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Short Communication

Epidemiological and TNFα polymorphism evaluation in patients with cryptococcal meningitis treated at a referral hospital in North Brazil

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Abstract

Introduction: The present study evaluated the epidemiology of cryptococcal meningitis and TNFα gene polymorphisms in patients at a reference hospital in northern Brazil. **Methods:** Samples from 25 patients infected with *Cryptococcus* spp. were collected to confirm the infection and to analyze the TNFα gene polymorphisms. **Results:** *Cryptococcus neoformans* was detected as the predominant etiological agent (100%) in HIV-positive patients. No genetic polymorphic changes were found. **Conclusions:** No correlation was observed between the analyzed TNFα polymorphisms and cryptococcal meningitis.

Keywords: Cryptococcal meningitis. Genetic polymorphisms. Cerebrospinal fluid. TNFα.

Cryptococcus species are pathogenic encapsulated yeasts that are commonly present in the environment; they are easily transported through the air and can infect humans when their spores are inhaled[1]. The immune response to *Cryptococcus* species, as to most fungi, is of the cellular type; the response mainly involves Th1 cells, which are responsible for the production of cytokines and chemokines, including tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and interleukins (IL10, IL12, IL6, and IL8), as well as the activation of calcium-independent nitric oxide synthase (iNOS)[2].

In studies that assessed the correlation between the degree of the immune response and the rate of infection, IL6, IFNγ, TNFα, and IL8 levels were shown to be significantly higher in survivors than in non-survivors, revealing the role of cytokines and chemokines in modulating the clinical outcome of cryptococcosis[2,3]. The genes responsible for the production of cytokines are polymorphic at specific sites, and certain polymorphisms located in coding and regulatory regions may change the expression and secretion of these cytokines, effectively altering the pattern of disease progression[4].

This study aimed to determine whether there were genetic polymorphisms in the TNFα gene in patients with cryptococcal meningitis hospitalized at the University Hospital in a tropical region of Brazil, to determine the epidemiological profile of predominant *Cryptococcus* species, and to analyze the morphological cellularity patterns and biochemical profiles in the cerebrospinal fluid (CSF) of these patients.

Twenty-five patients infected with *Cryptococcus* spp. were treated at the University Hospital João de Barros Barreto (HUJBB), located in the north Brazilian state of Pará within the administrative district of Belém, from December 2010 to December 2011. Patient samples were collected to confirm the infection through the analysis of CSF; 5 mL of whole blood was also collected for the analysis of genetic polymorphisms in the TNFα gene. A control group was established from the database of the Human Cytogenetic Laboratory of the Federal University of Pará (UFPA) and consisted of 120 healthy individuals; points of possible mutations in the TNFα gene of these individuals were analyzed.

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The study followed the precepts according to the Declaration of Helsinki and was approved by the CEP-HUJBB (research ethics committee of the University *Hospital João de Barros Barreto*) under protocol n ° 2161/10.

Biochemical analyses were performed automatically using Architect (Abbott Diagnostics, Illinois, USA) equipment belonging to the HUJBB. The reference values were standardized by the device, and the following values were adopted as normal: protein = 18 to 40 mg/dL and glucose = 40 to 75 mg/dL. The cellularity patterns were evaluated using a Fuchs Rosenthal hemocytometer or camera, with values between 0 and 5 cells per mm^3 adopted as normal, and values above the normal limits classified as hypercellularity. For CSF levels of protein and glucose (proteinorachia and glycorrhachia), the respective normal values adopted were 0-40 mg/dL and 40-80 mg/dL, and values below and above these normal limits were classified as hypo/hyperproteinorachia and hypo/hyperglycorrhachia.

The direct mycological examination of the CSF involving the analysis of the cellularity and the number of forms of *Cryptococcus* spp. was performed using a hemocytometer or a camera (Fuchs Rosenthal). CSF fungal cultures were carried out in Sabouraud agar medium, while the species (*C. gattii* or *C. neoformans*) were identified using CGB medium (canavanine-glycine-blue bromothymol agar). After cultivation, colony forming units (CFU) were classified as rare (0 to 20 CFU), frequent (21 to 180 CFU), or abundant (over 180 CFU).

