Virchows Arch (1999) 435:1–7

ORIGINAL ARTICLE

Karl Dhaene · Anja Verhulst · Eric Van Marck

SV40 large T-antigen and human pleural mesothelioma

Screening by polymerase chain reaction and tyramine-amplified immunohistochemistry

Received: 14 September 1998 / Accepted: 22 February 1999

Abstract DNA-like sequences of the p53 and pRB-inactivating simian virus 40 large T-antigen (SV40 LTag) have recently been found in mesotheliomas in the United States and several European countries. Nuclear expression of SV40 LTag, possibly in concert with detectable telomerase activity, could be responsible for immortalisation of (pre)malignant clones, as suggested by the mesothelioma-specific latency period. Depending on the antibody used, different results have been observed regarding the subcellular expression of SV40 LTag in mesotheliomas with SV40 LTag-like DNA sequences. In this study, we screened 28 Belgian mesothelioma tumour samples for the presence of SV40 LTag-like DNA and its gene product by polymerase chain reaction amplification, using the SV.for3/SV.rev primer set, and by tyramine-amplified immunohistochemistry, using the pAb419 and the pAb101 SV40 LTag antibodies. Amplicons were found in 13 of the 28 (46%) mesotheliomas. Cytoplasmic, but no nuclear, staining was found in 10 of these 13 cases. Although our study confirms the presence of SV40 LTag-like DNA sequences in Belgian mesotheliomas, we did not detect nuclear expression of the viral oncoprotein, which makes a pathogenic role of SV40 LTag in mesothelioma carcinogenesis questionable.

Key words Mesothelioma · SV40 · Telomerase · Immortalisation · Tyramine

Introduction

Malignant mesothelioma is an aggressive tumour arising from the serosal lining of the pleura, peritoneum, or pericardium. Although there is a wealth of epidemiological and experimental evidence supporting a strong association between asbestos exposure and pleural mesothelioma, 11–25% of cases are unrelated to asbestos exposure and only a minority of people exposed to high concentrations of asbestos develop mesothelioma [23, 25]. Other possible aetiological agents include ionising radiation, pleural scarring, and genetic susceptibility. An interesting new development worthy of study is the role of simian virus 40 (SV40) as a risk factor for mesothelioma. SV40 contaminated polio and adenovirus vaccines in the late 1950s and early 1960s and was accidentally inoculated into millions of people in the USA and possibly also in Europe [28]. Experimentally, SV40 has been shown to induce pericardial and pleural mesothelioma in hamsters [4].

The oncogenic capacity of SV40 resides in the ability of the SV40 Large T-antigen (SV40 LTag) to bind to and to inactivate nuclear tumour suppressor proteins like pRB and p53 [6, 20]. As evidenced by in vitro transformation studies, this property – though insufficient alone – paves the way for cellular immortality [34]. The existence of the latter phenotype in mesothelioma (pre)neoplastic cells can be deduced from the mesothelioma-specific decade-long latency period between asbestos exposure and disease onset, and from the detection of activity of the telomerase-ribonucleoprotein, to which immortalising capacities are also attributed on the basis of its ability to maintain the stability and integrity of chromosomal telomeres [9]. DNA sequences similar to sequences encoding the SV40 LTag oncogene (SV40 LTag-like DNA) and nuclear expression of the SV40 LTag oncoprotein have recently been detected in a high proportion of mesotheliomas in the United States, the United Kingdom and Italy (for review, see [3]). However, one study did not detect SV40 LTag-like DNA in mesotheliomas, whilst another revealed cytoplasmic but not nuclear staining when SV40 LTag-specific antibodies were used, making the transforming and oncogenic role of SV40 in mesothelioma questionable [13, 31].

These data prompted us to analyse samples of Belgian mesothelioma cases for the presence of a SV40-like agent. We therefore screened 28 mesotheliomas and 10

K. Dhaene · A. Verhulst · E. Van Marck (✉)
Laboratory of Pathology, University of Antwerp (UIA),
Department of Medicine, Universiteitsplein 1,
B-2610 Wilrijk – Antwerp, Belgium
e-mail: eric.van.marck@uza.uia.ac.be
Tel.: +32-3-821-3604, Fax: +32-3-821-4753

non-pleural lung tumours for the presence of SV40 LTag-like DNA by polymerase chain reaction (PCR) amplification and for the presence of the SV40 LTag protein, using two different anti-SV40 LTag antibodies in a tyramine-amplified immunohistochemical procedure.

Materials and methods

Samples were collected from the files held by the Department of Pathology of the University Hospital of Antwerp and had been diagnosed between 1990 and 1997. This study included frozen tissue material (21 cases) and archival formalin-fixed paraffin-embedded material (7 cases) from a total of 28 primary pleural mesotheliomas. The tumours were histologically subtyped on haematoxylin and eosin-stained sections, with the use of additional histochemical methods for neutral mucins and hyaluronic acid and a relevant panel of antibodies for primary exclusion of primary or secondary adenocarcinoma. Classification into 15 epithelial, 4 sarcomatous, and 9 mixed-type mesotheliomas was made according to the guidelines of Henderson et al. [15]. The mean age of the mesothelioma patients was 63 (range 41–86) years. Additionally, frozen materials from 10 bronchopulmonary carcinomas (5 squamous cell carcinomas and 5 adenocarcinomas) were investigated. The SV40 LTag-transformed murine endothelial cell line SVEC4–10 (American Type Culture Collection no. CRL-2181) was used as a positive control for the detection of SV40 LTag protein and DNA sequences.

