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Original Research

Association between GPX1 Pro189Leu polymorphism and the occurrence of bladder cancer in Morocco

K. Hadami[1,3], R. Ameziane El Hassani[1], A. Ameur[2], N. Dakka[3], M. Abbar[2], A. Al Bouzidi[2], M. Attaleb[1], M. El Mzibri[1*]

[1] Biology and Medical Research Unit, CNESTEN, Rabat, Morocco
[2] Military hospital Mohammed V, Rabat, Morocco
[3] Biochemistry and Immunology Laboratory, Faculty of Sciences, Rabat, Morocco

Abstract: Worldwide, Bladder cancer is the most frequent male malignancy. It is the third most common male malignancy in Morocco. The risk factors for developing bladder cancer are multiples including dietary conditions, environmental exposure and oxidative stress. GPX1 gene encoding for the human cellular antioxidant enzyme glutathione peroxidase1 is a key factor in the cell detoxification process. GPX1 Pro198Leu polymorphism is associated with a decrease of enzyme activity and may contribute to bladder cancer susceptibility. The present case-control study was planned to assess the presence of GPX1 Pro198Leu polymorphism in Moroccan population to determine whether it is associated with the risk of developing bladder cancer in Moroccan patients. A total of 32 patients with bladder cancer and 40 healthy controls were enrolled. Genotyping of the GPX1 Pro198Leu polymorphism was carried out by PCR amplification and DNA sequencing. Pro198Leu polymorphism was observed in both bladder cancer patients and healthy controls. No significant association between the polymorphism and bladder cancer occurrence was found (Pro/Leu vs. Pro/Pro: p=0.425; Leu vs. Pro: p=0.435). For the analysis of Pro198Leu polymorphism and progression of bladder cancer, no association was observed neither for stages (Pro/Leu vs. Pro/Pro: p=0.500; Leu vs. Pro: p=0.500) nor grades (Pro/Leu vs. Pro/Pro: p=0.415; Leu vs. Pro: p=0.427). Our results clearly showed no significant association between Pro198Leu polymorphism and risk of bladder cancer in our population, suggesting that the effect of this polymorphism on bladder cancer development might be a result of a combination with other genetic alterations and/or non-genetic variables such as diet and lifestyle factors.

Key words: Bladder cancer, antioxidant, GPX1, Pro198Leu, polymorphism, Morocco.

Introduction

Worldwide, bladder cancer is a major cause of morbidity and mortality. It ranks ninth in the global cancer incidence, accounting for approximately 429,000 new cases and 165,000 deaths occurred in 2012, and it is more prevalent in men than in women (1, 2). In Morocco, bladder cancer is the third most common cancer in men with an incidence nearly 11 times higher than in women. The age-standardized incidence rate (ASR) was 9.7 per 100,000 persons in men and 0.7 in women throughout the years 2006-2008 (3). The occurrence of bladder cancer increases steadily with age from 45 years with an average age of 65 years. The most frequent histological type is by far transitional cell (urothelial) carcinoma and it is usually diagnosed at stages I and II (3, 4).

Etiologically, tobacco smoking, occupational exposure to specific industrial chemicals, chronic urinary tract infections, pelvic radiation and diet intake are known to promote bladder cancer carcinogenesis (5-8). These potential risk factors are supposed to accumulate, directly or indirectly, reactive oxygen species (ROS) in cells, leading to cellular oxidative stress (9-12). Oxidative stress, which corresponds to an imbalance between oxidants and antioxidants in favor of oxidant systems, contributes to the different steps of carcinogenesis. Indeed, ROS are highly reactive molecules which can damage multiple cellular targets including membranes, proteins and nucleic acids. Their carcinogenicity results from the potential to mutate cancer-related genes, activate or deactivate signal transduction pathways, and alter the expression of growth- and differentiation-related genes (11, 12).

Human cellular glutathione peroxidase1 (GPX1, EC 1.11.1.9) is a selenium-dependent enzyme that play a key role in the antioxidant defense; it is composed by four identical subunits containing selenocysteine residues in a total molecular weight of 22–23 kDa (13). GPX1 enzyme eliminates hydrogen peroxide (H_2O_2) by reducing it into water and might protect cells and their environment from oxidative damage (11-13). GPX1 gene is located on chromosome 3p21.3 (14) and contains two exons (15). Within exon2, a single nucleotide polymorphism (rs1050450C>T) that results in a proline (Pro) to leucine (Leu) amino acid substitution at codon position 198 (Pro198Leu), close to the C-terminus of the protein, was observed. Several studies have reported that the presence of Leucine at position 198 affects the binding of selenium, a necessary element for GPX1 function, to the enzyme and therefore decreases enzyme activity (16-18). Furthermore, the relationship between Pro198Leu polymorphism and cancer risk has been evaluated, and discrepant results were obtained. Some studies indicated a significant association of Pro198Leu polymorphism with breast cancer (19-20), lung cancer

