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RESEARCH

Immunodetection of SV40 T/t-antigens in Human Osteosrcoma in a Series of Tunisian Patients

S. Ziadi · H. Boughamoura · M. Ben Maitig · R. Ben Gacem · S. Mestiri · L. Chaabani · M. Trimeche

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Abstract Osteosarcoma is a primary bone malignancy that typically occurs during adolescence but also has a second incidence peak in the elderly. The etiology of osteosarcoma is not well understood. Recent investigations have identified SV40 DNA sequences in osteosarcomas, suggesting that SV40 may contribute to tumor development. However, these studies also demonstrated geographical differences in SV40-positive osteosarcomas. The purpose of this study was to determine the prevalence and clinicopathological characteristics of SV40 positive osteosarcoma in Tunisian patients. Fifty-six formalin-fixed paraffin-embedded specimens of osteosarcomas were retrospectively investigated. Samples investigated were clinical cases examined between 1990 and 2004 in the Laboratory of Pathology at the University Hospital Farhat-Hached of Sousse (Tunisia). The search for SV40 was performed by immunohistochemistry using the Pab108 antibody for the detection of the viral oncoproteins: large T antigen and small t antigen (T/t-ag). SV40 status was correlated with clinico-pathological data. T/t-ag immunostaning was detected in the tumor cells in 31/56 (55.4%) osteosarcoma cases. SV40 positivity was more frequent (83%) in patients older than 40 years (5/6 cases) than in patients under 40 years (52%, 26/50), but the difference does not reach statistical significance ($p=0.33$). Moreover, the time between the onset of clinical symptoms and diagnosis was shorter for SV40 positive than SV40 negative cases ($p=0.08$). However, the viral status did not differ significantly according to gender, tumor size, histological subtype, tumor location, or metastases. This study documents the presence SV40 T/t-antigens in a proportion of osteosarcomas in Tunisian patients. The expression of these viral oncoproteins supports the hypothesis that SV40 may have a role in the pathogenesis of this tumor.

Keywords Osteosarcoma · Simian Virus 40 · Immunohistochemistry · Tunisia

S. Ziadi · R. Ben Gacem · S. Mestiri · L. Chaabani · M. Trimeche (✉)
Department of Pathology, Farhat Hached Hospital, Sousse 4000, Tunisia
e-mail: m_trimech@yahoo.fr

H. Boughamoura · M. Ben Maitig
Department of Orthopedics, Sahloul Hospital, Sousse, Tunisia

Introduction

Osteosarcoma is a common primary malignant tumor of bone in children and adolescents, mostly occurring in the second decade of life. Multiple molecular lesions underline the progression from normal bone to osteosarcoma. Coexisting alterations of the retinoblastoma susceptibility (pRb) and the p53 gene are known to be important events in this progression [1, 2]. P53 and pRb are two tumor-suppressor genes critical for cell growth, leading to abnormalities in cell-cycle regulation, increased cellular proliferation, and defects in the activation of apoptosis pathways. P53 and pRb are known to play a key role in the genesis of multiple tumors, as has been shown for osteosarcoma development [3–5].

Some investigations have identified DNA sequences of the simian virus 40 (SV40) in osteosarcomas suggesting that this virus contributes to the development of this tumor [5, 6]. SV40, which belongs to the polyomavirus family, is known to be a potent tumor-inducing virus [7, 8]. It is also capable of transforming many types of cells in tissue culture, including human cells [9]. SV40 is highly oncogenic in rodents and when inoculated subcutaneously, intracerebrally or intravenously in newborn hamsters induces soft tissue sarcomas, osteosarcomas, ependymomas and choroid plexus papillomas, and neoplasms of the hematopoietic system, such as lymphocytic leukemia, histiocytic lymphomas and rarely, B-cell lymphomas [10–12].

