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Association of *TNF* polymorphisms with *JAK2* (V617F) myeloproliferative neoplasms in Brazilian patients

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ABSTRACT

The classical chromosome Philadelphia-negative myeloproliferative neoplasms (MPNs) are a group of disorders that share clinical, hematological, and histological features. Proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) are elevated in patients with MPN. The aim of this study was to verify the association between the polymorphisms of *TNF* gene (-308G/A and -238 G/A) in BCR-ABL-negative MPN in our population. Blood samples obtained from MPN patients were genotyped for the *JAK2*V617F mutation and both *TNF* polymorphisms using PCR-RFLP. Thirty three (26.8%) patients with polycythemia vera (PV), 35 (28.7%) essential thrombocythemia (ET), 22 (17.7%) primary myelofibrosis (PMF), and 33 (26.8%) with unclassifiable MPN (MPNu) were included in the study. The *JAK2* V617F mutation was detected in 94 (76.42%) patients. Were observed a significant increase on the frequency of the *TNF*-238 GA genotype in MPN patients compared to controls (OR = 2.21, 95% CI = 1.02–4.80, P < 0.04). The distribution of the genotypes and allelic frequencies of *TNF*-308 was significantly different among the MPNs, *JAK2*V617F positive, PV and PMF, and controls. Our data has demonstrated that the polymorphisms on *TNF*-238 GA, *TNF*-308 GA were associated to MPN development in this population, triggered by *JAK2* V617F mutation.

1. Introduction

The myeloproliferative neoplasms (MPNs) are clonal disorders of hematopoietic stem-cells characterized by one or more myeloid lineages proliferation in bone marrow, leading to the accumulation of mature myeloid lineage that can be independent and/or hypersensitivity to cytokines for survival, proliferation and differentiation [1,2]. The classical chromosome Philadelphia-negative is a group of disorders that share clinical, hematological, and histological features, include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) [3].

A somatic activating mutation resulting in a valine to phenylalanine substitution at position 617 (V617F) in Janus kinase 2 (*JAK2*) has been identified in more than 95% of PV patients and in approximately half of the patients with ET and MF; however, it was not observed in healthy individuals [4]. The mechanism by which this mutated JAK initiates deregulated signals in cells is not fully elucidated. It is believed that *JAK2*V617F requires interactions with cytokine receptors to elicit its transforming signal [5]. Once activated, JAK family members lead to transcriptional regulation of STAT target genes that regulate a diverse array of cellular properties including growth, death, and differentiation [6].

Proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) are elevated in patients with MPN, but their contribution to disease pathogenesis is still unknown [7,8]. TNF-α is implicated in a variety of infectious and autoimmune diseases. Studies have shown that this clinical impact is partially due to the down regulation of cytokine levels. These data have highlighted new issues regarding the potential physiopathologic role of plasma cytokines in MPN [9,10].Genetic polymorphisms in the promoter region of the *TNF* gene are involved in the regulation of cytokine expression levels and have been associated with various inflammatory, hematological diseases, especially B-cell non-Hodgkin's lymphoma [11], chronic lymphocytic leukemia [12], multiple myeloma [13] and susceptibility to various types of cancers [14,15]. *TNF*-308 and *TNF*-238 have been the subject of many molecular epidemiological studies that investigated their importance in development and spread cancer [14–17].

The aim of this study was to verify the association between two polymorphisms in the promoter region of the *TNF*: -238 G > A (rs361525) and -308 G > A (rs1800629) in BCR-ABL-negative MPN in our population.

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2. Materials and methods

2.1. Study population

The study was approved by the local ethics committee (protocol number 318.552-COPEP-UEM), and all participants signed the informed consent. Blood samples were collected from 123 patients who received treatment at one of three regional oncologic services (Hospital do Câncer de Maringá, Instituto do Câncer de Londrina and Laboratório de Diagnóstico Molecular de Doenças Hematológicas — UNICAMP); these patients were previously diagnosed with BCR-ABL-negative MPN according to the 2008 WHO diagnostic criteria [1]. Blood samples were also collected from 123 healthy individuals (blood donors from the Hemocentro Regional de Maringá) in the same region and ethnicity and used as controls, considering that the *JAK2* V617F mutation is not found in healthy individuals [18,19].

2.2. JAK2 V617F mutation detection

DNA from patients was extracted from the biological specimens using a commercial kit according to the recommendations of the manufacturer (QIAampH DNA Blood Mini Kit, Qiagen). All blood samples obtained from the MPN patients were genotyped for the *JAK2* V617F mutation using a PCR-RFLP assay, as previously described [20,21]. To better visualize the post-digestion bands, we modified the described technique to include 2 U (1 mL) of BsaXI enzyme in each PCR amplicon digestion and used a 3% agarose gel with SYBR Green (Invitrogen Life Technologies, Grand Island, NY, USA) to run the post-digestion electrophoresis. The *JAK2* V617F positive samples presented a 460 bp band, while the negative samples contained two bands: 241 bp and 189 bp.

