Serologic investigation of undifferentiated nasopharyngeal carcinoma and simian virus 40 infection

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ABSTRACT: Background. The association between undifferentiated nasopharyngeal carcinoma (NPC) and Epstein–Barr virus (EBV) is well established. Nevertheless, available evidence suggests that other cofactors are required for the development of undifferentiated NPC. Several investigations reported simian virus 40 (SV40) footprints in human tumors of different histotypes.

Methods. Serum samples from patients with undifferentiated NPC (n = 64) and healthy subjects (n = 130) were analyzed by an indirect enzyme-linked immunosorbent assay (ELISA) with SV40 synthetic peptides to detect antibodies against viral peptide (VP) capsid proteins VP1, 2, and 3.

Results. Immunologic data indicate that in sera from patients with undifferentiated NPC, the prevalence of SV40 antibodies was 25%, whereas in controls it was 16%. This difference is not statistically significant (p > .05).

Conclusion. A similar prevalence of SV40 antibodies was detected in undifferentiated NPC and healthy subjects. Our serologic data suggest no association between undifferentiated NPC and SV40 infection. This investigation may stimulate further studies aimed at determining the possible contribution of other risk factors in the pathogenesis of undifferentiated NPC. ©2015 Wiley Periodicals, Inc. Head Neck 00:000–000, 2015

KEY WORDS: undifferentiated nasopharyngeal carcinoma, simian virus 40, enzyme-linked immunosorbent assay (ELISA), antibody

INTRODUCTION

Human undifferentiated nasopharyngeal carcinoma (NPC) is a tumor with a well-defined ethnic and geographic distribution. Undifferentiated NPC represents a rare malignancy with a low incidence in western countries with approximately 1 case in every 100,000 people. Distinct genetic and environmental risk factors are involved in undifferentiated NPC onset/progression. In almost all undifferentiated NPC, 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 whereas SV40 sequences were identified in EBV-immortalized lymphoblastoid cell lines3 and in B and T lymphocytes.4 Indeed, SV40, a small DNA tumor virus, has been found to be 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 patients with cancer. In experimental infections in vitro, SV40 is able to infect and transform both purified B cells and T cells.5 These results indicate that human lymphocytes may be vector of SV40 in other tissues of the host.6 In addition, SV40 was found to be mutagenic in different human cells.2,6 The viral oncoprotein large T antigen 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 content, it is worth recalling that in preneoplastic 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 undifferentiated NPC 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 patients with undifferentiated NPC and healthy controls.

**MATERIALS AND METHODS**

**Patients**

Serum samples of patients with undifferentiated NPC (n = 64) seen at the National Cancer Institute, Aviano, Italy, from 2010 to 2013 with histologically proven diagnosis of undifferentiated NPC, localized exclusively in the nose-pharyngeal area, were included in this study. Serum samples from healthy subjects (n = 130) with the same median age of patients with undifferentiated NPC (mean age, 57 years), 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 the patients and the healthy subjects. The study was approved by the County Ethical Committee, Ferrara, Italy.

**Immunologic test**

An indirect enzyme-linked immunosorbent assay (ELISA), developed and set up in our laboratories, was used as described before.7,8 Synthetic peptides, named viral peptide (VP)1B and VP2/3C, were used as antigens to detect human sera-specific immunoglobulin G (IgG) antibodies against SV40. They were VP1B, NH2-NPDEHQKGLSKLAAEKQFTDDSP-COOH and VP2/3C: NH2-IQNDIPRLTSQELERRTQRYLRD-COOH.

In previous studies, SV40 VPB 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 percentage of homology in amino acid sequences between human polyomaviruses and SV40-VPB/VP-C peptides, utilized in ELISA test, were reported before.8,9

The human peptide hNPS, a.a. sequence SFRNGVGTGMKKTSPQRK, was used as a negative control peptide. The synthetic peptides were synthesized by standard procedures and were purchased from UFPep-tides s.r.l., Ferrara, Italy.7–9

**Peptide coating.** Plates were coated with 5 μg of the selected peptide for each well and 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 minutes.

**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 3 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 solution (Sigma–Aldrich, Milan, Italy) and then read at the spectrophotometer (λ) of 405 nm. This approach detects the color intensity in wells in which the immunocomplexes were formed by optical density.

**Cutoff determination.** The cutoff was determined in each assay by an optical density reading of 2 negative controls, added to the SD and multiplied 3 times (+3SD). Sera with antibodies against SV40 were considered VP-positive upon reacting to both peptides of the late region and when sera that had been analyzed 3 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 were expressed as a percentage (%). To determine significances between the 2 groups we used the 2-sided chi-square test with Yates’ correction. All computational analyses were performed by Prism 4.0 (GraphPad software, La Jolla, CA).

The serologic profile of serum antibody reactivity to SV40 mimotopes was statistically analyzed using the unpaired t test. For all tests, we considered p values < .05 to be statistically significant.

**RESULTS**

Serum samples from patients with undifferentiated NPC (n = 64; median age, 57 years) together with the control represented by healthy subjects (n = 130; median age, 57 years) were analyzed by indirect ELISA for the presence of IgG class antibodies against SV40 VP mimotopes/epitopes. Designated VP1B and VP2/3C peptides.

