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ORIGINAL ARTICLE

High prevalence of antibodies reacting to mimotopes of Simian virus 40 large T antigen, the oncoprotein, in serum samples of patients affected by non-Hodgkin lymphoma

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Abstract A new immunological investigation was carried out to study the association between non-Hodgkin lymphoma and Simian virus 40 (SV40). To this end, a new indirect ELISA was employed with two mimotopes from SV40 large T antigen (Tag), the viral oncoprotein, to analyse for specific reactions to antibodies in sera from non-Hodgkin lymphoma patients and controls, represented by healthy subjects (HS) and breast carcinoma (BC) patients. This study allowed us to assay a new sera collection from non-Hodgkin lymphoma patients (NHL, $n = 254$). To verify the association between NHL and SV40 Tag, two totally independent cohorts were analysed: NHL1 $n = 150$ and NHL2 $n = 104$. The epidemiological survey included sera from HS1, $n = 150$; HS2, $n = 104$ and BC, $n = 78$. This new indirect ELISA revealed that antibodies against SV40 Tag mimotopes are detectable in NHL1 and NHL2 sera with a prevalence of 37 and 36%, respectively. The prevalence of SV40-antibodies detected in both NHL1 and NHL2 cohorts differs statistically from controls, at 19% for HS1 ($p < 0.01$), HS2 ($p < 0.05$) and BC patients ($p < 0.05$). This study, carried out with an immunological assay with specific Tag oncoprotein mimotopes of Simian virus 40, reports the presence of IgG antibodies against the large Tumour antigen in non-Hodgkin lymphomas for the first time. Our immunological data with two independent NHL cohorts show a statistically significant association between Simian virus 40 Tag and non-Hodgkin lymphoma. These results suggest that SV40-positive non-Hodgkin lymphomas could be treated differently from those tested SV40-negative.

Keywords NHL · SV40 · Infection · T antigen · Mimotope · Antibody

Abbreviations

a.a.	Amino acid
ABTS	2,2′-Azino-bis 3-ethylbenzthiazoline-6-sulfonic acid
BC	Breast cancer
BKPyV	BK polyomavirus
CI	Confidence interval
ELISA	Enzyme-linked immunosorbent assay
hNPS	Human neuro-peptide S
HS	Healthy subjects
IgG	Immunoglobulin G
JCPyV	JC polyomavirus
MCPyV	Merkel cell polyomavirus
NHL	Non-Hodgkin lymphoma
OD	Optical density
SV40	Simian virus 40
Tag	Large T antigen
tag	Small t antigen
VLP	Virus-like particle
VP	Viral protein
WHO/IARC	World Health Organization/International

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	Agency for Research on Cancer
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Introduction

Previous studies have been carried out to verify the association between Simian virus 40 (SV40) and different human lymphoproliferative disorders, including distinct types of non-Hodgkin lymphoma (NHL) [1–5]. Some groups indicated an association between NHL and SV40 by reporting a high prevalence of SV40 Tag coding sequences at up to 43% [1–3]. Other investigators have not confirmed the association [6]. Differing prevalence levels of SV40 infection, detected in human populations of distinct origin, could be due to living parameters and hygiene conditions [7]. The different prevalence of SV40 infection may account for SV40-positive or SV40-negative tumor samples, and SV40-positive or SV40–negative serum samples based on the IgG antibodies reactivity against the SV40 antigens [8]. Moreover, experimental data obtained in cell cultures using the SV40 infectivity neutralization assay employing human sera indicate that 12% carry neutralizing SV40 antibodies [9]. This technique is highly specific and is considered the gold standard in the field for evaluating the presence of SV40 serum antibodies [10]. However, this method is tedious, requires great lengths of time, high skill levels, needs several reagents, and has a high cost [11]. In short, it is not at all practical.

After its isolation, SV40 proved to be a powerful transforming agent and oncogenic in experimental animals [12]. SV40 inoculation in rodents by differing routes induced distinct tumours, including lymphoproliferative disorders [12, 13]. SV40 was isolated in 1960 from the early Salk intramuscular inactivated and Sabin oral attenuated antipoliomyelitis vaccines. SV40-contaminated poliovaccines were employed during the 1955–1963 period, as well as in subsequent years [14, 15].

SV40 transforming and oncogenic activities are due to two viral oncoproteins, known as Tag and small t (tag) antigens [12]. Tag binds the p53 and pRb family proteins, thus abolishing their tumour suppressor activities, whereas tag enhances Tag transformation by interacting with the cellular protein phosphatase-2A. Similarly, under the control of specific promoters, recombinant plasmids expressing Tag and transgenic mice carrying the Tag gene produce tumours in tissues where Tag is expressed. When taken together, these data indicate that SV40 is a potent oncogenic viral agent [12].

A World Health Organization/International Agency for Research on Cancer (WHO/IARC) meeting held in 2012 indicated that SV40 is not currently classifiable as a carcinogenic viral agent in humans [16], mainly due to a lack of robust epidemiological data.

