ORIGINAL ARTICLE

Antibodies reacting with Simian Virus 40 mimotopes in serum samples from patients with thalassaemia major

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Background. Simian virus 40 (SV40) is a small DNA tumour virus. Footprints of the virus have been detected in different humam lymphoproliferative disorders and in blood specimens of blood from healthy blood donors. This study was carried out to verify whether SV40 antibodies can be detected in serum samples from multiply transfused patients with thalassaemia major.

Materials and methods. An indirect enzyme-linked immunosorbent assay was employed, using SV40 specific synthetic peptides mimicking the antigens of the viral capsid proteins 1-2-3, to test for the presence of antibodies to SV40 in serum samples taken from patients affected by transfusion-dependent thalassaemia major (n=190) and healthy blood donors (n=251).

Results. The prevalence of antibodies against SV40 was higher in patients than in controls (24% *vs* 17%). The prevalence increased and was significantly higher in the older age group of patients affected by thalassemia major than in controls (38% *vs* 20%, p<0.04).

Discussion. The higher prevalence of serum antibodies against simian virus 40 in older, multiply transfused patients with thalassamia major than in controls suggests that this virus, or a closely related yet unknown human polyomavirus, could have been transmitted in the past by transfusion with whole blood. At the same time, our data indicate no significant differences in prevalence of SV40 antibodies in patients and controls of younger age thus suggesting that current transfusion methods with leucodepletion and filtered red cells are safe.

Keywords: thalassaemia, virus, SV40, antibody.

Introduction

Patients affected by thalassaemia major receive transfusion of red blood cells from the time of diagnosis, which is usually made in the first 2-3 years of life. As a consequence, they are exposed lifelong to the risk of acquiring blood-borne viral infections. The risk of the major blood transmissible viral infections, including hepatitis B and C viruses and human immunodeficiency virus, continues to decline because of specific analyses carried out in samples from blood donors. However, new viruses are becoming a concern. Recently, there have been small epidemics of West Nile and Chikungunya virus infections so that donor testing by nuclear antigens has been implemented in several countries[1].

Simian Virus 40 (SV40) is a viral agent of the Asian macaque (*Macacus rhesus*), which is its natural host. Accumulating data suggest that SV40 is also a human virus, able to spread by different routes. The presence of SV40 sequences and the expression of its viral antigens have been found in human neoplasms and normal tissues, including blood specimens, of children and adults, whereas specific antibodies against this virus were detected in serum samples of normal subjects and patients affected by tumours[2-8]. However, contrasting reports have appeared in the literature on the presence of SV40 in humans and its association with neoplasms[3,5,9,10]. As a consequence of these results, considerable debate has developed in the scientific community[2,3,5,11,12]. Although SV40 sequences and serum antibodies against this viral agent were detected in blood samples, no extensive data exist on the transmission of SV40 through blood transfusion[4,5,13]. We hypothesised that SV40 might be transmitted with blood transfusion. In order to test this hypothesis, we investigated the presence of anti-SV40 antibodies in multiply transfused patients with thalassaemia major.

Materials and methods

Patients

Serum samples were collected from thalassaemia patients. Written informed consent was obtained from patients treated between 2007 and 2013 at: (i) the Department of Paediatrics, University of Ferrara, (ii) Department of Internal Medicine, University of Milan and (iii) Department of Haematology, Sant'Eugenio Hospital, Rome. Italy. Control serum samples were obtained from blood donors[4-7]. Serum samples were analysed at the Sections of Microbiology, and Experimental Biology Cell and Molecular Genetics, University of Ferrara, for the presence of anti-SV40 antibodies by an indirect enzyme-linked immunosorbent assay (ELISA) employing SV40-specific synthetic peptides mimicking the VP1-3 antigens.

Since viruses are transmitted more easily with transfusions containing leucocytes, patients and controls were subdivided in three cohorts according to age: 20-30 years, 31-40 years and 41-50 years. The oldest cohort included patients born before 1965, when treatment with blood components was introduced and these patients had, therefore, received whole blood. The cohort aged 31-40 years included patients born between 1965 and 1985, who up to 1985, had received concentrated red blood cells. The youngest cohort, comprising patients born after 1985, the year when leucodepletion was introduced in our blood banks, had received only concentrated and filtered red blood cells. The study was approved by the County Ethical Committee of Ferrara.

