Journal of Applied Biomedicine

Original research article

Serum xanthine oxidoreductase and oxidative stress are associated with bladder cancer: a case-control study from Jordan

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Abstract
Xanthine oxidoreductase (XOR) is an oxidant enzyme that exists mainly in two distinct forms: the dehydrogenase form [xanthine dehydrogenase (XDH)] and the oxidized form [xanthine oxidase (XO)]. XO might contribute to tumorigenesis through direct metabolic activation of carcinogens and indirect generation of free radicals. Oxidative stress is one of the leading causes of bladder cancer (BC). Smoking and genetic susceptibility are also linked to oxidative stress and BC. This study investigated the association between XO serum levels and *XOR* genetic polymorphisms with BC. A case-control study was conducted among 109 BC patients and 109 controls matched by age, gender, body mass index, and smoking status. Serum levels of XO and 8-hydroxy-2'-deoxyguanosine (8-OhdG) were measured using ELISA, while thiobarbituric acid reactive substances (TBARS) and protein carbonyl (PC) were assessed using colorimetric assays. *XOR* single nucleotide polymorphisms were analyzed via tetra-primer ARMS-PCR. XO levels were significantly higher in BC patients than in controls [(5.11 ± 0.28 vs 3.83 ± 0.23) ng/ml, respectively ($p < 0.0006$)]. Among smokers, XO levels were also elevated in BC cases compared with controls [(5.29 ± 0.35 vs 3.41 ± 0.28) ng/ml, respectively ($p < 0.0001$)]. Oxidative stress biomarkers were elevated in BC patients compared with controls: 8-OHdG (19.39 ± 1.37 vs 16.32 ± 1.37 nmol/l), PC (8.88 ± 0.56 vs 4.42 ± 0.56) nmol/mg of protein, and TBARS (4.23 vs 3.15) μmol/ml, respectively ($p < 0.05$). Haplotype analysis showed that TGTCA, TGTA, TGA, and GTA were more frequent in BC patients and associated with increased BC status [4.17 (1.16–15.00), 1.84 (1.11–3.05), 1.62 (1.01–2.60), and 1.66 (1.02–2.71) fold increase in risk, respectively ($p < 0.05$)]. Elevated XO and oxidative stress markers are associated with BC, supporting their role in BC pathogenesis. Our findings suggest that they may act as potential diagnostic or therapeutic targets. However, mechanistic studies are required to clarify whether XO/oxidative stress markers contribute directly to carcinogenesis or reflects general redox imbalance in malignancy. Specific *XOR* haplotypes might serve as biomarkers for BC.

Keywords: Bladder cancer; Genetic polymorphisms; Oxidative stress; Xanthine oxidase

Highlights:
- Serum XO and oxidative stress biomarkers were elevated in BC.
- Novel *XOR* haplotypes were higher in BC patients.
- XO and oxidative stress markers appear as potential biomarkers in BC and/or therapeutic targets.

Abbreviations:
BMI: Body Mass Index. 8-OhdG: 8-hydroxy-2'-deoxyguanosine. EDTA: Ethylenediamine tetraacetic acid. ELISA: Enzyme-linked immunosorbent assay. HWE: Hardy-Weinberg equilibrium. LD: Linkage disequilibrium. PC: Protein carbonyl. ROS: Reactive Oxygen species. SEM: Standard error of the mean. SNPs: Single nucleotide polymorphisms. SPSS: Statistical Package for Social Sciences. TBARS: Thiobarbituric acid reactive substances. Tetra-ARMS PCR: Tetra-primer amplification refractory mutation system-polymerase chain reaction. UC: urothelial carcinoma. UV: Ultra-violet. XDH: Xanthine dehydrogenase. XO: Xanthine oxidase. XOR: Xanthine oxidoreductase

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http://doi.org/10.32725/jab.2025.015

Submitted: 2025-07-22 • Accepted: 2025-09-24 • Prepublished online: 2025-09-25
J Appl Biomed 23/3: 107–116 • EISSN 1214-0287 • ISSN 1214-021X

Introduction

Bladder cancer (BC) refers to any neoplasm that arises from the urinary bladder and is commonly initiated in the epithelial tissues (Shin et al., 2018). It is the 9th most frequent malignancy globally – 6th among men and the 17th among women – and the 9th leading cause of cancer-related deaths in men (Bray et al., 2024). In 2022, an estimated 613,791 new BC cases were reported worldwide (Bray et al., 2024).

Pathogenesis of BC is highly correlated with oxidative stress (Sawicka et al., 2020). Oxidative stress results from an imbalance between the oxidant and antioxidant systems, particularly their enzymes, leading to the accumulation of reactive oxygen species (ROS) (Mendes et al., 2021). ROS can cause irreversible damage to macromolecules like DNA, proteins, and lipids, promoting mutations involved in tumor initiation and progression through activating several signaling pathways involved in carcinogenesis and metastasis (Phaniendra et al., 2015). Studies have reported elevated levels of oxidative stress and reduced antioxidant capacity in both urine and serum samples isolated from BC patients (Galiniak et al., 2023; Sawicka et al., 2020; Wigner et al., 2021a).

