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A Pilot Study on Cytotoxic T Lymphocyte-4 Gene Polymorphisms in Urinary Schistosomiasis

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Urinary schistosomiasis is caused by the digenetic trematode *Schistosoma haematobium*, characterized by accumulation of eggs in the genitourinary tract. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) can play an important role in parasitic infection due to its major role as a negative regulator of T-cell activation and proliferation. This study was performed in patients with schistosomiasis and healthy controls to analyze the allele and genotype frequencies of four *CTLA-4* gene polymorphisms. The *CTLA-4* gene was amplified using Taqman real-time polymerase chain reaction, and allele and genotypes of 49 patients with schistosomiasis were analyzed using allelic discrimination analysis followed by subsequent direct sequencing. The results were compared with healthy control subjects. The frequencies of *CTLA-4* rs733618 A allele at position −1722 ($p=0.001$), rs11571316 C allele at position −1577 ($p<0.001$), and rs231775 A allele at position +49 ($p=0.002$) in the patient group were significantly higher than the control group. The rs733618 AA genotype ($p=0.001$), rs11571316 CC genotype ($p<0.001$), and rs231775 AA genotype ($p=0.007$) were also significantly overrepresented. Meanwhile, rs733618 AG genotype ($p=0.001$), rs11571316 CT genotype ($p=0.02$), and rs231775 GG genotype ($p=0.029$) were significantly decreased in the patients with schistosomiasis, as compared with the controls. No significant difference was observed in both allele and genotype of rs16841252. The results of this study suggest that the rs733618, rs11571316, and rs231775 polymorphisms in the *CTLA-4* gene may influence susceptibility to schistosomiasis infection in the Gabonese children.

Introduction

Schistosomiasis is a chronic helminth infection and is also known as bilharzias, bilharziosis, or snail fever. It remains one of the most prevalent parasitic diseases in the world. Schistosoma chronically infects over 200 million people in the developing world and an additional 600 million people are at risk of infection (Chitsulo *et al.*, 2000, 2004). It causes ~300,000 deaths per year in Africa alone (van der Werf *et al.*, 2003), and of the world's 207 million estimated cases of schistosomiasis, 93% occur in Sub-Saharan Africa (Steinmann *et al.*, 2006).

Urinary schistosomiasis is causes by *Schistosoma haematobium*. The helminth eggs may remain trapped within the bladder or the ureter walls, causing major pathological disorders in the urogenital system. It causes approximately two-thirds of the schistosomiasis cases in Sub-Saharan Africa (Hotez and Kamath, 2009). Possible consequences of *S. haematobium* infection include hematuria, dysuria and lesions of bladder, kidney failure, secondary sterility, and an elevated risk of bladder cancer. In particular, the highest parasite burdens are observed in preschool and school-aged children, often resulting in anemia, undernourishment, and growth stunting (Hotez *et al.*, 2008).

One of the hallmarks of chronic helminth infections is induction of T-cell hyporesponsiveness (Maizels *et al.*, 1993). Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is known to be a negative regulator of T-cell activation (Carreno *et al.*, 2000) and *CTLA-4* gene encodes a coinhibitory lymphocyte molecule that is preferentially expressed by regulatory T cells (Tregs) (Takahashi *et al.*, 2000). It is thought to be at least partially responsible for the hyporesponsive phenotype of Th2 effector cells that is often observed in helminth infection (Maizels *et al.*, 2004). For example, blockade of CTLA-4 during *Nippostrongylus brasiliensis* infection results in higher Th2 cytokine production and decreased parasite numbers (McCoy

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et al., 1997). In contrast, upregulation of expression of Treg-associated CTLA-4 in response to live *Brugia malayi* parasite in lymphatic filariasis patients might explain the decreased Th1 and Th2 cell frequencies (Babu *et al*., 2006).

