Sex and Foxp3 -924a/g Gene Polymorphism May Be Associated With the Clinical and Pathological Aspects of Chronic Viral Diseases

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Abstract

Background: The forkhead box protein 3 (FOXP3) transcription factor is one of the main markers of immunological suppression in different pathological profiles, and the presence of polymorphic variants may alter the gene expression of this factor. Despite descriptions of an association between the presence of the -924A/G polymorphism and chronic diseases, the role of the sex variant in this context has not yet been elucidated, as the FOXP3 gene is located on the human sex chromosome X.

Results: To contribute to this topic, 323 women and 373 men diagnosed with chronic viral infections or coronary artery disease and a healthy group of blood donors were genotyped for the -924A/G polymorphism. The -924A/G polymorphism was associated with clinical and pathological aspects and biomarkers of viral infections only in men, with functional differences between different infections.

Conclusions: A relationship is suggested between sex and FOXP3 polymorphisms, resulting in different biological repercussions.

Background

The forkhead box protein 3 (FOXP3) transcription factor is the main marker of regulatory T-cell (Treg) activation, a subpopulation specialized in the suppression of immune responses and the maintenance of homeostatic tolerance in different microenvironments (1). The impact of Treg frequency on the progression of chronic diseases is important because these cells mediate the inflammatory balance in an immunologically polarized environment (2).

FOXP3 gene is located on the short arm of the human chromosome X (Xp11.23; 21 kb) and consists of 11 exons (3). The relevance of single nucleotide polymorphisms (SNPs) located in the promoter region of the FOXP3 gene has been investigated due to they are involved in the transcription activation, as well as in the interaction with regulatory elements of the gene expression, possibly reflecting on the level of expression of FOXP3 and, thus, in the activation of Treg cells (4;5).

In this context, the polymorphism – 924A/G in the FOXP3 gene is related to functional changes that predispose individuals to diseases due to the mutant allele (G) changing the binding site of transcription factors that interact with the region (6). Although the knowledge about the association of this polymorphism with different diseases is based on the literature (7;8), the relationship between genetic variants of FOXP3 and sex, remain unclear. Thus, the present study evaluated the association of the – 924A/G polymorphism with clinical and pathological aspects and markers of progression of viral and non-viral chronic diseases between the sexes.

Results

The allele frequency of the – 924A/G polymorphism was similar between sexes, however statistical differences were observed between genders when assessing viral infections (Table 1).
Table 1
Association of sex and allele frequency of the $-924A > G$ polymorphism in patients with chronic diseases, stratified according to the clinical-pathological profile.

| Gender | Alleles ($-924A > G$) | Alleles ($-924A > G$) | Multiivariate analysis |
|--------|----------------------|----------------------|-----------------------|
|        | Female (%)           | Male (%)             |                       |
|        | $A$                  | $G$                  | Statistic#            | $A$                  | $G$                  | Statistic#            |                       |
|        |                      |                      |                       |                      |                      |                       |                       |
| Group  | Gender              |                      |                       |                      |                      |                       |                       |
|        | Female (323)         | Male (373)           | 0.07                  | 349 (54.0)           | 297 (45.9)           | -                     | 188 (50.4)           | 185 (49.6)           | -                     |
|        |                      |                      |                       | (3)                  | (8)                  |                       | (0)                  | (0)                  |                       |
| CVLD   |                      |                      |                       |                      |                      |                       |                       |                       |                       |
|        | 39 (12.0)            | 62 (16.6)            | 0.07                  | 39 (50.0)            | 39 (50.0)            | 0.47                  | 31 (50.0)            | 31 (50.0)            | 0.55                  | NS                    |
|        | (7)                  | (2)                  |                       | (0)                  | (0)                  |                       | (0)                  | (0)                  |                       |                       |
| HTLV-1 |                      |                      | 0.00                  | 52 (59.0)            | 36 (40.9)            | 0.62                  | 7 (33.3)             | 16 (66.6)            | 0.04                  |                       |
|        |                      |                      |                       | (9)                  | (1)                  |                       | (3)                  | (7)                  |                       |                       |
|        |                      |                      |                       | 1.20                 | (0.63                 |                       | 0.46                 | (0.18                 |                       |                       |
|        |                      |                      |                       |                     | -                      |                       |                     | -                      |                       |                       |
|        |                      |                      |                       |                      | 2.29                  |                       |                     | 1.18                  |                       |                       |
| CAD    |                      |                      | 0.03                  | 93 (51.1)            | 89 (48.9)            | 0.41                  | 68 (48.9)            | 71 (51.0)            | 0.35                  |                       |
|        |                      |                      |                       | (0)                  | (0)                  |                       | (2)                  | (8)                  |                       |                       |
|        |                      |                      |                       | 1.51                 | (1.06                 |                       | 0.43                 | (0.20                 |                       |                       |
|        |                      |                      |                       |                     | -                      |                       |                     | -                      |                       |                       |
|        |                      |                      |                       |                      | 2.13                  |                       |                     | 0.95                  |                       |                       |
|        |                      |                      |                       |                      |                       |                       |                       |                       |                       |                       |
|        |                      |                      |                       |                      |                       |                       |                       |                       |                       |