Xanthine oxidoreductase (XOR), a drug-metabolizing enzyme, has a paradoxical role in the pathogenesis of BC depending on its predominant isoform (Battelli et al., 2016a). It mainly exists in two distinct forms: the dehydrogenase [xanthine dehydrogenase (XDH)] and the oxidized form [xanthine oxidase (XO)] (Wigner et al., 2021a). In normal bladder tissue, XDH predominates at very low levels in the urinary bladder tissue for a detoxifying role (Battelli et al., 2019). In BC, XO levels and activity were raised at the tissue, serum, and urine levels, which increased production of free radicals (Gulec et al., 2003; Gunes et al., 2020; Moustafa, 2015). This XDH-to-XO shift is central to the role of XOR in bladder carcinogenesis (Chen and Meng, 2022). Excessive ROS levels can influence pro-tumorigenic and anti-tumorigenic signaling pathways in BC, leading to cell death. Conversely, reduced ROS levels are associated with cancer cell survival, invasion, metastasis, and oncogenesis (Metwally et al., 2011). In contrast to previous studies, a decrease in XOR (XDH/XO) levels/activity was found in BC cells (Alsabti, 1979; Chen and Meng, 2022).

The oxidative stress caused by XOR-related ROS has been verified in previous studies at different levels, including DNA, proteins, and lipids by measuring the serum and tissue levels of 8-hydroxy-2-deoxyguanosine (8-OhdG), protein carbonyl (PC), and thiobarbituric acid reactive substances (TBARS), respectively (Wigner et al., 2021b).

XO levels might be affected by several factors, including age, gender, race, smoking, environmental and genetic factors. Previous studies investigated the association between *XOR* polymorphism and cancer, especially breast, gastric, and brain cancers. It was found that gastric cancer, acute leukemia, multiple myeloma, and brain tumor are associated with rs207454, rs17011368, rs1884725, and rs494852, respectively (Bhatti et al., 2009; Liu et al., 2018; Van Ness et al., 2008; Zaruma-Torres et al., 2016). Furthermore, Rodrigues et al. (2014) reported that rs207454 and rs494852 are associated with breast cancer.

Upon reviewing the literature, it appears that there is debate and controversy about the expression pattern and level of XOR as it can exist in two interconvertible forms: XDH and XO, especially in different BC stages and grades (Metwally et al., 2011; Moustafa, 2015). In addition, limited evidence is available regarding the association between serum XO levels, oxidative stress biomarkers, and BC in the Jordanian population. Furthermore, the association between selected single nucleotide polymorphisms (SNPs) of the *XOR* gene and BC has not been explored in the literature, despite previous studies identifying links between *XOR* SNPs and other cancer types (Bhatti et al., 2009; Liu et al., 2018; Rodrigues et al., 2014).

The main aims of our study were to: (1) assess the association between serum XO levels and the allelic/genotypic distribution of selected *XOR*-related SNPs with BC among Jordanian population, and (2) evaluate the serum levels of redox homeostasis biomarkers-including 8-OHdG, PC, and TBARS in BC patients compared with controls.

Materials and methods

Ethical approval

This study was approved by the Jordan University of Science and Technology (JUST) Research Committee and the Institutional Review Board (IRB number 2021/141/11) of JUST and King Abdullah University Hospital – KAUH (Irbid/Jordan). The research was conducted following the Helsinki Declaration (Wigner et al., 2021b).

Study design and subject recruitment

This case-control study included 109 BC patients (cases) recruited from the urology clinic at KAUH between 2020 and 2022, and 109 control subjects without signs or symptoms of bladder disease, recruited from the family medicine clinic at the same institution. During enrollment, participants were interviewed to explain the study's purpose, data collection procedures, and blood sampling protocol. Written informed consent was obtained before participation. The same interviewer performed the data collection for both study groups.

The cases were patients recently diagnosed with BC who exhibited no clinically malignant disease other than BC. All enrolled patients had a confirmed diagnosis of urothelial carcinoma (UC). Patients with histological variants of UC or other epithelial BC (adenocarcinoma, squamous-cell carcinoma) were excluded. The subjects' electronic medical records were checked to obtain data about the BC clinical parameters. BC patients were stratified by tumor stage [non-invasive (n = 92; 86%) vs. invasive (n = 15; 14%)] and histological grade [low-grade (n = 57; 52%) vs. high-grade (n = 52; 48%)]. Of note, the non-muscle invasive BC cases (Ta, Tis, T1) included both low- and high-grade tumors (Ta: 39 low-grade, 15 high-grade, Tis: 1 low-grade, T1: 16 low-grade, 23 high-grade), while almost all muscle-invasive cases (T2–T4) were high-grade. Patients with nodal or metastatic disease (N+/M+) were also included.

The control group was matched with the BC group by age, gender, body mass index [BMI = weight (kg)/height^2 (meters)], and smoking status. During case-control recruitment, stratified sampling was applied to ensure comparable distributions of smoking status between patients and controls, thereby minimizing potential confounding by smoking, a major risk factor for BC. Subjects with a history of gout or taking XO inhibitors such as allopurinol were excluded from enrollment in both groups. XO inhibitors were reported to affect the expression level of XO or oxidative stress biomarkers, especially after chronic treatment with XO inhibitors (Nomura et al., 2014).

Blood sample collection

Two venous blood samples were obtained from each participant using two different blood tubes: 3 ml gel separator tubes and 3 ml ethylenediamine tetraacetic acid (EDTA) tubes. Samples in gel separator tubes were centrifuged at 4500 × g

for 5 min at room temperature within 2 h of collection. The serum was aliquoted into three 1.5 ml microcentrifuge tubes (Biologix, USA) and stored at –80 °C for downstream protein analysis. EDTA tubes were gently inverted to prevent clotting and whole blood was stored at –4 °C for DNA extraction.

