



Analysis of the *GSTM1*-null polymorphism in patients with pterygium from Goiânia, Goiás Brazil

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ABSTRACT. The first reports about pterygium date back to Hippocrates, and this disease still threatens vision health around the world. Pterygium is a formation of fibrous tissue consisting of highly vascularized epithelial and subepithelial tissue that grows excessively and with an abnormal shape on the cornea. Many physical and biological factors are associated with the pathogenesis of pterygium, including heat, dust, and other particles in the atmosphere, and immunological mechanisms, mechanisms involving extracellular matrix reorganization, growth factors, cytokines, apoptosis, and angiogenesis. The aim of this study was to further investigate the association between polymorphisms in *GSTM1* and the formation of pterygium. We collected peripheral blood samples from 90 patients diagnosed with pterygium and from 23 subjects without the disease in order to perform molecular analysis of the *GSTM1* gene. Subjects with one or two copies of the *GSTM1* allele had a normal genotype while those without any copies of the allele had a null genotype. The chi-square test or the Fisher exact test was performed in order

to investigate possible associations between the molecular analysis and the risk of pterygium. A significant difference between the frequency of the *GSTM1*-null genotype in patient and control groups was identified. However, sub-group analysis found that the *GSTM1*-null genotype was statistically significant in men, but not in women, and in Caucasians, but not in Brown or Black groups. Furthermore, the *GSTM1*-null genotype was not related to any of the risk factors analyzed: cases in family, occupational exposure, smoking, hypertension, and diabetes.

Key words: GST; *GSTM1*; Pterygium; Polymorphism

INTRODUCTION

Even though the first reports about pterygium date back to Hippocrates, its pathogenesis is not well-understood and it continues to threaten the vision health of many people around the world. The current description of the disease is similar to those from the past: a fibrous tissue mass is formed and moves across the surface of the cornea, assuming a triangular shape (Di Girolamo et al., 2004).

Pterygium is one of the most common known eye diseases. It is formed by fibrous connective tissue that is highly vascularized and grows abnormally on the cornea. Although pterygium can cause eye irritation in any phase of the disease, it is usually painless. Symptoms are related to foreign body and ocular burning sensations and red eyes. Signs of moderate to chronic conjunctivitis and thickening of the conjunctiva are also frequent and recurring symptoms. Diagnosis is performed by physical examination and is supplemented by biomicroscopy (Di Girolamo et al., 2004).

Pterygium is a benign condition of high worldwide prevalence and its high recurrence rate makes surgical procedures necessary (Golu et al., 2011). Countries located in the tropical zone show a pterygium prevalence of 22% while countries outside that area show a prevalence of 2% (Shiroma et al., 2009).

There is a higher incidence of pterygium in men, and this can be explained by different lifestyles between men and women: men generally spend more time outdoors than do women. Working outdoors, sun exposure, and other environmental factors such as dust can lead to chronic eye irritation and influence the onset of pterygium (Di Girolamo et al., 2004). Overall, the high correlation of the disease with ultraviolet (UV) radiation confirms its influence on the pathology of the disease. The anatomical structures around the eye (orbital rim, eyebrow, and nasal prominence) guarantee a degree of protection against the direct effects of UV rays. Nevertheless, protection is compromised when the exposure is through the bottom of the eye orbit or laterally (Golu et al., 2011). The negative effects of UV rays on the eyes are cumulative, and intense UV exposure means greater risk. Therefore, the use of sunglasses for eye protection is an important prophylactic measure, and it is important that sunglasses are used throughout the year and not only in the summer (Tung et al., 2010). The use of special lenses that filter UV rays with high efficiency is important when sun exposure is necessary and inevitable (Chui et al., 2008). Optical-quality sunglasses are able to block 100% of UV-A and UV-B radiation if they filter from 75 to 90% of the visible light (Said et al., 2007). Accessories such as hats and visors, among others may offer additional protection, especially if there is a need for prolonged sun exposure (Di Girolamo et al., 2004).

