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Frequency of Polymorphisms pro198leu in *GPX-1* Gene and ile58thr in *MnSOD* Gene in the Altitude Ecuadorian Population With Bladder Cancer

César Paz-y-Miño,* María José Muñoz,*† Andrés López-Cortés,* Alejandro Cabrera,* Adriana Palacios,* Bernardo Castro,* Nelson Paz-y-Miño,‡ and María Eugenia Sánchez*

*Instituto de Investigaciones Biomédicas, Facultad de Ciencias de la Salud, Universidad de las Américas, Quito, Ecuador
†Laboratorio de Genética Molecular y Citogenética Humana, Escuela de Ciencias Biológicas, Pontificia Universidad Católica del Ecuador, Quito, Ecuador
‡Departamento de Urología, Hospital Carlos Andrade Marín, Quito, Ecuador

Bladder cancer represents 8% of all malignancies diagnosed in men and 3% in women. The risk factors for developing bladder cancer, including the incidence rate, morbidity, and mortality, vary according to the ethnic group, exposition rate at work, age, gender, and tobacco consumption. Moreover, there is a risk of developing this carcinoma due to dietary conditions, demonstrating that certain enzymes neutralize oxidative compound derivative of carcinogens, which if not degraded, accumulate in the body and destroy epithelial cells of the bladder, causing an increase in the risk of developing this disease. The detoxifying enzymes inactivate dangerous chemical compounds and anions for the cell; that is, why it is important to know if the polymorphisms pro198leu in *GPX-1* and ile58thr in *MnSOD* are associated with bladder cancer. In this study, 120 individuals were analyzed as controls and 97 individuals with previously diagnosed bladder cancer. In the case of polymorphism pro198leu, highly significant differences were observed and individuals with this polymorphism presented a probability of developing bladder cancer 3.8 times greater than controls (OR = 3.8; 95% CI 2.16–6.78; $p < 0.001$). No significant differences in polymorphism ile58thr of *MnSOD* gene occurred when we compared the study population (OR = 2.1; 95% CI 1.26–3.49; $p > 0.05$). The results indicate that polymorphism of *GPX-1* gene influences the risk of developing bladder cancer in the Ecuadorian population, suggesting that more research on detoxifying genes in bladder cancer should be conducted.

Key words: *GPX-1*; *MnSOD*; pro198leu; ile58thr; Bladder cancer

INTRODUCTION

In industrialized countries, bladder cancer is the fourth most frequent cancer diagnosed in men, and the ninth most frequent cancer in women (1). Almost 357,000 new cases are reported every year, and bladder cancer is the most frequent type of cancer developing in the urinary tract. Globally it represents 3.3% of all tumors, and the risk of developing the disease is three times greater in men than in women (2). The incidence rate and the morbidity and mortality associated with bladder cancer vary according to ethnic group, gender, and age (2,3). In Ecuador, bladder cancer represents the 19th and 20th cause of death in men and in women, respectively (4,5).

Bladder cancer is related to tobacco addiction, being the principal risk factor in men (60%) and women (25%) (2,3, 6). The aromatic amines and the organic benzene derivatives found in cigarettes and the oxide reactive species such as molecular oxygen, hydrogen peroxide, and hydroxyl radicals (2,6,7) increase the risk of developing this neoplasm by 25%. Certain enzymes do not neutralize nor degrade the oxidative radicals, and accumulation of these compounds in the body causes the destruction of epithelial cells of the bladder, thereby increasing the risk of developing cancer (7).

Oxidative stress is characterized by an increase in the number of oxide reactive species and by the reduction of the activity of antioxidative enzymes, causing metabolic dysfunction, and damage to biologic macromolecules. In addition, the accumulation of oxide reactive species represents an important role in the pathogenesis of cardiovascular diseases, neurodegenerative disorders, chronic damage to the liver and kidneys, as well as the development of different types of cancer (8).

The enzymes that perform a detoxifying function deactivate compounds and anions that are dangerous for the cell. The defenses against oxide reactive species are glutathione peroxidase (*GPX*), catalase (*CAT*), and superoxide dismutase (*SOD*). *GPX* protects the cell from

Address correspondence to César Paz-y-Miño, M.D., D.B., Instituto de Investigaciones Biomédicas, Facultad de Ciencias de la Salud, Universidad de las Américas, Av. de los Granados y Colimes, 1er P., Quito, Ecuador 1712842. Tel/Fax: (593-2)3340229; E-mail: cpazymino@udla.edu.ec

oxidative damage, reducing levels of hydrogen peroxide and other organic peroxides (6,9). *GPX* is a detoxifying enzyme that depends on selenium; the *GPX* gene is located on chromosome region 3p21. This enzyme is the final regulator of the metabolic pathway that degrades oxide reactive species such as peroxide radicals (7,10).