Allelic variations in promoter regions of *CTLA-4* could potentially affect the gene expression by altering the transcription factor binding sites or other regulatory domains. The *CTLA-4* gene has been a primary candidate for genetic susceptibility to autoimmune diseases (Kristiansen *et al*., 2000). A recent study has shown that single-nucleotide polymorphisms (SNPs) located upstream of the transcription start site of *CTLA-4* strongly correlated with helminth infection (Fumagalli *et al*., 2010). Furthermore, one particular polymorphism at position +49A/G in the exon 1 region has been previously shown to have a functional effect on the expression level of CTLA-4 (Ligers *et al*., 2001; Mäurer *et al*., 2002). Therefore *CTLA-4* would be a suitable candidate gene for studies on polymorphisms in relation to disease susceptibility in schistosomiasis.

This study was performed in school-aged children with schistosomiasis and healthy controls to analyze the contribution of genotype and allele frequencies of *CTLA-4* gene polymorphisms to susceptibility of acquiring schistosomiasis.

Methods

Subjects

The study cohort comprised of 49 school-aged patients with schistosomiasis, 24 males and 25 females from Zilé, a village situated 15 km from Lambaréné. The area is rural and endemic for schistosomiasis (van Riet *et al*., 2007). Infection with *S. haematobium* was determined within 7 days prior to blood collection by examining a filtrated 10 mL of urine passed through a 10-μm filter (Millipore). Children were classified as infected if at least one *S. haematobium* egg was detected in the urine, or uninfected if three consecutive urine samples were negative. In addition, 52 unrelated and uninfected children (27 males and 25 females) were selected as controls. The study was approved by the "Comité d'Ethique Regional Independent de Lambaréné" (CERIL). Written, informed consents were obtained from parents or legal guardians of all subjects participating in the study.

DNA extraction and genotyping

Genomic DNA was extracted from blood spots of each participant using a QIAamp mini kit (Qiagen), according to the manufacture's instructions. Using public databases, including PubMed (National Center for Biotechnology Information), four SNPs were selected. Three *CTLA-4* SNPs, rs733618 (−1722 A/G), rs11571316 (−1577 C/T), and rs16840252 (−1477 C/T), and exon 1 SNP (i.e., rs231775 [+49 A/G]) were used in this study. Based on previous reports, these SNPs maybe expected to result in change of function or expression of the encoded protein (Ueda *et al*., 2003; Fernández-Mestre *et al*., 2009). All SNPs were genotyped using TaqMan assay in a 72-well rotor format (Corbett Research, Australia) at Universiti Sains Malaysia. All probes and primers were designed by the Assay-by-Demand service from Applied Biosystems (Table 1). Genotyping was performed in 20 μL reactions comprising 10 ng of genomic DNA, 1× TaqMan SNP Genotyping Assay (consisted of 200 nM of TaqMan MGB probes, FAM™ and VIC® dye-labeled, and 900 nM of primers), and 2× HotStarTaq Master Mix (contained 1.5 mM $MgCl_2$, 200 μM dNTPs, HotStarTaq DNA polymerase, and RNase-free water; Qiagen). Real-time polymerase chain reactions were thermally cycled with an initial denaturation step at 95°C for 15 min, followed by 40 cycles of 94°C for 30s, 60°C for 1 min, and 72°C for 1 min, and then a final extension step at 72°C for 10 min. Real-time fluorescence detection was performed during the annealing/extension step of each cycle. To ensure genotyping quality, positive control and non-template negative controls were included for each genotype in each run. Allelic discrimination was performed on a Rotor-Gene™ 6000 Multiplex System and data analysis was conducted with the software interface version 1.7. Subsequently, 10 randomly selected samples from each SNP were genotyped through direct sequencing for genotype confirmation and 100% concordance was obtained for every condition. Nucleotide changes were detected by visual inspection of chromatograms.

Statistical analysis

Statistical analysis was performed using PASW Statistic software 17.0 (SPSS Inc.). Allele and genotype frequencies were calculated on patient and control subjects by direct counting. Haplotype block and frequency were performed by using the default method of Gabriel *et al.* (2000) and based on the Haploview program output. Allele, genotype, and haplotype frequencies were compared with the controls using the chi-square test. The odds ratio and 95% confident intervals were calculated for each allele/genotype/haplotype in the patient and control groups. *p*-Value of <0.05 was considered as statistical level of significance.