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*** Corresponding author:** Mohammed EL Mzibri, Biology and Medical Research Unit, National centre of Energy, Nuclear Sciences and Techniques (CNESTEN), BP 1382 RP. Rabat, Maroc. Email: mzibri@yahoo.com

(21-22) and bladder cancer (31-35). In other studies, Pro198Leu polymorphism was not associated to breast cancer (24-26) and prostate cancer (27-29, 34).

Thus, the present case-control study was planned to assess the presence of GPX1 Pro198Leu polymorphism in Moroccan population to determine whether it is associated with the risk of developing bladder cancer in Moroccan patients.

Materials and Methods

Study specimens

Cancer cases. 32 fresh frozen urinary bladder biopsies were recruited for the variant analysis in the present study. The tumor samples were from biopsies collected for the routine diagnosis of bladder cancer from Urology department of the Military Hospital of Instruction Mohammed V in Rabat, Morocco. Tumor samples were collected by transurethral resection (TUR) or from cystectomy specimens. Each sample was divided into two portions: one portion was put in neutral buffered formalin and processed for routine histopathological examination in the Anatomopathology Department at the same hospital according to the World Health Organization (WHO) criteria and TNM (tumor node metastasis) classification; the other portion was stored at -80°C immediately after surgical removal, until DNA extraction.

Control cases. Human genomic DNA from 40 healthy peripheral blood samples collected at the National Blood Transfusion Center was used as control.

The study was conducted under the local ethical rules and informed consents were obtained from all patients.

DNA extraction

Genomic DNA was extracted from fresh frozen tissue specimens and from blood samples using the Isolate II Genomic DNA Kit (BIOLINE), according to manufacturer's protocol, and stored at -20°C until use. DNA concentration and purity were assayed using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific).

GPx1 Pro198Leu Genotyping

Pro198Leu polymorphism screening was carried out by PCR amplification and DNA sequencing (23). For PCR amplification, primers flanking a region in exon2 of GPX1 gene that contain the SNP rs1050450 C>T, GPX1-Ex2-F primer: 5'-CGCCACCGCGCTTATGACCG-3' and GPX1-Ex2-R primer: 5'-GCAGCACTGCAACTGCCAAGCAG-3' were used. PCR amplification was performed in a total volume of 25µL, containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200µM of each dNTP, 200nM of each primer, 0.25U Platinum Taq DNA polymerase (Invitrogen) and 100ng of genomic DNA. The mixtures were first denatured at 94°C for 7 min. Then, 35 cycles of PCR were performed with denaturation at 94°C for 30 s, primer annealing for 30 s at 60°C and primer extension for 30 s at 72°C. At the end of the last cycle, the mixtures were incubated at 72°C for 7 min. For every reaction, a negative control, in which DNA template was omitted from the amplification mixture, was included. PCR products were purified using the illustra ExoProStar 1-Step enzymatic clean up system (GE Healthcare Life Sciences), 1µl of 2X illustra ExoProStar 1-Step mix (Alkaline Phosphatase, Exonuclease I) was added to 6µl of PCR products, and the mixtures were incubated at 37°C for 15min, followed by incubation at 80°C for 15min to inactivate the enzymes. Sequencing of purified PCR products was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were performed in a final volume of 10µl, containing 1µl of 2.5X Big Dye ready reaction mix v.3.1, 10 pmol of forward primer and 100ng of purified PCR product. The mixtures were incubated at 96°C for 1min and 25 cycles were performed: denaturation at 96°C for 10s, primer annealing at 50°C for 5s and extension at 60°C for 4 min. The reactions were set to 30µl. To eliminate the excess of labeled ddNTPs, sequencing reaction products were purified using sephadex G-50 gel-exclusion chromatography (GE Healthcare Life Sciences). Direct sequencing of amplified PCR products was performed on an ABI 3130xL Genetic Analyzer (Applied Biosystems). The sequences were analyzed using Sequence Scanner v2.0 software (Applied Biosystems).

Statistical analysis

Statistical tests were performed using the OpenEpi software. Chi-square test with Yates' correction was used to evaluate the association of GPX1 genotypes with the occurrence of bladder cancer and to examine the correlation between GPX1 genotypes and cancer stage or grade. The statistical relationship was considered as significant if the derived p-value was <0.05. The estimated genotypic and allelic frequencies were associated with 95% confidence intervals (CI) calculated using the modified Wald test (Agresti-Coull).

