ORIGINAL ARTICLE

Serologic investigation on undifferentiated nasopharyngeal carcinoma and Simian Virus 40 infection

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ABSTRACT: Background**.** The association between the undifferentiated nasopharyngeal carcinoma (UNPC) and Epstein-Barr virus (EBV) is well established. Nevertheless, available evidence suggests that other co-factors are required for the development of UNPC. Several investigations reported Simian Virus 40 footprints in human tumors of different histotypes.

Methods. . Serum samples from UNPC affected patients (n=64) and healthy subjects, (HS) (n=130), were analyzed by anindirect ELISA with SV40 synthetic peptides to detect antibodies against viral capsid proteins VP1-2-3..

Results. Immunologic data indicate that in sera from UNPC patients the prevalence of SV40 antibodies was 25%, while in controls it was 16%. This difference is not statistically significant ($P > 0.05$). .

Conclusion. A similar prevalence of SV40 antibodies was detected in UNPC and HS. Our serologic data suggest no association between UNPC and SV40 infection. This investigation may stimulate further studies aimed at determining the possible contribution of other risk factors in the pathogenesis of UNPC.

KEY WORDS: undifferentiated nasopharyngeal carcinoma, Simian Virus 40, ELISA, antibody.

INTRODUCTION

Human undifferentiated nasopharyngeal carcinoma (UNPC) is a tumor with a well distinctive ethnic and geographic distribution. UNPC represents a rare malignancy with a low incidence in western countries with approximately 1 case every 100,000 people. Distinct genetic and environmental risk factors are involved in UNPC onset/progression. In almost all UNPC, latent Epstein-Barr virus (EBV) infection is detected. However, although EBV infection is an early pathogenic event, the detection of EBV latent infection in the high-grade, but not in low-grade dysplasia and normal epithelia suggest additional, still unknown factors promoting the cell transformation. Indeed, genetic and/or epigenetic alterations are required to favor cell immortalization, susceptibility to EBV entry and maintenance of permanent viral latency. [1]

Many studies detected Simian Virus 40 (SV40) footprints in different human tumors [2] while SV40 sequences were identified in Epstein-Barr virus (EBV)-immortalized lymphoblastoid cell lines (LCLs) [3] and in B- and T- lymphocytes.[4] Indeed SV40, a small DNA tumor virus, has been found associated at a high prevalence with some tumors, but it was also detected in normal tissues, although with a lower prevalence. SV40 DNA sequences were detected in circulating B lymphocytes of both healthy donors and cancer patients. In experimental infections in vitro, SV40 is able to infect and transform both purified B- and T-cells.[5] These results indicate that human lymphocytes may be vector of SV40 in other tissues of the host.[2] In addition, SV40 was found to be mutagenic in different human cells.[2, 6] The viral oncoprotein large T antigen (Tag) induces mutations and chromosomal damage, characterized by numerical and structural chromosomal alterations, such as gaps, breaks, dicentric and ring chromosomes, chromatid exchanges, deletions, duplications and translocations. [2, 6]

These data suggest that this small DNA polyomavirus may act together with EBV in the immortalization/transformation process of human epithelial cells. In this contents, it worth recall that in pre-neoplastic lesions specific genetic alterations were identified, such as 3p and 9p deletions, and p16 epigenetic inactivation. [1]

The possibility that SV40 might be associated, with EBV, in the development of human UNPC has not been investigated. On this ground, we carried out an immunologic study aimed at verifying whether antibodies against SV40 could be detected in serum samples from a cohort of UNPC patients and controls.

MATERIALS AND METHODS

Patients

Serum samples were of UNPC patients (n=64) seen at the National Cancer Institute, Aviano, Italy, from 2010 to 2013 with histologically proven diagnosis of UNPC, localized exclusively in the nose-pharyngeal area, were included in the study. Serum samples from healthy subjects (n=130) with the same median age of UNPC patients (57 ys), the control group, were obtained from our Clinical Laboratory Analysis. [7-9]

Serum samples were anonymously collected and coded with indication of the age and pathology. Informed written consent was obtained from patients and subjects. The study was approved by the County Ethical Committee, Ferrara, Italy.