Table 1. Position, Nucleotide Variations, Primer/Probe Assay, Frequencies Assay, and Distribution of Single-Nucleotide Polymorphisms

dbSNP ID	*Chromosome position*	*Alleles*	*MAF*[a]	*Cases* p[b]	*Control* p[b]	*Forward (F) and reverse (R) primer and probe assay*
rs733618	Chr2q33: 204439189	A/G	0.16	0.590	0.128	C_2415791_10 assay from Applied Biosystems
rs11571316	Chr2q33: 204439334	C/T	0.32	0.07	0.741	C_30981395_10 assay from Applied Biosystems
rs16840252	Chr2q33: 204439764	C/T	0.15	0.347	0.199	C_32900355_10 assay from Applied Biosystems
rs231775	Chr2q33: 204440959	A/G	0.33	0.475	0.663	C_2415786_20 assay from Applied Biosystems

The chi-square test was applied to test for deviations from Hardy–Weinberg equilibrium.
[a]Minor allele frequency.
[b]*p*-Value for a deviation from Hardy–Weinberg equilibrium.
MAF, minor allele frequencies.

Results

A total of 101 school-aged children participated in this study. The mean age (+SD) of infected children was 10.16 ± 2.44 years old (range = 5–15) while uninfected children was 10.71 ± 3.35 (range = 5–17) years old. The mean egg count (+SD) for the 49 infected children was 223.4 ± 419.1. There were no statistical differences of both gender and age between groups of patients and healthy controls. Genotyping of four SNPs located in the *CTLA-4* gene showed allele distributions in Hardy–Weinberg equilibrium in both the patient and control groups. The minor allele frequencies of the variants were 0.15%–0.33% (Table 1). The frequency of the following alleles were significantly higher in the patient group compared with the control group: rs733618 A allele at position −1722 (92% in patients vs. 75% in controls, $p = 0.001$), rs11571316 C allele at position −1577 (81% in patients vs. 55% in controls, $p < 0.001$), rs213775 A allele at position +48 (77% in patients vs. 56% in controls, $p = 0.002$; Table 2).

SNPs rs733618 AA genotype (85% in patients vs. 53% in controls, $p = 0.001$), rs11571316 CC genotype (67% in patients vs. 25% in controls, $p < 0.001$), and rs231775 AA genotype (61% in patients vs. 34% in controls, $p = 0.007$) were significantly overrepresented in the patient group. Meanwhile, rs733618 AG genotype (14% in patients vs. 44% in controls, $p = 0.001$), rs11571316 CT genotype (28% in patients vs. 61% in controls, $p = 0.02$), and rs231775 GG genotype (6% in patients vs. 21% in controls, $p = 0.029$) were significantly decreased in the patients with schistosomiasis, as compared with the control group. There was no statistically significant difference of both alleles and genotypes of rs16841252 between two groups of patients and controls (Table 3). In Table 4, four possible haplotypes were generated from haplotype block in the schistosomiasis cohort. No statistically significant association among haplotypes with schistosomiasis infection was observed.

Discussion

Schistosomiasis is a disease caused predominantly by the host's immune response to schistosome eggs (ova) and the disease manifestations they evoke (Pearce and MacDonald, 2002; Gryseels *et al*., 2006). In the case of *S. haematobium* infection, the pathology occurs mainly in the genitourinary tract where the schistosome eggs accumulate. Adults *S. haematobium* worms inhabit the vasculature surrounding the genitourinary tract leading to the deposition of schistosome eggs in the wall of the bladder and ureters (Burke *et al*., 2009). Blood loss through hematuria, intestinal and variceal bleeding, malnutrition, and the production of hemolytic factors by schistosomes can lead to anemia in schistosomiasis (Friedman *et al*., 2005). Anemia, in turn, has been associated with wasting in adults, and growth retardation and cognitive impairment in children (Gryseels *et al*., 2006).

