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ORIGINAL ARTICLE

Polymorphisms at exon 4 of *p53* and the susceptibility to herpesvirus types 6 and 1 infection in renal transplant recipients

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Summary

In order to replicate their own genome in the host nucleus, herpesviruses have to overcome the barrier presented by *p53* gene. Variants of codon 72 and codon 47 of exon four decrease the ability of *p53* to induce apoptosis. In order to investigate the influence of this germline inheritance on the susceptibility to herpesvirus type 6 (HHV6) and 1 (HHV1) infection, we examined 78 renal transplant recipients and 151 controls. HHV6 infection was more frequent among the renal transplant patients (35.89%) than in the control population (11.25%) ($P < 0.001$). HHV1 infection rate was similar in renal transplant patients (7.28%) and controls (2.56%). HHV6-positive cases were more frequent among patients with codon 72 of *p53* variants (60.71%) than among wild-type *p53* patients (28.20%) ($P = 0.001$) despite the higher frequency of codon 72 of *p53* wild-type variant in renal transplant patients compared with controls (64.1% vs. 36.4%; $P < 0.001$). The presence of a codon 72 of *p53* germline variant genotype increased the risk for HHV6 infection more than five times (OR = 5.479; 95% CI = 1.992–15.069). Our data suggest that codon 72 of *p53* polymorphism genotyping may be useful to screen for patients at higher risk for post-transplant infections hence identifying individuals that could benefit from preventive treatment.

Introduction

Renal transplant recipients are at an increased risk for developing many viral infections. As herpesviruses are widely distributed among human populations and persist in the host following primary infection, most transplant recipients will probably harbor multiple latent viruses. The immunosuppressive state post-transplant favors viral reactivation. In addition, patients that have escaped infection up to the time of transplant face the risk of acquiring cytomegalovirus or other herpesviruses from the donor organ or from blood products when receiving immunosuppressive agents to prevent or treat rejection [1]. These viral infections may be severe and threaten the outcome of the transplant recipients.

Cellular signaling plays a major role in different aspects of virus infection and pathogenesis [2,3]. Herpesviruses, like every infectious agent that requires the replication of its own genome in the host nucleus, have to overcome the barrier presented by *p53* tumor suppressor gene. Normal (wild-type) *p53* maintains the integrity of the genome by causing a pause in the cell cycle until damage has been repaired or by inducing apoptosis in cells posing a risk to the organism [4]. Both herpesvirus type 6 (HHV6) and herpesvirus type 1 (HHV1) have been shown to induce programmed cell death [5–7].

A critical region of *p53* for signaling apoptosis lies between codons 64 and 92, encoding a proline-rich region of the gene in which there is a common polymorphism resulting in either an arginine or a proline at codon 72 of

exon 4. Proline polymorphic alleles (P72) have a markedly poorer ability to induce apoptosis than arginine variants [4]. One source of this inferior apoptotic potential is the poorer ability of the proline variant to localize to the mitochondria [8]. The interaction of p53 with the pro-apoptotic mitochondrial membrane protein Bak and the consequent release of cytochrome c into cytosol are impaired [9]. More recently, another polymorphism site, at codon 47 of the same exon 4 of *p53*, was also demonstrated to significantly decrease p53 ability to induce apoptosis [10]. Codon 47 encodes proline in wild-type *p53*, but in a small subset of individuals it can encode serine (CCG-TCG) [10]. The serine 47 (S47) polymorphic variant, which replaces the proline residue necessary for recognition by proline-directed kinases, is a markedly poorer substrate for phosphorylation on serine 46 by p38 MAPK [10]. S47 was described to occur in *cis* with P72, suggesting both polymorphisms are linked [10].

We have recently demonstrated that HHV6 and HHV1 are associated with the risk for basal cell carcinomas and suggested that these viruses may play a role in the susceptibility to skin malignancies, perhaps through reactivation of a latent infection [11]. Unfortunately, the small number of immunocompromised subjects in this first study prevented any further insight into the role of their immunologic state. Deciphering cellular signaling, which is crucial for reactivation, may help us better understand herpesviruses pathogenesis. Hence, the present study was designed to investigate the influence of codon 72 and codon 47 of *p53* on the susceptibility to HHV6 and HHV1 infection in immunosuppressed patients.