To this aim, we reported seroepidemiological data employing a new Enzyme-linked immunosorbent assay (ELISA) with mimotopes representing epitopes of viral capsid proteins (VPs) 1, 2 and 3 and Tag [10, 11, 17–27]. Synthetic peptides were named VP B and C, and Tag A and D mimotopes, respectively. Seroepidemiological results obtained with these ELISAs indicated that the prevalence of IgG antibodies against SV40 VPs or Tag in the sera of normal subjects, and in oncologic patients affected by cancers not linked to SV40 infection, is in the range of 18%–20% [20, 28]. SV40 VP and Tag mimotopes used in ELISAs are specific for SV40 antibodies and do not cross-react with the homologous polyomaviruses BK (BKPyV) and JC (JCPyV), with hundreds of different genetic variants or with other human polyomaviruses [28]. The presence of specific SV40 Tag antibodies in sera from oncologic patients represents an important biomarker for addressing the association between human tumours and SV40 [29].

In this investigation, the association between NHL and SV40 Tag was studied by analysing the presence of serum IgG antibodies against SV40 Tag by indirect ELISA.

Materials and methods

Serum samples

The association between NHL and SV40 Tag was studied in two totally independent NHL serum collections. NHL sera were harvested in the same period, 2014–2016, and stored at −80 °C., until the time of analysis. To this purpose, sera from NHL patients ($n = 254$) were investigated by indirect ELISAs with SV40 Tag mimotopes [28]. Sera were from NHL1, $n = 150$; mean age 60 years old; NHL2, $n = 104$, mean age 52 years old. NHL sera were from the Haematology Section, University Hospital of Verona. Control groups are represented by serum samples from healthy subjects (HS, $n = 396$; HS1, $n = 150$, mean age 60 years old; HS2, $n = 104$, mean age 52 years old). To give robustness to our seroepidemiological data, we chose two independent NHL cohorts with two different mean ages: 60 years old and 52 years old. The two controls, HS1 and HS2, had the same mean age, 60 years old and 52 years old, respectively. The gender ratio was also similar in the NHL and HS cohorts. Sera from breast cancer patients (BC, $n = 78$; mean age 42 years old) were brought into this study as this tumour is not linked to SV40 [23]. The HS3, $n = 142$, mean age 42 years old, was the control cohort for BC. HS sera were collected anonymously at the Clinical Analysis Laboratory, University Hospital of Ferrara. Serum samples, $n = 728$, were stored at −80 °C., until the time of analysis and were

recorded with the age, gender and pathology of patients. The Ethic Committee of Ferrara approved the study.

Indirect ELISA

The indirect ELISA employed herein has been recently published [20, 28]. Briefly, mimotopes known as Tag A and D were employed in ELISA to detect SV40 Tag antibodies. *Coating phase*: immunologic plates (Nunc-immuno plate PolySorp, Thermo Fisher Scientific, Milan, Italy) were coated with 5 μg of the Tag A and Tag D peptide (sequences reported below) in each well, diluted in 100 μl of Coating Buffer 1×, pH 9.6 (Candor Bioscience, Wangen, Germany). Coated peptides were left at 4 °C for 16 h. Amino acid (a.a.) sequences of the two Tag A and Tag D synthetic peptides from a.a. residues 669–689 (21 a.a.) and from 659–682 (24 a.a.), respectively, are as follows:

Tag A: NH2-G S F Q A P Q S S Q S V H D H N Q P Y H I-COOH (Tag a.a. 669–689);
Tag D: NH2-H E T G I D S Q S Q G S F Q A P Q S S Q S V H D-COOH (Tag a.a. 659–682).

These two mimotopes were purchased, from UFPeptides s.r.l., Ferrara, Italy. *Blocking phase*: Coated peptides were rinsed twice with Tris-based Washing Buffer (Candor Bioscience, Wangen Germany). The blocking solution (200 μl) contained the casein and Tween detergent (Candor Bioscience, Wangen, Germany), pH 7.2, was added to each well. *Sera addition*. Wells were rinsed three times with Tris-based Washing Buffer (Candor Bioscience, Wangen, Germany). Then, diluted sera were added to wells. Control samples: positive controls were represented by (1) hyperimmune rabbit serum containing anti-SV40 Tag antibodies (diluted 1: 100) [28]; (2) six SV40 Tag-positive human sera, which reacted with SV40 VP mimotopes in previous investigations [11]; negative controls were (1) hyperimmune sera with anti-BKPyV and anti-JCPyV antibodies (diluted 1: 100); (2) three human sera found to be SV40-negative in a previous report [28]; (3) SV40 unrelated human neuropeptide S (hNPS) a.a sequence SFRNGVGTGMKKTSFQRAKS [11, 28]. Sera from NHL1-2, BC patients, and HS1-3 were diluted in Low Cross-Buffer pH 7.2 (Candor Bioscience, Wangen, Germany) initially at 1:20 and then at dilutions up to 1:640. Additional controls were constituted by secondary antibody only and wells void of both primary and secondary antibodies. Immuno-complex reactions were carried out at 37 °C for 90 min. *Secondary antibody addition.* Wells were rinsed twice with the washing buffer. Then, the secondary antibody was added to each sample. The solution contained goat anti-human or goat anti-rabbit IgG heavy (H) and light (L) chain-specific peroxidase-conjugate (Calbiochem-Merck, Darmstadt, Germany) diluted 1:10,000 in Low Cross-Buffer (Candor Bioscience, Wangen, Germany). *Optical density (OD) reading*. In the end, plates were washed 3 times and treated with 100 μl of 2,2′-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) solution (A 3219 Sigma-Aldrich, Milan, Italy) for the colorimetric reaction. Optical density (OD) was taken by a spectrophotometer ($\lambda = 405$ nm), as reported before [19, 28]. *Cut-off*. Cut-off values for each assay were obtained using the OD reading of the three negative control sera, added to the standard deviation (SD) and multiplied three times (+3 SD). The three SV40-negative control sera were selected from those below the cut-off value determined with second-degree polynomial regression by plotting the ranked net OD individual values for each peptide. A tendency curve was drawn from a second-degree polynomial regression for Tag A and D peptides, as published previously for MCPyV and BKPyV virus-like particles (VLPs) [28].