The *CTLA-4* gene is located at chromosome 2q33 and flanked by two closely related loci, *CD28* and *ICOS*. Notably, CTLA-4 is constitutively expressed on CD4+CD25+ Tregs, and such expression is important for Treg-mediated suppression of T-cell proliferation (Belkaid *et al*., 2002). Being transiently surface expressed, and released by activated T cells, it inhibits T-cell functions through a variety of direct and indirect mechanisms (Sansom and Walker, 2006; Schneider *et al*., 2006). The critical role of Tregs has been demonstrated in the mouse model in restraining the capacity of the immune response to both *Leishmania major* (Belkaid *et al*., 2002) and herpes simplex (Suvas *et al*., 2003). Kaur *et al.* (1997) showed that certain *CTLA-4* alleles are associated with leprosy, thus supporting the hypothesis that *CTLA-4* polymorphisms is involved in the host defense against infection. Moreover, infected lymphatic filariasis patients show higher levels of CTLA-4 expression in peripheral blood T cells than do uninfected controls, and *in vitro* IL-5 responses (associated with protection) are significantly enhanced in the presence of anti-CTLA-4 antibody (Steel and Nutman, 2003). Therefore, variants in the *CTLA-4* gene are very likely to influence signaling activities and to affect schistosomiasis susceptibility.

The current study showed significant results for the rs231775 allele and variants in the Gabonese cohort. Increased frequencies were observed in rs231775 A allele and AA genotype at the position +49 in the patient group, while frequency of the GG genotype in the same group was significantly decreased. Previous studies suggested that the *CTLA-4* +49 SNP may be a major determinant in infectious diseases. The +49A allele has been associated with increased risk for diseases resulting from dengue, Chagas's, leishmaniasis, hepatitis B virus (HBV)–related hepatocellular carcinoma (HCC), and lymphatic filariasis (Fernández-Mestre *et al*., 2009; Gu *et al*., 2010; Zulkarnain *et al*., 2011). Likewise, +49AA genotype was associated with an increased risk to two virus infection–related cancers, namely, HCC and cervical cancer, as compared with the GG genotype by 1.43-fold and 1.66-fold, respectively (Hu *et al*., 2010). The biological relevance of the +49AA genotype was demonstrated by Sun

Table 2. Allele Frequencies of Schistosomiasis Patients in Comparison with Normal Controls

			Patients (n = *49*)	*Controls* (n = *52*)		
dbSNP	*Position*	*Allele*	n *(%)*	n *(%)*	p-*Value*	*OR (95% CI)*
rs733618	−1722	A	91 (92.86)	79 (75.96)	**0.001**	**4.11 (1.72–9.80**
		G	7 (7.14)	25 (24.04)		0.24 (0.10–0.58)
rs11571316	−1577	C	80 (81.63)	58 (55.77)	**<0.001**	**3.53 (1.87–6.66)**
		T	18 (18.37)	46 (44.23)		0.28 (0.15–0.54)
rs16840252	−1147	C	80 (81.63)	92 (88.46)	0.235	0.58 (0.27–1.26)
		T	18 (18.37)	12 (11.54)		1.73 (0.79–3.75)
rs231775	+49	A	76 (77.55)	59 (56.73)	**0.002**	**2.64 (1.43–4.48)**
		G	22 (22.45)	45 (43.27)		0.38 (0.21–0.70)

OR, odds ratio; CI, confidence interval. Significant values ($p < 0.05$) are in bold.

Table 3. Genotype Frequencies of Schistosomiasis Patients in Comparison with Normal Controls