Synthetic peptides

Computer-assisted analyses allowed us to select two specific SV40 peptides, from the late viral region by comparing the three capsid proteins, VP 1-2-3 from SV40, with the amino acids of the human BK (BKV) and JC (JCV) polyomaviruses which are highly homologous with SV40, as well as with other, less homologous polyomaviruses[4,5]. Previous ELISA results indicated that the two SV40 peptides did not cross-react with the BKV and JCV hyperimmune sera that were employed as controls[4,5]. The two peptides belong to the VP1/VP2/VP3 viral capsid proteins (web site, http://www.ncbi.nlm.nih.gov/nuccore). The amino acid sequences of the two peptides, known as VP1 B and VP2/3 C, are as follows: VP1 B: NH2-NPDEHQKGLSKSLAAEKQFTDDSP-COOH VP2/3 C: NH2-IQNDIPRLTSQELERRTQRYLRD-COOH. VP1 B and VP2/3 C mimotopes were selected as they react specifically in indirect ELISA with the rabbit hyperimmune serum that had been experimentally immunised with SV40 (positive control serum). BKV and JCV hyperimmune sera did not react with VP1 B or VP2/3 C peptides (negative control sera). The amino acid residues of the two specific SV40 VP peptides show low homology with the BKV and JCV VP[4-6]. The synthetic peptides were synthesised using standard procedures and were purchased from UFPeptides s.r.l. (Ferrara, Italy)[4,5].

Indirect enzyme-linked immunosorbent assay

An indirect ELISA was developed and standardised to detect specific antibodies against SV40 in human sera using VP1 B and VP 2/3 C synthetic peptides[4,5]. 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). The peptide was then blocked with 200 µL/well of Blocking Solution (Candor Bioscience) at 37 °C for 90 minutes before the primary antibody was added. The different wells were covered with 100 µL of solution containing the following sera: positive-control, represented by immune rabbit serum containing anti-SV40 antibodies, negative controls represented by anti-BKV and anti-JCV immune sera, and three human serum samples which were found to be SV40 negative in our previous investigations[4,5]. The sera under analysis were diluted 1:20 in Low Cross-Buffer (Candor Bioscience). Each sample was analysed three times. The secondary antibody was added in a solution containing a goat anti-human IgG heavy and light chain specific peroxidase-conjugate (Calbiochem-Merck, Darmstadt, Germany) diluted 1:10,000 in Low Cross-Buffer. The samples were then treated with 100 µL of 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) solution (Sigma-Aldrich, Milan, Italy) and then read on the spectrophotometer (Thermo Electron Corporation, model Multiskan EX, Vantaa, Finland) at a wavelength (λ) of 405 nm. This approach detects the colour intensity by optical density (OD) in wells in which immunocomplexes are formed. The cut-off point was determined in each assay by an OD reading of three negative controls, which were added to the standard deviation and multiplied three times (+3SD). Sera with antibodies against SV40 were considered VP-positive if they reacted to both peptides of the late region and when sera that had been analysed three times by indirect ELISA gave the same positive result.

SV40 specificity of the indirect enzyme-linked immunosorbent assay employing synthetic peptides mimicking the VP antigens

In previous investigations, comparative computer-assisted analyses using the BLAST programme were carried out with the SV40 VP peptides B and C and the corresponding amino acid sequences of the new human polyomaviruses and hundreds of different BKV and JCV serotypes[4,5]. Results indicate a low homology for the BKV and JCV prototypes and other polyomaviruses[4,5].

Indirect ELISA data indicate that the two SV40 peptides B and C did not cross-react with the BKV and JCV hyperimmune sera (negative controls), as described before[4,5]. Briefly, hyperimmune sera against SV40 and BKV were obtained in rabbits that had been inoculated with purified viral stocks as previously reported[4]. The serum against JCV was kindly provided by Dr. Major (NIH, Bethesda, MD, USA)[4,5]. The anti-BKV immune serum was titered using a haemagglutination inhibition test employing human group O, Rh^+ red blood cells[4]. Anti-SV40 serum was titered by a neutralisation assay[4].

SV40 VP1 B and VP2/3 C mimotopes were selected as they react specifically in indirect ELISA with the rabbit hyperimmune serum that had been experimentally immunised with SV40 (positive control)[4,5]. The human peptide hNPS, amino acid sequence SFRNGVGTGMKKTSFQRAKS, was employed as a negative control peptide[4,5].

Serum samples tested by indirect ELISA diluted at 1/20 were considered SV40-positive when the OD was above 0.17-0.19, according to the spectrophotometric reading. Indeed, this cut-off point represents the value that discriminates SV40-negative samples (with OD below 0.17-0.19) from SV40-positive ones (with OD above 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.

Statistical analysis

Statistical analyses were performed using Prism software (GraphPad, San Diego, CA, USA). Data are presented as a percentage of positive samples. Differences among proportions were calculated by the chi-square test for independence in the contingency tables.