Over the last several years, different laboratories have reported the presence of SV40 in several types of human tumors, including brain tumors [13], pleural mesothelioma [14], osteosarcomas [5, 6, 15], lymphomas [16, 17], hepatocellular carcinoma [18], and papillary thyroid carcinoma [19]. We have recently reported a high prevalence of SV40 in diffuse large B-cell lymphomas and breast carcinomas occurring in Tunisian patients [17, 20]. To our knowledge, in Tunisia, no studies on the involvement of this virus in osteosarcoma have been achieved to date.

The oncogenic potential of SV40 is mediated by the two oncoproteins, the large T-antigen (T-ag) and the small t-antigen (t-ag), which display multiple functions. T-ag is a 90 kDa nuclear multifunctional phosphoprotein predominantly located in the nuclei of the infected cells. The main activity of T-ag for cell transformation and tumorigenesis is to target key cellular proteins, such as the tumor suppressor p53 and pRb family proteins, inactivating their functions [10, 21–23]. SV40 T-ag may also lead to transformation by inducing mutations to the cellular genome [24] or numerical and structural alterations of chromosomes [9, 25], such as gaps, breaks, dicentric and ring chromosomes, chromatid exchanges, deletions, duplications and translocations [26].

The principal role of the t-ag in transformation is to bind the catalytic (36 kDa) and regulatory (63 kDa) subunits of protein phosphatase 2A (PP2A) [21, 22], inactivating their function. Moreover, t-ag interacts with the centrosome and blocks mitosis in human cells [27], suggesting that it may disrupt cell cycle progression.

The process of rodent cell transformation induced by SV40 typically depends on the integration of the viral DNA into the host genome where it produces a high level of expression of the major viral oncogenic proteins, T-ag and t-ag [10, 21, 22, 28]. The expression of the two oncoproteins could be detected by immunohistochemistry with the specific monoclonal antibody Pab108 which recognizes an N-terminal epitope within the first 82 amino acids of the T/t-ag.

The aim of this study was to determine the prevalence and the clinico-pathological features of SV40-positive osteosarcomas in Tunisian patients.

Materials and Methods

Patients and Samples

Fifty-six cases of osteosarcomas were selected from the cases registered at the Department of Pathology at the Farhat-Hached Hospital of Sousse (Tunisia), from 1990 to 2004.

Cases were selected on the basis of the availability of complete clinical data and the availability of paraffin-embedded tissue specimens for histological and immunohistochemical analyses.

The medical records of these patients were reviewed and information about age, gender, duration of symptoms, size and site of tumors, and metastasis status.

Cases were reviewed to confirm the diagnosis and to classify the tumors according to the histological sub-types.

Immunohistochemical Staining

The detection of SV40 was performed by immunohistochemisty on formalin-fixed paraffin-embedded tissues sections using the Pab108 antibody (dilution 1:100; Santa Cruz Biotechnology, USA). The monoclonal antibody is directed against the large T and the small t antigens (T/t-ag) of SV40 and does not cross-react with the other polyomaviruses [Ref].

Briefly, 5 µm thick sections of paraffin embedded tissues were cut and dried overnight at 37°C, deparaffinized in toluene, and rehydrated through a series of graded alcohols. Then the sections were rinsed thoroughly with water and placed in Tris-buffered saline (TBS) (0.05 M Tris–HCl, 1.15 M NaCl, pH 7.6). Antigen retrieval was done with the appropriate buffer by boiling sections in water bath for 40 min until temperature reaches 98°C. Endogenous peroxidase activity was blocked with 5% H_2O_2 solution for 5 min, and sections were rinsed gently with TBS. The sections were then incubated with the appropriate primary antibody. After rinsing in TBS, described antibodies were detected using the EnVision+Labeled Polymer Kit (DakoCytomation) following the manufacturer's instructions. Sections were then washed three times with TBS, followed by application of the Diaminobenzidine Substrate Pack (DakoCytomation) according to the manufacturer's instructions. Finally, the slides were counterstained with hematoxylin, and mounted.

As positive control for SV40 T/t-ag detection, sections from a paraffin-embedded tissue of a transgenic adenocarcinoma of the mouse prostate (TRAMP) positive for SV40 T/t-ag expression were used (kindly provided by Professor P. Wikström, Department of Medical Bioscience, Umea University, Umea, Sweden).