It has been reported that the substitution of one nucleotide at the 198 codon of the *GPX1* gene results in the transversion of a thymine for a cytosine, resulting in a substitution of leucine (L) for proline (P) at the amino acid level. This change is associated with an increase in the risk of developing bladder and lung cancer in Caucasian populations, because of a reduction in enzymatic production and activity (7). In contrast, manganese superoxide dismutase (*MnSOD*) is the principal defense system in mitochondria because mitochondrial enzymes are important in the protection against oxidative stress at the cellular level, catalyzing the conversion of superoxide anion into molecular oxygen and hydrogen peroxide (10). The *MnSOD* gene is located in the 6q25 chromosome region; its sequence is present in nuclear DNA and it codifies the enzyme in the mitochondrial matrix (11). There are 151 polymorphisms reported for the *MnSOD* gene. Of those variations, one has a major incidence worldwide. This polymorphism involves a change of threonine (T) for isoleucine (I) at the 58 codon of exon 3. At the nucleotide level a transversion of thymine for cytosine occurs, affecting gene stability and reducing enzymatic production and activity, thereby being responsible for mitochondrial damage (12). The gene polymorphisms act as antioxidants, with manganese superoxide dismutase and glutathione peroxidase, diminishing their enzymatic capability of reducing oxide reactive species in the cell (7,10).

Due to the existence of substantial differences in bladder cancer incidence in different ethnic groups, it is important to determine the incidence of polymorphisms pro198leu (*GPX-1*) and ile58thr (*MnSOD*) in individuals of the Ecuadorian population with bladder cancer. Such a study is of great interest because it is the first report of polymorphic variants of these genes in Latin America and Ecuador; it also describes the incidence of these polymorphisms due to the ethnic and geographic heterogeneity of the Ecuadorian population, thereby demonstrating the different epidemiology of bladder cancer, as confirmed in other studies of *CF*, *NF2*, *hMSH2*, *BCR-ABL*, *RB1*, *CYP1A1*, and *SRD5A2* genes (13–18).

MATERIALS AND METHODS

Biological Samples

The study consists of a retrospective design, with 217 Ecuadorian male and female individuals being analyzed. Ninety-seven biopsies and surgical samples of fresh tumor tissue embedded in paraffin were obtained from individuals affected with bladder cancer. Tumor samples were collected from the Department of Urology of the Carlos Andrade Marín Hospital in Quito and the Department of Pathology of the Solón Espinoza Ayala Oncologic Hospital of Ecuador (SOLCA). For control individuals, 120 peripheral blood samples were obtained from donors older than 45 years. Each of them filled biomedical survey and informed consent forms.

Genotyping

DNA of affected individuals was extracted from tumor tissues embedded in paraffin using standard methodology by digestion with proteinase K. DNA extraction from peripheral blood of control individuals was accomplished using a Salting Out method and a DNA extraction kit: DNA Wizard® (Promega, Madison WI, USA) (19).

For the amplification of the 191-bp fragment of the *GPX-1* gene, a final volume of 25 µl was obtained for each PCR reaction. Each sample consisted of 10.85 µl of H_2O Milli-Q, 2.5 µl of PCR 10× Buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl) (Invitrogen, Carlsbad, CA), 0.8 µl of $MgCl_2$ at 25 mM, 2 µl of deoxynucleotide triphosphate at 200 mM, 2.5 µl of primers 5′-AAGGTG TTCCTCCCTCGTAGGT-3′ (forward) and 5′-CTACG CAGGTACAGCCGCCGCT-3′ (reverse) at 0.5 µM, 2.5 units of Taq polymerase (Invitrogen), and 4 µl of DNA sample at a concentration of 100 ng/µl. After preparation for the PCR reaction, samples were placed in a thermo cycler MJ Research PTC 200® (MJ-Research Inc., Watertown, MA) for amplification. The initial denaturation step lasted 8 min at 94°C, then 36 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and 9 min at 72°C were employed. For the amplification of the 145-bp fragment of the *MnSOD* gene, a final volume of 25 µl was employed for the PCR reaction. Each sample consisted of 10.85 µl of H_2O Milli-Q, 2.5 µl of PCR Buffer 10× (Invitrogen), 0.8 µl of $MgCl_2$ at 25 mM, 2 µl of dNTPs at 200 mM, 2.5 µl of primers 5′-ACTT CAGTGCAGGCTGAACAGC-3′ (forward) and 5′-CT GGTCCCATTATCTAATAGCTT-3′ (reverse) at 0.5 µM, 2.5 units of Taq polymerase (Invitrogen), and 4 µl of DNA sample at 100 ng/µl. For the amplification of the fragment, the initial denaturation step lasted 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, 1 min at 72°C, and 10 min at 72°C. The amplicons of the fragments of the *GPX-1* and *MnSOD* gene were resolved by electrophoresis in agarose gel at 2% with ethidium bromide. The amplicons were observed using an UV transilluminator.