Materials and methods

Subjects

The study was approved by the Research Ethics Committee of the University Hospital-School of Medicine of the State University of Campinas-São Paulo, and informed written consent was obtained from all individuals. The group of patients consisted of 78 kidney transplant recipients under standard maintenance immunosuppressive therapy that included azathioprine, cyclosporine, and prednisone (55%); mophetil mycophenolate, prednisone, and cyclosporine (24.2%) and tacrolimus, MMF, and prednisone (20.8%). They were consecutively recruited from patients transplanted from 06/2003 to 02/2005 that were under regular follow-up care at the renal transplantation outpatients' clinics. None of the patients presented unusual complications or any malignancy by the time of blood sample collection. A control group of 151 healthy individuals was selected from the general population of our region. Data on ethnic background, dietary habits and lifestyle, as well as age and sex, were considered in order to obtain a control group similar to the patients group. Patients and controls were classified into whites and nonwhites.

Methods

Blood specimens were obtained from all 229 subjects included in the study. HHV and genotyping were performed in blood withdrawn between 6 and 12 months after transplantation. DNA was extracted using a standard proteinase-K and phenol-chloroform protocol.

HHV6 and HHV1 identification

Herpesvirus type 1 and HHV6 were identified using previously described methods [11]. In summary, HHV1 sequences were obtained by polymerase chain reaction (PCR) using one set of primers that amplified a target sequence of 199 bp. In order to amplify HHV6 sequences, we used two sets of nested primers as schematically demonstrated in Fig. 1. The first set was designed to amplify a highly conserved sequence corresponding to the major capsid protein gene. It consisted of an outer pair of primers, A1 and A2, and an inner pair of primers, A3 and A4. Primers A1 and A2 defined a target sequence of 526 bp, whereas A3 and A4 amplified a 258-bp fragment. The second set of primers was designed to amplify the region shown to encode a putative large tegument protein, and consisted of an outer pair of primers, B1 and B2, and an inner pair, B3 and B4. Primers B1 and B2 defined a target sequence of 834 bp, while primers B3 and B4 amplified a 658-bp fragment. Positive HHV1 and HHV6 controls were obtained from patients with active clinical infection and positive serological tests.

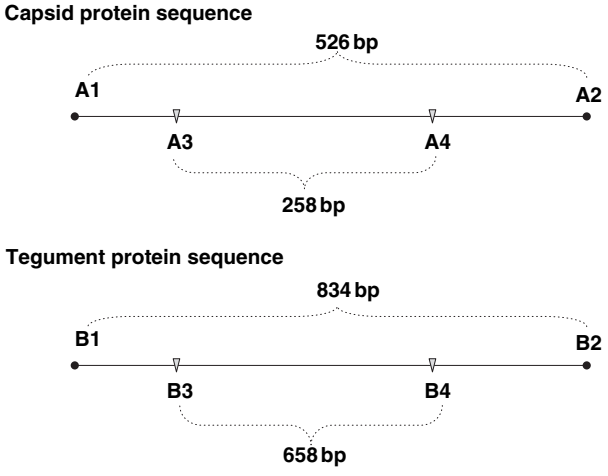

Figure 1 Schematic representation of the sets of primers used to detect herpesvirus type 6.

Determination of codon 72 of p53 polymorphism
For the identification of the polymorphism at codon 72 of the *p53* gene, we used an allele-specific PCR reaction with two sets of primers, one to amplify the Arg allele and the other to amplify the Pro allele, as previously described [12].

Determination of codon 47 of p53 polymorphism
For the identification of the polymorphism at codon 47 of the *p53* gene, we used a PCR-RFLP assay with primers (5′CACCCATCTACAGTCCCCC′/5′ACCGTAGCTGC CCTGGTAG3′) that amplified a fragment of 241 bp. The PCR was performed in 25 μl volumes of a mixture containing 100 ng DNA, 10 μM of each primer, 10 mM Tris–HCl (pH 8.0), 0.1 mM of each dinucleotide triphosphate, 2.0 mM $MgCl_2$ and 0.5 U Taq DNA polymerase. Amplifications were carried out for 35 cycles of 94 °C for 30 s, annealing temperatures 60.5 °C for 50 s and 72 °C for 1 min, with an initial denaturation step of 94 °C for 5 min and a final extension step of 72 °C for 10 min using an MJ PTC–200 PCR system thermocycler. PCR products were digested with *bcn*I (*Cau*II) and analyzed on a 3.0% agarose gel.

Six samples from each assay were directly sequenced and confirmed to be the PCR-predicted variants. Positive and negative control samples were included in all PCR and RFLP runs to detect possible contamination problems, gel loading, and typing inconsistencies.