Another cause of degenerative diseases of the eye such as pterygium is oxidative stress. Oxidative stress is understood as an imbalance between the formation and removal of oxidizing agents in a tissue, caused by excessive production of reactive oxygen species or by a decrease in endogenous antioxidants. Free radicals, such as superoxide anion, hydroxyl, alkoxy, peroxy, and hydroperoxy groups are chemical species that contain unpaired electrons in their outer electron orbitals, whereas reactive oxygen species are any oxidizing compounds (Everecklioglu et al., 2003).

Reactive oxygen species are formed in several different situations in an organism: for example, in the production and regulation of nucleic acids, the detoxification of xenobiotics, during the actions of immune system cells following an inflammatory response, in transducing cellular signals, and resulting from environmental influence (exposure to UV radiation). One of the most important mechanisms of production of reactive oxygen species is energy metabolism. The excess of reactive compounds causes damage to many tissues through their reaction with cellular structures, contributing to aging and chronic and autoimmune diseases (Solomon, 2006).

Glutathione S-transferases (GSTs) belong to a family of multifactorial enzymes with the function of catalysis of the nucleophilic attack of glutathione in its reduced form (GSH) (Hayes et al., 2005). GSTs are one of the enzyme types responsible for protection against the oxidation caused by reactive oxygen species. In humans, the GST family can be divided into the classes α , μ , π , σ , κ , ϵ , and θ . The μ family locus, commonly called (M), is located on chromosome 1 (1p13.3). There are three alleles: *GSTM1* (0), *GSTM1* (A), and *GSTM1* (B). *GSTM1* (0) corresponds to a deletion and the homozygote expresses neither the mRNA nor the corresponding protein. The *GSTM1* (A) and (B) alleles encode monomers that form homo- and heterodimeric enzymes. *GSTM1* 0/0 is usually called the GSTM-null polymorphism (Tsai et al., 2004).

Classes μ (*GSTM1*) and θ (*GSTT1*) are polymorphic genes and, according to Scianni et al. (2008), they are homozygous in about 50 and 24% of the different ethnic populations, respectively. The null genotype is a consequence of unequal recombination. The deletion of 15 kb contains the entire gene (Hayes et al., 2005). Some authors have suggested that human *GSTT1* and *GSTM1* take part in the deactivation of reactive oxygen species that would otherwise be involved in the cellular processes of inflammation, aging, and degenerative diseases (Bolth and Thier, 2006).

Tsai et al. (2004) demonstrated that the *GSTM1*-null genotype was associated with an increased susceptibility to early onset of pterygium, although it was not associated with late-onset pterygium cases. The authors suggested that *GSTM1* might be an important antioxidant involved in the metabolism of xenobiotics (Young et al., 2010).

It has been proposed that cumulative DNA damage might contribute to the development of certain diseases. For example, patients with DNA repair deficiencies are more likely to develop neoplastic diseases. Deletion of *GSTM1*, which characterizes the null genotype, is associated with the absence of enzymatic function and an increase in cytogenetic damage (Tsai et al., 2004). The *GSTM1*-null genotype has also been associated with an increased risk of developing pterygium. The expression of *GSTM1* is thought to be important in protecting corneal cells against reactive oxidants, as these cells exhibit marked photosensitivity. Subjects with the *GSTM1*-null genotype were reported to show more intense inflammatory reactions after exposure to UV radiation, and they had a higher prevalence of skin cancer (Tung et al., 2010). Such genetic studies are an important step in understanding the pathogenesis of pterygium. Genetic markers can be used to identify patients at risk and to develop preventive measures for those patients that could reduce the likelihood of developing pterygium (Weiss

et al., 2005). Accordingly, the aim of the present study was to analyze the relationship between the *GSTM1*-null polymorphism and pterygium.