The genotype of the individuals affected with bladder cancer and control individuals was determined by the

polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. The *Apa*I restriction enzyme was used to identify the pro198leu variant, while the *Pvu*I restriction enzyme was employed to identify the ile58leu variant.

To have the restriction enzymes cut at the restriction sites, the products from the PCR amplification must be digested for 2 h at 37°C with 5 U of enzyme, using *Apa*I for the *GPX-1* gene and *PvuI* for the *MnSOD* gene (New England Biolabs, Beverly, MA). After digestion with the enzymes, analysis of the genotype was performed by electrophoresis in agarose gel using 5% ethidium bromide, and observing the gel under UV light.

In the pro198leu polymorphism of the *GPX-1* gene, when the restriction enzyme does not recognize the restriction site the DNA is not mutated; this can be observed as one digestion product of 191 bp in the agarose gel, thus referring to a homozygous individual (P/P). When three digestion products are observed in the agarose gel (191, 117, and 74 bp) the individual is heterozygous (P/L), and when two digestion products are observed in the agarose gel (117 and 74 bp), the individual is homozygous (L/L). For the ile58thr polymorphism in the *MnSOD* gene, when the restriction enzyme does not recognize the restriction site, a fragment of the 145 bp can be observed in the agarose gel, thereby indicating a homozygous individual (I/I). If three digestion products are observed in the gel (145, 124, and 21 bp) the individual is heterozygous (I/T). When two digestion products are observed (124 and 21 bp), the individual is homozygous (T/T).

Statistical Analysis

All information obtained from the studied individuals was kept in a database and statistical analyses were performed using PASW Statistics 17 for Windows (SPSS, Chicago, IL). Allelic frequency of the analyzed variants was calculated, and genotypic frequencies were also calculated. Chi-square analysis was performed to determine significant differences between affected and healthy individuals and pro198leu and ile58thr polymorphisms. The relative risk of developing disease in the presence of the studied polymorphisms between affected and control individuals was determined using the odds ratio test (OR). Data were analyzed using a 2×2 contingency table.

RESULTS

Clinical-Pathological Parameters

The clinical-pathological parameters considered in this study were age, gender, smoking, and histological subtype.

The average age in control individuals at the time of blood sample collection was 68 years of age in men and 64 years of age in women, whereas the average age of individuals with bladder cancer was 69 for men and 67 for women. But, according to this, 34% of the healthy individuals and 73% in affected cases were older than the average age, whereas 66% of the controls and 27% of the cases were younger than the average age.

As for gender, the group of healthy individuals consisted of 33% of women and 67% of men, while the group of affected individuals consisted of 43% of women and 57% of men. With respect to histological subtype, 89% of the affected individuals presented with transitional cell carcinoma, being 86% men and 93% women. Only 1% of the individuals presented with adenocarcinoma, with 2% being men and 0% being women; 6% of the affected individuals presented urothelial papillary carcinoma, with 7% being men and 5% being women; and 4% of affected individuals presented with squamous cell carcinoma, with 5% being men and 2% being women (Table 1).

Concerning smoking as a risk factor to develop bladder cancer, 74% and 56% of affected individuals and healthy individuals, respectively, used to smoke, whereas 26% of affected and 44% of controls never smoked.

Allelic Frequency of pro198leu and ile58thr Polymorphisms

In the group of individuals with bladder cancer, 28 patients were homozygous (P/P) patients for the normal allele of the *GPX-1* gene, 19 were heterozygous (P/L), and 50 individuals were homozygous for the (L/L) allele. In the control group, 73 individuals were homozygous (P/P), 42 were heterozygous (P/L), and 5 were homozygous for the (L/L) allele. The frequency of the (P/P) allele was 0.39 and 0.79 for affected and control groups, respectively, while the frequency of the (L/L) was 0.61 for the affected group and 0.21 for the control group (Table 2).