Results

In the first step of our investigation, an indirect ELISA was employed to test serum samples taken from patients affected by transfusion-dependent thalassaemia major (n=190; female=105, male=85) and control, healthy blood donors (n=251; female=33, males=218). The patients were aged 20 to 50 years, with a median age of 31 years. The median age of the controls was the same.

Serum samples had been diluted at 1/20, for reactivity to SV40 epitopes from VP1, peptide B. The prevalence of serum samples from patients with thalassaemia which reacted with the SV40 VP1 B mimotope was 24% overall (45/190). The prevalence in subjects aged 20-30, 31-40, and 41-50 years was 19% (11/58), 22% (23/103), and 38% (11/29), respectively (Table I).

The overall prevalence of serum samples from healthy blood donors, employed as controls, which reacted with the SV40 VP1 B mimotope was 17% (44/251). In this instance, the prevalence in subjects aged 20-30, 31-40, and 41-50 years was 10%, 19%, and 20%, respectively (Table I).

In the ELISA experiments, the human peptide hNPS[14] was employed as a negative control peptide. Data indicate that this negative control peptide did not react with 89 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 same assay was then used to detect IgG class serum antibodies against SV40 VP2/3 epitopes, which are known as VP2/3 C peptide. It turned out that serum samples reacted with the SV40 VP2/3 C peptide as had been detected previously for the VP1 B peptide in the respective cohorts of patients and controls. Conversely, seronegative samples for the SV40 VP1 B peptide failed to react with SV40 VP2/3 C epitopes. The exceptions were negligible and consisted of a few serum samples which were found to be negative for VP1 B, while testing positive for VP2/3 C peptide, and *vice versa*. The difference was not statistically significant ($p>0.05$) (Tables I and II).

Table I - Prevalence of immunoglobulin G (IgG) antibodies reacting with simian virus 40 (SV40) viral peptide (VP) mimotopes.

Age (years)	Number of samples	Number of positive samples (%)		
		VP B	VP C	VP (B+C)
20-30	58	15 (26)	12 (21)	11 (19)
31-40	103	25 (24)	28 (27)	23 (22)
41-50	29	12 (41)	11 (38)	11 (38)
20-50	Total 190	52 (27)	51 (27)	45 (24)

Human sera were from thalassemia patients. Statistical analysis was performed with the chi-square test. The different prevalence of SV40 antibodies between the cohorts of thalassaemia patients was not statistically significant ($p>0.05$).

Table II - Prevalence of immunoglobulin G (IgG) antibodies reacting with SV40 viral peptide (VP) mimotopes.

Age (years)	Number of samples	Number of positive samples (%)		
		VP B	VP C	VP (B+C)
20-30	50	8 (16)	6 (12)	5 (10)
31-40	90	22 (24)	20 (22)	17 (19)
41-50	111	25 (22)	22 (20)	22 (20)
20-50	Total 251	52 (21)	51 (20)	44 (17)

Human sera were from blood donors. Statistical analysis was performed with the chi-square test. The different prevalence of SV40 antibodies between the cohorts of blood donors was not statistically significant ($p>0.05$).

In this study, only samples that tested positive for both the VP1 B and VP2/3 C peptides were considered SV40 positive.

Combining these data, the overall prevalence for SV40 antibodies was 17% (Table I). SV40-positive sera tested by indirect ELISA diluted 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 below 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.

The two indirect ELISA, with two distinct VP peptides, gave overlapping results, thus confirming the presence of anti-SV40 VP antibodies in human sera from patients and controls (Tables I and II, Figure 1).

The seroprevalence of SV40 in human sera was analysed in detail, with regards to age, gender and geographic region of the patients and blood donors. Indirect ELISA data indicated that the prevalence of SV40 antibodies is similar in cohorts of individuals with the same age and gender, despite coming from different regions in Italy (data not shown).

In this investigation, in each of the three age groups the patients affected by thalassaemia major had an higher prevalence of SV40 antibodies compared to the healthy blood donors of the same age. However only the difference in prevalence of SV40 antibodies in the oldest group was statistically significantly different (patients, 38% versus healthy blood donors, 20%; p=0.04). The results are summarised in Tables I and II, Figure 1.

The serological profiles of serum antibody reactivity to SV40 mimotopes are presented in Figure 2.