Serum analysis for XO and oxidative stress biomarkers

The level of XO in the collected serum samples was determined using the Human XO ELISA Kit (MBS2533395) (MyBioSource, San Diego, USA). Serum 8-OHdG level was measured using the ELISA kit (MBS808265) (MyBioSource, San Diego, US). PC was measured using a commercially available PC content kit (MBS2540497) (MyBioSource, San Diego, USA), and total protein was measured using the total protein assay (MBS254845) (MyBioSource, San Diego, US). The level of TBARS was detected using the TBARS Microplate Assay Kit (MBS8305390) (MyBioSource, San Diego, USA). All procedures were conducted according to the manufacturer's instructions.

SNPs analysis

DNA extraction and quantification

Genomic DNA was extracted from EDTA blood using the GF-1 Blood DNA Kit (Vivantis, Selangor Darul Ehsan, Malaysia). DNA samples were stored at –20 °C for subsequent Tetra-ARMS PCR experiments.

SNPS target information

Five SNPs were analyzed; four in the *XOR* gene (rs207454, rs1884725, rs17011368, rs494852) and one intergenic SNP (rs206833). SNPs were selected based on literature reviews linking them to cancer or chronic diseases. Sequences were verified via the National Center for Biotechnology Information (NCBI) database (Suppl. Table S1).

Tetra-primer amplification refractory mutation system-polymerase chain (Tetra-ARMS PCR)

Primers were designed using Biotech and PRIMIER1 websites and then genotyped using Tetra-ARMS-PCR. The mutation sites were arranged asymmetrically regarding the common (outer) primers to facilitate the separation of allele-specific amplicons with different product lengths by standard agarose gel electrophoresis. Gradient PCR was conducted on a thermal cycler to obtain the ideal annealing temperature for each primer set. Then, the best ratio between the inner and outer primers was selected as 1:1, 2:1, or 3:1 (Table 1). All SNPs and related primer sequences with the optimum annealing temperature and PCR products are presented in Table 2.

Tetra-ARMS-PCR was carried out in a final volume of 20 µl containing 1.5 µl of DNA, and 10 µl of 2×Go Taq® Green Master Mix was used to prepare 20 µl of PCR products in a 200 µl sterile tube (Table 1). All Tetra-ARMS-PCR steps were carried out under sterile conditions using a PCR cabinet. Thermal cycling was carried out in a Thermal Cycler as follows: (i) initial denaturation at 95 °C for 12 min, (ii) denaturation at 95 °C for 0.15 min, (iii) certain annealing temperature for each SNP for 0.30 min, (iv) extension at 72 °C for 2 min for 30 cycles, and (v) a final extension at 72 °C for 10 min (Table 2). Subsequently, PCR products were separated on a 3% agarose gel, stained with ethidium bromide (10 mg/ml) using an electrophoresis separator, and visualized under UV light. To validate and confirm the results of Tetra-ARMS-PCR, at least three samples for each SNP were sequenced using the Sanger sequencing technique. For further information regarding this technique, Medrano and de Oliveira (2014) efficiently proved this technique and clarified the guidelines in detail.

Gel electrophoresis

Before sequencing, 10 µl of the PCR product was loaded onto a 3% agarose gel containing ethidium bromide (10 mg/ml, ROTH). Horizotal gel electrophoresis was performed at 120 V for 60 min using a standard power supply. PCR products were visualized under ultraviolet (UV) light using a UV transilluminator.

Validation of the assay

Tetra-ARMS PCR results were validated using Sanger sequencing at the (Princess Haya Biotechnology Center, JUST). For each SNP, a minimum of three samples were sequenced. For pre-sequencing preparation, the outer PCR fragment of each SNP was amplified using primers specific to the annealing temperature of the respective SNP. Amplified products were stored at –4 °C during shipping for sequencing.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) 26 (Inc, Chicago, Illinois, USA) statistical analysis program. Differences between parameter values of the two groups were analyzed using Student's *t*-test, with values expressed as mean ± standard error of the mean (SEM). The categorical baseline demographic and clinical data of the two groups were compared using the chi-square test.

Genotype distribution and allelic frequencies were analyzed and aligned with previously published control data. Hardy–Weinberg equilibrium (HWE) testing for BC cases and

Table 1. Information about the PCR reaction of each SNP

Materials	SNPs				
	rs17011368	rs207454	rs1884725	rs494852	rs206833
NFW (µl)	6.7	6.7	6.1	6.7	6.1
MM (µl)	10.0	10.0	10.0	10.0	10.0
Fo (µl)	0.3	0.3	0.3	0.3	0.3
Fi (µl)	0.6	0.6	0.9	0.6	0.9
Ro (µl)	0.3	0.3	0.3	0.3	0.3
Ri (µl)	0.6	0.6	0.9	0.6	0.9
DNA (µl)	1.5	1.5	1.5	1.5	1.5

Note: NFW: nuclease-free water; MM: master mix; Fo: forward outer; Fi: forward inner; Ro: reverse outer; Ri: reverse inner

