JJBS

Volume 12, Number 5,December 2019
ISSN 1995-6673
Pages 603 - 608

Jordan Journal of Biological Sciences

Lack of Association of Human Prostate Cancer with Exon 1 and -116 C/G Promoter Polymorphism on the X-Box DNA Binding Protein-1 Gene

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Received March 2, 2019; Revised March 20, 2019; Accepted March 27, 2019

Abstract

The X-box binding protein-1 (XBP1), a critical gene in the endoplasmic reticulum stress response, has been linked to many cancers in several studies. Recent studies indicate that the upregulation of XBP1 promotes cell proliferation and invasion of prostate cancer (PC). This research is aimed at the measurement of the frequencies of Exon 1 and -116 C/G promoter polymorphism on the XBP1 gene as well as the investigation of this polymorphism as a predisposing genetic marker to assess possible strategies for screening families at the risk of developing PC. Blood samples of seventy patients with PC and seventy healthy individuals were evaluated using TaqMan genotyping technique and direct DNA sequencing analysis. Overall, the sequencing of exon1 of the XBP1 showed that there was no mutation, neither in PC subjects nor in the control. Additionally, there was no significant statistical difference between the PC cases with -116C/G polymorphism of XBP1 and the control subjects in the genotype (P = 0.674) and allele frequencies (P = 0.436). The present study is the first report to discuss the risk factors associated with mutations in XBP1 in PC progression. The results suggest no significant relevance between -116C/G and exon 1 (rs5762809 and rs2228260), and PC susceptibility in the Jordanian population.

Keywords: DNA sequencing, Polymorphism, Prostate cancer, X-box binding protein-1 gene.

1. Introduction

Prostate cancer (PC) is one of the most commonly diagnosed cancers and the second leading cause of cancer-related deaths among men, representing ~9 % of all cancer deaths in men worldwide with > 29,430 deaths in 2018 (Tao *et al*., 2015; Siegel *et al*., 2018). Worldwide, a distinct geographical variation in the incidence of PC has been reported (Benafif *et al*., 2018). According to the latest WHO data published in 2017, PC deaths in Jordan reached 191 or 0.72% of total deaths (WHO, 2017). Although PC mortality rate is decreasing in high income countries, the incidence and burden of the disease are steadily increasing globally, resulting in further challenges in the allocation of limited health care resources (Pishgar *et al*., 2018). Information is limited concerning the efficacy of available screening tests in men predisposed to developing PC (Giri *et al*., 2018). The pathogenesis underlying PC remains out of reach. However, the epidemiologic observations have revealed that pathogenesis of PC reflects both genetic and environmental factors. For example, familial PC studies suggested that PC has a substantial inherited predisposition; they have found that the risk tends to increase with the increased numbers of affected relatives (Alberti, 2010; Kral *et al*., 2011; Al Olama *et al*., 2014). In addition, the study of cohort twins between 44,788 pairs of Sweden, Denmark, and Finland indicated that more than 40 % of the cases of the PC disease were attributed to inheritance (Lichtenstein *et al*., 2000).

Previous research has revealed that both endoplasmic reticulum stress (ERS) and the unfolded protein response (UPR) activation are implicated in tumorigenesis (Corazzari *et al*., 2017; Doultsinos *et al*., 017). UPR involves three main signaling pathways: protein kinase RNA-like ER kinase (PERK), inositol requiring kinase1α (IRE1α), and activating transcription factor 6 (ATF6) (Corazzari *et al*., 2017; Doultsinos *et al*., 017). The activated IRE1 α sensor is responsible for the non-conventional splicing of unspliced XBP1 (XBP1u) mRNA to the active form spliced XBP1 (XBP1s) through its endoribonuclase activity (Moore and Hollien, 2015). XBP-1s enters the nucleus and induces the transcription of genes correlated with the protein-folding capacity and the ER-associated degradation. XBP1s is a central UPR effector, and previous studies indicate that the up- regulation of

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** Abbreviations: ER, endoplasmic reticulum; GWAS, genome wide association studies; OR, odds ratio; PCR, polymerase chain reaction; PC, prostate cancer; PSA, prostate-specific antigen; SNPs, single nucleotide polymorphisms; UPR, unfolded protein response; XBP1, X-box binding protein-1

XBP1s promotes cell proliferation and invasion of cancerous cells. To the authors' knowledge, no independent study has yet assessed the potential of mutations on the XBP1 gene of human PC cell lines or clinical samples as useful markers in prostate oncology. For this goal the present study was carried out.