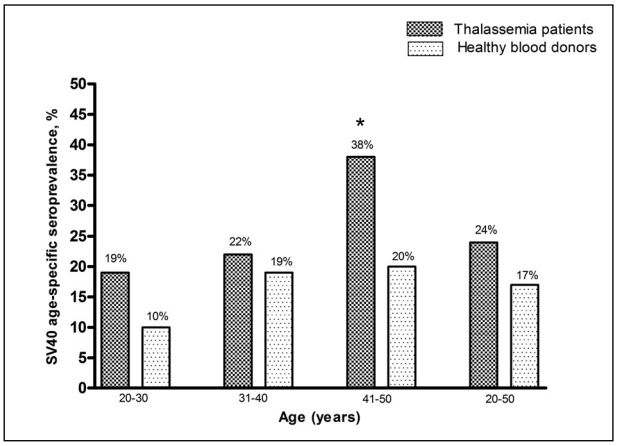

Figure 1 - Prevalence of SV40-positive serum samples in patients with thalassaemia and in healthy blood donors.
Statistical analysis was performed with the chi-square test. Statistical analysis revealed significant differences in the prevalence of SV40 between thalassaemia patients aged 41-50 years old and the cohort of healthy blood donors of the same age (p=0.04). No statistically significant differences in SV40 seroprevalence were found between the other cohorts of thalassaemic patients and their control groups of blood donors. Significance levels are indicated in the figure (*p<0.05).

Discussion

SV40 is an oncogenic virus that was inadvertently administered to human populations worldwide in the 1960s by contaminated vaccines, mainly anti-polio vaccines, produced in naturally infected monkey kidney cells naturally infected with SV40[2,3,11,12]. Accumulating data suggest that SV40, spreading by different routes, is able to infect humans as well as Asian macaques. SV40 sequences have been found in blood specimens from normal individuals[13,15] as well as in tumours of different histotypes, including non-Hodgkin's lymphomas[9,10,16]. However, it is worth mentioning that other groups failed to detect SV40 footprints in human specimens[2].

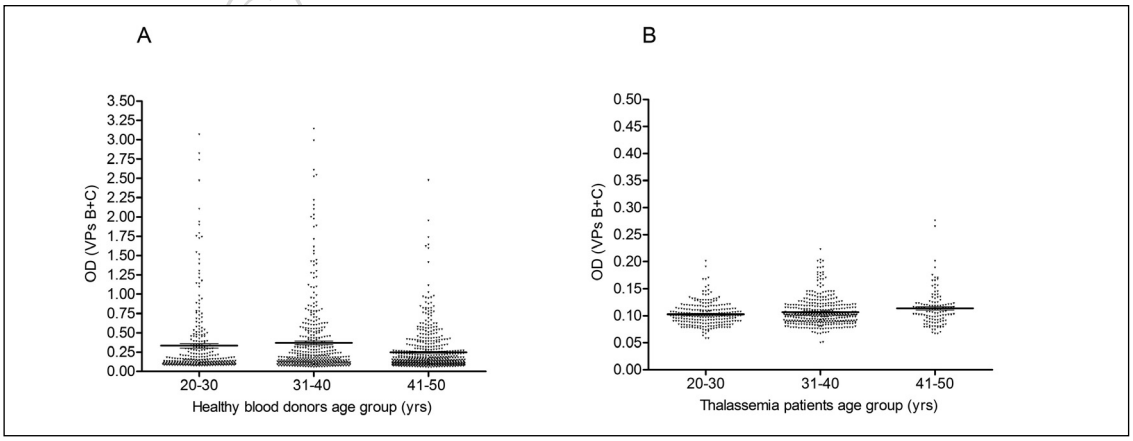

Figure 2 - Serological profile of serum antibody reactivity to SV40 mimotopes VP B+C.
Immunological data are from healthy blood donors and from patients affected by thalassaemia. Results are presented as values of OD readings at λ 405 nm, of serum samples diluted at 1:20, detected in indirect ELISA. 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 of the mean (SEM) for each group of subjects analysed.

In previous investigations we showed that linear peptides VP1 B and VP2/3 C resemble natural SV40 linear epitopes, constituting a docking site for the serum antibody repertoire directed against both linear and conformational antigens[4-7]. On these grounds, serum samples from patients and blood donors were analysed for their reactivity to SV40 epitopes from viral capsid proteins using an indirect ELISA. The findings indicate that the overall prevalence of SV40 antibodies in patients affected by thalassaemia major and in healthy controls, aged from 20-50 year old, is 24% and 17%, respectively. It is important to note that the prevalence of SV40 in human sera detected by this immunological study does not differ substantially from that reported in previous studies carried out in the USA and UK using neutralisation testing against SV40 infectivity, which is considered the gold standard for measuring the presence of the SV40 antibody[17-19]. A similar prevalence of SV40 sequences in normal individuals was found in earlier investigations by polymerase chain reaction assays (PCR) in peripheral blood mononuclear cell/buffy coat samples[13,20-22].