Statistical analysis
Statistical analysis was conducted using SAS (Statistical Analysis System, version 8.1, SAS Institute Inc, Cary, NC, USA, 1999–2000). Associations were assessed using 2×2 or $2 \times n$ contingency table analysis and chi-squared or Fisher's exact tests were used to examine homogeneity between cases and controls regarding gender, color, viral infection, and genotypes. Kruskal–Wallis test was used to compare age among the different groups. The odds ratio and 95% CI provide a measure of the strength of association, e.g. indicating the increase in odds of a given genotype patient presenting a viral infection compared with the control population. All tests were conducted at the $P = 0.05$ level of significance.

Results

Both patients and controls groups were statistically similar regarding sex (31 males and 47 females vs. 66 males and 85 females), age (42.76 ± 12.86 vs. 38.87 ± 14.85 years old), and color (71 whites and seven nonwhites vs. 132 whites and 19 nonwhites), demographic and lifestyle characteristics, including alcohol consumption, cigarette smoking, dietary habits, education and exercise, UV, and possible chemicals exposure.

Table 1. Codons 72 and 47 of exon 4 of *p53* genotypes distribution among the control individuals and renal transplant patients.

Codon	Genotypes	Controls, *n* (%)	Renal transplant, *n* (%)	*P*
Codon 72	*Arg/Arg*	55 (36.42)	50 (64.1)	**<0.0001**
	Arg/Pro	94 (62.25)	26 (33.33)	**<0.0001**
	Pro/Pro	2 (1.32)	2 (2.56)	0.2194
Codon 47	*Pro/Pro*	106 (70.19)	61 (78.2)	0.2130
	Pro/Ser	45 (29.8)	17 (21.79)	0.2130
	Ser/Ser	0	0	
Codon 72 + 47	Variants	30 (19.86)	6 (7.69)	**0.0206**
Total of cases		151	78	

Table 1 summarizes data of the overall proportions of the *p53* codon 72 and codon 47 genotypes in the control population and in the renal transplant patients. Genotypes were not in Hardy–Weinberg equilibrium neither in the patients nor in the control group. Renal transplant patients showed an increased frequency of the wild-type Arg/Arg variant of codon 72 of *p53* (64.1%) in comparison with the control population (36.4%) (*F*; $P < 0.001$). Codon 47 of *p53* wild-type Pro/Pro variant appeared in similar proportions in the renal transplant population (78.2%) and the controls (70.19%). S47 occurred in 45 whites and 17 nonwhites individuals. Only 36 (29.03%) out of the 124 individuals presenting P72 variants also had S47 variant. The number of individuals presenting the combined P72 and S47 variants was higher in the control group (19.86%) than in the renal transplant patients (7.69%) (*F*; $P = 0.0206$).

Herpesvirus type 6 and HHV1 infections were detected in 28 and two transplant patients, respectively, as demonstrated in Table 2. HHV6 infection was more frequent among the renal transplant patients (35.89%) than in the control population (11.25%) (*F*; $P < 0.0001$), but there was no difference in the incidence of HHV6 positivity among the sub-groups of patients treated with different immunosuppressive drugs. There was no statistical difference between the prevalence of HHV1 infection among the control population (7.28%) and the renal transplant patients (2.56%).

There was no difference in the number of HHV6 or HHV1 infected patients between S47 and wild-type codon 47 of *p53* cases. Also, HHV1 infection rate was similar in individuals with P72 and wild-type codon 72 of *p53* genotype cases. However, HHV6 positive cases were more frequent among renal transplant patients with P72 variants (17 out of the 28 HHV6 positive cases = 60.71%) than in patients presenting the wild-type Arg/Arg genotype (11 out of the 50 cases = 22%) (*F*; $P = 0.001$). In fact, the presence of a germline P72 genotype increased

Table 2. Estimates of relative risk for herpes virus type 6 (HHV6) and type 1 (HHV1) infection associated with exon 4 of p53 genotypes.

	Controls		Renal transplant					
Genotypes	HHV6	HHV1	HHV6	HHV1	OR (CI)	P HHV6	OR (CI)	P HHV1
Codon 72								
ARG/ARG	5	2	11	2	1*	–	1*	–
ARG/PRO	12	9	15	0	4.835 (1.732–13.494)	0.004	0.366 (0.016–7.915)	0.543
PRO/PRO	0	0	2	0	17.174 (0.768–384.03)	0.058	3.880 (0.144–104.41)	1.000
P72 variants	12	9	17	0	5.479 (1.992–15.069)	0.001	0.340 (0.015–7.348)	0.533
Codon 47								
PRO/PRO	15	9	22	2	1*	–	1*	–
PRO/SER	2	2	6	0	0.966 (0.314–2.975)	1.000	0.680 (0.031–14.848)	1.000
SER/SER	0	0	0	0	–	–	–	–
S47 variants	2	2	6	0	0.966 (0.314–2.975)	1.000	0.680 (0.031–14.848)	1.000

*Used as the reference group.

the risk for HHV 6 infection more than five times (OR = 5.479; 95% CI = 1.992–15.069).