MATERIAL AND METHODS

All subjects that took part in the present study answered a social and economic questionnaire containing basic information, including name, ethnicity, and drinking alcohol consumption and/or smoking habits. All patients signed an informed consent form. We collected peripheral blood samples from 90 patients diagnosed with pterygium, and from 23 subjects free from the disease to form the control group. Blood samples were collected at the Hospital Banco de Olhos in the city of Goiânia, Goiás. The molecular analysis to determine the *GSTM1* genotype was performed at Replicon-PUC-Goiás.

Genomic DNA was extracted and purified using the Illustra™ blood genomic Prep Mini Spin Kit (GE Healthcare®, USA). The DNA samples were quantified using a NanoVue™ Plus (GE Healthcare) according to manufacturer instructions. DNA samples that were at a minimal concentration of 5 ng/μL were used in the analysis.

After extraction, the DNA samples were subjected to amplification by polymerase chain reaction (PCR) to detect allele-specific polymorphism of the *GSTT1*, according to the protocol of Frare et al. (2013). PCR products were subjected to electrophoresis on a 2% agarose gel with an electric field of 10 V/cm, and then stained with ethidium bromide (5 mg/mL). The gel was visualized and documented by UV transillumination on an ImageMaster™ VDS® (Amersham Pharmacia Biotech, GE Healthcare, Munich, Germany). Subjects with one or two copies of the *GSTT1* allele have a normal genotype while those without any copies of the allele have a null genotype. The number of alleles was determined by electrophoresis gel (Frare et al., 2013). Genotypes were recorded in spreadsheets using the Microsoft Excel® 2003 software (Microsoft Corp., Redmond, WA, USA). Subsequently, the chi-square test or the Fisher exact test was performed in order to investigate possible associations between molecular analysis results and the incidence of pterygium. We used BioEstat® 3.0 (Mamirauá/MCT-CNPq Civil Society, Belém, Brazil) to perform the analysis.

RESULTS

The frequencies of the *GSTM1* gene polymorphism were analyzed in the patient (case: patients with pterygium) and control (subjects free from the disease) groups as shown in Table 1. In the patient group, containing 90 individuals, the *GSTM1* gene could be detected in 17.6% (15/90) of the subjects, whereas 83.3% (75/90) carried the null polymorphic genotype. In the control group (containing 23 subjects) we detected 43.5% (10/23) positive for the *GSTM1* gene and 56.5% (13/23) with the null polymorphic genotype. The results show a statistically significant difference confirming a positive relationship between the *GSTM1*-null genotype and pterygium ($P = 0.0056$).

Table 2 shows the genotype distribution of *GSTM1* in relation to gender in the patient and control groups. Analysis of the *GSTM1* polymorphism in women in the patient group detected 20% (10/50) of the patients with the *GSTM1*-present genotype and 80% (40/50) with the null polymorphic genotype. Of females in the control group, 33.3% (4/12) had the *GSTM1*-present genotype and 66.7% (8/12) had the *GSTM1*-null genotype. This result was not statistically significant ($P = 0.2631$).

Analysis of the *GSTM1* polymorphism in men demonstrated that 12.5% (5/40) of the patient group had the *GSTM1*-present genotype, while 87.5% (35/40) had the null polymorphic genotype. In the control group, 54.5% (6/11) of the men had the *GSTM1*-present genotype and 45.5% (5/11) had the null polymorphic genotype. The difference in this frequency was statistically significant ($P = 0.0071$), which supports the hypotheses of a higher incidence of pterygium in men.

Table 1. Distribution of the *GSTM1* polymorphism in patient and control groups.

	GSTM1-present		GSTM1-null		P
	N	%	N	%	
Patients (N = 90)	15	16.7	75	83.3	0.0056
Controls (N = 23)	10	43.5	13	56.5	

P value from the chi-square test.

Table 2. Distribution of the *GSTM1* polymorphism in relation to gender in the patient and control groups.

Gender	GSTM1-present		GSTM1-null		Total		P
	N	%	N	%	N	%	
Female							
Patients	10	20.0	40	80.0	50	100.0	0.2631
Controls	4	33.3	8	66.7	12	100.0	
Male							
Patients	5	12.5	35	87.5	40	100.0	0.0071
Controls	6	54.5	5	45.5	11	100.0	

P value from the Fisher exact test.