Results

Histopathological data

The demographic characteristics of the 32 bladder cancer patients showed that 84.37% were men (n=27) and 15.62% were women (n=5), the mean age of patients was 49 with extreme ages at 42 and 78 years old. The pathological analysis revealed that among the 32 cases, 31cases were urothelial carcinomas (UC) (96.87%) and one case was adenocarcinoma (3.12%). The tumor staging revealed that among the 31 UC cases, 4 were classified as Ta (12.90%), 14 as T1 (45.16%), 5 as T2 (16.12%) and only one case was staged as T4 (3.22%). The tumor grading showed that among 31 UC cases, 9 cases were classified as low grade (29.03%), whereas 15 were high grade (48.38%), the stage and the grade of 7 other UC cases were undetermined (22.58%) (Table 1).

Detection of Pro198Leu polymorphism

Successful amplification and direct sequencing of GPX1 exon2 was obtained for both bladder cancer specimens and controls. The figure 1 shows an example of nucleotide sequence of GPX1 exon2 obtained in cancer and control cases. Direct sequencing analysis of exon2 revealed rs1050450 C>T substitution resulting in Pro198Leu in both bladder cancer specimens and controls.

Table 2 summarizes the obtained genotypic and allelic frequencies in both cancer cases and healthy controls. In bladder cancer cases, 62.50% of cases (20/32) have the Pro/Pro genotype and 37.50% (12/32) have the

Table 1. Clinico-pathological data of bladder cancer cases.

Characteristics	N	frequency (%)
Sex		
Male	27	84.37
Female	5	15.62
Urothelial carcinoma	31	96.87
Stage		
Ta	4	12.90
T1	14	45.16
≥T2*	5	16.12
T4	1	3.22
Undetermined	7	22.58
Grade		
low	9	29.03
high	15	48.38
Undetermined	7	22.58
Adenocarcinoma	1	3.12

*≥T2: advanced stages, T2 and beyond.

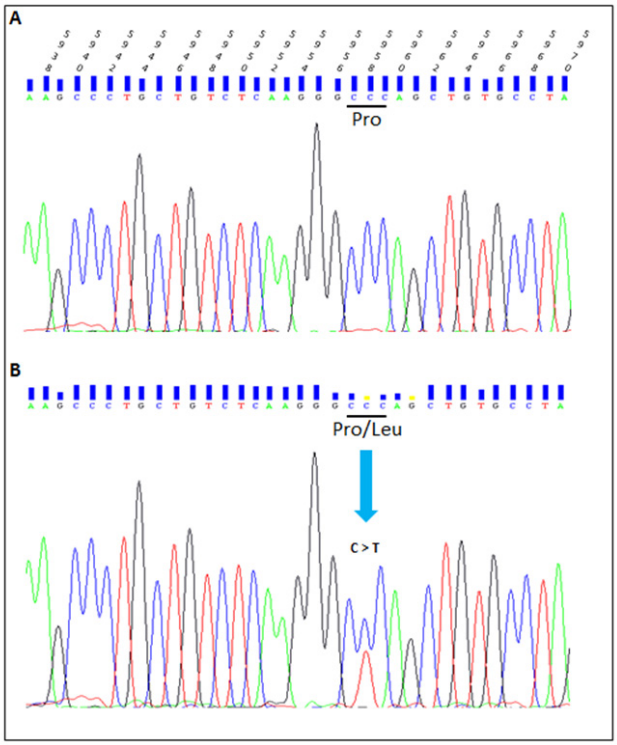

Figure 1. DNA sequence chromatograms of GPX1 exon2. **A:** DNA sequence chromatogram of unaffected members; there is no C>T transition, **B:** DNA sequence chromatogram of carries; there is a single base C>T transition in exon 2 that causes Pro198Leu substitution at heterozygote form.

Pro/Leu genotype, whereas in control cases, Pro/Pro and Pro/Leu genotypes were found in 57.50% (23/40) and 42.50% of cases (17/40), respectively. No Leu/Leu genotype was found neither in cancer cases nor in controls. Statistical analysis showed that no significant difference in the frequencies of Pro198Leu was observed between cancer cases and healthy controls (p=0.425).

Analysis of allelic frequencies showed that Proline allele was present in 81.25% of cancer cases and in 78.75% of control cases. The frequency of Leucine allele was observed in 18.75% and 21.25% of cancer and healthy control cases, respectively. No statistically significant difference of GPX1 allelic frequencies was observed between cancer and control groups (p=0.435).