Since the problem of false-positive results with PCR is well documented, we took great care to avoid contamination in this study. Three different laboratories were used for DNA extraction, PCR set-up and post-PCR analysis. All reagents other than *Taq* polymerase and primer stocks were pretreated with ultraviolet light, and dedicated pipettes with filtered tips were used for each stage of the analysis. Genomic DNA was extracted from the frozen samples and from the SVEC4–10 cell line using a standard proteinase K-phenol-chloroform extraction method. For PCR analysis of the archived samples, unstained 5-µm sections were deparaffinised and digested with proteinase K without ionic detergents, followed by boiling of the obtained crude lysates as described by Frank et al. [12]. Crude lysates were diluted further 1:10 prior to PCR amplification.

SV40 LTag DNA amplification was performed with the primers designed by Bergsagel et al. [1]: SV.for3 (5′ - TGA GGC TAC TGC TGA CTC TCA ACA - 3′) and SV.rev (5′ – GCA TGA CTC AAA AAA CTT AGC AAT TCT G – 3′). These primers amplify the RB-pocket-binding domain of SV40 LTag (105 bp). Hot-start PCR reactions were performed in a volume of 50 µl containing 1.5 mM $MgCl_2$, 50 mM KCL, 10 mM Tris-HCl pH 8.5, 50 µM of each dNTPs, 25 pM of each primer; 100 ng of genomic DNA or 20 µl of diluted crude lysate of the frozen and the archival samples, respectively, were analysed. Reactions were run in a Perkin-Elmer 9600 Gene Amp PCR System with cycling conditions as follows: 2 U of Taq DNA polymerase (Promega Benelux) were added after an initial denaturing step at 94°C during 3 min, and thermocycling was continued by cycling 45 times at 94°C for 1 min, followed by 63°C for 1 min, and 72°C for 1 min. The reactions were terminated by a 5-min final extension step at 72°C. PCR products were electrophoresed on a 10% non-denaturing polyacrylamide gel in 0.5× TBE buffer and stained with ethidium bromide. Each PCR series was carried out with one negative (omission of template) and one positive (DNA from SVEC4–10 cells) control. The quality of DNA in cases negative for SV40 LTag amplification was assessed using primers AG1 and AG2, which are specific for the amplification of the ubiquitous 379-bp human A gamma-globin gene sequence [19].

All tissues were examined immunohistochemically using two different anti-SV40 LTag antibodies: the anti-SV40 LTag mouse monoclonal antibody pAb419 Ab-1 (Oncogene Science, Calbiochem, Nottingham, UK), applied by us in a conventional peroxidase-based technique, is reactive with both the N-terminal region of the 94-kDa SV40 Large T antigen and the 21-kDa SV40 small t antigen and stains nuclei of SV40 infected cells diffusely. The anti-SV40 LTag pAb101 (Santa Cruz Biotechnology, Santa Cruz, Calif.), applied by us in a tyramine-amplified immunohistochemical procedure using ‘home-made’ biotinylated tyramine based on the method described by King et al. [18], reacts with the 94-kDa SV40 Large T antigen but is non-cross-reactive with the 21-kDa SV40 small t antigen and produces punctuate nuclear staining in SV40 infected cells.

Frozen sections 5 µm thick were cut, mounted on poly-L-lysine-coated slides, air-dried (30 min), and fixed in acetone at 4°C (10 min). The antigen retrieval procedure for paraffin-embedded tissues consisted in pressure-cooking in citrate buffer (0.01 M, pH 6.0) for 5 min. All slides were immersed in methanol supplemented with 1% H_2O_2 for 30 min to block the endogenous peroxidase. After rehydration and rinsing in phosphate-buffered saline (PBS), non-specific binding was blocked with normal goat serum (dilution 1:5, Dako, Glostrup, Denmark) dissolved in PBS supplemented with 2% bovine serum albumin for 20 min. The sections were incubated either overnight at 4°C (pAb419 antibody, dilution 2,5 µg/ml) or for 1.5 h at room temperature (pAb101 antibody, dilution 2 µg/ml). For conventional peroxidase testing, the sections were overlaid with peroxidase-conjugated goat anti-mouse polyclonal antibody (Rockland, Gilbertsville, Pa.) diluted 1:200 as the secondary antibody. In the technique using home-made biotinylated tyramine, sections were washed in PBS (3×5 min), and biotinylated goat anti-mouse antibody (Rockland) diluted 1:400 was applied for 30 min. The sections were again washed 3 times, followed by application of the first streptavidin ABC/horseradish peroxidase (sABC/HRP) complex (Dako, prepared according to the manufacturer’s instructions) for 30 min. Sections were washed 3 times in TNT buffer (0.1 M Tris-HCl; pH 7.6; 0.15 M NaCl; 0.05% Tween 20), incubated with home-made biotinylated tyramine (see below) (dilution 1:160) for 10 min, then incubated again with the second sABC/HRP complex. With each technique, peroxidase was revealed by incubating the sections with liquid DAB solution (Dako). The specificity of the immunohistochemical reactions was controlled as follows: (1) omitting the first antibody; (2) substituting the primary antibodies with an unrelated monoclonal antibody of the same IgG2a kappa isotype at the same concentration but directed against an unrelated antigen (monoclonal mouse anti-human amyloid A component antibody, IgG2a kappa isotype, Dako). Sections were counterstained with Carazzi’s haematoxylin and mounted in DPX (Fluka Chemika-Biochemica, Buchs, Switzerland).