Over the past decade and with the emergence of new technologies, identifying genetic variations, such as Single Nucleotide Polymorphisms (SNPs) offered the possibility of developing a novel biomarker (Botstein *et al*., 1980; Daly *et al*., 2017). Moreover, previous studies found that approximately 30 % of the estimated heritability of PC can be attributed to SNPs and more than 100 of them have been genotyped (Al Olama *et al*., 2014; Broeck *et al.*, 2014; Benafif *et al*., 2018).

2. Materials and Methods

2.1. Patients and Control Subjects

This study consisted of two hospital-based case-control groups: seventy Jordanian patients with PC from the Department of Pathology at the King Abdullah University Hospital (KAUH) (Irbid, Jordan) (mean age = 69.9 ± 9 years) and age-matched (mean age = 61.6 ± 12.0 years). The subjects were recruited between September 2017 and February 2018. The histopathological diagnosis was conducted by specialized pathologists according to the TNM staging system (stage I-IV) by the American Joint Committee on Cancer (AJCC). The study protocols were approved by the Jordanian Ministry of Health (CODE: MOH REC 170106) and the Institutional Review Board of the KAUH (IRB number 130/1/2902). A written informed consent was obtained from each recruited participant before enrollment. Information on age, PSA level, Gleason score at diagnosis and other sociodemographic characteristics of the study subjects were obtained through a direct questionnaire survey.

2.2. Cell Lines and Cell Culture

The human PC cell lines (PC3, DU145 and LNCaP) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained and cultured in a DMEM (Dulbecco's modified Eagle's medium; Hyclone, Logan, UT, USA) medium supplemented with a 10 % heat-inactivated fetal bovine serum (FBS), 100 units/mL of penicillin, 100µg/mL of streptomycin and 1 % amphotericin B (25µg/mL) (Wel GENE Inc.). The cells were cultured in a humidified atmosphere with 5 % CO_2 at 37°C.

2.3. SNP Selection and Genotyping

DNA was extracted from the blood samples and the cultured cells using a Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. All the primers and restriction enzyme used in this study were designed manually. The location and fidelity of restriction enzyme and primers' sequence were checked using the following software:

- http://primer3.ut.ee/,https://genome.ucsc.edu/,
- http://ensembl.org/Homo_sapiens/Gene,
- and http://www.labtools.us/nebcutter-v2-0/.

Primers for rs2269577 SNP were designed: 5′-GTTTCAGGACCGTGGCTATG-3′ (forward primer) and 5′-TCAGTCTGGAAAGCTCTCGG-3′ (reversed primer). A total of 50 ng of genomic DNA was amplified in a 25 µL of a final volume PCR reaction containing 0.4 µM of each primer and 12.5µL of the green master mix (GoTaq®Green Master Mix, Promega, USA). The amplification was performed at 95°C for five minutes with an initial denaturation, followed by thirty-five cycles of 95°C for thirty seconds, 52°C for thirty seconds, and 72°C for thirty seconds, and a final extension of five minutes at 72°C. The amplified fragments of 190 bp of PCR products were digested with the *BstEII* restriction enzyme.

2.4. Sequence Analysis

Primers for Exon1 were designed: 5′-GTTTCAGGACCGTGGCTATG-3′ (forward primer) and 5′-TCAGTCTGGAAAGCTCTCGG-3′ (reversed primer). These primers were designed for flanking the exon 1, as well as 186 bp upstream (containing putative regulatory elements) and 48 bp from intron 1 sequences of XBP1 gene. The products were amplified by polymerase chain reactions (PCR) with a touchdown program (95°C for 5 min; 40 cycles of 95°C for 30 s, 52°C for 30s, 72°C for 30 s, 72°C for 5 min). After amplification, the products were purified using a MEGA quick-spin Total Fragment DNA Purification Kit (Intron, Korea) and directly sequenced on ABI 3730XL DNA Analyzer (Applied Biosystems). All the variants identified by sequence analysis were checked against the dbSNP data (version 129) for determining the novelty of variants, and the novel variants.