CVLD - Chronic Viral Liver Diseases;
CAD - Coronary Artery Disease;
CG - Control Group;

# - $x^2$ test. Odds ratio (Confidence interval - 95%);

* - $0.05 \geq p \geq 0.01$;

** - $0.01 \geq p \geq 0.001$. 
| Gender | Alleles (−924A > G) | Multi-variate analysis |
|--------|---------------------|-----------------------|
|        | Female (%)          | Male (%)              |                        |
|        | A               | G       | Statistic# | A               | G       | Statistic# |
|        | Female (%)        | Male (%) |            | Female (%)        | Male (%) |            |
| CG     | 149 (46.1)        | 149 (39.9) | 165 (55.4) 133 (44.6) | 82 (54.3)   67 (45.7) |

**Inflammation (CVLD)**

|        | A0-A1     | A2-A3     | Statistic# |
|--------|-----------|-----------|------------|
|        | 24 (61.5) | 15 (38.4) | 0.52       |
|        | 43 (69.3) | 19 (30.6) |            |

|        | F0-F1     | F2        | Statistic# |
|--------|-----------|-----------|------------|
|        | 20 (51.2) | 8 (20.5)  | 0.82       |
|        | 29 (46.7) | 17 (27.4) |            |

CVLD - Chronic Viral Liver Diseases;

CAD - Coronary Artery Disease;

CG - Control Group;

# - $x^2$ test. Odds ratio (Confidence interval − 95%);

* − 0.05 $p$ 0.01;

** − 0.01 $p$ 0.001.
| Gender       | Alleles (− 924A > G) | Alleles (− 924A > G) | Multi variate analysis |
|--------------|----------------------|----------------------|-----------------------|
|              | Female % (n)         | Male % (n)           |                       |
|              | A                    | G                    |                       |
|              | A                    | G                    |                       |
| F3-F4       | 11 (28.2)            | 16 (25.8)            | 11 (50.0)             |
| HTL V-1     | 27 (61.3)            | 13 (61.9)            | 32 (59.2)             |
|             | 32 (59.2)            | 22 (40.7)            | 0.86                  |
| HAM/TS P    | 17 (38.6)            | 8 (38.1)             | 20 (58.8)             |
| Risky infections (CAD) | 78 (85.7) | 121 (87.0) | 80 (86.0) |
|             | 80 (86.0)            | 74 (85.0)            | 60 (88.2)             |
|            | 13 (14.2)            | 18 (12.9)            | 13 (14.9)             |
|            | 13 (13.9)            | 13 (14.9)            | 8 (11.7)              |
| CVLD - Chronic Viral Liver Diseases; |
| CAD - Coronary Artery Disease; |
| CG - Control Group; |

In the present study, no significant association between sex and chronic viral liver diseases was observed, although the prevalence of males was higher among those infected (Table 1). In males the allele A was associated with advanced fibrosis, with a relative risk of 4 fold in the bivariate analysis. However, the risk
decreased and was not significant according to the multivariate analysis (Table 1). The viral load (VL) was high among males carrying allele A (p: 0.0007) as compared to carriers of the allele G (Fig. 1A), but the transaminase ratio (AST/ALT) was not significant between alleles and sex, although in males this rate is increased (Fig. 1B). The levels of gamma-glutamyl transferase (GGT) in males with allele G were higher than in those with allele A, but without significant differences (Fig. 1C).

Our data indicate that females are more associated with HTLV-1 infection than the −924A/G polymorphism itself, although the allele A has been associated as a protective factor against infection, especially in males (Table 1). Again in men, variant A was also associated with low levels of proviral load (p: 0.0396), T CD4⁺ lymphocytes (p: 0.0483) and IL-8 cytokine (p: 0.0549); the elevation of the levels of the cytokine IL-10 was a trend observed, however, with low statistical power (p: 0.1102) (Fig. 1D-G). The T CD8⁺ lymphocytes count was similar between genotypes/sexes (data not shown).