The TNFα gene, located on chromosome 6, locus 6p21.3, was analyzed for the presence or absence of polymorphisms in the regions 308, 238, 863, and 1031 using rs 1800629 (-308), rs 361525 (-238), rs 1800630 (-863), and rs 1799964 (-1031). For this analysis, the following steps were performed: DNA extraction from 200 µL of whole blood through phenol-chloroform and ethanol precipitation; DNA quantification; primer design in which two primers were designed, primer pair 1 encompassed mutations- 1031 and 863 (256 bp in size) and had the sequences F:5′ AGGGATATGTGATGGACTCACC and R: 3′: GTCCTGGAGGCTCTTTCACTC; and primer pair 2 encompassed mutations- 308 and 238 (382 bp in size) and had the sequences F: 5′: AGGGATATGTGATGGACTCACC and R: 3′: GTCCTGGAGGCTCTTTCACTC; PCR; and 2% agarose gel electrophoresis in which 3 µL of dye mixed with 4 µL of DNA was loaded in the gel and run at 60 V, 250 mA, and 250 watts for 30 min, followed by staining with SYBR Safe (Life Technologies, CA, USA).

The amplified regions were subjected to direct sequencing in triplicate using the chain termination method[5]. All laboratory procedures were based on assays using the Big DyeTM Thermator kit (v.3.1) (Life Technologies, CA, USA), with AmpliTaq® DNA (Life Technologies, CA, USA). The primers 1 and 2 and the cycling conditions were the same as in the PCR step[5]. The sequencing solution was composed of 14.4 µL of water, 3 µL of Save Money, 0.8 µL of the forward primer, 0.8 µL of Big Dye, and 1 µL of the PCR solution. The sequencing results were obtained in the forward direction in the electropherogram and in FASTA format. Sequences were visualized in Chromas Pro version 1.33 software (TechnelysiumPtyLtd., Australia) and analyzed for the presence of variations compared to the consensus sequence of *Homo sapiens* TNFα. The nucleotide sequences were edited using the BioEdit program.

The following morphological cellularity and biochemical profile results were obtained: 80% of the samples showed hypercellularity (values above 5 cells/mm^3), with a mean of 154 cells/mm^3 and an equivalent standard deviation (SD) of 135.85, which is considered high because of the variability of the quantity of cells (samples with few and many cells). In 88% of the samples, mononuclear cells (MNCs) predominated, with a mean of 135 cells/mm^3 and SD = 29.86, which is considered low because the MNC levels on average were very close to each other.

Regarding high levels of protein, hyperproteinorachia (>40 mg/dL) was observed in 76% of the patients (19 CSF samples); the mean protein level was 75.82 mg/dL with SD = 65.52. Regarding the reduction of glucose, hypoglycorrhachia (<40 mg/dL) was found in 76% of the samples, with a mean glucose level of 35.12 mg/dL and SD = 8.33.

CFUs were observed in all samples, with 48% (12 samples) having the rare CFU growth profile, composed of 50% *C. gattii* and 50% *C. neoformans*. Moreover, 40% (10 samples) presented the frequent CFU profile, of which 40% were *C. gattii* and the other 60% were *C. neoformans*, and only one sample (4%) presented the abundant CFU profile, composed of *C. gattii*. Finally, 8% (two samples) could not be classified quantitatively.

Among the species of *Cryptococcus* isolated from the CSF samples, 48% (12) were *C. neoformans*, 44% (11) were *C. gattii*, and two samples (8%) were unidentifiable. Regarding HIV infection among the patients analyzed, 44% were HIV-positive, while 56% were HIV-negative. In 100% of the HIV-positive patients, the isolated fungal species was *C. neoformans*. Regarding the HIV-negative patients, *C. gattii* was identified in 78.57% (11), and *C. neoformans* was identified in 7.14% (1), and 14.29% (2) were unidentifiable (**Table 1**).

The following epidemiological results were obtained: 52% of the patients were male, and 48% were female. Regarding age, most were adolescents and adults, with 64% of the patients belonging to the age group of 14 to 39 years old; 20% of the patients were 1 to 13 years old, and 16% were older than 40 years. The mean age of the patients analyzed was 27.66 years, with SD = 13.91.

Regarding the geographical origin of the patients analyzed, all patients were from the state of Pará, with 56% of the patients from the metropolitan area of Belém, including Ananindeua (four cases) and Marituba (two cases); 44% of the patients were from municipalities in the interior of the State of Pará.

In the present study, in addition to analyzing the cellular and biochemical patterns of CSF, we investigated the possible association of cytokine gene polymorphisms in the context of *Cryptococcus* spp. infection. Several polymorphisms of these inflammatory response genes have already been associated with inflammatory and infectious diseases; however, to our knowledge, this is the first study to investigate possible changes in genes encoding cytokines in patients with cryptococcal meningitis.