Biotinylated tyramine was prepared according to the method described by Kerstens et al. [17]. Briefly, 100 mg of sulfosuccinimidyl 6-(biotinamido)hexanoate (Pierce, Rockford, USA) and 30 mg tyramine hydrochloride (Sigma Chemical Company, St.Louis, Mo.) were added to 40 ml of 50 mM borate buffer (pH 8.0). The solution was stirred overnight at room temperature and filtered (0.45 µm filter). This solution was dissolved (optimal dilution 1/160, experimentally determined) in Tris-HCl buffer (pH 7.6) containing 0.03% H_2O_2.

Results

Table 1 summarises the results of the detection of SV40-like DNA sequences in respect of the histopathological subtype and patient data. Thirteen of the 28 (46%) mesothelioma samples contained sequences of the predicted length using the primers SV.for3 and SV.rev (Fig. 1). There was an equal distribution of SV40-like DNA sequences in samples taken from frozen (10/21) and those taken from paraffin-embedded tissue (3/7). The mean age of the subjects with SV40-like DNA in the tumour samples was 65 years (range 41–80 years). A 105-bp band was also seen in 1 adenocarcinoma of the lung,

Table 1 SV40 LTag DNA detection and protein immunoreactivity profiles of mesothelioma and lung carcinoma specimens *(MM* mesothelioma, *LC* lung carcinoma, *f* frozen section, *p* formalin fixed paraffin-embedded section, *E* epithelial, *Mix* mixed, *S* sarcomatous, *SQ* squamous carcinoma, *A* adenocarcinoma, *C* cytoplasmic, *N?* presumed nuclear, *ND* not done)

Case no.	Sex – age years	Tumour type	γ-Globin DNA	SV40 LTag DNA	pAb419 Anti-LTag	pAB101 Anti-LTag
MM1 (f)	F - 61	E	+	–	–	–
MM2 (f)	F - 66	E	+	–	C	–
MM3 (f)	M - 61	E	ND	+	C	–
MM4 (f)	M - 68	Mix	+	–	–	–
MM5 (f)	M - 59	Mix	ND	+	C	–
MM6 (f)	M - 67	S	+	–	–	–
MM7 (f)	M - 80	S	ND	+	C	–
MM8 (f)	M - 41	E	ND	+	C	–
MM9 (f)	F - 67	E	ND	+	C/N?	–
MM10 (f)	M - 63	Mix	+	–	–	–
MM11 (f)	M - 78	E	+	–	–	–
MM12 (f)	F - 80	Mix	+	–	–	–
MM13 (f)	M - 53	Mix	ND	+	C/N?	–
MM14 (f)	M - 86	E	+	–	C	–
MM15 (f)	M - 69	Mix	ND	+	C/N?	–
MM16 (f)	M - 65	S	ND	+	–	–
MM17 (f)	F - 64	Mix	+	–	–	–
MM18 (f)	M - 53	Mix	+	–	C	–
MM19 (f)	M - 69	E	ND	+	C	–
MM20 (f)	M - 63	S	+	–	–	–
MM21 (f)	M - 79	E	ND	+	–	–
MM22 (p)	M - 55	E	+	–	–	–
MM23 (p)	M - 65	E	+	–	–	–
MM24 (p)	F - 63	E	+	–	–	–
MM25 (p)	M - 48	E	ND	+	–	–
MM26 (p)	F - 64	E	ND	+	C	–
MM27 (p)	M - 78	Mix	ND	+	C	–
MM28 (p)	F - 42	E	+	–	–	–
LC1 (f)	M - 61	SQ	+	–	–	–
LC2 (f)	F - 73	SQ	+	–	–	–
LC3 (f)	F - 60	SQ	+	–	–	–
LC4 (f)	M - 75	SQ	+	–	–	–
LC5 (f)	M - 69	SQ	+	–	–	–
LC6 (f)	M - 64	A	+	–	–	–
LC7 (f)	M - 75	A	+	–	–	–
LC8 (f)	M - 48	A	+	–	–	–
LC9 (f)	M - 60	A	+	–	–	–
LC10 (f)	M - 59	A	ND	+	–	–