Only those samples reacting with both mimotopes A and D were considered SV40 Tag-positive. Our representations revealed an inflection point at 0.19–0.18 for peptide A and peptide D, respectively.

Statistical analysis

The prevalence of SV40-positive samples from NHL patients and controls was determined using Chi-square with Yates' correction. The serologic profile of the reactivity to SV40 mimotopes was statistically analysed using the Anova and Tukey's multiple comparisons test. All computational analyses were performed with Prism 6.0 (GraphPad software, San Diego, CA). For all tests, p was considered to be statistically significant when <0.05.

Results

Detecting SV40 Tag antibodies using indirect ELISA in sera from non-Hodgkin lymphoma-affected patients

This investigation was carried out to study the association between NHL and SV40 Tag. To this purpose, sera from two independent NHL cohorts, known as NHL1 and NHL2, were investigated to detect antibodies against SV40 Tag epitopes. This study employed a new indirect ELISA with synthetic peptides, which mimic SV40 Tag antigens known as A and D mimotopes.

Initially, NHL1 sera diluted to 1/20 were tested for reactivity to SV40 Tag A peptide. NHL1 serologic profiles are shown in Fig. 1. NHL1 sera found to be SV40 Tag A positive had a prevalence of 40% (Table 1), as their OD is in the range of 0.19–0.89 (Fig. 1). Then, NHL1 sera were analysed in indirect ELISA for their reactivity with SV40