Immunologic test

An indirect ELISA, developed and set up in our laboratories, was employed as described before. [7, 8] Synthetic peptides, named VP1 B and VP2/3 C, were used as antigens to detect in human sera specific IgG antibodies against SV40. They were:

VP1 B, NH2- NPDEHQKGLSKSLAAEKQFTDDSP- COOH;

VP2/3 C: NH2- IQNDIPRLTSQELERRTQRYLRD- COOH.

In previous studies, SV40 VP B and C peptides were comparatively analyzed through BLAST software, with the corresponding amino acid sequences of 166 BKV, 112 JCV and 4 HPyV 10 serotypes, as well as the other known polyomaviruses. The low percent of homology in amino acid sequences between human polyomaviruses and SV40-VPB/VPC peptides, utilized in ELISA test, were reported before. [8,9]

The human peptide hNPS, a.a. sequence SFRNGVGTGMKKTSFQRAKS was employed as a negative control peptide. The synthetic peptides were synthesized by standard procedures and were purchased from UFPeptides s.r.l., Ferrara. Italy. [7-9]

Peptide coating. Plates were coated with 5 µg of the selected peptide for each well, diluted in 100 µl of Coating Buffer (Candor Bioscience, Wangen, Germany). *Peptide blocking.* Blocking was made with 200 µl/well of the Blocking Solution (Candor Bioscience, Wangen, Germany) at 37°C for 90 min. *Primary antibody adding.* Different wells were covered with 100 µl containing the following sera: positive-control, represented by the immune rabbit serum containing anti-SV40 antibodies, negative controls represented by the immune sera anti- BKV and anti-JCV, and human serum samples under analysis diluted at 1:20 in Low Cross-Buffer (Candor Bioscience, Wangen, Germany). Each sample was analyzed three times. *Secondary antibody adding.* The solution contained a goat anti-human IgG heavy and light chain specific peroxidase-conjugate (Calbiochem-Merck, Darmstadt, Germany) diluted 1:10,000 in Low Cross-Buffer. *Dye treatment and spectrophotometric reading.* Samples were treated with 100 µl of 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) solution (Sigma-Aldrich, Milan, Italy) and then read at the spectrophotometer (λ) of 405 nm. This approach detects the color intensity in wells where the immunocomplexes were formed by optical density (OD). *Cut-off determination.* The cut-off was determined in each assay, by an OD reading of two negative controls, added to the standard deviation and multiplied three times (+3SD).

Sera with antibodies against SV40 were considered VP-positive upon reacting to both peptides of the late region and when sera which had been analyzed three times by indirect ELISA testing gave the same positive result.

Statistics

The prevalence of SV40-positive serum samples from oncologic patients was compared with the prevalence detected in healthy individuals. All data are expressed as a percentage (%). To determine significances between two groups we used two-sided chi-square test with Yates' correction. All computational analyses were performed by Prism 4.0 (GraphPad software, La Jolla, CA, USA).

The serologic profile of serum antibody reactivity to SV40 mimotopes was statistically analyzed using Unpaired t-test. For all tests, we considered *P* values < 0.05 to be statistically significant.

RESULTS

Serum samples from UNPC patients (n=64, median age 57 ys), together with the control represented by healthy subjects (n=130, median age 57 ys) were analyzed by indirect ELISA for the presence of IgG class antibodies against SV40 VP mimotopes, designated VP1 B and VP2/3 C peptides.