TABLE 1: Frequency of the *Cryptococcus* species found in the CSF analyses

	Species					
	C. neoformans		*C. gattii*		None identified	
Patients	**n**	**%**	**n**	**%**	**n**	**%**
HIV-positive	11	100	0	0%	0	0%
HIV-negative	1	7.14	11	78.57	2	14.29
Total	12	48	11	44	2	8

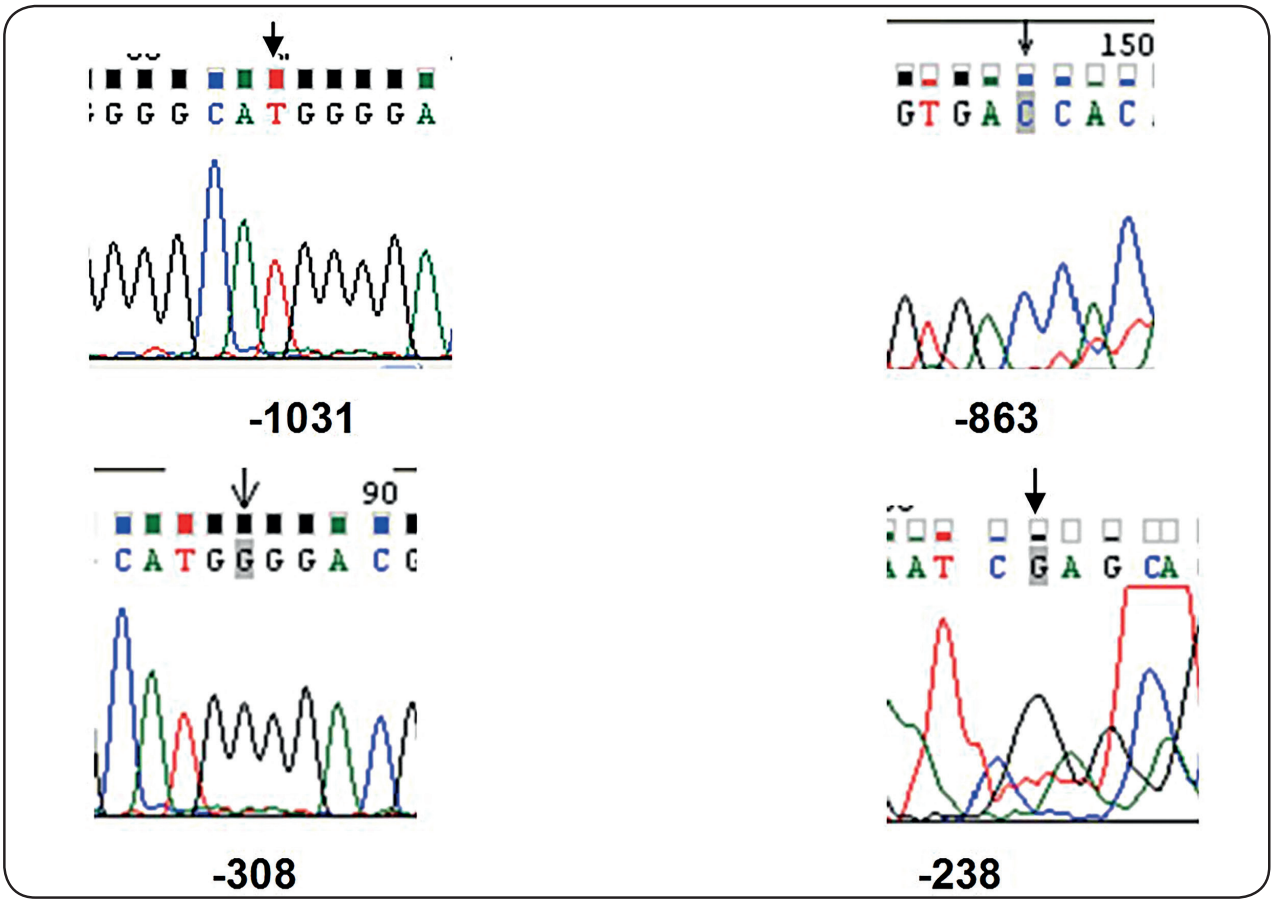

FIGURE 1: Schematic representation of the chromatograms of the loci evaluated for the presence of polymorphisms.