			Patients (n = *49*)	*Controls* (n = *52*)		
dbSNP	*Position*	*Genotype*	n *(%)*	n *(%)*	p-*Value*	*OR (95% CI)*
rs733618	−1722	AA	42 (85.7)	28 (53.8)	**0.001**	**0.194 (0.07–5.12)**
		AG	7 (14.3)	23 (44.2)	**0.001**	**4.759 (1.81–12.55)**
		GG	0 (0.0)	1 (2.0)	0.329	–
rs11571316	−1577	CC	33 (67.3)	13 (25.0)	**<0.001**	**0.194 (0.08–0.45)**
		CT	14 (28.6)	32 (61.5)	**0.02**	**3.627 (1.59–8.28)**
		TT	2 (4.1)	7 (13.5)	0.098	3.656 (0.72–18.54)
rs16840252	−1147	CC	34 (69.4)	40 (76.9)	0.392	1.471 (0.61–3.57)
		CT	12 (24.5)	12 (23.1)	0.868	0.925 (0.37–2.31)
		TT	3 (6.1)	0 (0.0)	0.07	–
rs231775	+49	AA	30 (61.2)	18 (34.6)	**0.007**	**0.355 (0.15–0.75)**
		AG	16 (32.7)	23 (44.2)	0.232	1.6363 (0.73–3.68)
		GG	3 (6.1)	11 (21.2)	**0.029**	**4.114 (1.07–15.78)**

OR, odds ratio; CI, confidence interval. Significant values ($p < 0.05$) are in bold.

et al. (2008) who showed that, upon stimulation, T cells carrying the +49AA genotype had significantly lower activation and proliferation rates compared with T cell carrying the +49GG genotype. On the other hand, the +49AA genotype was increased in patients with localized cutaneous leishmaniasis patients, as compared with patients with diffused cutaneous leishmaniasis, indicating that this genotype, which was previously associated with the normal proliferation of T cells, may confer protection against development of diffused cutaneous leishmaniasis (Fernández-Mestre *et al.*, 2009).

The A to G mutation at position +49 leads to a nonsynonymous amino acid change from threonine to alanine in the peptide leader sequence of the CTLA-4 protein, thus the change in the polarity of the amino acid may potentially alter the function of the protein (Thio *et al.*, 2004). A previous study reported that the +49G allele has been associated with improved clearance of hepatitis C virus infection after alpha interferon-based therapy (Yee *et al.*, 2003). In another study enterovirus infection of 71 meningoencephalitis patients had significantly more GG genotype of *CTLA-4* +49 polymorphism than those without meningoencephalitis (Yang *et al.*, 2001). However the current study did not find any significant differences of the G allele and GG genotype at this position between schistosomiasis patients and controls.

The present data also indicated an increased frequency of major alleles in the *CTLA-4* gene at positions −1722 and −1577 in the patients with schistosomiasis as compared with the control group. Moreover, −1722 AA and −1577 CC genotypes in the patient group were significantly overrepresented, whereas frequencies of heterozygous genotypes for both SNPs were significantly decreased. The role for the promoter SNP −1722 has been studied in HBV recovery and has a strong linkage association with the SNP at position +49 (Yee *et al.*, 2003). Theoretically, since both SNPs are in the promoter region of the gene, they may alter the transcriptional regulation of *CTLA-4*. To the best of our knowledge, this is the first report on the association between *CTLA-4* gene polymorphism and schistosomiasis.

There were several limitations of our study. First, the population was small, involving 49 patients and 52 healthy controls. Thus a much larger sample size will be needed to confirm our results, and to detect larger number of SNPs and haplotypes. Second, the present cohort comprised school-aged children with schistosomiasis who were compared with healthy school-aged children. To clearly define the association between *CTLA-4* polymorphisms and schistosomiasis, it will be necessary to conduct a case-control study comparing schistosomiasis patients with gender-, ethnic-, and disease severity–matched patients with other infectious diseases. In conclusion, this pilot study showed that *CTLA-4* polymorphisms maybe involved in the pathophysiology of schistosomiasis in Gabonese school children.

Table 4. Haplotype Frequencies of Schistosomiasis Patients in Comparison with Normal Controls

Block 1				
SNP	*rs733618*	*rs11571316*	*rs16840252*	*Haplotype frequencies*[a]
Allele	A/G	C/T	C/T	
Hap 1	A	C	C	0.371
Hap 2	A	T	C	0.322
Hap 3	G	C	C	0.158
Hap 4	A	C	T	0.148

[a]Haplotype frequencies were not significant by chi-square test, $p > 0.05$.

SNP, single-nucleotide polymorphism.

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Disclosure Statement

No competing financial interests exist.

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