The distribution of Pro198Leu genotypes in the 31 urothelial carcinomas according to stages and grades of bladder carcinoma is reported in Table 3. In the early stages (Ta-T1), the Pro/Pro and Pro/Leu genotypes were detected respectively in 55.56% (10/18) and 44.44% (8/18) of cases, whereas in advanced stages (≥ T2), Pro/Pro was detected in 66.66% of cases (4/6) and Pro/Leu in only 33.33% (2/6) of cancer cases. In terms of cancer grade, Pro/Pro and Pro/Leu genotypes were detected in both low and high grades. In low grades, Pro/Pro and Pro/Leu genotypes were detected respectively in 55.56% (5/9) and 44.44% (4/9) of cancer cases. In high grades, Pro/Pro was detected in 60% (9/15) and Pro/Leu in 40% (6/15) of cases. Statistical analysis showed that

Table 2. Genotypic and allelic frequencies in bladder cancer cases and controls.

Cases	N	Genotype		p	Allele		p
		Pro/Pro % (95%CI)	Pro/Leu % (95%CI)		Pro % (95%CI)	Leu % (95%CI)	
Bladder cancer	32	62.50 (45.21-77.12)	37.50 (22.88-54.79)	0.425	81.25 (69.87-89.09)	18.75 (10.91-30.13)	0.435
Controls	40	57.50 (42.18-71.5)	42.50 (28.5-57.82)		78.75 (68.49-86.38)	21.25 (13.62-31.51)	

Table 3. Genotypic and allelic frequencies according to clinical stage and grade.

UC cases (N=31)		N	Genotype		p	Allele		p
			Pro/Pro %(95%CI)	Pro/Leu %(95%CI)		Pro %(95%CI)	Leu %(95%CI)	
Stage	Ta-T1	18	55.56 (33.7-75.46)	44.44 (24.54-66.3)	0.500	77.78 (61.67-88.53)	22.22 (11.47-38.33)	0.500
	≥T2	6	66.67 (29.58-90.75)	33.33 (9.25-70.42)		83.33 (54-96.51)	16.67 (3.49-46)	
Grade	Low	9	55.56 (26.63-81.16)	44.44 (18.84-73.37)	0.415	77.78 (54.25-91.53)	22.22 (8.466-45.75)	0.42
	High	15	60 (54.25-91.53)	40 (8.47-45.75)		80 (62.33-90.86)	20 (9.14-37.67)	
ND		7	71.42 (35.24-92.44)	28.58 (7.56-64.76)		85.71 (58.81-97.24)	14.29 (2.76-41.19)	

there's any significant association between Pro198Leu polymorphism and cancer stage (Pro/Leu *vs* Pro/Pro: p=0.500) or tumor grade (Pro/Leu *vs* Pro/Pro: p=0.415).

Allele frequency analysis showed that Leu allele prevails in both early (22.22%) and advanced (16.67%) stages, and in both low (22.22%) and high (20%) grades. There was no significant difference in the distribution of the polymorphism between the clinical stages (Leu *vs* Pro: p=0.500) and grades (Leu *vs* Pro: p=0.427).

Discussion

During last decades, a growing interest was given to the role of oxidative stress in carcinogenesis. Currently, there is evidence that oxidative DNA damage and impairment in DNA repair response are implicated in cancer development and progression. Accordingly, several studies have investigated the association between GPX1 Pro198Leu polymorphism, affecting the GPX1 function, and cancer development, and results are controversial depending on the study populations.

The association between Pro198Leu polymorphism and breast cancer development has been well documented. A strong association was reported in Denmark and USA (19, 20), whereas other studies conducted in UK and USA didn't observe any significant association between Pro198Leu polymorphism and breast cancer among Caucasian women (24-26). Other studies suggested that Pro198Leu polymorphism is linked with risk of lung cancer (21, 22) but not with prostate (27-29) and colorectal (30) cancers.

Great interest was also given to the association between Pro198Leu polymorphism and bladder cancer risk. Accordingly, some studies have found that the Pro198Leu polymorphism is associated with an increased risk of this disease. Ichimura *et al*. showed that Pro/Leu genotype was significantly associated with bladder cancer in Japanese population (213 patients and 209 normal controls) (OR 2.63, 95% CI 1.45-4.75, p=0.001), and it was significantly correlated to advanced tumor stage (Ta-1 vs. T2-4, OR 2.58, 95% CI 1.07-6.18, p=0.034) but not with tumor grade (31). Furthermore, Paz-y-Miño *et al*. found that Ecuadorian population with this polymorphism (97 cases and 120 controls) 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) but the risk was higher with Leu/Leu genotype (52%) than Pro/Leu genotype (19%) (32).