In the polymorphism analysis, a total of 50 genomic sequences (25 patients with 2 fragments each) of the TNFα gene from patients with fungal meningitis were amplified and read using the Chromas Pro version 1.33 program to investigate nucleotide alterations. The reference sequence of the TNFα gene was obtained from the NCBI (ID: 7124). By comparing sequences between individuals, the absence of somatic mutations at the referred l loci was found in the study group. Comparisons of the study group gene sequence with the NCBI sequence were performed.

No alterations were identified in the TNFα gene sequences analyzed, as the patients were identified as wild homozygotes, -1031 (C/T), -863 (A/C), -308 (G/A), and -238 (A/G), for the analyzed loci (**Figure 1**).

The controls that were collected from the database of the Human Cytogenetics Laboratory of the Federal University of Pará totaled 120 cases; 55% were female, 45% were male, and the mean age was 41 years (SD = 11.17). The same loci, -1031, -863, -308, and -238, were analyzed, and no polymorphisms were detected.

In summary, the CSF samples evaluated in this study showed hypercellularity, with a predominance of MNCs, hyperproteinorachia, and hypoglycorrhachia. A similar pattern was reported in a previous study, which also found

hypercellularity, hyperproteinorachia, and hypoglycorrhachia in samples from patients with cryptococcosis[6].

We attributed the results indicating rare fungal CFUs to previous antifungal use that effectively inhibited colony growth, as well as to the milder form of infection that does not present major clinical repercussions. These data are representative of the prognosis and possible therapeutic approaches because some protocols recommend using the quantitative CFU values in CSF as a cut-off point to determine whether patients should be treated with either prolonged induction amphotericin therapy or consolidation therapy with doses of fluconazole[7].

The frequency of isolates containing *C. neoformans* and *C. gattii* differed from the findings in the literature, wherein the predominance of *C. gattii* was previously described in Pará[8]. This predominance of *C. neoformans* in our samples was probably because of the large number of HIV-positive patients[9]. The high frequency of these patients (44%) is attributed to the fact that the HUJBB is a reference hospital for the treatment of AIDS in the State of Pará.

Most HIV-negative patients were infected with *C. gattii* (79%). Although cryptococcosis is classically considered a systemic opportunistic mycosis, it may also affect phenotypically normal patients. Therefore, this was a heterogeneous population ranging from apparently normal hosts to those with significant immune impairment. Although these patients appeared to be a homogeneous group, outcomes and complications may be more severe in this group of patients, including permanent neurological sequelae[1].

Even though there was little difference with respect to the prevalence of the disease between men and women, our results revealed that the disease was predominant in men (52%); this was similar to the results reported by Tavares and Marinho[10]. In relation to the age groups, Moreira et al.[9] described a higher occurrence of cases in the age group between 30 and 60 years, which differed from the findings in this study. However, our data corroborated a study performed at the University *Hospital of Uberlândia*, which found a higher prevalence of cryptococcosis cases in the 20–40 year age group[11].

Diniz et al.[12] did not report a specific trend between urban and rural areas; moreover, in this study, the proportion of cases in the metropolitan and inland areas was similar, with a slight predominance (56%) in the hospitalization of this population in metropolitan areas over the interior of the state.

Although polymorphisms in the TNF-α gene have been reported in the literature and associated with the susceptibility to and/or severity of different human infectious diseases[13], we did not find any variation of alleles with respect to the wild type sequence for both primer pairs 1 and 2. This lack of evidence of mutations does not exclude mutations in other loci of this gene. Our results suggest that the loci- 863, 1031, 308, and 238 retained the nucleotide sequences of the wild type TNFα gene.

Therefore, in this study, the variation of cytokine levels was not the result of mutations. Another possibility is that individuals were homozygous or heterozygous for the respective loci analyzed, different from what has been observed previously in infectious diseases such as tuberculosis[14] and hepatitis B[15], where variations in loci resulted in susceptibility to or protection against the disease.

Although no association was found between polymorphisms in the TNFα gene and cryptococcal meningitis, we cannot conclusively state that there is no relationship between this disease and other polymorphisms in cytokine-producing genes, as cytokine levels have been found to be altered in cryptococcal meningitis. Because no other studies on cytokine gene polymorphisms in this type of meningitis have been performed, further research is needed to clarify the role of other genes in the context of this disease.

In conclusion, the epidemiological results of this study indicated that cryptococcal meningitis occurred more frequently in males and in the young age group and that the most prevalent species was *C. neoformans*, which was closely related to HIV infection. The highest prevalence of *C. gattii* infection was observed in HIV-negative patients. The evaluation of polymorphisms in the studied group revealed no association between genetic factors in the TNFα gene and a predisposition to fungal disease.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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