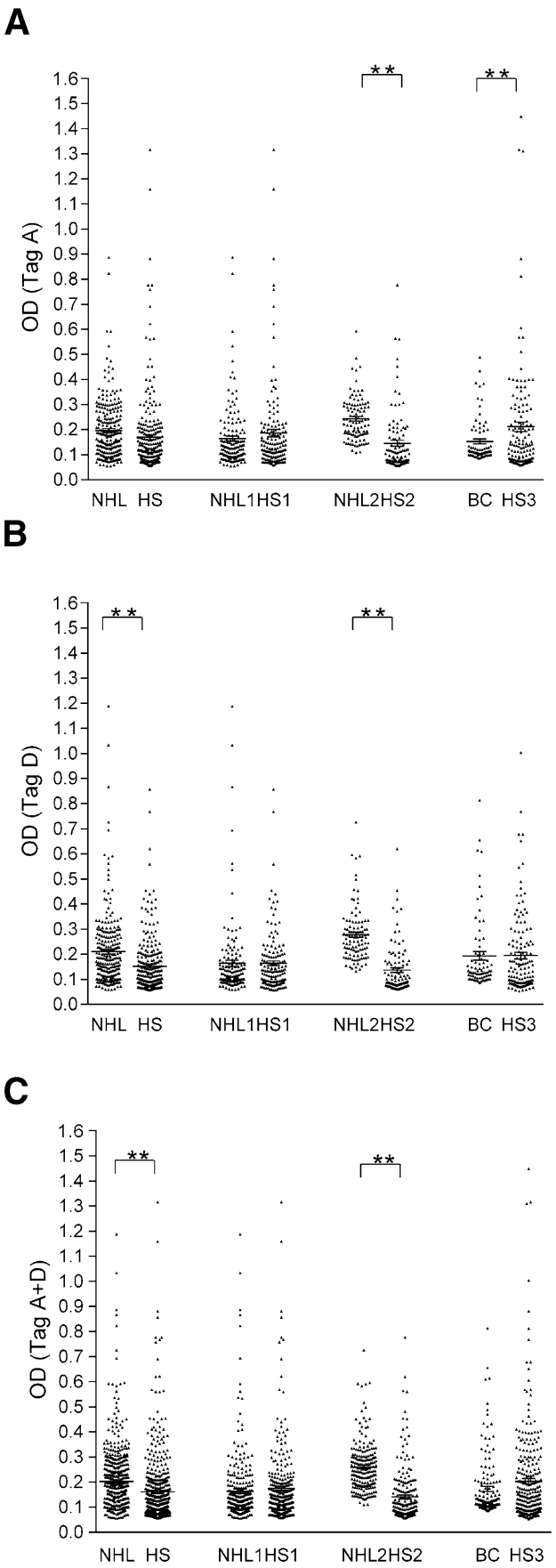

Fig. 1 Serologic profile of serum antibody reactivity to SV40 Tag ▶ mimotope A (**a**), mimotope D (**b**) and Tag mimotopes A + D (**c**). Immunologic data are from the total NHL and the two independent NHL1 and NHL2, BC patients and healthy subjects (HS total, and HS1, HS2, together with HS3). Results are presented as OD values. In the scatter dot plotting, each plot represents the dispersion of individual sample OD values to a mean level, indicated by the *long horizontal line* inside the scatter with the standard error of the mean (SEM) marked by a *short horizontal line* for each age group. Data were analysed with one-way Anova analysis and Tukey's multiple comparisons test (OD mean, 95% CI). **a** High OD values for antibodies against SV40 mimotope Tag A in NHL2 patients (0.24 OD, 95% CI 0.23–0.26) versus HS2 (0.14 OD, 95% CI 0.12–0.17, $^{**}p < 0.0001$). The mean OD of sera against SV40 mimotope Tag A in BC (0.15, 95% CI 0.13–0.17) was lower than that detected in healthy females (HS3) (0.21, 95% CI 0.17–0.25, $^{*}p < 0.05$). **b** High OD values for antibodies against SV40 mimotope Tag D in NHL patients (0.21 OD, 95% CI 0.19–0.23) versus HS (0.15 OD, 95% CI 0.14–0.17, $^{**}p < 0.0001$). High OD values for antibodies against SV40 mimotope Tag D in NHL2 patients (0.27 OD, 95% CI 0.26–0.29) versus HS2 (0.14 OD, 95% CI 0.11–0.16, $^{**}p < 0.0001$). (C) OD value for antibodies against SV40 mimotopes both peptides Tag A and Tag D was higher in NHL (0.20 OD, 95% CI 0.19–0.21) versus HS (0.16 OD, 95% CI 0.15–0.17, $^{**}p < 0.0001$); similarly, high OD values for antibodies against both peptides Tag A and Tag D were detected in NHL2 patients (0.26 OD, 95% CI 0.25–0.27) versus HS2 (0.14 OD, 95% CI 0.13–0.15, $^{**}p < 0.0001$)

Tag D mimotope. SV40 Tag D positive samples had the same prevalence (41%) (Table 1) which revealed for the Tag A peptide. Positive samples in this test had OD ranging from 0.18 to 1.19 (Fig. 1). In our study, only NHL1 sera ($n = 55$) testing positive for both mimotopes A and D, which reached a prevalence of 37%, were considered SV40-positive (Table 1; Fig. 2).

Then, the indirect ELISA was used to analyse the other independent NHL2 cohort. In this instance, NHL2 sera ($n = 104$) reacted with mimotopes A and D with a prevalence of 38% and 39%, respectively (Table 1; Fig. 2). Tag-positive samples were only those reacting with both peptides. Prevalence was 36% in this NHL2 cohort. The majority of NHL serum samples ($n = 162/254$; 64%) found to be Tag A negative did not react with SV40 Tag D antigen. A minority of sera ($n = 17/254$; 7%) gave discordant results. Indeed, 7 samples reacting with the Tag A peptide failed to react with Tag D mimotope; vice versa, 10 other sera tested Tag D positive while not recognizing Tag A peptide. The prevalence for Tag A (39%) and Tag D (40%) peptides does not differ statistically ($p > 0.05$). Similarly, the prevalence of 39% and 40% found for mimotopes A and D, respectively, is not statistically significant when compared to the prevalence of 36% detected for samples which were positive for both peptides, Tag A + Tag D (Table 1; Fig. 2). The positive control sample, represented by the hyperimmune anti-Tag rabbit serum, recognized both Tag A and Tag D mimotopes, with OD 2.9 and 2.2, respectively. The SV40 positive human sera detected herein

Table 1 Prevalence of IgG antibodies reacting to SV40 Tag mimotopes

Patient/subject	Number of serum samples	Mean age (years)	Male (%)	Number of positive samples (%)			*p* value
				Tag A	Tag D	Tag (A + D)	
NHL	254	60	61	99 (39)	102 (40)	92 (36)	<0.0001***
NHL1	150	60	54	60 (40)	61 (41)	55 (37)	0.0013
NHL2	104	52	70	39 (38)	41 (39)	37 (36)	0.013
HS	254	57	63	67 (26)	58 (23)	49 (19)	
HS1	150	60	66	41 (27)	36 (24)	29 (19)	
HS2	104	52	62	26 (25)	22 (21)	20 (19)	
BC	78	42	–	15 (18)	17 (22)	15 (19)	ns
HS3	142	42	–	34 (24)	34 (24)	28 (20)	