The risk of coronary heart disease was associated with male gender. The frequency of the polymorphism was not associated with pathological profile as the risk of infection history (Table 1). CRP levels were low and similar between sexes and alleles (data not shown).

**Discussion**

There is controversy regarding the genetic changes caused by the −924A/G polymorphism; however, regarding the immunological aspect, studies suggest that the allele A favors an anti-inflammatory profile, while the allele G favors a pro-inflammatory profile (9;6).

It seems counterintuitive to associate an anti-inflammatory factor with the risk of tissue aggression; however, these findings corroborate previous studies that suggest that variant A favors the persistence of viral infection in patients with fibrosis (10). With the increase in VL, there is a long-term pro-fibrogenic tendency induced by chronic inflammation and continuous response to healing (11), resulting in the fluctuation of pre-cirrhotic biomarkers (12), such as GGT. The transaminase ratio was stable between genotypes and sexes, indicative of chronic infection, but not relevant for estimating the stage of fibrosis (13). Recent studies by our group show that in liver fibrosis, viral load, in fact, is more associated with the histological profile than liver integrity enzymes (14).

In the multivariate analysis, the allele A was also associated with advanced fibrosis; however, the decrease in the statistical probability is indicative of other factors influencing liver histopathology. In recent publications, we discard alcoholism as a behavioral factor associated with liver fibrosis (14).

In HTLV-1 infection, the high prevalence of infected women is an epidemiological fact observed in different populations studied (15;16), and is related to the effectiveness of sexual transmission from male to female. With the present study, we suggest that not only transmissivity, but also sex-linked immunogenetic factors can influence susceptibility to HTLV-1 infection. The observed associations suggest that intrinsic mechanisms regulate the action of FOXP3 variants between sexes. Studies show that, in women, epigenetic processes modulate the expression of FOXP3 and alter the susceptibility to
diseases (17); the normal development of cells carrying mutant genes is a consequence of mixed chimerism (18), which is suggested in the present study due to the marked frequency of heterozygous women (data not shown). In men, changes in FOXP3 tend to be more relevant due to heredity (19), regulation induced by the Y chromosome (20) and sex hormones (21).

Although there are no studies on the association of the polymorphism with HTLV-1 infection, it is suggested that, in men, the anti-inflammatory tendency induced by the allele A reduces chronic immune hyperactivity, typically seen in the pathogenesis of the infection, reflecting the decrease in the proviral load and the cytokines of cellular immunity (22). The relationship of the polymorphism with the T CD4⁺ lymphocytes and IL-8 is indicative of the constitution of an atypical pro-inflammatory immune network (23), however, already observed in HTLV-1 infection, mainly in patients with Adult T-cell leukemia (ATL) (24).

In the present study, we did not associate the polymorphism with the clinical and pathological aspects of HTLV-1 infection. However, given the prevalence of asymptomatic patients observed, a cohort study in patients with allele A in this clinical group can clarify whether, in the long term, there will be polarization for a pro-inflammatory profile and changes in the pathogenesis of infection.

Only males were associated with the risk of CAD, however, unrelated to the history of Chlamydia infection. The history of Chlamydia pneumonia prevailed and CRP levels fluctuated, but both were not associated with sex and polymorphism. Recent reports confirm that men are generally more likely to develop CAD than women, with the highest death rate in middle age. Women, on the other hand, are at greater risk of stroke, which usually occurs at older ages (25).

**Conclusion**

In conclusion, the results of the present study indicate a relationship between sex and the polymorphism −924A/G in gene FOXP3, in which gene variants seem to be more associated with men and with different immunological roles that vary between chronic viral infections.

**Methods**

A total of 323 females and 373 males diagnosed with chronic viral liver disease (CVLD), human T-cell leukemia virus type 1 (HTLV-1) infection or coronary artery disease (CAD) and healthy groups of blood donors, all from regional reference centers in the state of Pará, Brazil (Tropical Medicine Nucleus of the Federal University of Pará, Foundation of Hemotherapy and Hematology Center of Pará (HEMOPA) and Institute of Health Sciences of the Federal University of Pará), were evaluated. Participants were informed about the objectives of the study and, after agreeing to participate, signed a consent form. The study was approved by the ethics committees of the participating entities (CAAE: 73782017.8.0000.0018; CAAE: 0011.0.324.000–09 and CAAE: 31223214.2.0000.0018). All patients were not undergoing treatment.
The inclusion/exclusion criteria and the biological data for patients CVLD were established in our previous study (10; 14).