2.5. Statistical Analysis

Statistical analyses were conducted using the Statistical Package of the Social Sciences software version 15.0 (SPSS, Inc., Chicago, IL). Comparisons between-group differences in continuous variables were evaluated using the Pearson Chi square and goodness of fit test ($P \leq .05$). The association between -116C/G and exon 1 polymorphisms on XBP1and the risk of PC were estimated by odds ratio (OR) with a 95 % confidence interval (95 % CI). Hardy–Weinberg allele frequency percentages for the prostate cancer patients and the control group and allele frequency were calculated according to the following equation: (A) is the major allele and (a) is the minor allele

Frequency of allele A= p = f (AA) + 1/2 (Aa)

Frequency of allele a= q = f (aa) + 1/2 (Aa).

The observed and expected numbers for the healthy control and patients for the -116 C→G mutant genotype (GG) were as follows: (31 and 28.5) and (26 and 28.5), respectively. For the heterozygous genotype (GC), the values were (35 and 35.5) and (36 and 35.5), respectively. For the wild-type genotype (CC), the observed and expected numbers were (4 and 6) as well as (8 and 6) for the control and PC patients, respectively. No significant difference was observed between the actual and expected distributions of the -116 C→G SNP between the PC patients and control (*P*-value > 0.05).

3. Results

Table 1 shows the frequencies of the -116 of XBP1 genotypes in patients and controls, and the corresponding odds ratios. Neither the GC, nor the GG genotypes were significantly more frequent in early onset prostate cases than in the controls (OR= 0.514, 95% = CI 0.14-1.86 and 0.419, 95% CI= 0.11-1.55, respectively), while the lowest frequency was for homozygous (CC) genotype (11.43 %)

in PC patients, and (5.7 %) in controls. In this study, possible interactions between the various XBP1 polymorphisms and PC risk have been investigated. There was no evidence of any interaction between the XBP1 genotypes (OR= 0.514, 95 % CI= 0.014-1.86, *P*=0.31).

Table 1. Distribution of genotypes and allelic frequencies among PC patients and control.

XBP1 (-116 G→C) Genotypes	Prostate Cancer Number (%)	Control Number (%)(1)	OR(2)	95% CI(3)	*p*-Value
GG	26 (37.14%)	31 (44.3%)	0.419	0.11 - 1.55	0.193
GC	36 (51.43%)	35 (50%)	0.514	0.14- 1.86	0.311
CC	8 (11.43%)	4 (5.7%)	1.01	0.42 - 2.75	0.099
Allele Frequencies					
G	88 (62.9%)	97 (69.3%)	0.750	0.46 - 1.32	0.26

% (1) = Hardy–Weinberg allele frequency percentages for prostate cancer patients and control group, OR (2) = odd ratio, C.I (3) = confidence interval at 95%.

To explore potential interactions between genes and the environment, the relationship between genotype and the risk of PC at different levels depending on the selected properties was examined. The risk of developing prostate cancer according to the PC patients' variables (age, Gleason score, PSA level, metastasis and treatment) was studied. Results shown in Table 2 reveal that there were no significant differences between the frequencies of genotypes and alleles of XBP1 -116C→G polymorphism and PC patients' variables. Furthermore, no significant difference between the patients' age and genotypes and alleles' frequencies was recorded with p-values of 0.31 and 0.27, respectively. Data presented in Table 2 indicate that the stratification analysis of the PC group on the basis of Gleason score frequencies of the CC+CG genotype and alleles of XBP1 (-116 C→G) polymorphism was not associated with significant increases in Gleason score (*P*-values = 0.14 and 0.99, respectively). Similarly, there was no significant association between the PC patients' PSA level and genotypes and alleles of XBP1 (-116 C→G) frequencies with *P*- values of 0.44 and 0.19, respectively. Chi square analysis for association showed nonsignificant association between the presence or absence of metastasis of PC in patient and the genotypes and alleles of XBP1 (-116 C→G) polymorphism frequencies (*P*-values 0.86 and 0.65, respectively). There was no significant difference between GG, GC and CC genotypes and C, G allele frequencies according to the treatment type in the PC patients and control group with *P*- values of 0.58 and 0.32, respectively