Human sera were from non-Hodgkin lymphoma (NHL), breast cancer (BC) patients, and healthy subjects (HS1, HS2 and HS3) controls. The prevalence of SV40 antibodies in all NHL sera was statistically higher than detected in all HS, with the same mean age (***$p < 0.0001$) and BC patients ($p = 0.0076$). The prevalence of SV40 antibodies in NHL1 sera was statistically higher than detected in HS1, which represents the control with the same mean age ($p = 0.0013$). The prevalence of SV40 antibodies in NHL2 sera was statistically higher than detected in the HS2, the control with the same mean age ($p = 0.013$). The prevalence of SV40 antibodies in sera from BC patients does not differ statistically (ns) from that of healthy females (HS3), with the same mean age of 42 years old ($p > 0.05$).Statistical analysis was performed using Chi-square with Yates' correction. The *p* values shown are based on the Tag (A + D) positive numbers

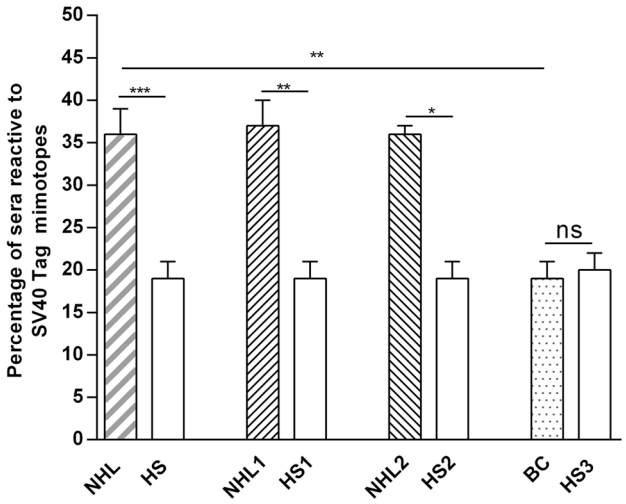

Fig. 2 Prevalence of SV40 Tag-positive serum samples from NHL and BC patients, and three cohorts of healthy subjects (HS1, HS2, HS3). In order to compare SV40 Tag antibody prevalence in NHL, two different groups of healthy subjects (HS) were chosen with the same median age and gender (HS1, HS2). Statistical analyses revealed significant differences in SV40 prevalence between NHL and the relative cohort of healthy subjects (HS) (***$p < 0.0001$) and BC patients (**$p < 0.01$). The prevalence of SV40 antibodies in NHL1 sera was statistically significant higher than that detected in HS1, which represents the control with the same mean age (**$p < 0.01$). The prevalence of SV40 antibodies in NHL2 sera was statistically higher than that detected in the HS2, the control with the same mean age (*$p < 0.05$). No statistically significant difference in SV40 seroprevalence was found between BC patients and their control group of healthy subjects (HS3). (***$p < 0.0001$; **$p < 0.001$, *$p < 0.05$)

displayed ODs at up to 0.876, whereas human sera that did not react with SV40 Tag mimotopes had ODs at less than 0.18. The human negative control, the neuro-peptide known as hNPS, tested-negative in all sera, with OD in the range of 0.052–0.085, which is an OD revealed in SV40-negative samples (data not shown).

Our data indicated that the 36% prevalence of serum antibodies against SV40 Tag epitopes detected in NHL sera (NHL1 + NHL2) is statistically higher than detected in controls represented by HS cohorts (19%; ***$p < 0.0001$) and BC (19%; **$p < 0.01$) (Table 1; Fig. 2). SV40 Tag antibodies prevalence in NHL1- and NHL2-affected patients (37% and 36% respectively) is significantly higher than that detected in their controls represented by HS1 and HS2 (19%; **$p < 0.01$, *$p < 0.05$,) (Table 1; Fig. 2).

The total number of NHL patients, from the two NHL1 and NHL2 cohorts, were divided into two different new age groups, younger NHL patients in the range of 15–50 years old (median age = 40 years old), and older NHL patients in the range of 51–92 years old (median age = 66 years old). The presence of SV40 Tag antibodies in these two different age groups of oncologic patients was observed with a prevalence of 29% and 38%, respectively, which is statistically non-significant ($p > 0.05$; Table 2). In younger NHL patients and HS, with a similar age (40 years old), the prevalence of SV40 Tag antibodies (29% vs. 17%) did not differ statistically ($p > 0.05$; Table 2). On the other hand, the prevalence of SV40 Tag serum antibodies in older NHL patients, with a range age

Table 2 Prevalence of IgG antibodies reacting to SV40 Tag mimotopes in different age cohorts