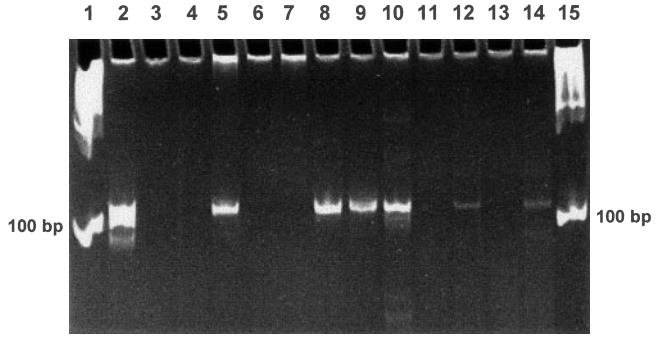
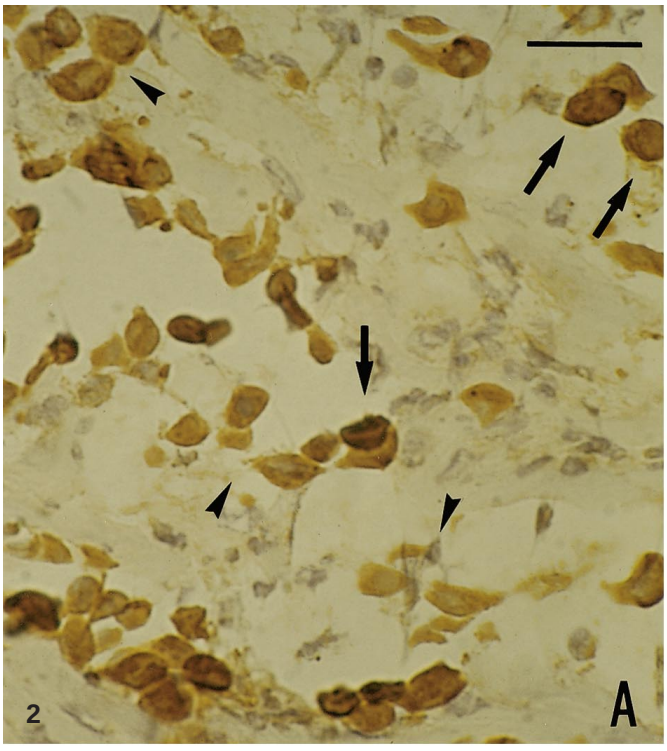
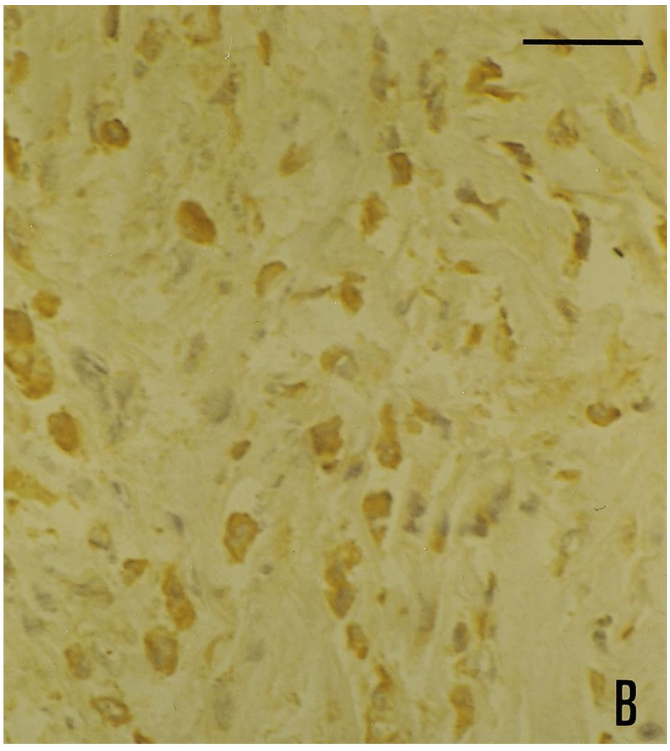


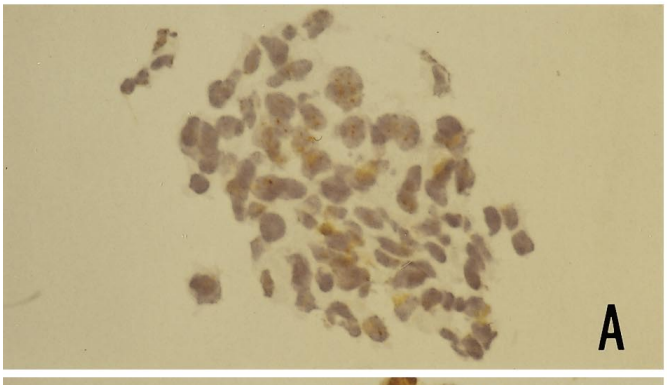
Fig. 1 Representative results demonstrating SV40 LTag primer set-specific DNA amplification in mesothelioma specimens using SV.for3 and SV.rev primers. *Lanes 1, 15* 100-bp DNA ladder (Gibco BRL), *lane 2* SV40 LTag DNA-positive control (SVEC4–10 cells), *lane 3* negative PCR control (template omitted), *lanes 4–14* mesothelioma specimens. A 105-bp band is visible in mesothelioma cases 15, 19, 21, 9, 8 and 16 (*lanes 5, 8, 9, 10, 12* and *14*). PCR products were electrophoresed on a 10% nondenaturing polyacrylamide gel in 0.5× TBE buffer and stained with ethidium bromide

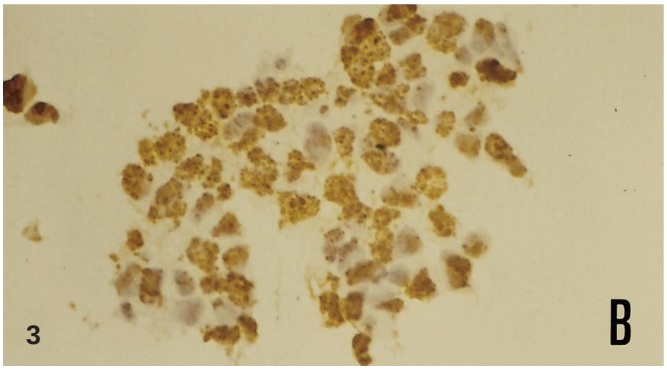
whereas the other 9 lung carcinomas did not prove positive for SV40-like DNA. All of the DNAs that did not contain SV40-like sequences appeared suitable for PCR analysis, since a 379-bp Ig gamma gene sequence could be amplified in the consecutive control reaction [data not shown].