Table 2 Association between the frequencies of genotypes and alleles of XBP1 -116C→G polymorphisms and prostate patients' variables.

	rs2269577 Genotypes				rs2269577 Allele Frequencies		
Variable	GG Number (%)	GC Number (%)	CC Number (%)	*p*-value	C (%)	G (%)	*p*-value
PC Patient's Age							
40-60	4 (15.4%)	9 (25%)	1 (12.5%)		11 (39.3%)	17 (60.7%)	
61-80	22 (84.6%)	23 (63.9%)	6 (75%)	0.31	35 (34.3%)	67 (65.7%)	0.27
>80	0 (0%)	4 (11.1%)	1 (12.5%)		6 (60%)	4(40%)	
PC Patient's Gleason Score							
5-6	5 (22.7%)	14 (38.9%)	1 (12.5%)		16 (40%)	24 (60%)	
7-8	12 (54.6%)	11 (30.6%)	6 (75%)	0.14	23 (39.7%)	35 (60.3%)	0.99
9-10	5 (22.7%)	11 (30.6%)	1 (12.5%)		13 (38.3%)	21 (61.7%)	
PC Patients' PSA Level							
0.0-4.0	10 (40%)	17 (51.5%)	5 (62.5%)		27(55.1%)	37 (44.6%)	
4.1-10.0	7 (28%)	5 (15.2%)	0 (0%)	0.44	5 (10.2%)	19 (22.9%)	0.19
>10.0	8 (32%)	11 (33.3%)	3 (37.5%)		17 (34.7%)	27 (32.5%)	
PC Patient's Metastasis Status							
Yes	5 (20.8%)	6 (17.7%)	1 (12.5%)	0.86	8 (33.3%)	16 (66.7%)	0.65
No	19 (79.2%)	28 (82.3%)	7 (87.5%)		42 (38.9%)	66 (61.1%)	
PC Patients' Treatment							
Hormonal Therapy	15(62.5%)	23(67.6%)	6 (75%)		35 (39.8%)	53 (60.2%)	
Hormonal & Chemo/ Radiotherapy	6 (25%)	6 (17.7%)	0 (0%)	0.58	6 (25%)	18 (75%)	0.32
Radical Prostatectomy	3 (12.5%)	5 (14.7%)	2 (25%)		9 (45%)	11 (55%)	

Figures 1and 2 show the *BstEII*-digested products of the DNA from the subjects and cell lines, respectively, electrophoresed on 3 % high-resolution agarose gel. Three types of bands, namely 190 bp, 103 bp and 87 bp are resolved. The two bands of 103 bp and 87 bp signify the normal CC genotype; three bands of 190 bp, 103 bp and 87 bp indicate the heterozygous CG genotype, while the 190 bp indicates the mutant GG genotype.

To identify prostate susceptibility-related genetic variants, sixty-six of the PC subjects and control subjects were screened for mutations in XBP1 exon1 by direct nucleotide sequencing. The sequencing analysis revealed no noteworthy mutation.