With respect to ile58thr, the group of affected individuals with bladder cancer consisted of 43 homozygous individuals for the (I/I) allele, 47 heterozygous individuals for the (I/L) allele, and 7 homozygous individuals for the (T/T) allele. In the group of control individuals, 75 individuals were found to be homozygous for the (I/I) allele, 47 were heterozygous for the (I/T) allele, and no individuals for (T/T). The allelic frequency of the (I/I) allele was 0.68 for the group of affected individuals and 0.32 for control group. The (T/T) allelic frequency was 0.82 for the affected individual group and 0.18 for the control group (Table 3).

Statistical Analysis

Ninety-seven affected individuals and 120 control individuals were evaluated. The chi-square test was per-

Table 1. Clinical-Pathological Parameters From Study Population

Parameters	Affected	Controls	Odds Ratio
Age	71 (>68)	41 (>66)	0.6, 95% CI 0.334–1.020, $p < 0.05$
	26 (<68)	79 (<66)	
Sex			5.3, 95% CI 2.9–9.5, $p < 0.001$
Male	55	83	
Female	42	37	
Smoking			2.3, 95% CI 1.28–4.07, $p < 0.05$
Smoker	72	67	
Nonsmoker	25	53	
Histotype	Male	Female	
Histotype	Male	Female	
Transitional cell carcinoma	47 (55%)	39 (45%)	
Adenocarcinoma	1 (100%)	0 (0%)	
Urothelial papillary carcinoma	4 (67%)	2 (33%)	
Squamous cell carcinoma	3 (75%)	1 (25%)	

formed at a significance level of 0.001 with 2 degrees of freedom. The pro198leu polymorphism had a chi-square of 69.9 ($p < 0.001$), and the ile58thr polymorphism a chi-square of 0.25 ($p > 0.05$).

The OR test was performed in a contingence table of 2×2 to determine the relative risk of acquiring bladder cancer due to the presence of the variants in affected and control individuals, and according to the genotype of each of the groups. The pro198leu polymorphism presented an OR of 3.8 (95% CI 2.1–6.8; $p < 0.001$), while the ile58thr polymorphism presented an OR of 2.1 (95% CI 1.3–3.5; $p > 0.05$) (Table 4). Moreover, this test was performed to determine the relative risk of develop the disease due to old age (OR = 5.3; 95% CI 2.9–9.5; $p < 0.001$), sex (OR = 0.6; 95% CI 0.33–1.02; $p < 0.05$), and smoking (OR = 2.3; 95% CI 1.28–4.07; $p < 0.05$) (Table 1).

DISCUSSION

DNA finds itself in constant risk of being fragmented by endogenous and exogenous sources; that is why cells develop DNA repair systems to prevent damage caused by oxide reactive species and other agents that cause fragmentation (12,22). With this in mind, detoxifying genes like *GPX-1* and *MnSOD* were studied and described. These genes encode enzymes involved in the control of the balance and regulation of oxidative stress within cells, which have been shown to be involved in the development of bladder, lung, and breast cancer (7,20,21). The study presents the analysis of two genes using PCR-RFLP technique to determine the presence of the polymorphisms pro198leu in the *GPX-1* gene and ile58thr in the *MnSOD* gene in Ecuadorian individuals affected with bladder cancer.

The relationship between the presence of the pro198leu variant of the *GPX-1* gene and its association with the risk of developing bladder cancer was determined. This polymorphism is related to the development of carcinomas due to an elevated significant difference between the affected population and the control group. The presence of the pro198leu polymorphism had a positive rela-

Table 2. Genotype Distribution and Allele Frequency of the pro198leu Polymorphism

Group	Genotype	Individual (%)	Genotypic Frequency	Allele Frequency
Affected	P/P	28 (29%)	0.29	0.39
($n = 97$)	P/L	19 (19%)	0.19	
	L/L	50 (52%)	0.52	0.61
Control	P/P	73 (61%)	0.61	0.79
($n = 120$)	P/L	42 (35%)	0.35	
	L/L	5 (4%)	0.04	0.21

P/P, proline/proline; P/L, proline/leucine; L/L, leucine/leucine.

Table 3. Genotype Distribution and Allele Frequency of the ile58thr Polymorphism

Group	Genotype	Individuals (%)	Genotypic Frequency	Allele Frequency
Affected	I/I	43 (44%)	0.44	0.68
($n = 97$)	I/T	47 (49%)	0.48	
	T/T	7 (7%)	0.07	0.32
Controls	I/I	75 (62%)	0.63	0.82
($n = 120$)	I/T	45 (38%)	0.37	
	T/T	0 0	0.0	0.18

I/I, isoleucine/isoleucine; I/T, isoleucine/threonine; T/T, threonine/threonine.