Patient/subject	Median age (range) (years)	Number of patient/subject	Number of positive samples (%)			*p* value
			Tag A	Tag D	Tag (A + D)	
NHL	63 (15–92)	254	99 (39)	102 (40)	92 (36)	<0.0001**
	40 (15–50)	59	18 (31)	20 (34)	17 (29)	ns
	66 (51–92)	195	81 (41)	82 (42)	75 (38)	<0.01*
HS	58 (22–100)	254	67 (26)	58 (23)	49 (19)	
	45 (22–50)	88	22 (25)	17 (19)	15 (17)	
	60 (51–91)	166	45 (27)	41 (25)	34 (20)	

Human sera were from non-Hodgkin lymphoma (NHL) and healthy subjects (HS) stratified by cohorts of different age groups. The prevalence of SV40 antibodies in all NHL patients was statistically higher than that detected in all HS (**$p < 0.0001$). The prevalence of SV40 antibodies in NHL patients with a age range of 15–50 years old was not statistically different than detected in the HS with the same age range of 22–50 years old (ns; $p > 0.05$). The prevalence of SV40 antibodies in NHL patients with a age range of 51–92 years old was statistically higher than detected in the HS with the same age range of 51–91 years old (*$p < 0.01$). The prevalence of SV40 antibodies in NHL patients with an age range of 15–50 years old was not statistically different than that detected in NHL patients with the age range of 51–92 years old ($p > 0.05$). The presence of SV40 Tag antibodies in these two different age groups of oncologic patients was observed with a prevalence of 29% and 38%, respectively, which is statistically non-significant ($p > 0.05$). Statistical analysis was performed using Chi-square with Yates' correction

ns no statistically significant

of 51–92 years old (38%) was statistically higher than that detected in HS with the same age range of 51–91 years old (20%) with a **$p < 0.01$ (Table 2).

These data show that SV40 Tag antigens are recognized by specific human IgG antibodies, with an increasing prevalence observed in older NHL patients.

The prevalence of SV40 Tag antibodies in sera from patients with several NHL subtypes does not differ statistically **** ($p > 0.05$), (Table 3). NHL has many subtypes, with two main categories: high-grade or aggressive and low-grade or indolent (Table 4). The prevalence of SV40 Tag antibodies in indolent NHL subtype was

Table 3 Serum samples from non-Hodgkin lymphoma patients and different NHL subtypes

Patient/subject	Number of serum samples	Number of Tag (A + D) positive samples (%)
NHL subtypes		
Lymphoplasmacytic lymphoma	1	1 (100)
Lennert lymphoma T	1	1 (100)
Immunoblastic lymphoma	4	2 (50)
Nodal marginal zone lymphoma	14	7 (50)
T Cell large granular leukaemia/lymphoma	8	4 (50)
Mantle cell lymphoma	20	9 (45)
Splenic marginal zone B-cell lymphoma	21	9 (43)
Follicular NHL lymphoma	33	14 (42)
Diffuse large B-cell lymphoma	48	19 (40)
Aggressive lymphoma	3	1 (33)
Low grade	18	6 (33)
Mucosa-associated lymphoid tissue lymphoma	3	2 (33)
Unclassified	27	9 (33)
Anaplastic large cell lymphoma	9	2 (22)
B-CLL	19	3 (16)
Marginal zone B-cell lymphoma	19	3 (16)
Burkitt's lymphoma	4	0 (–)
Peripheral T-cell lymphoma	2	0 (–)
NHL	254	92 (36)**
HS	254	49 (19)

Human sera were from non-Hodgkin lymphoma (NHL) and healthy subjects (HS). The prevalence of SV40 Tag antibodies in all NHL patients is statistically significant higher that detected in HS (**$p < 0.0001$). Statistical analysis was performed using Chi-square with Yates' correction

Table 4 Prevalence of immunoglobulin G antibodies reacting to SV40 Tag mimotopes in patients affected by indolent and aggressive NHL subtypes

Sera	Number of patients/subjects	Mean age patients/subjects (range) years	Male (%)	Number of positive sample (%)			*p* value (%)
				Tag A	Tag D	Tag (A + D)	
NHL	254	60 (15–92)	61	99 (39)	102 (40)	92 (36)	<0.0001
Indolent	159	62 (21–88)	63	64 (40)	65 (41)	61 (38)	<0.001*
Aggressive	68	53 (15–88)	62	24 (35)	26 (38)	22 (32)	>0.05
UNK	27	66 (27–92)	44	11 (41)	11 (41)	9 (33)	
HS	254	57 (22–91)	63	67 (26)	58 (23)	49 (19)	
HS1	150	60 (22–87)	66	41 (27)	36 (24)	29 (19)	
HS2	104	52 (23–91)	62	26 (25)	22 (21)	20 (19)	

