor DNA being available. Nevertheless, Southern blot based evidence of the integrated state of the viral DNA has been noted in 3 out of 69 human papillary thyroid carcinomas (67), and 5 out of 10 cases of osteosarcoma studied by Mendoza *et al*., (69).

4.3. Cellular evidence of SV40 activity in tumor cells

The mode of action of SV40 is well characterised and SV40 gene products are used as laboratory tools to extend cellular lifespan.

A study by Carbone *et al*., (70) examined the potential biological effect of SV40 in mesothelioma by investigating (i) p53 expression levels; (ii) whether p53 mutations were apparent; (iii) potential Tag and p53 interactions. Carbone's survey of 51 mesotheliomas revealed that 50% showed p53 immunopositivity. In addition this correlated with co-expression of SV40 Tag which was confirmed by in situ hybridisation used to detect the messenger RNAs of the two proteins.

P53 protein is normally rapidly turned over due to its association with Mdm2 that regulates its degradation by a proteosome pathway (71,72). P53 immunopositivity indicates that the protein has been stabilised in some way. This can occur by interaction with other proteins such as Tag or by the acquisition of mutations both of which prevent p53 degradation.

Single-strand conformational polymorphism (SSCP) analyses of exons 5-9 of p53 from 25 mesotheliomas were performed. These exons are considered to be "hotspots" for p53 mutation. SSCP analysis showed that there were no detectable p53 mutations in 23/25 of the mesotheliomas tested. This implied that the cells were tolerating high levels of wild type p53, a phenomenon which has been observed in a subset of epithelial tumors (73). However the stabilisation in mesothelioma seems to be due to the presence of SV40 Tag. Indeed anti p53 antibodies co-immunoprecipitated Tag from lysates derived from mesotheliomas. SV40 Tag derived from lysates was able to bind *in vitro* translated p53 protein. As a final control p53 in Tag expressing cells failed to induce expression of p21, a cyclin dependent kinase inhibitor which mediates the G1/S cell cycle checkpoint.

SV40 Tag is unusual in that it can also interact with and inactivate other tumor suppressors. These include members of the Rb gene family pRb (the retinoblastoma protein), p107 and p130. In a related study (74) Rb family proteins were shown to be overexpressed in mesothelial cells which contained SV40 DNA sequences. A subset of these were tested and possessed Tag protein detectable by immunoprecipitation. This study was not as robust as Carbone's as interaction between Rb proteins and Tag was not shown. However, *in vitro* translated pRb, p107 and p130 did interact with Tag immunoprecipitated from mesothelioma cells.

Zhen *et al*., (75) have recently independently demonstrated physical complexes of Tag with p53 and pRb in multiple mesothelioma and brain tumour extracts. The pathogenic significance of these complexes is however dependent on the extent of their distribution within the tumour cell population as well as the degree of cell transformation activity associated with them. The work of Ramael *et al*., (76), Shivapurkar *et al*., (77) and Waheed *et al*., (78) has thrown useful light on this questions. Shivapurkar *et al*., (77) using a laser capture microdissection approach have shown SV40 DNA to be exclusively distributed to tumour cell populations in a large number of mesothelioma samples. Ramael *et al*., (76) using a combination of an in situ DNA hybridisation method and immunocytochemistry have shown SV40 DNA and viral proteins (e.g. small t) to be distributed within mesothelioma tumour samples in the same proportion of the tumour cells. Finally, the work of Waheed *et al*. (78) using Tag specific anti-sense oligonucleotide transfection of several SV40 DNA positive primary human mesothelioma cell lines, has suggested that the Tag/p53/pRb complexes are biologically active and also widely dispersed amongst the tumour cells.

5. PERSPECTIVE

The main conclusion to be drawn from this review is that before one can attribute a plausible causal link between SV40 and mesothelioma, it is important to ensure that SV40 DNA is adequately, reproducibly and reliably detected in the biopsy material available for analysis. Secondly, if adequate amounts of fresh tissue are available, it is important to analyse the biological and physical state and activity of the SV40 DNA associated with the tumor cells.

The presence of SV40 DNA in human pleural mesotheliomas has been verified by multiple independent laboratories. The lack of detection shown by two laboratories appears to be due to either the lower sensitivity of the overall methodological approach used, or demographic differences associated with tissues selected for analysis. For a reliable and reproducible detection of SV40 in human tumor samples it is necessary to use a sufficient amount of efficiently extracted good quality DNA, in conjunction with a high sensitivity PCR technique.

The epidemiological cohort data when adjusted for age show rising trends in the incidence of CNS tumors and mesothelioma selectively in the population exposed to polio/adenovirus vaccines contaminated with SV40 virus.

There is also sufficient cellular evidence to suggest functional and potentially oncogenic presence of SV40 DNA in human mesothelioma tumour cells.

The causal link between SV40 and mesothelioma and its association with the known carcinogenic involvement of asbestos however still needs to be examined further in detail. Nonetheless the recent paper of Bocchetta *et al*., (79) has thrown some useful light on this issue. They have shown for the first time that the infection of human mesothelial cells by SV40 is different from the semipermissive infection thought to be characteristic of most other human cells. Thus mesothelial cells are uniformly infected but yet lysed by the viral genome presence. More remarkably, it has been noted that asbestos is able to complement replication-defective (ori-) SV40 mutants in the cell transformation process. Overall these data offer the first mechanistic explanation for the ability of SV40 to transform mesothelial cells preferentially and provide supportive evidence for a possible co-carcinogenic association of asbestos and SV40 in the causation of mesothelioma. One possible way forward for examining the *in vivo* significance of these data is to set up an animal model in which the combined carcinogenic potential of asbestos and SV40 is tested directly using *in vivo* induction of pleural mesothelioma as the end point. For this it may be efficacious as well as pertinent to introduce the two carcinogens simultaneously through inhalation of SV40 coated asbestos fibres.

The oncogenic effect of SV40 in animal models has been found to require prolonged survival of virally transfected cells to allow virus induced transformation and immortalisation to take place. This is likely to be permitted at least in part by a lack or suppression of an adequate cytotoxic T-cell response against the oncogenic early antigens of the virus. Boosting of the cell mediated immunity may therefore help to destroy not only the early virally infected state but also neoplastically transformed cells, as well as established tumors (80). This could have important implications for a more rational approach to both prevention and treatment of human mesothelioma, a tumor for which currently there is no effective treatment.

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