Table 2. *XOR* SNPs information and related primers

SNP ID	Primer name	Primer sequence (5`→3`)	Tm (°C)	Ta (°C)	PCR product size (bp)
rs207454	*Fi*	AGGAGAGGAGATGGCCATCAGGCAAGT	71.3	65	Outer: 275 T allele: 182 G allele: 148
	Ri	GGTCAGGGTTCCTGAAAACCTCACTCCC	73.1		
	Fo	TCACACTATGAGCTGGGCAAGGACAACA	70.1		
	Ro	TCAGATAATGAGGACCTGGTGCAGGTGG	71.6		
rs1884725	*Fi*	GCAATTACCTGATTCAGAAAAGGAACTTTG	60.1	52	Outer: 380 G allele: 183 A allele: 255
	Ri	AATTCCCACCAAGTTTGGAATAAGCGTT	62.6		
	Fo	GGTTGATCCCAGATCTCTGAGTCTCACT	66.4		
	Ro	CAAGGATGTCCTGTTTTGATGTGAACAT	61.4		
rs17011368	*Fi*	TCAGGTCCATAAAAGGAGTTGTTCTTGAT	66.5	55	Outer: 343 T allele: 220 C allele: 180
	Ri	AAACTTCTTTGTGATCCTCAGGATGATG	65.7		
	Fo	TCTTGAATTTAATTTGCAAACCAGGG	61.6		
	Ro	AATGGAGGAATATGATTCTCTGGGCT	64.7		
rs494852	*Fi*	AATAAGATGAAAGCTCTGCCCTTCCAT	65.3	57	Outer: 426 T allele: 288 C allele: 195
	Ri	ACTTTGGTTTTCTCAGTTGACTGCAGTATG	68.0		
	Fo	GTTCCTTAGCTTCCAATCACTTTGCTTC	67.2		
	Ro	GCTCAAGTTAAGACTCTGCTGCAAATCA	67.2		
rs206833	*Fi*	CCTTCTGAGAGCACTGTCCCAGGACGA	71.4	57	Outer: 340 A allele: 224 G allele: 173
	Ri	GGTGTTTTTTGGCCAACAGAGATTCAGCAC	65.9		
	Fo	ATAGAGCAACTGTGCTGTGCGAAGGGACT	68.1		
	Ro	CTTCAGGCTTTGGAGAGTCCAAGGTGAGC	69.6		

Note: Fi: forward inner; *Ri:* reverse inner; *Fo:* forward outer; *Ro:* reverse outer; Tm: Temperature of melting; Ta: annealing temperature

controls was performed using SNPStats (https://snpstats.net/start.htm). Genotype and allele frequencies were obtained using SNPStats (https://www.snpstats.net/preproc.php), and groups were compared using chi-square analysis (https://www.socscistatistics.com/tests/chisquare2/default2.aspx). Haplotype analysis and linkage disequilibrium estimates were conducted using SHEsis Plus (http://shesisplus.bio-x.cn/SHEsis.html). For all data, a p-value of 0.05 or lower was considered statistically significant.

Results

Study population

Baseline characteristics of the study subjects are summarized in Table 3. The majority of BC subjects were male (84%) with a mean age of 65.12 years, and 56% were smokers. Most patients presented with non-muscle-invasive BC (n = 94; Tis = 1%; Ta = 49%; T1 = 36%), while a smaller proportion had muscle-invasive disease (n = 15; T2 = 6%; T3 = 5%, T4 = 3%). The distribution between low-grade (n = 57; 52%) and high-grade (n = 52; 48%) tumors was approximately equal.

Subjects of the control group who had no BC or signs of bladder disease were matched with cases in terms of gender, age, smoking status, and BMI. Among the control group, 84% were males and the average age was 62.57 years. Among the controls, 56% were smokers. No significant differences were observed between cases and controls regarding age, gender, smoking status, or BMI (p > 0.05).

Comparison of the serum level of XO between BC patients and controls

The mean of XO serum levels was significantly higher in the BC group than in the controls [(5.111 ± 0.283 vs 3.832 ± 0.229) ng/ml, respectively (p = 0.0005)] – Fig 1A.

Subgroup analysis of XO level according to smoking status

Subgroup analysis for the difference in serum level of XO between controls and BC patients was carried out according to smoking status (Table 4). Among smokers, there was a significant increase in the mean of XO serum levels in BC patients compared with controls [(5.29 ± 0.35 vs 3.41 ± 0.28) nmol/l, respectively; (p < 0.0001)]. In comparison, no significant differences in the mean of serum XO were observed between the controls and BC patients among non-smokers [(4.36 ± 0.36 vs 4.87 ± 0.46) nmol/l, respectively (p > 0.05)]. When comparing XO level within the control group, smokers had significantly lower XO levels than non-smokers [(3.41 ± 0.28 vs 4.36 ± 0.36) ng/ml, respectively (p = 0.0367)]. Among BC patients, smokers had higher XO levels than non-smokers, but this was statistically not significant [(5.29 ± 0.35 vs 4.87 ± 0.46) ng/ml, respectively (p = 0.461)].

Comparison of the serum level of oxidative biomarkers in BC patients with the control group

We compared the serum levels of oxidative stress biomarkers, including 8-OHdG, PC, and TBARS, between the BC group and the control group. The mean levels of all biomark-

Table 3. Characteristics of study participants

Variable	Control (n = 109)	BC (n = 109)	p-value[1]
Age (years)	62.57 ± 1.68	65.12 ± 1.68	0.13
Gender (n) (%)			
Male	92 (84%)	92 (84%)	1.00
Female	17 (16%)	17 (16%)	
BMI[2] (kg/m^2)	28.60 ± 4.82	28.18 ± 0.59	0.49
Smoking status			
Smoker	61 (56%)	61 (56%)	1.00
Non-smoker	48 (44%)	48 (44%)	
Tumor stage			
Non-invasive (Tis, Ta, T1)	–	94 (86%)	–
Invasive (T2, T3, T4)	–	15 (14%)	
Tumor grade			
Low	–	57 (52%)	–
High	–	52 (48%)	

Note: n (number of participants); BMI (body mass index); [1] p-values were calculated by Pearson's chi-squared for gender and smoking status, while student t-test was used for age and BMI. [2] BMI = weight (kg)/height^2 (meters).