Cryostat and paraffin sections from 28 mesothelioma specimens and from 10 lung carcinomas were evaluated for SV40 LTag expression, using the pAb419 and pAb101 anti-SV40 LTag antibodies in conventional and tyramine-amplified immunohistochemistry, respectively. With the pAB419 antibody, 13 mesotheliomas showed strong cytoplasmic staining in a conventional peroxidase-based procedure, whereas no staining could be detected in any of the lung carcinomas. The percentage of cells with cytoplasmic staining varied from 30% to 80%, and varied within areas of a given specimen. In the mesotheliomas with cytoplasmic immunoreactivity the signal was confined to the tumour cells lining the tubulopapillary structures. Immunoreactivity was not seen in the surrounding stromal compartment (Fig. 2 A). Cytoplasmic staining was also detected in sarcomatous mesothelioma (Fig. 2B). In 10 of the 13 specimens with cyto-









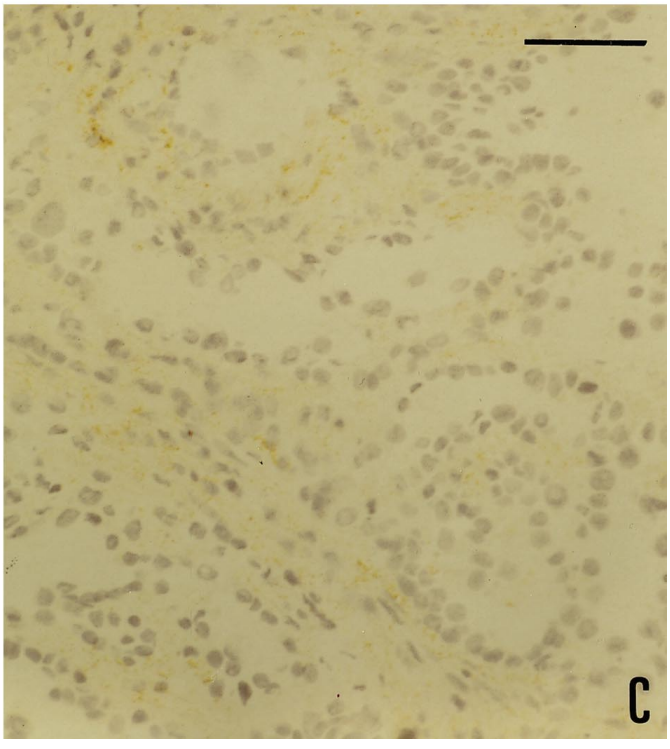


Fig. 2A, B Immunoperoxidase staining with the anti-SV40 LTag mouse monoclonal pAb419 antibody in mesothelioma tumour sections. **A.** Case 9. Cytoplasmic immunoreactivity (*arrowheads*) in tubulo-papillary lining cells with possible nuclear staining (*arrows*) in some cells. Surrounding reactive stromal cells are negative. Original magnification ×400, *scale bar* 25 µm. **B** Case 7. Cytoplasmic staining only in sarcomatoid tumour cells. Original magnification ×400, *scale bar* 25 µm

Fig. 3A–C Immunohistochemical staining with the anti-SV40 LTag mouse monoclonal pAb101 antibody. **A** Immunoperoxidase staining showing discrete punctuate nuclear staining in 60% of SVEC4–10 control cells. Original magnification ×400, *scale bar* 35 µm. **B** 'Home-made' tyramine-amplified immunohistochemistry showing coarse granular staining in nearly 100% of SVEC4–10 cell nuclei. Original magnification ×400, *scale bar* 35 µm. **C** Case 9. Negative 'home-made' tyramine-amplified immunohistochemical staining in mesothelioma. Original magnification ×250, *scale bar* 50 µm

plasmic staining, SV40-like sequences were detected. No staining was found in 15 mesotheliomas, 12 of which also proved negative by PCR analysis. In the remaining 3 cases an immunohistochemical signal could not be detected although they were classified positive using the SV40 primer set. In 3 mesothelioma cases with detectable amplicons and cytoplasmic staining, it seemed possible that strong cytoplasmic staining was masking diffuse nuclear reactivity (Fig. 2A). We therefore re-examined our samples with the pAb101 antibody. In preparative experiments, we tested the pAb101 antibody in a conventional nonamplified procedure and found a discrete punctuate staining pattern in a proportion of the SVEC4–10 control cells (Fig. 3A). On application of tyramine-based signal amplification, however, heavy coarse granular staining was found in nearly 100% of the nuclei of the control cells (Fig. 3B). Using this technique, we did not detect nuclear staining in any of the cases that had been found to be negative or to have cytoplasmic staining only when the pAB419 antibody was used (Fig. 3 C) in the immunohistochemical session referred to above.