The differing prevalence of SV40 antibodies, revealed in serum samples from NHL indolent and aggressive subtypes. The prevalence of SV40 Tag antibodies in NHL was statistically higher than detected in the HS control (**$p < 0.0001$). The prevalence of SV40 antibodies in indolent NHL subtype was statistically higher than detected in the HS1 control cohort with a similar mean age (*$p < 0.001$). The prevalence of SV40 Tag antibodies in aggressive NHL subtype was not statistically higher than detected in the HS2 control cohort with a similar mean age (*$p > 0.05$). The prevalence of SV40 Tag antibodies in sera from patients with indolent and aggressive NHL subtypes does not differ statistically (°$p > 0.05$). Statistical analysis was performed using Chi-square with Yates' correction

UNK unknown subtypes

statistically higher than that detected in the HS1 control cohort with a similar mean age (*$p < 0.001$), (Table 4). On the other hand, the prevalence of SV40 Tag antibodies in aggressive NHL subtype was not statistically higher than that detected in the HS2 control cohort with a similar mean age (*$p > 0.05$), (Table 4).

The OD values of all patients (NHL) and in NHL2 are higher than those detected in controls (HS and HS2, respectively). The OD value of serum antibodies against SV40 mimotopes A and D was higher in NHL (0.20 OD, 95% CI 0.19–0.21) versus HS (0.16 OD, 95% CI 0.15–0.17, **$p < 0.001$). Similarly, high levels of antibodies against both peptides Tag A and Tag D were detected in NHL2 patients (0.26 OD, 95% CI 0.25–0.27) versus HS2 (0.14 OD, 95% CI 0.13–0.15, **$p < 0.0001$), (Fig. 1). The serologic profile of NHL1 patients was not statistically significantly compared to HS1. No positive results were found with the SV40-unrelated human neuro-peptide S, which was used as a negative control (data not shown).

NHL sera ($n = 20$), which were found to be SV40-positive for both Tag A and Tag D peptides, with an OD in the 0.49–0.65 range, were serially diluted from 1/20 to 1/640 to determine their Tag antibody titer. These sera contained antibodies against SV40 Tag, which remained detectable at 1/80 dilution (Fig. 3). The titer of SV40 Tag antibodies in NHL sera did not differ greatly for the two Tag mimotopes. Our serological experiments, replicated three times by independent operators, gave overlapping results.

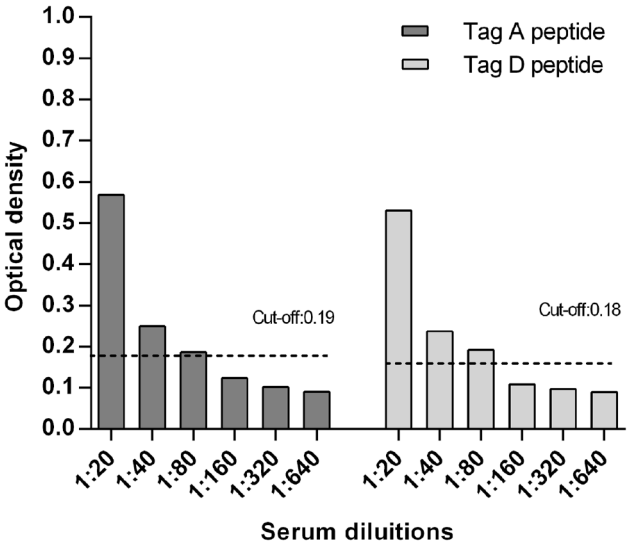

Fig. 3 Titer of SV40-positive sera. Sera ($n = 20$) from NHL patients found to be SV40-positive for both Tag A and Tag D peptides were serially diluted from 1/20 to 1/640 and further investigated by indirect ELISAs. These assays indicated that sera carry antibodies against SV40 Tag that remain positive at 1/80 dilution. This result indicated that the titer of SV40 Tag antibodies in positive sera from NHL patients does not greatly differ for the two Tag A and Tag D peptides

Detecting SV40 Tag antibodies using indirect ELISA in controls represented by sera from HS and BC patients

Control sera from HS and BC patients were analysed using indirect ELISA with two SV40 Tag A and D mimotopes

(Table 1). The total number of HS were divided into three different age groups: HS1; HS2; HS3; as indicated in MM. Accordingly, the prevalence of antibodies in different control age groups was recorded (Table 1; Fig. 2). HS1, HS2 and BC sera tested SV40 Tag-positive for both peptides (Tag A + Tag D) with an overall prevalence of 19%. It is noteworthy that the prevalence of SV40 antibodies in BC patients (19%) and healthy females (HS3) (20%), with the same median age, 42 years old, does not differ statistically (Table 1). Serologic profiles of control sera (total HS, and the single cohort of HS1, HS2, HS3, BC) are shown in Fig. 1. Optical density values, mean OD, of HS, which include all HS of the three cohorts HS1, HS2 and HS3 analysed as a single cohort, are similar and were not statistically significant (Fig. 1). The mean OD of sera against SV40 mimotope Tag A in BC (0.15, 95% CI 0.13–0.17) was lower than that detected in healthy females (HS3) (0.21, 95% CI 0.17–0.25, $*p < 0.05$).