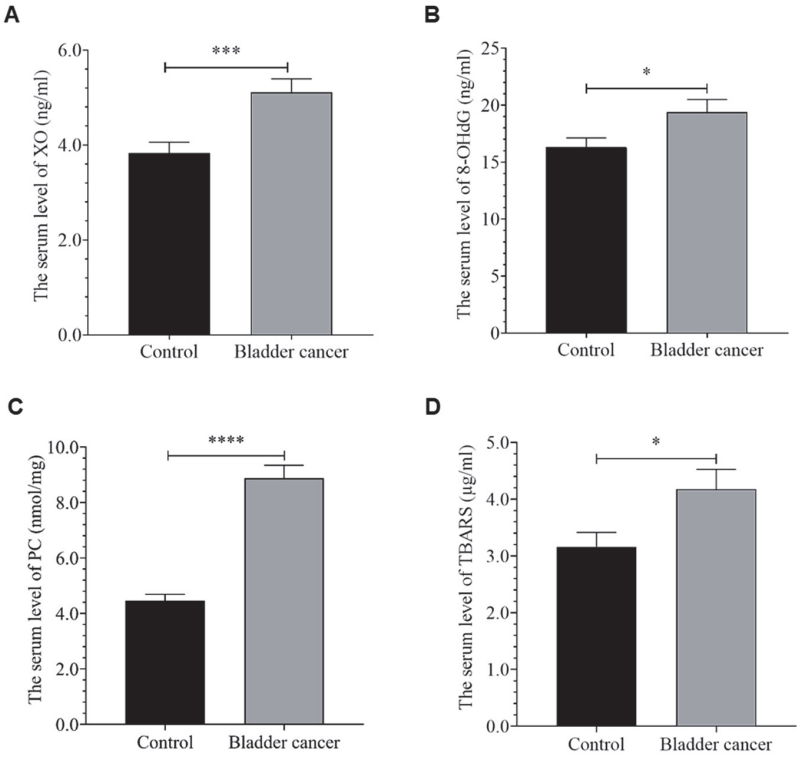

Fig. 1. Comparison of the serum level of xanthine oxidase (XO) and oxidative stress biomarkers. Differences between (**A**) XO, (**B**) 8-hydroxy-2'-deoxyguanosine (8-OhdG), (**C**) Protein carbonyl (PC), and (**D**) Thiobarbituric acid reactive substances (TBARS) between BC patients and controls; data was presented as mean ± SEM (standard error of the mean); p-value was calculated using student t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

ers were significantly higher in the BC group than in controls, 8-OHdG [(19.39 ± 1.37 vs 16.32 ± 1.37) nmol/l, respectively (p = 0.026)] (Fig 1B), PC [(8.88 ± 0.56 vs 4.42 ± 0.56) nmol/mg of protein, respectively ($p < 0.0001$)] (Fig 1C), and TBARS [(4.225 vs 3.149) µmol/ml, respectively (p = 0.0255)] (Fig 1D).

Association between the serum level of XO and oxidative biomarkers with tumor stage and grade

The serum levels of XO were significantly lower in invasive tumors (T2–T4) compared with non-invasive tumors (Tis, Ta, T1) [(3.40 ± 0.48 vs 5.38 ± 0.31) ng/ml, respectively (p = 0.01)]

(Table 5). Moreover, the serum levels of the investigated oxidative biomarkers (8-OHdG, PC, TBARS) were also evaluated according to tumor stage and grade (Table 5) of BC. We observed a significant increase in the mean of 8-OHdG in invasive tumors (T2, T3, T4) compared with non-invasive tumors [(31.35 ± 6.42 vs 19.64 ± 1.56), respectively (p = 0.01)]. The serum levels of 8-OHdG were also significantly higher among high-grade tumors compared with low-grade tumors [(22.29 ± 1.57 vs 16.59 ± 1.48) ng/ml, respectively (p = 0.009)]. In comparison, no significant differences were observed between the serum levels of other oxidative biomarkers (PC, TBARS) with either the tumor stage or grade (p > 0.05).

Table 4. Serum XO levels by smoking status in bladder cancer patients and controls

Subject's group	Sub-group	XO (ng/ml) ± SEM	p-value[1]
Smoker (n = 122)	Control (n = 61)	3.41 ± 0.28	<0.0001*
	BC (n = 61)	5.29 ± 0.35	
Non-smoker (n = 96)	Control (n = 48)	4.36 ± 0.36	0.38
	BC (n = 48)	4.87 ± 0.46	
Control (n = 109)	Smoker (n = 61)	3.41 ± 0.28	0.0367*
	Nonsmoker (n = 48)	4.36 ± 0.36	
BC (n = 109)	Smoker (n = 61)	5.29 ± 0.35	0.461
	Nonsmoker (n = 48)	4.87 ± 0.46	

Note: Data was presented as mean ± SEM (standard error of the mean); n (number of participants); [1] p-value was calculated using student t-test; * indicates statistical significance