Statistical Analysis

Statistical analysis was carried out with SPSS software package (version 17.0). Pairwise correlations between SV40 status and clinicopathological parameters were investigated by chi-square test or Fisher's exact test where appropriate.

Results

Clinico-Pathological Findings

The mean age of the patients at the time of diagnosis was 20 years, ranging from 7 to 80 years. The gender distribution was similar. The most common site of the primary tumor was the distal femur and proximal tibia (38 cases) followed by the humerus (4 cases) the mandible (3 cases), the maxilla (3 cases) the fibula (3 cases) and 5 different other locations: calcaneus orbit, scapula vertebrae and sacrum. The size of the tumors ranged from 3 to 25 cm, with a median size of 12 cm.

The mean time elapsed between onset of symptoms and diagnosis was 7 months (range 1 to 40 months).

Of the 56 patients only eight had a pulmonary metastasis at the time of diagnosis. The histopathological features showed varied degrees of cellularity, but in all cases there was evidence of osteoid formation.

The conventional type of osteosarcoma is the most common including: osteoblastic subtype (42 cases), fibroblastic (6 cases) and chondroblastic subtype (4 cases). The other was classified as telangiectatic osteosarcoma (4 cases).

Prevalence and Characteristic of SV40 Positive Osteosarcomas

Immunohistochemical analysis revealed that T/t-ag protein was positive in 31 cases (55.4%). The signal was strong, mainly nuclear, and showed subtle variations in intensity from one cell to another. In SV40 positive cases, T/t-ag immunostaining was noted in tumor cells whereas no expression was observed in stromal cells (Fig. 1).

Comparison of the clinicopathological features of SV40-positive and –negative groups is summarized in Table 1.

In the group of patients aged under 40 the SV40 was found in half the cases (26/50), however the SV40 was detected in almost all tumors occurring in patients older