In the first step of our investigation, serum samples from oncologic patients affected by UNPC were analyzed by indirect ELISA for the presence of IgG class antibodies against SV40 VP mimotopes/epitopes. Indirect ELISA testing was employed to assay serum samples, which had been diluted at 1/20, for reactivity to SV40 epitopes from VP1, VP1 B peptide. Serum samples reacting with the SV40 VP1 B mimotopes reached an overall prevalence of 25%. Then, the same assay was addressed to detect IgG class serum antibodies against SV40 VP2/3 epitopes, which are present in the VP2/3 C peptide. Serum samples reacted with the SV40 VP2/3 C peptide with a similar prevalence, 27%, as had been detected previously for the VP1 B peptide. Conversely, seronegative samples for the SV40 VP1 B peptide failed to react with SV40 VP2/3 C epitopes. The exceptions were negligible represented by a few serum samples which were negative for VP1 B peptide, while testing positive for VP2/3 C peptide, and vice-versa. The difference was not statistically significant ($P > 0.05$) (Table 1). In the ELISA experiments, the human peptide hNPS was employed as a negative control peptide. [9] Data indicate that this negative control peptide does not react with SV40-positive sera. The OD value was usually in the range of 0.088-0.098, which is consistent with the OD for SV40-negative sera.

The two indirect ELISAs, with the two distinct VPs B and C peptides gave overlapping results, thus confirming the presence of anti-SV40 VPs antibodies in human sera from patients affected by UNPC (Table 1).

In our investigation only those samples found positive for both B and C peptides were considered SV40-positive.

Altogether our immunologic data indicate that combining the SV40-positive sera (*n* = 64), both for the VP1 B and VP2/3 C peptides, the overall prevalence was 25% (Table 1; Figure 1). No positive results were obtained with human peptide used as a control, which had an OD of less than 0.1. SV40-positive sera tested by indirect ELISA diluted at 1/20 had a general cut-off, by spectrophotometric reading, in the range of 0.17-0.19 OD. This cut-off represents the value that discriminates SV40-negative (sample bellow OD 0.17-0.19) from SV40-positive samples (above OD 0.17-0.19). The positive control, represented by the SV40 hyperimmune serum, had an OD of up to 1.8, while the two JCV and BKV hyperimmune sera, which were employed as negative controls, had an OD of less than 0.1.

To verify whether human sera (n= 130) from healthy subjects contain IgG antibodies reacting to SV40 antigens/peptides, the same indirect ELISA employing synthetic peptides corresponding to SV40 VPs epitopes was used. Serum samples diluted at 1:20, from healthy subjects with the same median age (57 yrs) of UNPC patients, were tested for reactivity to two SV40 peptides from VP1/VP3 capsid proteins, VP1 B and VP2/3 C peptides (Table 1). The prevalence of SV40 antibodies IgG class, detected in the cohort of healthy individuals, had a prevalence of 16% (Table 1; Figure. 1).

Also in these series of immunologic assays, seropositive samples to SV40 VP2/3 C peptide were the same samples found positive for the SV40 VP 1 B peptide. Conversely, seronegative samples to SV40 VP1 B peptide failed to react with SV40 VP 2/3 C peptide. A few serum samples were found negative for VP 1 B, while testing positive for VP 2/3 C peptide. SV40-positive sera, diluted at 1:20, had a general value of approximately 0.15 OD.

Specifically, in serum samples from UNPC patients (*n* = 64) the prevalence of specific SV40 VPs antibodies was 25% while in the control group (*n* = 130) was 16%. This difference is not statistically significant (P>0.05, Chi square test with Yates' correction), (Table 1, Figure1).

Serologic profiles of serum antibody reactivity to SV40 mimotopes are presented in Figure 2. The difference in OD mean value of sera from UNPC and Healthy subjects for SV40 B peptide, was

statistically significant, ($P<0.05$, Unpaired t test).

DISCUSSION

In our investigation, serum samples from UNPC patients and normal individuals were analyzed for their reactivity to SV40 VP mimotopes using indirect ELISA. Sera from UNPC patients reacted against SV40 VP antigens with a higher prevalence (25%) compared to the cohort control represented by healthy subjects (HS) (16%). However, the difference is not statistically significant.