Table 5. Tumor stage/grade and the serum levels of XO

Oxidative stress biomarkers	Tumor characteristic		n (%)	Mean ± SEM	p-value[1]
XO (ng/ml)	Tumor invasiveness (stage)	Non-invasive (Tis, Ta, T1) Invasive (T2, T3, T4)	94 (86%) 15 (14%)	5.38 ± 0.31 3.40 ± 0.48	0.01*
	Tumor grade	Low High	57 (52%) 51 (48%)	5.51 ± 0.38 4.73 ± 0.41	0.16
8-OHdG (ng/ml)	Tumor invasiveness (stage)	Non-invasive (Tis, Ta, T1) Invasive (T2, T3, T4)	94 (86%) 15 (14%)	19.64 ± 1.56 31.35 ± 6.42	0.01*
	Tumor grade	Low High	57 (52%) 51 (48%)	16.59 ± 1.48 22.29 ± 1.57	0.009*
PC (nmol/mgprot)	Tumor invasiveness (stage)	Non-invasive (Tis, Ta, T1) Invasive (T2, T3, T4)	94 (86%) 15 (14%)	11.78 ± 1.67 23.42 ± 11.04	0.55
	Tumor grade	Low High	57 (52%) 51 (48%)	9.00 ± 0.66 10.03 ±1.41	0.50
TBARS (µg/ml)	Tumor invasiveness (stage)	Non-invasive (Tis, Ta, T1) Invasive (T2, T3, T4	94 (86%) 15 (14%)	4.49 ± 0.48 6.62 ± 1.54	0.11
	Tumor grade	Low High	57 (52%) 51 (48%)	3.88 ± 0.42 4.48 ± 0.49	0.35

Note: Data was presented as mean ± SEM (standard error of the mean); n (number of participants); [1] p-value was calculated using student t-test; * indicates significant differences

Multivariate analysis of XO serum level and the serum levels of oxidative stress biomarkers with BC

Although BC patients were not significantly older than controls in univariate analysis, serum levels of XO and oxidative stress biomarkers (8-OHdG, TBARS, PC) could be influenced by age, gender, BMI, and smoking status. Therefore, multivariate regression analysis was conducted to assess whether XO or oxidative stress biomarkers significantly affected BC status after adjusting for potential confounders. We revealed that serum XO significantly increased in BC by approximately 1.2-fold (Table 6) (OR = 1.196; 95% CI: 1.031–1.389; p = 0.018). Additionally, PC emerged with significant elevation in BC (p < 0.0001).

The association between allele and genotype frequencies of selected XOR SNPs and BC

In this study, we investigated the association of four XOR SNPs (rs207454, rs1884725, rs17011368, rs494852) and an intergenic SNP (rs206833) with BC. Alleles and genotypes were identified using the Tetra-ARMs PCR technique on DNA extracted from all study subjects (n = 218). The distribution of allele and genotype frequencies is presented in Table 7. Genotype frequencies in both BC patients and controls satisfied HWE (p > 0.05), with no significant differences observed between groups (p > 0.05).

Multiple inheritance models (codominant, dominant, recessive, over-dominant, and log-additive) were analyzed using

the SNPStats web tool (Suppl. Table S2). No significant associations were found between the studied SNPs and BC across any genetic model ($p > 0.05$).

Haplotype results

Linkage disequilibrium analysis and haplotype construction were performed on all five SNPs using SHEsis software. Four haplotypes were identified that significantly increased in BC ($p < 0.05$, haplotype frequency >0.03). The XOR-related SNPs (rs207454, rs1884725, rs17011368, and rs494852) and intergenic SNP (rs206833) were arranged by chromosomal position on chromosome 2. The TGTCA haplotype conferred a 4.17-fold increased susceptibility of BC ($p = 0.01$; OR = 4.17; 95% CI: 1.16-15.00) (Table 8). Additionally, three other haplotypes (TGTA, TGA, and GTA) increased in BC by 1.84-, 1.62-, and 1.66-fold, respectively ($p < 0.05$; OR >1). Notably, haplotype analysis excluding the intergenic SNP (rs206833) showed no significant differences between BC patients and controls.

Table 6. Multivariate regression analysis of study subjects

Variable	OR	95% CI		*p*-value[1]
		Lower	Upper	
Age	1.034	0.996	1.072	0.079
Male	1.577	0.511	4.864	0.428
BMI	0.993	0.906	1.087	0.876
Smoking status	1.014	0.433	2.377	0.974
XO (ng/ml)	1.196	1.031	1.389	0.018*
8-HOdG (ng/ml)	1.006	0.968	1.046	0.753
PC (µmol/mgprot)	1.331	1.168	1.518	<0.0001*
TBARS (µg/ml)	1.074	0.925	1.248	0.349

Note: OR: odds ratio, CI: confidence interval; [1] *p*-values were calculated by binomial logistic regression analysis; * indicates a significant difference

Table 7. Genotype and allele frequencies of each SNP in BC patients and control subjects

SNP[1]	Genotype/Allele[1]	Control (*n* = 109)	BC (*n* = 109)	HWE	*p*-value[2]
rs207454	TT	84 (77%)	82 (75%)	0.37	0.54
	TG	24 (22%)	27 (25%)		
	GG	1 (1%)	0 (0%)		
	T	192 (88%)	191 (88%)	–	0.88
	G	26 (12%)	27 (12%)		
rs1884725	GG	68 (62%)	74 (68%)	0.81	0.38
	GA	34 (31%)	32 (29%)		
	AA	7 (7%)	3 (3%)		
	G	170 (78%)	180 (83%)	–	0.22
	A	48 (22%)	38 (17%)		
rs17011368	TT	98 (90%)	95 (87%)	0.76	0.52
	TC	11 (10%)	14 (13%)		
	CC	0 (0%)	0 (0%)		
	T	207 (95%)	204 (94%)	–	0.53
	C	11 (5%)	14 (6%)		
rs494852	CC	54 (50%)	52 (48%)	0.43	0.95
	CT	42 (38%)	44 (40%)		
	TT	13 (12%)	13 (12%)		
	C	150 (69%)	148 (68%)	–	0.83
	T	68 (31%)	70 (32%)		
rs206833	GG	72 (66%)	62 (57%)	0.69	0.37
	GA	31 (29%)	40 (37%)		
	AA	6 (5%)	7 (6%)		
	G	175 (80%)	164 (75%)	–	0.20
	A	43 (20%)	54 (25%)		