Taken together, the data from the ELISA indicate that natural SV40 infection occurs at a lower prevalence in both patients and normal individuals compared with the high prevalence of infection of the two well-characterised human BK and JC polyomaviruses, which is in the range of 60-90%. The prevalence of anti-SV40 serum antibodies increases with age, reaching 38% and 20% in patients and controls, respectively, aged from 41-50 year old.

It has been shown that SV40 is present in the urine, stool, tonsil and blood specimens of children and adults suggesting that different ways of transmission are responsible for SV40 infection by the urinary, oro-faecal, respiratory and haematic routes[23-26]. These data seem to confirm the findings in earlier studies on subjects administered SV40-contaminated vaccinèes by different routes. Indeed, in these vaccines, SV40 was detected/isolated after a number of days or weeks, either in stools or from throats due to the administration of contaminated vaccines as an oral or nasal spray[27,28]. However, these findings differ from those of other immunological studies in which SV40 antibodies were either absent or their prevalence was very low[29,30]. These previous findings were affected by some degree of cross-reactivity. Indeed, VLP or VP1 employed as antigens contain many epitopes in common with SV40, BK and JC polyomaviruses. In these investigations, the immune sera were pre-absorbed with BKV and JCV antigens in an attempt to give SV40 specificity to the assay. This procedure may eliminate/reduce the presence of SV40 antibodies in analysed sera[31]. Until now technical artefacts did not allow an estimation of the real prevalence of antibodies against SV40 in the population. SV40 synthetic peptides, employed as mimotopes/antigens in ELISA, were selected because they do not cross-react with BKV and JCV. This ELISA allowed us to estimate the prevalence of specific SV40 antibodies against the structural protein antigens in serum samples from healthy blood donors[4], children[6], elderly subjects[8], patients with mesothelioma[5] and those with breast cancer[7]. This result, together with data obtained in other several studies, mainly carried out using PCR techniques, suggested that SV40 may be transmitted in humans by horizontal infections.

It was found that the prevalence of antibodies against SV40 was higher in the cohort of transfusion-dependent thalassaemia patients than in the controls. The increase in prevalence of antibodies against viruses with age is a common feature. On the other hand, one may speculate that in the past SV40 could have been transmitted by transfusion with whole blood. Indeed, the difference in the prevalence between thalassaemic patients and controls was statistically significant only in the group aged 41-50 years. The significantly higher prevalence in the oldest cohort could be due to the fact that these patients received whole blood in their childhood and, subsequently, non-filtered red cells. Intracellular viruses including SV40, can be transmitted more easily with transfusions containing leucocytes. It is also possible that thalassaemia patients acquired SV40 antibody passively from blood transfusions.

Recent investigations reported that older thalassaemic patients may develop specific tumours. Iron accumulation and hepatitis B and C viruses are considered among carcinogenic agents[32-34]. One may speculate that SV40, which is a small DNA tumour virus, could be an additional oncogenic risk factor for the oldest thalassaemia cohort.

Our study has several weaknesses. In fact it is a retrospective study and we do not have proof of absence of SV40 antibodies before the onset of transfusion or that SV40 was present in the blood that these patients received.

The higher prevalence of serum antibodies in older thalassaemia patients compared to controls suggests that transfusion of whole blood may be associated with the acquisition of SV40 or antibodies against the virus, but a possible confounding of the association by other factors makes interpretation and conclusion difficult. In addition, due to the age distribution in the thalassemia population, the number of patients in the older cohort is small, and this may add a bias in the analysis of the results. However, the transfusion of packed red cells or filtered blood appears to be safe as there is no significant increase in SV40 seropositivity in thalassaemic subjects managed in this way compared with the rate

in controls. Other routes of SV40, or a closely related yet undiscovered human polyomavirus, transmission/infection might be much more common than current transfusion practice.

Authorship contribution

Caterina Borgna-Pignatti, Alfredo Corallini and Mauro Tognon: conception and design of study; collection, assembly, analysis and interpretation of data; manuscript writing and final approval of manuscript.

Marcella Felletti, Giuliana Turlà, Elisa Mazzoni, Cristina Malaventura, Maria Domenica Cappellini, Paolo Cianciulli, and Gian Luca Forni: conception and design of study; provision of study material or patients; data analysis and interpretation; manuscript writing and final approval of manuscript.

Caterina Borgna-Pignatti and Elisa Mazzoni contributed equally to the work.

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The Authors declare no conflicts of interest.

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