Table 4. Statistical Analysis

pro198leu	P/P	P/L	L/L	Chi-Square	Odds Ratio
Affected	29%	19%	52%	69.9, $p < 0.001$	3.8, 95% CI 2.1–6.8, $p < 0.001$
Control	61%	35%	4%		
ile58thr	I/I	I/T	T/T	Chi-Square	Odds Ratio
Affected	44%	49%	7%	0.25, $p > 0.05$	2.1, 95% CI 1.3–3.5, $p > 0.05$
Control	62%	38%	0%		

tionship to the risk of developing bladder cancer when the group of affected individuals was compared to the control group (OR = 3.8; 95% CI 2.1–6.8; $p < 0.001$), demonstrating that the presence of the allelic variant (L/L) deteriorates the enzymatic activity of glutathione peroxidase, allowing the accumulation of oxidative reactive species in the body, thereby conferring risk of developing bladder cancer in the Ecuadorian population. Individuals with the L/L variant have a four times greater risk of developing the disease than individuals with the P/P variant, as demonstrated by other studies (7,21). There are various scientific reports obtained in different populations around the world, and an association between these variants with the risk of developing the neoplasm were found. The incidence of these polymorphisms varies between different ethnic groups, for example: the allelic frequency of L/L in the Japanese population is 0.05, and in the Caucasian population it is 0.36 (7,20). Additionally, it has been determined that variants in different populations increases 2.6 times of developing bladder cancer and 1.43 times of developing breast cancer (20).

Referring to age, it has been determined that the risk to acquire bladder cancer is relatively greater in old aged individuals (OR = 0.6; 95% CI 0.334–1.020; $p < 0.05$), being a risk factor to develop this disease. Moreover, it was determined that men are at five times more risk to develop this type of cancer than women (OR = 5.3; 95% CI 2.9–9.5; $p < 0.001$). According to tobacco consumption, it has been determined that individuals who used to smoke are at two times more risk to develop bladder cancer than individuals that never smoke (OR = 2.3; 95% CI 1.28–4.07; $p < 0.05$).

As a result of the ethnic differences, the distribution of polymorphisms is affected; some studies have found that the risk of developing bladder cancer when a significant incidence of the L/L allelic variant exists, with the proportion of homozygous individuals for the L/L allele being low for the Asian population and high for the Caucasian population (7,20). These findings have been corroborated for the Ecuadorian population, due to the L/L genotype being present in a high proportion of individuals diagnosed with bladder cancer ($n = 51$; 51%).

The *MnSOD* gene acts as a primary source of defense against cellular oxidants, regulating mitochondrial transport. Low levels of *MnSOD* gene activity may cause oxidative stress, leading to the development of cancer, diabetes, and neurodegenerative diseases like Parkinson's and Alzheimer's (12).

It has been reported that the frequency of the ile58thr variant of the *MnSOD* gene does not have a high significance (21). Comparing the values reported with the values obtained in the present study, we confirmed that the incidence of the T/T allele maintains a low level within the Ecuadorian population. We have shown statistically that there is no significant difference between the bladder cancer group and the control group. Calculating the related risk of this polymorphism, a negative relationship between the tendency to develop bladder cancer was found (OR = 2.1; 95% CI 1.2–3.6; $p > 0.05$), concluding that the individuals who present the pro198leu variant have an increased risk of developing bladder cancer; contrary to what we found for ile58thr polymorphism and being different from that reported by other authors (11).

The ile58thr polymorphism of the *MnSOD* gene varies within different populations, with an incidence of 11% in Japanese populations and 30% in Chinese populations, compared to the Caucasian population, which has a 62% of incidence (23,24).

It has been reported that the expression level of the manganese superoxide dismutase enzyme, codified by the *MnSOD* gene, varies within tissues and shows an increment in individuals with brain, skin, and lung tumors; for this reason, the present study is important because it allows a determination of the presence of mutations of this gene in the Ecuadorian population with bladder cancer and demonstrates that genes of cystic fibrosis (*CF*), retinoblastoma (*RB*), *NF2*, *CYP1A1*, *BCR-ABL*, *hMSH2*, and *SRD5A2* have a different behavior as reported in other populations (13–18). Studies of detoxifying genes is an important contribution to an understanding of the genetics of bladder cancer in Ecuador and other populations.

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