Note: n (number of participants), HWE (Hardy–Weinberg Equilibrium); [1] Analysis was performed using the SNPStats browser; [2] *p*-values were calculated by Pearson's chi-squared test

Table 8. Haplotype association of the five SNPs in bladder cancer and control subjects

Haplotype	rs207454	rs1884725	rs17011368	rs494852	rs206833	Control	Bladder cancer	OR (95%CI)	*p*-value[1]
1	T	G	T	C	A	3 (0.01)	12 (0.05)	4.17 (1.16~15.00)	0.01*
2	T	G	T	–	A	29 (0.13)	48 (0.22)	1.84 (1.11~3.05)	0.01*
3	T	G	–	–	A	36 (0.16)	53 (0.24)	1.62 (1.01~2.60)	0.04*
4	–	G	T	–	A	33 (0.15)	50 (0.22)	1.66 (1.02~2.71)	0.03*

Note: OR: odd ratio; CI: confidence interval; [1] *p*-value were calculated by the Pearson Chi-square test; * indicates a significant difference

Discussion

This study demonstrated that elevated serum levels of XO, oxidative stress biomarkers (8-OHdG, PC, TBARS), and genetic variations in *XOR* are associated with BC. To the best of our knowledge, this is the first study in Jordan to establish a link between BC and elevated serum XO levels. Additionally, it is the first study to report an association between *XOR*-related genetic haplotypes and BC.

Upon reviewing the literature, we found that the XOR enzyme exhibits variable expression and level patterns in different tumors, with downregulation observed in some cancers (such as gastrointestinal, colorectal, breast and liver cancers), and upregulation in others (including prostate cancer, laryngeal well-differentiated squamous cell carcinomas, and BC) (Chen and Meng, 2022). In addition, XOR has a dual role in cancer development and progression, exhibiting both pro-tumorigenesis and antitumor properties (Battelli et al., 2016b). According to the ongoing debate and controversy regarding the expression patterns and levels of XOR and XO, particularly across different cancer stages and grades, we have investigated the serum levels of XO in patients with BC from Jordan. In this study, XO serum levels were significantly higher in patients with BC compared with controls. This finding aligns with the literature that has linked high serum XO levels with BC pathogenesis (Gulec et al., 2003; Gunes et al., 2020; Metwally et al., 2011). Additionally, XO inhibition has been shown to reduce tumor growth and metastasis in breast cancer (Oh et al., 2019). Our findings also demonstrated a significant elevation of XO serum levels in non-invasive tumors (Tis, Ta, T1) compared to invasive tumors (T2, T3, T4). However, no significant association was identified between XO and tumor grade. Our findings support those of a study by Alsabti (1979), where a significant gradual decrease in XO serum levels with advanced BC stage and grade were reported.

While the inclusion of metastatic cases introduces some heterogeneity into our cohort, it also reflects the full clinical spectrum of BC and may enhance the generalizability of our findings. Based on these findings, we suggest that the XO enzyme could be a potential drug target for controlling tumor invasiveness, particularly in early stages, regardless of BC grade. In addition, it might serve as a potential diagnostic/prognostic marker for BC patients at early stages. Although our study demonstrated an association between oxidative stress, elevated XO, and BC, translation of redox-modulating therapies into oncology has been limited. Approved XOR inhibitors such as allopurinol, febuxostat, and topiroxostat are widely used for hyperuricemia and tumor lysis syndrome, and preclinical studies suggest potential anticancer effects (Fahmy et al., 2020; Joshi et al., 2021; Spina et al., 2015; Takai et al., 2014). Allopurinol and febuxostat have been shown to reduce oxidative stress, induce apoptosis in prostate and lung cancer models, and enhance the activity of chemotherapeutic agents (Mi et al., 2020; Yasuda et al., 2008). Febuxostat also demonstrated reno-protective effects in cisplatin-treated models (Fahmi et al., 2016). Despite such findings, no antioxidant or XOR-targeted therapy has entered routine oncological practice, largely due to the dual role of ROS, which contributes to both tumorigenesis and therapy-induced cytotoxicity. Future strategies should focus on targeting specific ROS sources, exploiting synthetic-lethal vulnerabilities, and integrating predictive biomarkers. In addition, future studies combining both protein expression and enzymatic activity assays would provide deeper insight into the mechanistic role of XOR in BC. Our results underscore the therapeutic relevance of XOR in BC, warranting further biomarker-driven and translational studies.

Mounting evidence suggests that XO levels can be influenced by smoking which contributes to DNA damage and impairs antioxidant mechanisms (Shah et al., 2015). Strope and Montie (2008) reported that more than 50% of BC patients are smokers, highlighting this risk factor's critical role in BC development.