Discussion

We screened mesothelioma tumour samples from Belgian patients for the presence of sequences and translation product of the SV40 LTag oncogene. This study was intended as a first screening test and, for the sake of comparison with the data of other workers, we chose the SV.for3/SV.rev primer pair that has been used by several other groups in the highly sensitive PCR assay. Further, the transforming capacity of SV40 LTag depends on its inhibitory binding to p53 and pRB tumour suppressor proteins, and according to this the oncoprotein should be expressed in the cellular nucleus. Up to the present, only groups led by or collaborating with Carbone and one French group have complemented their PCR results with immunohistochemical protein expression studies of mesothelioma tumour sections [2, 8, 13, 32]. We have chosen to work with the two different anti-SV40 LTag antibodies reported by these groups. To make immunohistochemistry as sensitive as possible, we applied homemade tyramine-based immunodetection after modifications of the original protocol [17, 18]. In SV40 LTag-transformed cells the pAb101 antibody produced a discrete punctuate nuclear signal, which we found could be greatly amplified by a tyramine-based procedure.

We detected the 105-bp amplicon in 46% of our mesothelioma cases. The frequency of detection is in keeping with the findings of the other groups and confirms the presence of SV40 LTag-like DNA in Belgian mesothelioma patients. The route of infection of these human tumour samples is difficult to understand, and this discussion indirectly raises the question of whether SV40 is more common in human cancers than in normal healthy tissue. Human exposure to SV40 has been documented in the United States, where about 98 million people were immunised between 1955 and 1962 [28]. It is likely, though not substantiated, that the same contaminated Salk parenteral vaccine was administered in Belgium. All patients from which PCR positive samples were derived were born before 1962 and thus could have been infected by contaminated polio vaccines. In contrast, Martini et al. found SV40 DNA in many normal tissue specimens, including 24% of circulating blood cells and 45% of seminal fluid samples from healthy donors, suggesting that SV40 might be a common human polyomavirus that is prevalent in the general community [22]. In a recent retrospective serological survey individuals born after 1962 had positivity rates of about 10% for SV40-neutralising antibody, which did not differ statistically from the rates (15%) in older age groups, born before 1962 [16]. Taken together, these data indicate that there could be an alternative source of human infection by SV40, which currently makes it impossible to attribute an iatrogenic or a natural background origin of infection to those mesotheliomas in which we detected SV40 LTag-like DNA. In this preliminary study, we did not examine normal human tissues including normal mesothelium or lung tissue, leaving the issue of whether the virus is as common in some normal tissues as it might be in some cancers unresolved.

All lung carcinomas but one were found to be negative in the PCR assay. In their series, Carbone et al. detected SV40-like DNA in 2 lung carcinomas out of 25 primary and secondary lung tumours [2]. Since none of the adenocarcinomas investigated tested positive, they proposed that positivity for SV40 could be helpful, together with other markers currently used, in differentiating mesotheliomas from primary and metastatic adenocarcinomas. A more recent study also detected SV40 viral sequences in 7 of 18 (38.8%) mesothelioma specimens, but in none of 18 lung cancers investigated [27]. In contrast, Galateau-Salle et al. found no statistically significant difference in the occurrence of DNA sequences between malignant mesothelioma and bronchopulmonary carcinoma, demonstrating that positivity for SV40 does not support a diagnosis of mesothelioma [13]. At present, the debate concerning diagnostic options using SV40 DNA detection and the question as to why mesothelium would specifically be targeted is the controversial issue, which is basically related to the question of the strength of association between SV40 LTag and mesothelioma carcinogenesis. From the results of our rather small series, we can only say that SV40 seems to be more common in mesothelioma than in lung carcinoma. Recent results suggest that sequence differences at the carboxy end of SV40 LTag may contribute to tissue-specific SV40 replication. SV40 LTag probably needs to interact with host cell transcription factors in the process of switching from early to late transcription, and transcription factors vary in a cell-type- and tissue-specific fashion [26]. Nonetheless, we should not exclude the possibility of artificial amplification of a tumour-specific cellular homologue of LTag using this primer pair [3].

To date, only one group has found diffuse nuclear (and also some cytoplasmic) and punctuate nuclear staining with the pAb419 and pAb101 antibodies, respectively [32]. Using the pAb419 antibody, we found strong cytoplasmic staining in 10 out of 13 samples that had detectable SV40 LTag DNA sequences. In some tumour cells of 3 mesothelioma cases we could not rule out the possibility that presumed nuclear reactivity was simulated by strong cytoplasmic staining, and we therefore repeated our series using the pAb101 antibody. We found no nuclear foci in our mesothelioma sections. At first sight, the immunohistochemical results are puzzling, but they fit in to some degree with the observations of others. While sequences related to SV40 LTag were present in 47.5% of mesotheliomas, Galateau-Salle et al. also found cytoplasmic staining only, and no nuclear staining, with the pAb419 antibody in both sections and cell lines exhibiting SV40 LTag DNA sequences [13]. The observed cytoplasmic staining with pAb419 can be of interest, because the epitopes that are recognised by this antibody are also present on the SV40 small t antigen, which is found predominantly in the cytoplasm of infected and transformed cells [10]. Since the primer set that we and others used is directed towards a sequence unique to the SV40 *LTag* gene and does not amplify the SV40 small *t* gene, this putative cross-reactivity awaits further investigation. The pAb101 antibody, however, is non-cross-reactive with the SV40 small t antigen, according to the vendor's information, which means that, given the amplification procedure, the presence of SV40 LTag oncoprotein is improbable in our cases.