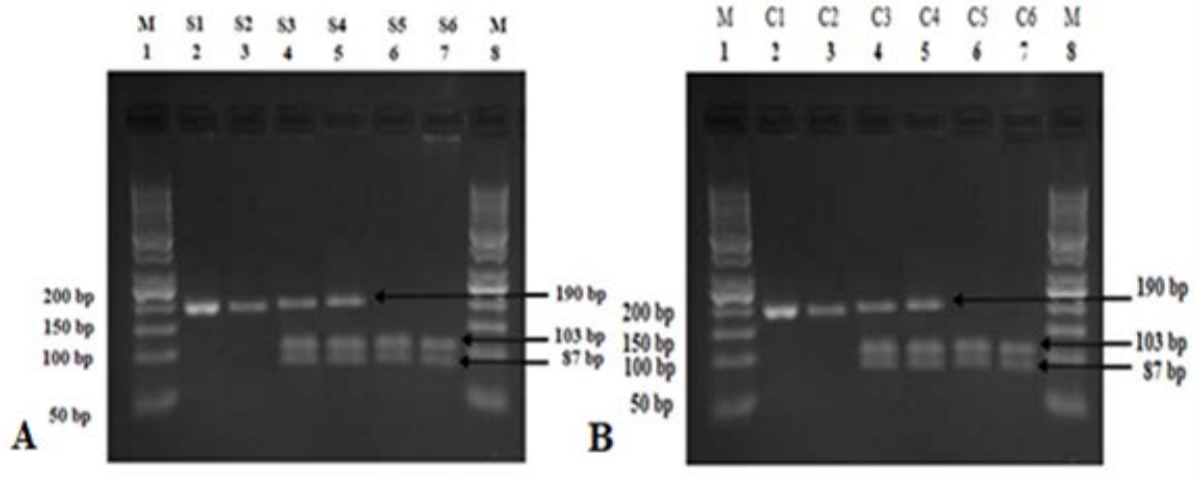

Figure 1. PCR-RFLP analysis of XBP1 (-116C→G) SNP. A. The *BstEII*-digested products of the DNA from Prostate cancer subjects (PC). Samples separated by 3 % agarose gel electrophoresis. Lane 1:50 bp DNA ladder. The remaining lanes (S1 through S6) represent the *BstEII* digested PCR products of PC samples. B. Control samples separated by 3 % agarose gel electrophoresis. Lane 1:50 bp DNA ladder. The remaining lanes (C1 through C6) represent the digested PCR products of control samples. The two bands of 103 bp and 87 bp signify the normal CC genotype; three bands of 190 bp, 103 bp and 87 bp indicate the heterozygous CG genotype, while the 190 bp indicates the mutant GG genotype.

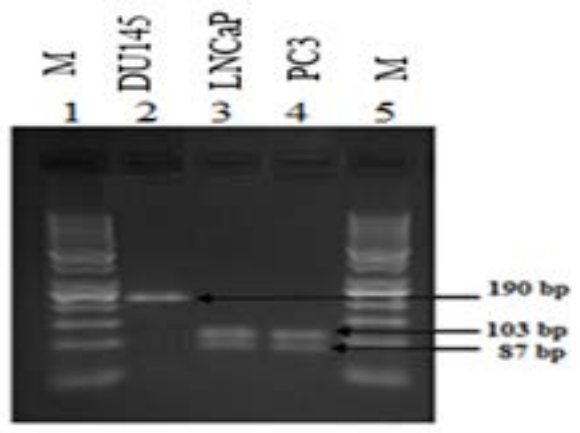

Figure 2. PCR-RFLP analysis of XBP1 (-116C→G) SNP of PC cell lines. The *BstEII*-digested products of the DNA from cell lines were separated by 3% agarose gel electrophoresis. Lane 1:50 bp DNA ladder. Lane 2: DU145 cell line. Lane 3: LNCaP cell line. Lane 4: PC3 cell line. The genotypic analysis of the three cell lines shows that the genotype of the DU145 was GG, while the genotype CC appeared in both LNCaP and PC3 cell lines.

4. Discussion

This study is an attempt to identify PC susceptibility genes and variants as reliable biomarkers associated with the increased cancer risk that has been challenging and unsuccessful (Wallis and Nam, 2015; Alvarez-Cubero *et al.*, 2016; Lynch *et al*., 2016).