Besides confirming previous data indicating that SV40 is present in the adult population, our findings indicate that SV40 infection is not associated with UNPC. The prevalence of SV40 antibodies in serum samples from UNPC patients and HS seems to confirm earlier data obtained in investigations carried out by PCR techniques, which indicated SV40 sequences in tumor and normal tissues. [3, 6, 9, 10]

It has been shown that SV40 is present in the urine, stool, tonsil and blood specimens of carriers suggesting that different routes of transmission are responsible for SV40 infection. [11-19] At present, SV40 infection seems to occur independently from early contaminated vaccines. Indeed, many studies reported SV40 sequences, serum antibodies against SV40 and SV40 isolation/rescue from subjects too young or too old to have been vaccinated with SV40-contaminated vaccines. Altogether these investigations suggest that SV40 may be contagiously transmitted in the human population either directly by person-to-person contacts or indirectly by the oro-fecal and other routes. [2, 6]

In this study, the cohort of healthy individuals showed an overall prevalence of the IgG class of SV40 antibodies is in the range of 16%. It is important to note that the SV40 prevalence in human sera from healthy subjects, detected by our immunologic study, does not differ substantially from that reported by a previous study carried out in the U.S. using neutralization testing against SV40 infectivity, which is considered the gold standard for measuring the presence of the SV40 antibody with neutralization activity. [17] In addition, SV40 sequences were detected in earlier studies with a similar prevalence (16%) by PCR assays in healthy blood donors. [20]

The onset/progression of UNPC, like other cancers, is associated with specific gene mutations .[21-23] However, the agents responsible for the occurrence of mutations/chromosome alterations are poorly understood, particularly those acting as initiators in the early phases of UNPC development.

One may speculate that SV40 is a co-factor in UNPC among patients who do not mount an immune response to this small DNA tumor virus.

Although EBV is strictly associated with UNPC, SV40 seems not to be an additional co-factor of pathogenic relevance.[2, 6] It is also possible that our sample size is still to small to draw final conclusion on the putative role of SV40 in the onset/progression of UNPC.

Our results may stimulate further studies to investigate addition risks factors, other than EBV, with the aim to better elucidate the UNPC pathogenesis.

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Figure's Legend

Figure 1. Prevalence of SV40-positive serum samples from undifferentiated nasopharyngeal carcinoma (UNPC) patients and healthy subjects (HS). To compare the SV40 prevalence of UNPC, with that detected in HS, employed as controls, HS were chosen with the same median age and gender. Statistical analyses revealed not significant differences in SV40 prevalence between UNPC and the relative cohort of HS ($P>0.05$), statistical analysis was performed by the $\chi 2$ test with Yates' correction.

Figure 2. Serologic profile of serum antibody reactivity to SV40 mimotopes VP1 B (A) and VP2/3 C (B) and VPsB-C (C). Immunologic data are from patients affected by UNPC and from healthy subjects. Data are OD values at 405 nm of serum samples diluted 1:20, detected in indirect ELISA. In scatter dot plotting, each plot represents the dispersion of OD values to a mean level indicated by the line inside the scatter with standard error mean (SEM) for each group of subjects analyzed (Mean OD ± SEM). The difference of OD mean value for reactivity with VP1 B is statistically significant lower in sera from patients with UNPC (*$P < 0.05$). Statistical analysis was performed by the Unpaired t test .