The absence of expression of SV40 LTag DNA sequences seems not to be concordant with the supposed oncogenic role of SV40 LTag. However, recent experiments indicate that SV40 LTag can induce irreversible chromosomal and genetic alterations, leading to a loss of dependence on the viral oncoprotein for maintenance of the transformed state [11]. Variable SV40 LTag expression in tumour cells has also been described in prostate and breast tumours developing in SV40-transgenic mice [21]. In their mesothelioma series, Galateau-Salle et al. performed PCR hybridisation with SV40-specific probes and found low hybridisation indices [13]. They suggested that very few copies are present within the tissue samples or that the sequences had been diluted following the cell division associated with tumour growth. Their and our observations are consistent with a so-called hit-and-run mechanism, on the basis of which SV40 LTag might initially have affected a larger proportion of mesothelioma cells than can now be observed [33]. It is noteworthy that recent data have revealed that different sets of primers for the SV40 genome can result in a different percentage of positive results when human mesotheliomas are tested, with highest amplification rates (71%–100%) for the SV.for3/SV.rev primer pair [14, 26]. It is not known at present why different sets of primers, SV.for3 and SV.rev in particular, result in different percentages of positive samples. Perhaps these primers can amplify LTag sequences from other papova viruses, such as the BK virus, a ubiquitous human virus with LTag at gene level similar to SV40 LTag, or other (unknown) recombinant viruses [24].

Given the extremely long latency period that characterises mesothelioma, our current and previous finding of two life-span-prolonging agents, SV40 LTag and telomerase, respectively, is interesting since both, at least in an in vitro setting, seem to induce the immortal phenotype in a concerted action. In vitro, SV40 LTag-infected cells bypass senescence or mortality stage 1 (M1), thereby acquiring an extended life-span up to crisis or mortality stage 2 (M2). Only after abrogation of M2-controlling mechanisms cells really gain the immortal phenotype [29, 30, 35]. Recent research revealed that the latter event, which is characterised by ultra-short cellular telomeres, coincides with reactivation of telomerase [5]. Therefore, the classic hypothesis of telomerase reactivation states that the latter is induced by peri-crisis telomere exhaustion [36]. Thus, the exact molecular basis for telomerase deregulation is speculative. The prediction is that one or more tumour suppressor genes prevent activation of telomerase in normal human cells, and that these wild-type genes may be eliminated as a consequence of the chromosome instability produced by the extremely short telomeres during p53/pRb-defective life-span extension [7].

Although it is tempting to speculate that, at least in a subgroup of mesothelioma cases with detectable virus-like sequences, SV40 LTag or other (unknown) LTags, through inhibitory binding to p53 and pRB and hence induction of telomere exhaustion and genetic instability, might have helped to eliminate or inactivate in vivo those genes responsible for M2, say telomerase inhibitory genes, we realise that our data do not constitute hard evidence. Much more important is that the tally of SV40 LTag mesothelioma papers has led to a renewed interest in the subject of viral oncogenesis in carcinogenesis in general and mesothelioma carcinogenesis in particular, which should also focus on disturbances of telomere dynamics. Meanwhile, the exact nature of the amplified sequences and their oncogenic role remains to be determined. These first data have prompted us to repeat our analyses with an extended battery of primers spanning the entire SV40 genome, and to propose future multicenter studies with interlaboratory controls.

Acknowledgements The authors would like to thank Dr. Roland Hübner for constructive comments on an earlier version of the manuscript, and Frank Rylant and Lilian Moeneclaey for excellent technical support.

References

1. Bergsagel DJ, Finegold MJ, Butel JS, Kupsky WJ, Garcea RL (1992) DNA sequences similar to those of simian virus 40 in ependymomas and choroid plexus tumors of childhood. N Engl J Med 326:988–993
2. Carbone M, Pass HI, Rizzo P, Marinetti M, Di Muzio M, Mew DJ, Levine AS, Procopio A (1994) Simian virus 40-like DNA sequences in human pleural mesothelioma. Oncogene 9:1781–1790