Table 3 shows the *GSTM1* genotype distribution among different ethnic groups: Caucasian, and patients with brown or black skin. Analysis of the *GSTM1* gene in the Caucasian patients found that 12% (6/50) had the *GSTM1*-present genotype and 88% (44/50) had the *GSTM1*-null genotype; 50% (8/16) of subjects in the control group showed *GSTM1*-present genotype and 50% (8/16) showed the *GSTM1*-null genotype. This result was statistically significant ($P = 0.0030$) and it supports the idea that the disease is more frequent in Caucasian patients.

Table 3. Comparison between skin color and the *GSTM1* polymorphism in patient and control groups.

Groups	Patients		Controls		P
	N	%	N	%	
*Caucasian					0.0030
GSTM1-present	6	12.0	8	50.0	
GSTM1-null	44	88.0	8	50.0	
Total	50	100.0	16	100.0	
*Brown skin					0.5538
GSTM1-present	5	21.7	1	33.3	
GSTM1-null	18	78.3	2	66.7	
Total	23	100.0	3	100.0	
*Black skin					0.6683
GSTM1-present	4	23.5	1	25.0	
GSTM1-null	13	76.5	3	75.0	
Total	17	100.0	4	100.0	

P value from the Fisher exact test. *As reported by the patient.

In patients with brown skin, 21.7% (5/23) of the subjects belonging to the patient group had the *GSTMI*-present genotype and 78.3% (18/23) had the polymorphic null genotype. In the control group, 33.3% (1/3) had the *GSTMI*-present genotype and 66.7% (2/3) had the *GSTMI*-null genotype. The results for this pseudo-ethnic group showed no statistically significant difference ($P = 0.5538$).

The analysis of *GSTMI* polymorphisms in patients with black skin found that 23.5% (4/17) of the subjects in the patient group showed the normal genotype and 76.5% (13/17) showed the null *GSTMI* genotype. One quarter (25%; 1/4) of the subjects in the control group showed the normal genotype and 75% (3/4) showed the null polymorphic genotype. No statistically significant difference was identified between the patient and control groups ($P = 0.6683$).

Furthermore, the *GSTMI*-null genotype was not found to be related to the other risk factors analyzed: cases in family, occupational exposure, smoking, drinking, hypertension, and diabetes.

DISCUSSION

Pterygium is a serious public health problem. It is often diagnosed at a routine eye exam and prevention campaigns against pterygium are rarely performed. Several studies have been conducted in order to identify a molecular genetic component of this disease (Jaworski et al., 2009). One candidate, the *GSTMI* gene product, plays a key role in protecting tissues against oxidative stress that could lead to diseases such as pterygium (Tung et al., 2010).

Our analysis of *GSTMI* polymorphic variants found a statistically significant difference between the *GSTMI*-null genotype in patient and control groups when the frequencies between these groups were compared. A separate study comprising 127 patients confirms our results; they found that the null genotype of the *GSTMI* gene is associated with the onset of pterygium (Tsai et al., 2004). However, two independent studies in China (Young et al., 2010) and in Taiwan (Tung et al., 2010) analyzed the *GSTMI*-null polymorphism in patients with pterygium and found no significant differences. The discrepancy in the results might be explained by the populations used in these studies. The Brazilian population is characterized by miscegenation, and has ancestral roots in Europe, Africa, and America with various migration patterns (Suarez-Kurtz, et al., 2014). In China (Young et al., 2010) and Taiwan (Tung et al., 2010), the populations are more homogeneous, having an Asian origin.

In our study, we found that the presence of the *GSTMI*-null genotype was related to the onset of pterygium in men. However, there was no significant difference in the prevalence of pterygium in women for this polymorphism. A study conducted in Australia (Sherwin et al., 2013) showed that the frequency of the *GSTMI*-null genotype is more frequent in the male population of that country, a result that corroborates the results in the present study. Other studies also corroborate our results.