Discussion

More effective immunosuppressive therapies have been steadily decreasing the rate of acute rejection after renal transplantation. Unfortunately, more aggressive immunosuppression exposes the renal transplant recipient to more frequent infectious complications. Detectable reactivations of HHV6 occur in one-third to two-thirds of all transplant [13]. We found 35.89% of our renal transplant patients to be infected with HHV6, a rate very similar to most reports on the incidence of post-transplant HHV6 infection in solid organ transplants [14]. Likewise, in the majority of patients seropositive for herpes simplex virus (HSV)-1 or -2, virus replication occurs after transplantation, although only a minority of patients develops symptoms [13]. The clinical significance of reactivation of these viruses in renal transplant recipients is still not clear, but they certainly may represent a serious threat to the immunosuppressed transplant recipient. Infection with HHV6 induces marked immunodepression, may be a co-factor of HIV progression, and an association with cytomegalovirus disease and with fungal infection in transplant recipients has also been reported [15–17]. Prior to the onset of antiherpetic drug prophylaxis, recurrent HSV1 or HSV2 infections accounted for as much as 70–80% of severe mucocutaneous diseases in allogenic bone marrow or blood progenitor cell transplant recipients and affected a similar number of solid organ transplant recipients [17–19].

The *p53* gene plays a critical role in cell cycle control, facilitating DNA repair activities and protecting against DNA damages [20]. Every infectious agent that requires the replication of its own genome in the host nucleus has to overcome the barrier presented by *p53*. Alterations in the level, function, and localization of *p53* caused by herpesviruses have been reported in various studies and it has been suggested that herpesviruses require the recruitment of *p53* in order to replicate [21–23]. Also, HHV6 was shown to induce apoptosis [24–26].

The ability of *p53* to induce apoptosis is significantly reduced by two polymorphisms, at codons 47 and 72 of exon 4 [10]. We found a higher prevalence of HHV6 infection among individuals with the less effective P72 alleles, suggesting that HHV6 infection is facilitated by the presence of codon 72 polymorphism. Arresting cells, rather than destroying them, may be an evolutionary-developed advantage for HHV6. Indeed, hiding from the immune system and keeping infected cells alive may prevent the immune reactions initiated by the liberation of viral proteins from apoptotic cells. Hence, infection of cells that are less able to undergo apoptosis may favor HHV6 survival. Recent *in vitro* experiments suggest that HHV6 has a mechanism to retain *p53* within the cytoplasm that protects the infected cells from apoptosis, corroborating previous reports of alterations in the level, function, and localization of *p53* produced by herpesviruses [27].

We were not able to identify a higher risk of viral infection among individuals with the S47 polymorphism, perhaps because of the relatively small number of patients with this polymorphism. There is very little data on S47 polymorphism. It was described in <5% of African-Americans and in none of the 69 Caucasians included in a first prevalence report [28]. More recently, genotyping 200 African-Americans, Li identified an even lower prevalence of S47 variant in only 1% of the subjects and found the S47 allele in *cis* with the proline 72 polymorphism (P72) suggesting that S47 and P72 were linked [10]. Our data indicate a much higher prevalence of 27.07% of the S47

variant among the Brazilian population. Furthermore, only 29% occurred in *cis* with P72, contradicting the previous reports. Because our population presents a highly heterogeneous ethnic background, we classified our subjects in white and nonwhite. The ethnic mixture of our population composed of people with different heritages and relatively recent immigration flows, besides the relatively small number of individuals we studied, may have contributed to the observed lack of Hardy–Weinberg genotypic equilibrium. The fact that the majority of the individuals with S47 were white suggests that broader studies, including a larger number of Caucasians, are needed to establish the real prevalence of S47.

In conclusion, we demonstrated that the germline inheritance of P72 increases the risk for HHV6 infection. Hence, a simple genotyping procedure using a peripheral blood sample may help identify transplant recipients that could benefit from preventive treatment in a disease and infection management program for transplant patients [29].

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