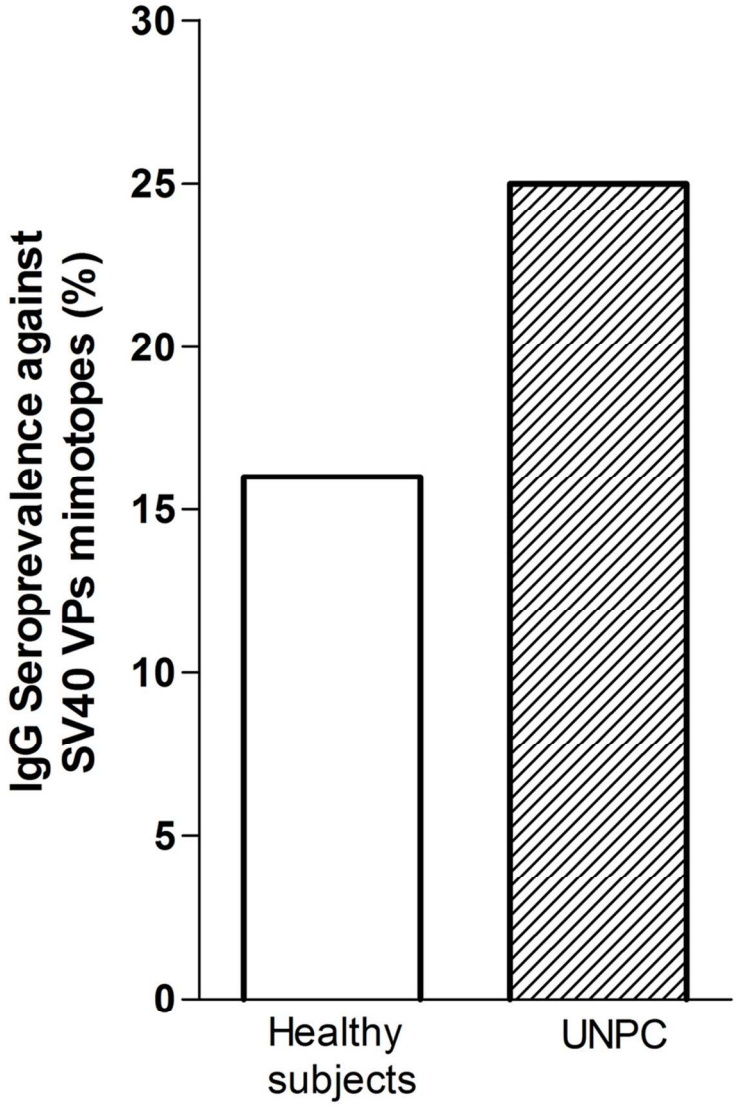
Conflicts of Interest:

All authors declare no financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work, including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Table 1. Prevalence of immunoglobulin G antibodies reacting with Simian Virus 40 (SV40) viral peptide (VP) mimotopes.

Human Sera	Number of Patients/ subjects	Median Age (y)	Male (%)	Number of Positive Samples (%)		
				VP B	VP C	VPs (B+C)
UNPC patients	64	57	81	16 (25)	17 (27)	16 (25)
Healthy subjects	130	57	65	21 (16)	24 (18)	21 (16)

Human serum samples were from undifferentiated nasopharyngeal carcinoma (UNPC) patients and healthy subjects. The different prevalence of SV40 antibodies, between the cohorts of UNPC patient and healthy subjects, was not statistically significant ($P>0.05$). Statistical analysis was performed using the χ 2 test with Yates' correction.



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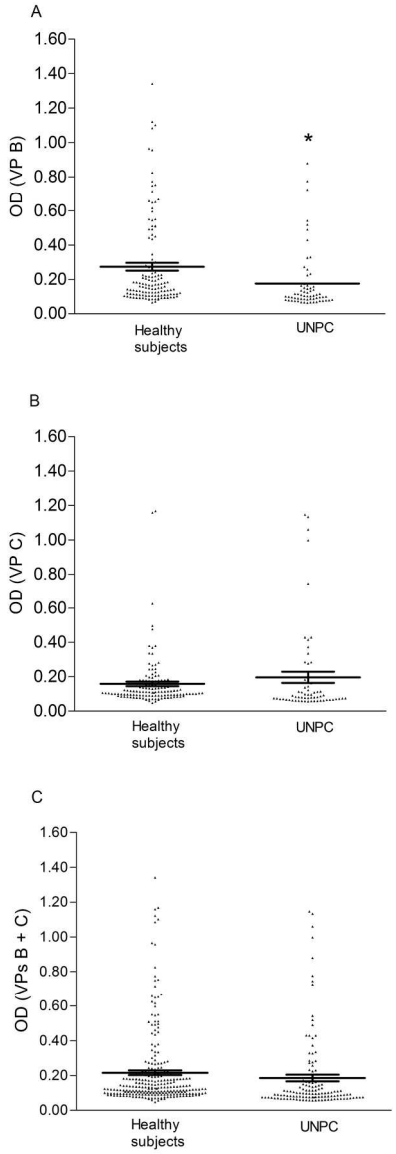

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149x392mm (300 x 300 DPI)