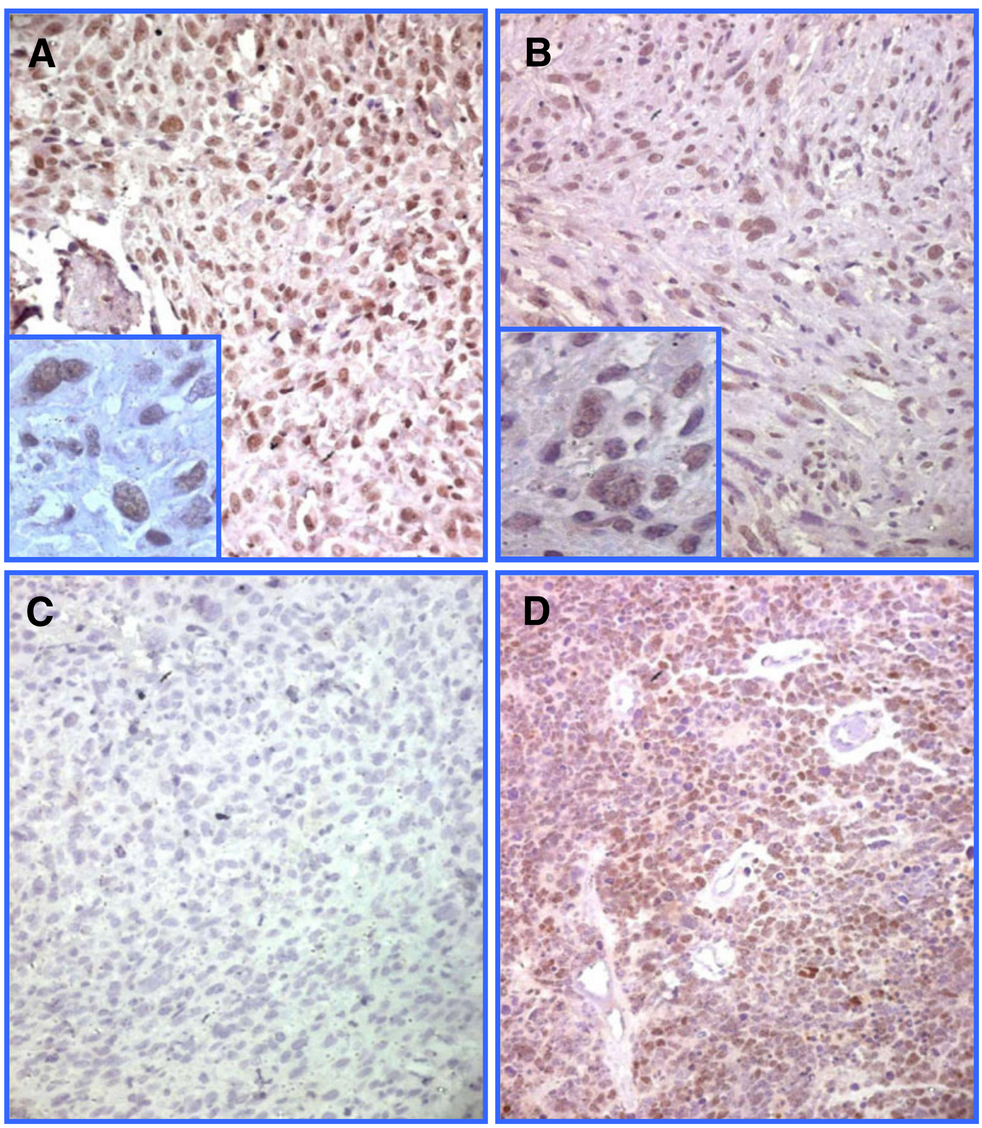

Fig. 1 Representative examples of immunohistochemical staining for the detection of the SV40 T/t-antigens using the Pab108 antibody. Panels **a** and **b:** positive osteosarcoma cases (Original magnification × 200). Panel **c:** a negative osteosarcoma case (Original magnification × 100). Panel **d:** positive control (SV40-positive transgenic adenocarcinoma of mouse prostate)

Table 1 Clinico-pathological features of SV40-positive and -negative osteosarcomas

Variables	Number of SV40-positive cases	Number of SV40-negative cases	P-value
Age (years)			
Range	9-80	7-42	
≤ 20	19	17	0.33
21–40	7	7	
>40	5	1	
Gender			
Male	16	12	0.78
Female	15	13	
Time to symptoms onset			
≤ 6 months	24	14	0.08
> 6 months	7	11	
Tumor size			
Range	6-23	3-25	
Median	11.9	12.4	0.75
Site of tumor			
Cranium and face	4	3	0.91
Trunk	2	1	
Members	25	21	
Histological subtypes			
Osteoblastic	22	20	0.76
Fibroblastic	4	2	
Chondroblastic	3	1	
Telangiectasic	2	2	
Metastasis			
Yes	5	3	0.72
No	26	22	

P-values were calculated by χ^2 or Fisher's exact tests (two sided) and were considered to be statistically significant for $p<0.05$

than 40 years (5/6 cases, 83%), but the difference does not reach statistical significance ($p=0.33$).

Moreover, the time between the onset of clinical symptoms and diagnosis was shorter for SV40 positive cases than SV40 negative cases. Indeed, this period was less than 6 months in 77.4% (24/31) of SV40 positive cases and in only 56% (14/25) of SV40 negative cases. But the difference does not reach statistical significance ($p=0.08$).

However, the viral status did not differ significantly according to the gender, the tumor size, the histological subtype, the tumor location, or the presence of metastases.

Discussion

The simian virus SV40 (SV40), a potent DNA oncogenic polyomavirus, has been detected in several human tumors [29–31]. However, a causative role for this virus has not been convincingly established. The oncogenic potential of SV40 is thought to be through its primary viral genes products, T/t-antigens, responsible for SV40 replication and SV40-mediated cell transformation.