2.3. Analysis of the genetic polymorphism of TNF-238 and TNF-308

DNA from patients and controls was extracted from the biological specimens using a commercial kit according to the recommendations of the manufacturer (QIAampH DNA Blood Mini Kit, Qiagen). *TNF* promoter polymorphisms were detected by using a PCR-RFLP assay. A 107-bp fragment encompassing the −308 variant site was amplified by PCR using standard reagents, technique, and primers (5-AGG CAAT AG GTTTTGAGGGGCCAT-3) and (5-TCCTCCCTGCTCCG ATTCCG-3) for the −308 variant and 152-bp fragment (5-ATCTGGA GGAAGCGGTA GTG-3), and (5-AGAAGACCCCCCTCG GAACC-3) for the −238 variant allele. To detect *TNF*-308 variant alleles, the PCR product was digested with Thermo Scientific Fast Digest NcoI restriction enzyme at 37 °C, according to the manufacturer's recommendations. The presence of the A allele was detected by the observation of a 107-bp band, whereas the presence of the G allele was noted by the observation of a 87-bp and 20-bp band. To detect the -238 variant alleles, the PCR product was digested with Thermo Scientific Fast Digest MspI restriction enzyme at 37 °C using an according to the manufacturer recommendations. The presence of the A allele was detected by the observation of a 152-bp band, whereas the presence of the G allele was noted by the observation of a 133-bp and 19-bp band. The visualization of the bands in a 3% agarose gel with SYBR Green (Invitrogen Life Technologies, Grand Island, NY, USA).

2.4. Statistical analysis

The absolute and relative frequencies of the *JAK2* V617F mutation in the MPN patients were calculated. The allelic and genotypic frequencies for the polymorphism of the *TNF*-238 and -308 of the patients and controls were compared using a Chi-Square test with Yates' correction, for 2×2 contingency tables, considering a 95% confidence interval (CI). The results were considered statistically significant when the P-value was below 5%.

3. Results

We examined 123 Brazilian patients with MPN, 50 men (40.66%) and 73 women (59.34%), including 33 (26.8%) with PV, 35 (28.7%) ET, 22 (17.7%) PMF, and 33 (26.8%) with unclassifiable MPN (MPNu). The *JAK2* V617F mutation was detected in 94 (76.42%) patients. The genotype and allele frequency of *TNF*-238 and -308 polymorphisms in patients with MPN and healthy controls are shown in Table 1. We observed a significant association between the GA genotype frequency of *TNF*-238 of MPN when compared to the control groups (OR = 2.21, 95% CI = 1.02–4.80, P < 0.04). However, the comparison of genotype and allele frequency *TNF*-238 stratified by *JAK2* V617F mutation status and MPN disease entities (PV, ET, PMF and MPNu) with the control population did not show association.

Overall, the distribution of the genotype and allelic frequencies of *TNF*-308 G/A was significantly different between the MPN patients, JAK2V617F-Positive, PV and PMF with control populations. However, the comparison of the genotype and allelic frequencies of *TNF*-308 G/A of *JAK2*V617F-negative MPN patients, ET and MPNu with the control population did not show association (Table 2).When the haplotype frequencies were analyzed, the -238G/-308A was more frequent in patients with MPNs (OR = 1.54, CI = 1.12–2.11, P = 0.007), even as to -238G/-308A (OR = 1.64, CI = 1.17–2.29, P = 0.003) and -238A/-308A (OR = 6.00, CI = 1.29–27.97, P = 0.02) in MPN patients with *JAK2*V617F mutation positive compared to controls. However, the -238G/-308G was more frequent in the control group when compared to MPN patients overall (OR = 0.66, CI = 0.49–0.87, P = 0.004) and with MPN patients with *JAK2*V617F mutation positive (OR = 0.63, CI = 0.47–0.85, P = 0.003).

4. Discussion

This is the first case–control study that investigated the association of polymorphisms of *TNF* promoter genes with MPN patients' susceptibility.

The proinflammatory cytokine TNF-α is a mediator of the immune response involved of immune inflammatory reaction, although TNF-α has a beneficial function in the host defense against these diseases, its uncontrolled excessive production can also contribute to the disease pathogenesis [22]. Single-nucleotide polymorphisms (SNPs) in the promoter regions of *TNF* genes can regulate gene expression to change the connection of initial transcription factors [23]. *TNF* promoter polymorphisms have been intensively studied as a potential determinant of disease susceptibility in numerous diseases, where TNF-α levels were thought to be important. The substitution of G to A at position -308 in the *TNF* promoter is associated with an increased TNF-α production [16,24], while the substitution of G to A at position -238 is associated with a decreased TNF-α production [25].

The GA genotype, representative of the *TNF*-238 G/A and -308 G/A, was found to be significantly higher in our sample of patients, providing a risk of 2.21 and 1.82, respectively, to develop the MPN.

The *TNF*-238 promoter polymorphism has been associated with a decreased susceptibility to various types of cancers including gastric carcinoma, uterine cervical carcinoma, colorectal carcinoma, and renal cell carcinoma [26], in contrast to the *TNF*-238 A allele, that has been-associated with certain autoimmune and infectious diseases [27,28]. According to our results, no significant association with the-238 A allele was found, but the -238 GA genotype was considered to be a risk factor for the development of MPN.

On the other hand, several researchers showed association between the *TNF*-308A allele and an increased susceptibility to non-Hodgkin's lymphoma, breast carcinoma, uterine endometrial cancer, and prostate cancer [29,30]. The *TNF*-308A allele is known to up-regulate the transcription of the *TNF* gene, these findings show the critical role of TNF-α in tumor promotion [16]. Our results revealed that the *TNF*-308 polymorphism had a possible impact on the incidence of MPN, due to the