A group of studies from Beijing (Ma et al., 2007) and from Ethiopia (Meseret et al., 2008) showed that pterygium is more frequent in men. However, a Chinese study analyzed the *GSTMI*-null polymorphism but found no significant difference between men and women (Young et al., 2010). As for the incidence of pterygium itself, the discrepancies in the published results might be explained by the different populations used in each study. Unlike the Brazilian population, the Australian population originates primarily from Europe (Sherwin et al., 2013), the population from Beijing has an Asian origin (Ma et al., 2007), and the population of Ethiopia is mostly Semitic (Meseret et al., 2008).

A study conducted in Barbados (Luthra et al. 2001) also showed that ethnicity might be related to the prevalence of pterygium. They analyzed 4709 patients and found that the prevalence of pterygium in that country is related to the Black population. Pterygium is 2.5 to 3.0 times more frequent in the Black patients compared to the rest of the population (Luthra et al. 2001). In contrast, a group from Spain showed that the prevalence of pterygium was higher in Caucasians, which supports our results (Viso et al., 2011).

In the present study, we observed that the presence of the *GSTM1*-null genotype did not increase the possibility of developing the disease in patients who had ocular abnormalities in the family. Unlike our results, a study in Africa (Anguria et al., 2012) showed that cases of pterygium in the family can contribute to increasing the frequency of the disease in subsequent generations. Again, the discrepancy in results might be due to the different populations studied. The miscegenistic Brazilian population (Suarez-Kurtz et al., 2014) supports considerable genetic heterogeneity, whereas the African population studied was more homogeneous with respect to their genetic characteristics (Anguria et al., 2012).

We observed that the *GSTM1*-null genotype did not increase the possibility of developing pterygium when there was occupational exposure. A study in Malaysia (Cajucum-Uy et al., 2010) supports our results. They found no significant differences when comparing pterygium and occupational exposure in that population. On the other hand, a research group from Barbados (Luthra et al., 2001) showed that pterygium was twice as frequent in patients who had occupational exposure. The authors claimed that it was very important to invest in prevention programs that encouraged people who work while exposed to the sun to use proper equipment in an attempt to decrease the chance of developing pterygium.

In this case, we believe that any discrepancies in results might be explained by the small sample size of this research, which represent a genotypic *GSTM1* profile of the central Western Brazil. The present study has shown that the presence of the *GSTM1*-null genotype did not increase the possibility of developing pterygium in patients who smoke. These results are supported by those from a group of scientists from India (Asokan et al., 2012), who evaluated the effect of smoking in relation to pterygium. A further study conducted with Latin American patients living in the United States also found no significant difference regarding pterygium and smoking (West and Muñoz, 2009).

We found that the presence of the *GSTM1*-null genotype did not increase the possibility of developing pterygium when the patient suffered from hypertension. Similarly, a study conducted in China (Zhao et al., 2013) followed 4439 patients for 10 years, and found that there was no relationship between the onset of the disease and hypertension. We also found that the presence of the *GSTM1*-null genotype did not increase the risk of developing pterygium when the patient had diabetes, and, in addition, we demonstrated that pterygium was not related to alcohol consumption. A study group from China (Zhong et al., 2012) assessed several parameters regarding the onset and development of pterygium such as height, weight (not evaluated by our group), hypertension, drinking, smoking, and diabetes. Their results support our results: there was no statistical significance between pterygium and diabetes or alcohol consumption. The results overall, therefore, demonstrate that the *GSTM1*-null genotype is not related to the following risk factors: cases in family, occupational exposure, smoking, hypertension, and diabetes.

Our research shows that a significant difference between the frequency of the *GSTM1*-null genotype in patient and control groups was identified. However, sub-group analysis found that the *GSTM1*-null genotype was statistically significant in men, but not in women, and in

Caucasians, but not in Brown or Black groups. Furthermore, the *GSTM1*-null genotype was not related to any of the risk factors analyzed: cases in family, occupational exposure, smoking, hypertension, and diabetes.

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