Studies from several countries (USA, Europe) have successfully detected SV40 DNA by PCR-based methods in variable proportions of osteosarcoma, ranging from 10% (Houston, USA) [6] to 70% of specimens (Italy) [5, 6, 32, 33]. However, the detection of SV40 DNA sequences in osteosarcoma does not necessarily indicate a causative role of SV40 in the development of those sarcomas. Therefore, the role of SV40 in osteosarcoma development has yet to be elucidated. Additionally, data on the SV40 incidence in different populations of the investigated geographical regions are rarely found, making a clear decision about the role of SV40 in osteosarcoma difficult.

Previous investigators analyzing SV40 in osteosarcomas used mainly conventional PCR, followed by Southern-blot hybridization and occasional sequencing. A few studies have investigated SV40 oncoproteins expression in human cancers [20, 34–37]. In the current study we investigated SV40 T/t-ag protein expression in osteosarcomas by immunohistochemistry using a specific mouse monoclonal antibody (Pab108). This antibody recognizes an N-terminal epitope within the first 82 amino acids of the T-ag and t-ag, and therefore displays nuclear and cytoplasmic staining. Pab108 antibody is specific for SV40 and does not cross-react with other polyomaviruses (JCV and BKV). This antibody worked well on paraffin wax embedded sections of tissues fixed in 10% buffered formalin or in Bouin's liquid.

Using Pab108 antibody, SV40 was found positive in 31 (55.4%) of our osteosarcoma cases. The signal was strong and mainly nuclear and it was observed specifically in cancer cells but not in the stromal cells. The expression of SV40 T/t oncoproteins in osteosarcomas suggests that SV40 is not a passenger virus but may have an active role in bone cancerogenesis.

Some studies detected SV40 DNA not only in tumor but also in blood samples of osteosarcoma patients, indicating the possibility that the virus can circulate in these individuals [1, 15, 38]. Other investigators establish that authentic natural strains of SV40 can be found associated with human osteosarcomas, with the tumor-related viral DNAs displaying archetypal, non-duplicated regulatory regions and variable T-ag C-terminal sequences [39].

On the other hand the frequency of SV40 positivity reported for osteosarcoma has varied from less than 10% of tumors tested in the United states [6] to greater frequencies, reaching over 70% of specimens positive in Italy [5, 6, 32, 33]. In the current study we found SV40 in 55.4% of cases. These differences in results may reflect varying prevalence of SV40 infections in different geographic regions, technical issues related to sample processing, characteristics of the specific patient populations that were the sources of specimens.

There was a recent report describing plasmid contamination of human specimens in the laboratory [40]. The authors suggested that plasmid contamination could be the source of some SV40 sequences detected in human material. However, the detection of the viral T/t-ag oncoprotein expression in the tumor cells in our patients suggests that SV40 is not a passenger virus. Indeed, SV40 oncogenicity and transforming ability are strictly dependent on the expression of the early region gene product encoding for T-ag which could complexes with the tumor suppressor's p53 and pRb, inactivating their functions; alters the integrity and stability of the host cell genome, including numerical and structural chromosomal aberrations [41, 42]. Theses mechanisms play a key role in osteosarcoma development.

The source of SV40 infection in the human osteosarcoma patients is unknown. The major source of known human exposure to SV40 was immunization with SV40-contaminated poliovaccines in the period from 1955 to 1963, during which large number of people worldwide were inadvertently exposed to live SV40. A recent study indicates that some poliovirus vaccines were contamined with SV40 after 1963 [43]. In Tunisia, antipoliomyelitis vaccination campaigns were initiated in early 1960s; however, we don't know whether SV40 contaminated poliovaccine lots were used.

In the current study, we found that patients with SV40-positive osteosarcomas occurred among individuals born after the period of use of SV40-contaminated vaccines in Tunisia. This observation is in agreement with studies of other human malignancies which found that SV40 was associated with some cancer cases in children and young adult patients not exposed to contaminated poliovirus vaccines, suggesting that SV40 has entered the human population and become a new and emergent pathogen [13, 16, 35, 39, 44].

In summary, this study documents the presence SV40 T/t-antigens in a fraction of osteosarcomas in Tunisian patients. The expression of SV40 T/t oncoproteins in human osteosarcoma supports the hypothesis that SV40 may have a role in the pathogenesis of this sarcoma. Further analysis is required to confirm these findings ant to determine the role of SV40 in the development or progression of osteosarcoma.