Patients HTLV-1* were classified as asymptomatic or with paraparesis (HTLV-I-associated myelopathy/tropical spastic paraparesis - HAM/TSP) according to pre-established criteria (26). The quantification of the proviral load was based on previously described protocols (27). Quantification of lymphocytes TCD4* and CD8* was performed by immunophenotyping in flow cytometry (FACSCountTM Reagents - TriTEST™/TruCount, BD Biosciences, San Jose, CA, EUA). Pro-inflammatory (TNF-α, TNF-β, IFN-γ, IL6 and IL8) and anti-inflammatory (IL-10) cytokine levels were determined by immunoenzymatic assays (Human ELISAReady-SET-Go, EBioscience, Inc., San Diego, CA, USA).

Patients with coronary disease had arterial obstruction presenting with severe arterial obstruction with or without ischemia (infarction) and another group of patients with cardiac valvulopathy, presenting with volume overload and cardiac pressure. The history of Chlamydia infection in these patients was obtained by immunoenzymatic assays (NovaTec Immundiagnostica GmbH, Germany); and C-reactive protein (CRP) levels were measured by immunoturbidimetry (DiaSys, Waterbury, CT, USA).

Genotyping of the polymorphism was performed by real-time PCR (C_15942641_10 - Applied Biosystems, Foster City, CA, USA) with established temperature and cycling protocols (10).

The chi-square and Fisher's exact tests were used to determine Hardy-Weinberg equilibrium and allele frequency; Student's t-test, the Mann-Whitney test, ANOVA and the Kruskal-Wallis test were used to determine the association between the polymorphism and biomarkers. Statistical analysis was performed using BioEstat 5.0 (28) and GraphPad Prism 6.1, with a significance level of 95% (p ≤ 0.05).

**Abbreviations**

-924A/G  
Polymorphism of the substitution of adenine base for a guanine base in the promoter region (-924) of the FOXP3 gene; **ALT**:Alanine transaminase; **AST**:Aspartate transaminase; **CAD**:Coronary Artery Disease; **CVLD**:Chronic Viral Liver Diseases; **CRP**:C-reactive protein; **FOXP3**:Forkhead box protein 3; **GGT**:Gamma-glutamyl transferase; **HAM/TSP**:HTLV-I-associated myelopathy/tropical spastic paraparesis; **HTLV-1**:Human T-cell leukemia virus type 1; **Treg**:Regulatory T-cell; **VL**:Viral load.

**Declarations**

**Ethics approval and consent to participate**

The present study was submitted to and approved by the Research Ethics Committee of the Tropical Medicine Nucleus of the Federal University of Pará (CAAE: 73782017.8.0000.0018), Foundation of Hemotherapy and Hematology Center of Pará (HEMOPA) (CAAE: 0011.0.324.000-09) and Institute of
Health Sciences of the Federal University of Pará (CAAE: 31223214.2.0000.0018). All individuals who agreed to participate in the study signed an informed consent form.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The original data sets generated and analyzed during this study are made available by the corresponding author upon reasonable request.

**Competing interests**

The authors declare no competing financial interests exist.

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**Authors’ contributions**

LMSP, MWSM and RBHC performed the molecular analysis, interpretation of results. LMSP writing of the article. SD, MAFQ and INA participated in the sample collection, biochemical, histopathological profile and virological analysis. SRSSC and MSS performed medical consultations, patient interviews and clinical information collection. ANMRS, MOGI, RI and ACRV idealized the project. RI and ACRV guided and reviewed the article. All authors read and approved the final manuscript.

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Figures
Figure 1

Association of the -924A/G polymorphism with biomarkers of chronic viral diseases according to sex: In men with chronic viral liver diseases and advanced fibrosis, the viral load was higher in patients with the allele A (A); the ratio of transaminases (AST/ALT) was higher in men, however, with no differences between alleles (B); the level of GGT fluctuated between genotypes/sexes (C). In men infected with HTLV-1, the proviral load (D), TCD4+lymphocytes (E) and the level of IL-8 (F) were low in allele A carriers; the level of IL-10 was biased lower in patients with the allele A (G), but without statistical significance.