Discussion

SV40 Tag coding sequences and the expression of SV40 Tag oncoprotein have been reported by many researchers in different human tumours including lymphoproliferative disorders of distinct types [1–5]. It should be recalled that other studies did not confirm these results [6]. The reason for these discrepancies is unknown. The differing techniques employed in distinct investigations may account for the positive and negative association data between SV40 and human cancers of different histotypes, including NHL [1–6]. Some authors used virus-like particles (VLPs) or soluble VP1 as antigens in their immunological studies [15]. These SV40 VP1 antigens contain many epitopes in common with other closely related human polyomaviruses, such as BKPyV and JCPyV [11, 15]. Publications have shown that these polyomaviral antigens are responsible for some cross-reactivity, thus reducing the specificity of the immunological data [30]. In other investigations, in an attempt to reduce the non-specific reactivity of serum antibodies, the samples under analysis were pre-adsorbed with homologous viral antigens [31]. However, it turned out that SV40-positive samples present at low prevalence in analysed sera had abolished their reactivity with VLPs [31]. It is highly likely that by adopting this procedure even the SV40 antibodies were lost [32]. One may infer that these negative data are an artefact of the methods employed [15]. To circumvent these non-specific approaches, sensitive and specific immunologic assays based on indirect ELISAs with synthetic peptides corresponding to SV40 VP and Tag mimotopes were developed and set up. These new indirect ELISAs with SV40 VP and Tag mimotopes allowed us to detect and quantify SV40 VP antibodies in sera from NHL patients [26], other oncologic patients [10, 18, 19], and healthy subjects, respectively [28].

Herein, we report the results obtained using the new specific immunological test employed to evaluate the association between NHL and SV40. These new data show an association between NHL and SV40. Our immunological results indicate that SV40 Tag antibodies are detected at a higher prevalence (36%) in NHL patients than in HS (19%). These data are in agreement with the previous study where NHL patients had a higher prevalence (43%) of SV40 VP antibodies [26]. A different prevalence of antibodies against SV40 VP and SV40 Tag is not statistically significant ($p > 0.05$). In agreement with our immunological data, earlier investigations reported the association by detecting a higher prevalence of SV40 Tag coding sequences in NHL specimens compared to controls [1–5].

Our immunological results are important because data on the association between NHL and SV40 are confirmed and extended [1–5, 26]. Moreover, the detection of SV40 Tag antibodies in NHL patients is significant, because in experimental conditions this viral oncoprotein has a causal role in tumour onset [12, 13, 16].

The prevalence of IgG serum antibodies against SV40 Tag, detected in NHL patients stratified by age: 15–50 years old and 51–92 years old, was 29% and 38%, respectively. This different prevalence is not statistically significant ($p > 0.05$). However, these data show that SV40 Tag antigens are recognized by specific human IgG antibodies, with an increasing prevalence in older NHL patients.

On the other hand, the prevalence of SV40 serum antibodies in older NHL patients (38%) was statistically higher than that detected in HS with the same age range of 51–91 years old (20%) ($p < 0.01$) (Table 2).

In addition, our serologic profiles shown in Fig. 1 indicate low OD values in NHL1 patients and HS1, whereas the OD values detected in NHL2 patients are higher than those in HS2, with a statistically significant difference in prevalence ($p < 0.0001$). Our hypothesis is that NHL1 patients have less IgG titer, as OD values, compared to NHL2 patients. Indeed, OD values are higher in NHL2 (Table 2). These results can be ascribed to the different mean ages of the two NHL cohorts, which are older in NHL1 (60 years old) than in NHL2 (52 years old). It is possible that in older patients, SV40 Tag antibodies reach lower OD values because of a physiological decline in the immune system due to age.

The prevalence of SV40 Tag antibodies in sera from patients with indolent (38%) and aggressive (32%) NHL subtypes does not differ statistically ($°p > 0.05$), while the prevalence of SV40 antibodies in indolent NHL subtypes (38%) was statistically higher than that detected in the HS1 control (19%; $p < 0.001$) with a similar mean age.

In other studies, we showed that in experimental conditions, SV40 infects and transforms both B and T lymphocytes producing viral progeny with a low titer [33, 34].

These data suggest that B and T lymphocytes are only semi-permissive to SV40.

It should be noted that this is the first report on immunological data obtained with NHL serum samples which reacted to two different SV40 Tag antigens/mimotopes. In previous investigations, SV40 Tag antibodies were detected in experimental animals which developed SV40-induced tumours [12, 13]. In other studies carried out with different human cancers, including NHLs, SV40 Tag was detected by immunohistochemistry methods with specific monoclonal antibodies [4, 5, 15, 35, 36].

SV40 Tag ELISA, alongside SV40 VPs, may provide researchers with standardized assays to study an association between NHL and SV40. Our immunological results on this association are not proof that SV40 is the causal agent of NHL. An alternative interpretation, for instance, could be that our data result from a closely related human polyomavirus, which at present remains unknown.

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Compliance with ethical standards

Conflict of interest The authors have no financial conflicts of interest.

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