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References

1. Scholz RB, Kabisch H, Weber B et al (1992) Studies of the RB1 gene and the p53 gene in human osteosarcomas. Pediatr Hematol Oncol 9:125–137
2. Szendroi M (2006) Bone tumours. Int Orthop 30:435–436
3. Crawford LV, Cole CN, Smith AE et al (1978) Organization and expression of early genes of simian virus 40. Proc Natl Acad Sci USA 75:117–121
4. De Luca A, Baldi A, Esposito V et al (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
5. Mendoza SM, Konishi T, Miller CW (1998) Integration of SV40 in human osteosarcoma DNA. Oncogene 17:2457–2462
6. Carbone M, Rizzo P, Procopio A et al (1996) SV40-like sequences in human bone tumors. Oncogene 13:527–535
7. Cicala C, Pompetti F, Carbone M (1993) SV40 induces mesotheliomas in hamsters. Am J Pathol 142:1524–1533
8. Shein HM, Enders JF, Levinthal JD (1962) Transformation induced by simian virus 40 in human renal cell cultures. II. Cellvirus relationships. Proc Natl Acad Sci USA 48:1350–1357
9. Ray FA, Peabody DS, Cooper LJ et al (1990) SV40 T antigen alone drives karyotype instability that precedes neoplastic transformation of human diploid fibroblasts. J Cell Biochem 42:13–31
10. Barbanti-Brodano G, Martini F, De Mattei M et al (1998) BK and JC human polyomaviruses and simian virus 40: natural history of infection in humans, experimental oncogenicity, and association with human tumors. Adv Virus Res 50:69–99
11. Diamandopoulos GT (1972) Leukemia, lymphoma, and osteosarcoma induced in the Syrian golden hamster by simian virus 40. Science 176:173–175
12. Coe JE, Green I (1975) B-cell origin of hamster lymphoid tumors induced by simian virus 40. J Natl Cancer Inst 54:269–270
13. Bergsagel DJ, Finegold MJ, Butel JS et al (1992) DNA sequences similar to those of simian virus SV40 in ependymomas and choroid plexus tumors of childhood. N Engl J Med 326:988–993
14. Carbone M, Pass HI, Rizzo P et al (1994) Simian virus 40-like DNA sequences in human pleural mesothelioma. Oncogene 9:1781–1790