In our study, among smokers, a significant increase in XO serum levels was observed in BC patients compared to the control group. These findings suggest that the effect of smoking on XO serum levels may be significantly influenced by BC status, indicating a possible synergistic interaction between smoking and tumor biology in amplifying the serum levels of XO. Notably, smoking status was found to differentially influence serum XO levels in controls and BC patients. Among controls, non-smokers exhibited significantly higher XO levels than smokers, consistent with evidence that tobacco smoke constituents can modulate redox enzymes and alter systemic oxidative balance (Kim et al., 2013; Shah et al., 2015). In contrast, no significant difference in XO levels was detected between smokers and non-smokers within the BC group, implying that tumor-related dysregulation of oxidative pathways may overshadow the independent effect of smoking. Taken together, these observations indicate that smoking status exerts divergent effects on XO regulation depending on disease context – suppressing XO activity in otherwise healthy individuals but having no detectable impact once BC is established. These findings align with previous evidence linking XO to ROS generation and cancer progression (Kim et al., 2013). They also highlight the potential of XO as a biomarker or therapeutic target in smoking-associated BC. Further mechanistic studies are warranted to clarify how smoking interacts with XOR pathways during cancer initiation versus progression. For example, experiments on bladder epithelial cell cultures exposed to cigarette smoke extract could determine whether smoking downregulates XO expression under normal conditions, and whether transformation into malignant cells reverses this effect by inducing oxidative pathways. Similarly, animal models

combining tobacco exposure with chemically induced BC could clarify how smoking interacts with XOR activity during cancer initiation versus progression.

Several studies have found a proportional relationship between oxidative stress biomarkers and BC development and progression (Soini et al., 2011; Wigner et al., 2021b). In our study, a significant reduction in XO level with tumor invasiveness suggests a potential downregulation of oxidative enzyme activity as the disease progresses. This decrease may reflect metabolic adaptations in more aggressive tumors, where altered redox balance plays a role in tumor survival. In contrast, 8-OHdG levels were significantly elevated in both invasive and high-grade tumors, consistent with its established role as a marker of oxidative DNA damage and tumor aggressiveness. Meanwhile, there is no significant association between protein and lipid damage levels with tumor stage or grade, indicating limited diagnostic value compared to 8-OHdG. This discrepancy may be attributed to the uneven distribution of patients across tumor-stage groups and the limited sample size within tumor-grade groups.

Haplotype analysis revealed distinct *XOR*-related haplotypes associated with BC in our study population. Individuals carrying the TGTCA haplotype exhibited an increased susceptibility to BC, which included the rs1884725-G and rs206833-A alleles. Additionally, other haplotypes (TGTA, TGA, and GTA) were significantly elevated among BC patients in the Jordanian population. Notably, the intergenic SNP rs206833, located in an enhancer region between the *XOR* and *SRD5A2* genes (Minoshima et al., 1995), was consistently present in all significant haplotypes, suggesting its role in BC. Intergenic regions, such as the one harboring rs206833, often contain regulatory elements (e.g., promoters, enhancers) that influence gene expression (Minoshima et al., 1995). The inclusion of rs206833 within identified haplotypes in BC implies that this variant may alter the expression of neighboring genes (*XOR* and *SRD5A2*), potentially contributing to BC pathogenesis. However, our findings should be interpreted carefully due to the study's limited sample size. Larger-scale replication studies are necessary to validate these associations. Further functional investigations are also warranted to elucidate the mechanistic role of the rs206833-containing enhancer region and its impact on XOR expression and enzymatic activity.

Our findings contribute to the growing body of evidence suggesting that BC is influenced by both environmental factors and genetic variations. These results enhance our understanding of BC pathophysiology and provide a foundation for developing novel pharmacological and non-pharmacological approaches to prevent or slow BC progression.

Conclusion

Haplotype analysis identified novel haplotypes elevated in BC. Our findings highlight the potential role of serum XO levels and oxidative stress biomarkers in BC oncogenesis. Interestingly, serum XO levels were significantly lower in invasive tumors compared with non-invasive tumors. This may indicate metabolic reprogramming in advanced disease, where increased XO levels may reflect a redox imbalance that supports tumor progression. Although our study cannot establish causality, these findings suggest that XO expression might carry diagnostic or prognostic relevance, warranting confirmation in larger, stage-stratified cohorts. Additionally, large-scale clinical studies with extended follow-up periods are needed to clarify the relationship between XO and disease progression, particularly across different cancer stages. The quantification of oxidative stress biomarkers may provide a valuable tool for assessing tumor progression. Such biomarkers could also guide the design of targeted antioxidant therapies in BC patients. Future research should investigate redox homeostasis in BC patients with the histopathological features of the tumor microenvironment, including tumor budding and inflammatory infiltration. These studies may provide deeper insights into the interplay between oxidative stress, genetic factors, and BC pathogenesis. However, definitive conclusions regarding the functional role of XO require further validation through *in vitro* studies using BC cell lines.

Ethical approval

This study was approved by the Jordan University of Science and Technology (JUST) Research Committee and the Institutional Review Board (IRB number 2021/141/11) of JUST and King Abdullah University Hospital – KAUH (Irbid/Jordan). The research was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants before their inclusion in the study.

Authors' contributions

Conception: *L.E.* and *N.A.*; Funding acquisition: *L.E.*; Subjects recruitment: *A.A.* and *O.H.*; Molecular experiments: *A.A.*; Interpretation or analysis of data: *L.E.*, *A.A.*, *N.A.* and *H.B.*; Writing original draft: *L.E.*, *A.A.*, and *M.A.*; Revision of important intellectual content: *O.H.*, *M.A.*, and *H.B.*; Supervision: *L.E.*, *N.A.*, and *O.H.* All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index.

Acknowledgements

The authors would like to thank all the bladder cancer patients and control subjects who participated in this study. Special thanks to staff members of the urology and oncology clinics of KAUH.

Availability of data and materials

Data is available from the corresponding author upon reasonable request.

Declaration on the use of AI

None.

Funding

The study was supported by the Deanship of Research at Jordan University of Science and Technology (Grant number 20210352).

Conflict of interest

The authors have no conflict of interest to declare.

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