An increasing number of SNPs had been suggested to be implicated in the development and progression of PC (Broeck *et al*., 2014). However, studies are not directly comparable because of the different classifications of the disease risk and different Prostate specific antigen (PSA) levels used (Colicchia *et al*., 2017).

rs2269577 (C→G) is a promoter variant and a disease-causing mutation of XBP1 that exerts functional effects on XBP1 activity itself therefore resulting in an abnormal XBP1 expression, which can be involved in an abnormal splicing and DNA damage that might affect the clinical outcome in PC patients (Knight, 2003). Recently, association studies in cancer have focused on several candidate genes. The present study is focused upon the XBP1 gene. It could not prove any statistically significant difference in the genotypic frequency of the (−116 C/G) polymorphism between the PC patients and control group. Moreover, the results of the current study did not find any significant association between rs2269577 genetic variations on the XBP1 gene and patients' age, presence or absence of metastasis, Gleason score, PSA level and treatment type. XBP1 is a critical transcription factor induced by ER stress as a major regulator of the unfolded protein response (UPR). There are few reports about the incidence of promoter mutations in several diseases including Alzheimer (Liu *et al*., 2013), the bipolar disorder (Masui *et al*., 2006), diabetes (Liu *et al*., 2015), inflammatory bowel diseases (Kaser *et al*., 2008), psychiatric illnesses (Cheng *et al*., 2014), multiple myeloma (Carrasco *et al*., 2007), and schizophrenia (Jonsson *et al*., 2006). A previous study (Hou *et al*., 2004) which is consistent with the results of the present study, observed no relationship between XBP1 (-116C→G) polymorphism and the bipolar disorder when normal controls were compared with bipolar disorder patients. These results support many studies on various populations with different disorders who could not find a strong correlation of this polymorphism. One possible explanation for the inconsistencies among different population groups is the ethnic characteristics. In addition, they are likely to be due to a number of factors including social and environmental factors as well as hereditary genetics. (Tanaka *et al*., 2001; Benafif *et al*., 2018).

To the best of the authors' knowledge, this is the first study to be conducted on men using SNPS in the XBP1 gene. It does not provide evidence that (C-116G) polymorphism in this gene is significantly associated with a high risk of PC. However, this study has the advantage of being conducted on a homogeneous population of the same ethnicity. This excludes the influences on allele sequences that may arise from different ethnic groups.

However, there are several limitations to be considered in the interpretation of the results of this study. First, it is limited by time and geographical factors: all subjects were recruited from a single institution, and the sample size was relatively moderate, which has limited the study to the common variants. The authors have been faced with several instrumental and administrative routine applications and reluctance in collaboration from a number of public and private hospitals. Thus, the investigated population may not be representative because the issue of selection bias cannot be ruled out due to hospital-based controls. To limit the potential selection bias, the authors recruited samples by matching the controls to the cases based on age. The use of blood donors as population controls has been criticized on the grounds that blood donors differ from the general population in several factors, including their medical history and the medical histories of their parents (Golding *et al*., 2013). This might introduce a bias in the interpretation of the results and lead to spurious disease associations. It is true that in Jordan, male blood donors have not been screened for PC, and their family history of the disease also is unknown. Therefore, these donors may be affected with PC later in life or carry variants with reduced penetrance that is associated with the disease. The collection of control samples from people with an assessed medical history is often not feasible for individual research groups, as it is both time-consuming and expensive. While blood donors may not optimally represent the genomic constitution of the general population, they do, however, provide a set of controls that is readily available. To reduce the bias for accuracy, sufficiently large numbers of controls should be analyzed. Second, no information on other factors such as occupational exposure and certain dietary components was available in our research; these variables might interact with XBP1 genotypes or act as potential confounding factors.

5. Conclusions

This is the first study to be conducted on men using SNPS in the XBP1 gene. It does not provide evidence that (– C-116G) allele in this gene is significantly associated with a high risk of PC. Although no mutations in the exon1 of the XBP1 were found, there may be other mutations in XBP1 that may have beneficial, deleterious, or neutral effects, depending on their location in the gene. However, since this study does not include the whole gene, further studies should be conducted to analyze the whole gene in order to identify other genetic alterations that can help determine an effective treatment plan.

Acknowledgments

We thank all the participants who took part in this research. This work was financially supported by the Deanship of Scientific Research and Graduate Studies at Yarmouk University under grant number (28/2015).

Conflict of Interest

The authors declare that they have no conflicts of interest.

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