3. Carbone M, Rizzo P, Pass HI (1997) Simian virus 40, poliovaccines and human tumors: a review of recent developments. Oncogene 15:1877–1888
4. Cicala C, Pompetti F, Carbone M (1993) SV40 induces mesotheliomas in hamsters. Am J Pathol 142:1524–1533
5. Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, Bacchetti S (1992) Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 11:1921–1929
6. DeCaprio JA, Ludlow JW, Figge J, Shew JY, Huang CM, Lee WH, Marsilio E, Paucha E, Livingston DM (1988) SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell 54:275–283
7. De Lange T (1998) Telomeres and senescence: ending the debate. Science 279:334–335
8. De Luca A, Baldi A, Esposito V, Howard CM, Bagella L, Rizzo P, Caputi M, Pass HI, Giordano GG, Baldi F, Carbone M, Giordano A (1997) The retinoblastoma gene family pRb/p105, p107, pRb2/p130 and simian virus-40 large T-antigen in human mesotheliomas. Nat Med 3:913–916
9. Dhaene K, Hübner R, Kumar-Singh S, Weyn B, Van Marck E (1998) Telomerase activity in human pleural mesothelioma. Thorax 53:915–918
10. Ellman M, Bikel I, Figge J, Roberts T, Schlossman R, Livingston DM (1984) Localization of the simian virus 40 small t antigen in the nucleus and cytoplasm of monkey and mouse cells. J Virol 50:623–628
11. Ewald D, Li M, Efrat S, Auer G, Wall RJ, Furth PA, Hennighausen L (1996) Time-sensitive reversal of hyperplasia in transgenic mice expressing SV40 T antigen. Science 273:1384–1386
12. Frank TS, Svoboda NS, Hsi ED (1996) Comparison of methods for extracting DNA from formalin-fixed paraffin sections for nonisotopic PCR. Diagn Mol Pathol 5:220–224
13. Galateau-Salle F, Bidet P, Iwatsubo Y, Gennetay E, Renier A, Letourneux M, Pairon JC, Moritz S, Brochard P, Jaurand MC, Freymuth F (1998) SV40-like DNA sequences in pleural mesothelioma, bronchopulmonary carcinoma, and non-malignant pulmonary diseases. J Pathol 184:252–257
14. Griffiths DJ, Nicholson AG, Weiss RA (1998) Detection of SV40 sequences in human mesothelioma. Dev Biol Stand 94:127–136
15. Henderson DW, Shilkin KB, Whitaker D, Atwood HD, Constance TJ, Steele RH, Leppard PJ (1992) The pathology of malignant mesothelioma, including immunohistology and ultrastructure. In: Henderson DW, Shilkin KB, Le P. Langlois S, Whitaker D (eds) Malignant mesothelioma. Hemisphere, New-York Washington Philadelphia, pp 69–139
16. Jafar S, Rodriguez-Barradas M, Graham DY, Butel JS (1998) Serological evidence of SV40 infections in HIV-infected and HIV-negative adults. J Med Virol 54:276–284
17. Kerstens MJ, Poddighe PJ, Hanselaar AG (1995) A novel in situ hybridisation amplification method based on the deposition of biotinylated tyramine. J Histochem Cytochem 43:347–352
18. King G, Payne S, Walker F, Murray GI (1997) A highly sensitive detection method for immunohistochemistry using biotinylated tyramine. J Pathol 183:237–241
19. Lednicky JA, Stewart AR, Jenkins JJ, Finegold MJ, Butel JS (1997) SV40 DNA in human osteosarcomas shows sequence variation among T-antigen genes. Int J Cancer 72:791–800
20. Lin JY, Simmons DT (1991) The ability of large T antigen to complex with p53 is necessary for the increased life span and partial transformation of human cells by simian virus 40. J Virol 65:6447–6453
21. Maroulakou IG, Anver M, Garrett L, Green JE (1994) Prostate and mammary adenocarcinoma in transgenic mice carrying a rat C3(1) simian virus 40 large tumor antigen fusion gene. Proc Natl Acad Sci USA 91:11236–11240
22. Martini F, Iaccheri L, Lazzarin L, Carinci P, Corallini A, Gerosa M, Iuzzolino P, Barbanti-Brodano G, Tognon M (1996) SV40 early region and large T antigen in human brain tumors, peripheral blood cells, and sperm fluids from healthy individuals. Cancer Res 56:4820–4825
23. McDonald JC, McDonald AD (1996) The epidemiology of mesothelioma in historical context. Eur Respir J 9:1932–1942
24. Monini P, de Lellis L, Barbanti-Brodano G (1995) In: Barbanti-Brodano G, Bendinelli M, Frieman H (eds) DNA tumor viruses oncogenic mechanisms. Plenum, New York, pp 51–73
25. Pairon JC, Orlowski E, Iwatsubo Y, Billon GM, Dufour G, Chamming's S, Archambault C, Bignon J, Brochard P (1994) Pleural mesothelioma and exposure to asbestos: evaluation from work histories and analysis of asbestos bodies in bronchoalveolar lavage fluid or lung tissue in 131 patients. Occup Environ Med 51:244–249
26. Pass HI, Donington JS, Wu P, Rizzo P, Nishimura M, Kennedy R, Carbone M (1998) Human mesotheliomas contain the simian virus-40 regulatory region and large tumor antigen DNA sequences. J Thorac Cardiovasc Surg 116:854–859
27. Procopio A, Marinacci R, Marinetti MR, Strizzi L, Paludi D, Iezzi T, Tassi G, Casalini A, Modesti A (1998) SV40 expression in human neoplastic and non-neoplastic tissues: perspectives on diagnosis, prognosis and therapy of human malignant mesothelioma. Dev Biol Stand 94:361–367
28. Shah K, Nathanson N (1976) Human exposure to SV40: review and comment. Am J Epidemiol 103:1–12
29. Shay JW, Wright WE (1989) Quantitation of the frequency of immortalization of normal human diploid fibroblasts by SV40 large T-antigen. Exp Cell Res 184:109–118
30. Shay JW, Pereira-Smith OM, Wright WE (1991) A role for both RB and p53 in the regulation of human cellular senescence. Exp Cell Res 196:33–39
31. Strickler HD, Goedert JJ, Fleming M (1996) Simian virus 40 and pleural mesothelioma in humans. Cancer Epidemiol Biomarkers Prevention 5:473–475
32. Testa JR, Carbone M, Hirvonen A, Khalili K, Krynska B, Linnainmaa K, Pooley FD, Rizzo P, Rusch V, Xiao G (1998) A multi-institutional study confirms the presence and expression of simian virus 40 in human malignant mesothelioma. Cancer Res 58:4505–4509
33. Wiman KG, Klein G (1997) An old acquaintance resurfaces in human mesothelioma. Nat Med 3:839–840
34. Wright WE, Shay JW (1992) The two-stage mechanism controlling cellular senescence and immortalization. Exp Gerontol 27:383–389
35. Wright WE, Pereira-Smith OM, Shay JW (1989) Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. Mol Cell Biol 9:3088–3092
36. Wynford TD, Kipling D (1997) Cancer and the knockout mouse. Nature 389:551– 552