15. Yamamoto H, Nakayama T, Murakami H et al (2000) High incidence of SV40-like sequences detection in tumour and peripheral blood cells of Japanese osteosarcoma patients. Br J Cancer 82:1677–1681
16. Vilchez RA, Madden CR, Kozinetz CA et al (2002) Simian virus 40 and non-Hodgkin lymphoma. Lancet 359:817–823
17. Amara K, Trimeche T, Ziadi S et al (2007) Presence of simian virus 40 DNA sequences in diffuse large B-cell lymphomas in Tunisia correlates with aberrant promoter hypermethylation of multiple tumor suppressor genes. Int J Cancer 121:2693–2702
18. Wong NA, Rae F, Herriot MM et al (2003) SV40 Tag DNA sequences, present in small proportion of human hepatocellular carcinomas, are associated with reduction survival. J Clin Pathol 56:904–909
19. Pacini F, Vivaldi A, Santoro M et al (1998) Simian virus 40-like DNA sequences in human papillary thyroid carcinomas. Oncogene 15:665–669
20. Hachana M, Trimeche M, Ziadi S et al (2009) Evidence for a role of the Simian Virus 40 in human breast carcinomas. Breast Cancer Res Treat 113:43–58
21. Garcea RL, Imperiale MJ (2003) Simian virus 40 infection of humans. J Virol 77:5039–5045
22. Barbanti-Brodano G, Sabbioni S, Martini F et al (2004) Simian virus 40 infection in humans and association with human diseases: results and hypotheses. Virology 318:1–9
23. Sheppard HM, Corneillie SI, Espiritu C et al (1999) New insights into the mechanism of inhibition of p53 by simian virus 40 large T antigen. Mol Cell Biol 19:2746–2753
24. Theile M, Strauss M, Luebbe L et al (1980) SV40-induced somatic mutations: possible relevance to viral transformation. Cold Spring Harb Symp Quant Biol 44:377–382
25. Stewart N, Bacchetti S (1991) Expression of SV40 large T antigen, but not small t antigen, is required for the induction of chromosomal aberrations in transformed human cells. Virology 180:49–57
26. Tognon M, Casalone R, Martini F et al (1996) Large T antigen coding sequences of two DNA tumor viruses, BK and SV40, and nonrandom chromosome changes in two glioblastoma cell lines. Cancer Genet Cytogenet 90:17–23
27. Gaillard S, Fahrbach KM, Parkati R et al (2001) Overexpression of simian virus 40 small-T antigen blocks centrosome function and mitotic progression in human fibroblasts. J Virol 75:9799–9807
28. Hurault de Ligny B, Godin M et al (2003) Virological, epidemiological and pathogenic aspects of human polyomaviruses. Presse Med 32:656–658
29. Jasani B, Cristaudo A, Emri SA et al (2001) Association of SV40 with human tumours. Semin Cancer Biol 11:49–61
30. Klein G, Powers A, Croce C (2002) Association of SV40 with human tumors. Oncogene 21:1141–1149
31. Gazdar AF, Butel JS, Carbone M (2002) SV40 and human tumours: myth, association or causality? Nat Rev Cancer 2:957–964
32. Heinsohn S, Scholz RB, Weber B (2000) SV40 sequences in human osteosarcoma of German origin. Anticancer Res 20:4539–4545
33. Butel JS, Jafar S, Stewart AR (1998) Detection of authentic SV40 DNA sequences in human brain and bone tumours. Dev Biol Stand 94:23–32
34. Galateau-Salle F, Bidet P, Iwatsubo Y et al (1998) SV40-like DNA sequences in pleural mesothelioma, bronchopulmonary carcinoma, and non-malignant pulmonary diseases. J Pathol 184:252–257
35. Malkin D, Chilton-MacNeil S, Meister LA et al (2001) Tissue specific expression of SV40 in tumors associated with Li-Fraumeni syndrome. Oncogene 20:4441–4449
36. Zhen HN, Zhang X, Bu XY et al (1999) Expression of the simian virus 40 large tumour antigen (Tag) and formation of Tag–p53 and Tag–pRb complexes in human brain tumors. Cancer 86:2124–2132
37. Martinelli M, Martini F, Rinaldi E et al (2002) Simian virus 40 sequences and expression of the viral large T antigen oncoprotein in human pleomorphic adenomas of parotid glands. Am J Pathol 161:1127–1133
38. Heinsohn S, Szendroi M, Bielack S et al (2009) Evaluation of SV40 in osteosarcoma and healthy population: a Hungarian-German study. Oncol Rep 21:289–297
39. Lednicky JA, Stewart AR, Jenkins JJ 3rd et al (1997) SV40 DNA in human osteosarcomas shows sequence variation among T-antigen genes. Int J Cancer 72:791–800
40. López-Ríos F, Illei PB, Rusch V et al (2004) Evidence against a role for SV40 infection in human mesotheliomas and high risk of false-positive PCR results owing to presence of SV40 sequences in common laboratory plasmids. Lancet 364:1157–1166
41. Atkin SJ, Griffin BE, Dilworth SM (2009) Polyoma virus and simian virus 40 as cancer models: history and perspectives. Semin Cancer Biol 19:211–27
42. Gjoerup O, Chang Y (2010) Update on human polyomaviruses and cancer. Adv Cancer Res 106:1–51
43. Cutrone R, Lednicky J, Dunn G et al (2005) Some oral poliovirus were contaminated with infectious SV40 after 1961. Cancer Res 65:10273–10279
44. Lednicky JA, Garcea RL, Bergsagel DJ, Butel JS (1995) Natural simian virus 40 strains are present in human choroid plexus and ependymoma tumors. Virology 212:710–717