In the first step of our investigation, serum samples from oncologic patients affected by undifferentiated NPC were analyzed by indirect ELISA for the presence of IgG class antibodies against SV40 VP mimotopes/epitopes. Indirect ELISA testing was used to assay serum samples, which had been diluted at 1/20 for reactivity to SV40 epitopes from VP1 and VP1B peptides. Serum samples reacting with the SV40 VP1B 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 VP2/3C peptide. Serum samples reacted with the SV40 VP2/3C peptide with a similar prevalence, 27%, as had been...
detected previously for the VP1B peptide. Conversely, seronegative samples for the SV40 VP1B peptide failed to react with SV40 VP2/3C epitopes. The exceptions were negligible and were represented by a few serum samples that were negative for VP1B peptide, whereas testing positive for VP2/3C peptide, and vice-versa. The difference was not statistically significant ($p > 0.05$; Table 1). In the ELISA experiments, the human peptide hNPS was used as a negative control peptide. Data indicate that this negative control peptide does not react with SV40-positive sera. The optical density value was usually in the range of 0.088 to 0.098, which is consistent with the optical density for SV40-negative sera.

The 2 indirect ELISAs, with the 2 distinct VPs B and C peptides, gave overlapping results, thus confirming the presence of anti-SV40 VP antibodies in human sera from patients affected by undifferentiated NPC (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 VP1B and VP2/3C peptides, gave the overall prevalence of 25% (Table 1; Figure 1). No positive results were obtained with human peptides used as controls, which had an optical density of $<0.1$. SV40-positive sera tested by indirect ELISA diluted at 1/20 had a general cutoff, by spectrophotometric reading, in the range of 0.17 to 0.19 optical density. This cutoff represents the value that discriminates SV40-negative (sample below optical density 0.17–0.19) from SV40-positive samples (above optical density 0.17–0.19). The positive control, represented by the SV40 hyperimmune serum, had an optical density of up to 1.8, whereas the 2 JCV and BKV hyperimmune sera, which were used as negative controls, had an optical density of $<0.1$.

To verify whether human sera ($n = 130$) from healthy subjects contain IgG antibodies reacting to SV40 antigens/peptides, the same indirect ELISA using synthetic peptides corresponding to SV40 VPs epitopes was used. Serum samples diluted at 1:20, taken from healthy subjects with the same median age (57 years) of patients with undifferentiated NPC, were tested for reactivity to 2 SV40 peptides from VP1/VP3 capsid proteins, VP1B and VP2/3C 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 VP2/3C peptide were the same samples found positive for the SV40 VP1B peptide. Conversely, seronegative samples to SV40 VP1B peptide failed to react with SV40 VP2/3C peptide. A few serum samples were found negative for VP1B, while testing positive for VP2/3C peptide. SV40-positive sera, diluted at 1:20, had a general value of approximately 0.15 optical density.

Specifically, in serum samples from patients with undifferentiated NPC ($n = 64$), the prevalence of specific SV40 VP antibodies was 25% whereas in the control group ($n = 130$) was 16%. This difference was not

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**TABLE 1. Prevalence of immunoglobulin G antibodies reacting with simian virus 40 viral peptide mimotopes.**

<table>
<thead>
<tr>
<th>Human sera</th>
<th>No. of patients/subjects</th>
<th>Mean age, y</th>
<th>Male %</th>
<th>VP B (%)</th>
<th>VP C (%)</th>
<th>VPs B + C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with undifferentiated NPC</td>
<td>64</td>
<td>57</td>
<td>81</td>
<td>16 (25)</td>
<td>21 (16)</td>
<td></td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>130</td>
<td>57</td>
<td>65</td>
<td>17 (27)</td>
<td>24 (18)</td>
<td>21 (16)</td>
</tr>
</tbody>
</table>

Abbreviations: VP, viral peptide; NPC, nasopharyngeal carcinoma.

Human serum samples were from patients with undifferentiated NPC and healthy subjects. The different prevalence of simian virus 40 (SV40) antibodies, between the cohorts of patients with undifferentiated NPC and healthy subjects, was not statistically significant ($p > 0.05$). Statistical analysis was performed using the chi-square test with Yates’ correction.
Statistically significant ($p > .05$, chi-square test with Yates’ correction; Table 1; Figure 1). Serologic profiles of serum antibody reactivity to SV40 mimotopes are presented in Figure 2. The difference in the optical density mean value of sera from undifferentiated NPC and healthy subjects for SV40B peptide was statistically significant ($p < .05$, unpaired $t$ test).

DISCUSSION

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

In addition to confirming previous data indicating that SV40 is present in the adult population, our findings indicate that SV40 infection is not associated with undifferentiated NPC. The prevalence of SV40 antibodies in serum samples from patients with undifferentiated NPC and healthy subjects seems to confirm earlier data obtained in investigations carried out by polymerase chain reaction 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 orofecal 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 United States 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 polymerase chain reaction assays in healthy blood donors.20

The onset/progression of undifferentiated NPC, 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 undifferentiated NPC development.24 One may speculate that SV40 is a cofactor in undifferentiated NPC among patients who do not mount an immune response to this small DNA tumor virus.
Whereas EBV is associated with undifferentiated NPC, SV40 does not seem to be a cofactor of pathogenic relevance for that type of tumor.2,6 It is also possible that our sample size is still too small to draw a final conclusion on the putative role of SV40 in the onset/progression of undifferentiated NPC.

Our results may stimulate further studies to investigate additional risk factors, other than EBV, with the aim to better elucidate the undifferentiated NPC pathogenesis.24

REFERENCES