Of particular interest, Kucukgergin *et al.* have indicated that the Leu/Leu genotype of GPX1 was associated with a significantly higher risk of bladder cancer than the Pro/Pro genotype in Turkish population (157 patients and 224 healthy controls), and it was more frequently observed in bladder cancer patients with high-stage tumors than those with low-stage tumors (33). These findings were confirmed by two Chinese meta-analyses suggesting that carriers of the variant T allele were associated with a significantly increased risk of bladder cancer (Leu vs. Pro, OR 2.111, 95% CI 1.020-4.368, heterogeneity (p< 0.001); Pro/Leu and Leu/Leu vs. Pro/Pro, OR 1.876, 95% CI 1.011-3.480, heterogeneity (p< 0.001)) (34, 35).

Our study enrolled 32 bladder cancer patients and 40 healthy controls. The characteristics of the 32 patients showed that 27 of them were men and 5 were women. This differential risk for the pathology between the two sexes is supposed to be related to rate exposure to risk factors, specifically tobacco smoking and alcohol consumption (40).

In the present study, we found no association of GPX1 Pro198Leu polymorphism with bladder cancer development (Pro/Leu vs. Pro/Pro: p=0.425; Leu vs. Pro: p=0.435) as well as no overall association with stages (Pro/Leu vs. Pro/Pro: p=0.500; Leu vs. Pro: p=0.500) and grades (Pro/Leu vs. Pro/Pro: p=0.415; Leu vs. Pro: p=0.427) of bladder cancers. In spite of the relatively lower sample size of our study, our results are in agreement with previously reported data in Egyptian population which was performed on 612 cases and 618 matched population-based controls, suggesting that the common genetic variation in GPX1 gene are not associated with overall bladder cancer risk (CT: OR 0.91, 95% CI 0.72-1.17; TT: OR 1.02, 95% CI 0.64-1.64) (36). Interestingly, the Leu/Leu genotype, reported as highly aggressive form, was not found in our study.

These discrepant results could be attributed to the study population, with specific genetic aspects, and the sample size. Other potential confounding factors should be also considered including the technique used for point mutation analysis (e.g., RFLP may have a higher rate of false positives vs. DNA sequencing or Real-Time PCR assays) (36). In addition, Van Huis-Tanja *et al.* have reported a significant discordance between formalin-fixed, paraffin-embedded tissue and blood genotype to assess the polymorphism of GSTP1, another gene implicated in the cellular antioxidant defense (37).

Interestingly, some studies have reported that the combination of the GPX1 Pro198Leu polymorphism and other point mutations may have a synergistic effect on disease risk. *In vitro* functional analyses indicated that the combination of polymorphisms (Ala^5/Ala^6 and Pro198Leu) of the GPX1 gene had a 40% decrease in enzyme activity (23). Furthermore, studies of Cox *et al*. on breast cancer clearly showed that GPX1 Por198Leu polymorphism was not associated with cancer development; but combination with another polymorphism, Ala16Ala genotype of MnSOD, was associated with significantly increased breast cancer risk (OR 1.87, 95% CI 1.09–3.19) (38).

Moreover, it was reported that gene-environment interaction can affect the genotype polymorphism. Of obvious interest, it would be to examine more polymorphisms and more genes along the same pathway, as well as non-genetic variables such as plasma antioxidant levels or lifestyle factors which affect oxidative stress, including cigarette smoking, consumption of alcohol and dietary antioxidants or supplements. Indeed, accumulating evidence has demonstrated that not all individuals having genetic alterations develop bladder cancer. Differences in the occurrence of bladder cancer among persons with the same GPX1 genotype may be attributable to carcinogen exposures and dietary factors that have been shown to increase carcinogen-derived oxidative radicals. Hansen *et al.* studied whether the GPX1 Pro198Leu polymorphism and several lifestyle factors predict colorectal cancer risk (39). They observed a higher risk associated with alcohol consumption and smoking among homozygous GPX1 198Leu carriers, with inci-

dence rate ratios for colorectal cancer of 1.45 (95% CI 1.17-1.81, p=0.02) per 10g alcohol intake per day and 2.56 (95% CI 0.99-6.61, p=0.02) among ever smokers compared with never smokers at enrolment.

In conclusion, the 198Leu genotype is rare in Morocco and the GPX1 Pro198Leu polymorphism is not associated alone to bladder cancer risk, suggesting that the effect of this polymorphism on bladder cancer development is a result of a combination with other genes' polymorphisms or other non-genetic variables such as diet and lifestyle factors. Other genetic and epigenetic alterations disturbing the redox homeostasis in bladder tumors in our specimens are under investigation in our laboratory.

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