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The Impact of VDR-FokI Polymorphism in Iraqi Patients with Prostate Cancer and Prostate Benign Hyperplasia

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Abstract

The polymorphism in the vitamin D receptor gene FokI position is used to evaluate the polymorphism impact on the levels of vitamin D, testosterone and prolactin hormones in the sera of patients with prostate cancer and benign prostatic hyperplasia vs. healthy controls. The vitamin D receptor gene Fok1 restriction site was amplified and examined by TaqMan RT-PCR technique. It was found that the TT genotype played a protective effect in 70% and 50% in prostate cancer and benign prostatic hyperplasia patients respectively. While, the CC genotype was found to be 100% disease-attributed genotype in both prostate cancer and benign prostate hyperplasia. Also, the distribution of genotypes (TT, TC and CC) was not consistent with Hardy Weinberg equation in the patients with prostate cancer as a significant difference was found by chi-square test (X2 >3.84) at P ≥0.05 between the observed and expected frequencies. But wasn't seen in patients with BPH or control group. The level of vitamin D was significantly affected by the genotype CC of VDR-FOK I in prostate cancer patients compared with TT and TC genotypes. There were no significant differences in Vit. D level among the three genotypes in the patients with BPH and the healthy control group. In association with genotypes, the levels of testosterone and prolactin did not differ significantly among the studied groups. It could be concluded that the vitamin D receptor FokI polymorphism is associated with Iraqi prostate cancer patients more than in benign prostate hyperplasia with vitamin D deficiency in blood serum.

Keywords: Benign prostate hyperplasia, Prostate cancer, Prolactin, Testosterone, *VDR-Fok1* Polymorphism.

Introduction

The steroid, thyroid, and retinoid nuclear receptor superfamily include the vitamin D receptor[1,2]. In response to its ligand, Vitamin D [1,25-(OH)2 D3], the receptor produces anti-proliferative, anti-inflammatory, and pro-angiogenesis effects in the tissues that express the receptor. Depending on the type of cell and the microenvironment in which the cell is located, these effects may have an anti-tumor effect [3,4].

Structurally, the receptor is made up of two domains: An N-terminal DNA binding domain and a C-terminal vitamin D binding domain [5]. When vitamin D binds to the C-terminus, it forms a

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heterodimer with the retinoid X receptor (RXR) and triggers the activation of genes downstream. The promoters of the responsive genes contain a CpG responsive element (Vitamin D receptor element[6,7]. It is primarily expressed in the cytoplasm of osteocytes, the gut, the kidney, and the liver as a receptor associated with vitamin D metabolic processes to control calcium and phosphate transfer[8]. Additionally, immunological cells, cutaneous tissues, cardiovascular tissues, and the neurological system all express VDR[9]. A large gene on the chromosome located at 12q13.11, has 11 exons and spans approximately 75 kb, encodes for the receptor protein [7,10,11]. the polypeptide chain is encoded by exons 2 through [9] of VDR gene [12]. The initial polymorphic sites in the vitamin D receptor were historically given names for the restriction endonucleases that were employed to find the allelic variations [13]. The most significant starting codon in the second exon is represented by the first identified polymorphism, *Fok*I (T/C), which is positioned in the coding region. The other polymorphism variant, which is inherited as a haplotype because it is located at the beginning of the eighth exon, is BsmI (A/G), ApaI (G/T), TaqI (T/C), as well as the Tru9I (G/A), and EcoRV [10]. Among all these polymorphic sites, only *Fok*I reduces the length of the produced protein and forms truncated protein[14].

The full length of VDR is a 427-amino acid protein (denoted "f" allele or "ATG" allele) to indicate the presence of the *Fok*I restriction site or "M1" for translation from the first methionine in the primary sequence) or a 424- truncated -amino acid protein (denoted "F" allele or "ACG" allele for the absence of the *Fok*I site or named "M4" to indicate translational initiation from the methionine at the fourth position in the primary sequence) are produced as a result of the transition of thymine-to-cytosine [15,16]. The F allele possesses higher transcriptional activity than f allele and it was associated with a higher risk of cardiovascular disease, hypertention[14,17], thalassemia[15] , systematic lupus erthymatosus [16], Osteoarthritis[18] and higher susceptibility to ovarian cancer[19].The relation of the F and f alleles of *Fok*I position, with cancer is still controversial, so this study aims to determine the frequency of the *Fok*I variant in Iraqi patients with benign prostatic hyperplasia and prostate cancer in comparison to healthy controls, as well as the association between the *Fok*I SNP and serum levels of vitamin D, testosterone, and prolactin in the study populations.

Materials and Methods

Clinical samples

This study was conducted between February 2018 to January 2019 in Baghdad, Iraq. It included 75 participants; twenty-five individuals were diagnosed with prostate cancer (PCa) and twenty-five were diagnosed with benign prostate hyperplasia (BPH). Their ages ranged from 45-86 and (46-91) years, respectively. The patients were treated at Medical City/Ghazi Al-Hariri hospital. Patients undergoing chemotherapy or radiotherapy, those who had undergone prostatectomy, those with various malignancies, those with any form of inflammation, and patients with diabetes were all disqualified from this study. There were 25 healthy volunteers in the control group, ranging in age from 41 to 86. The donation was approved by the patient and the controls.

Blood Samples collection

Five ml of venous blood samples were collected from patients diagnosed with prostate cancer (PCa), BPH, and healthy individuals serving as the control group. Two ml of the blood was transferred to EDTA tubes to prevent blood clotting, while three ml of blood were transferred to a silicone gel tube glass to get serum for the hormonal tests.

Measurement of Vitamin D and Hormones concentrations

The concentrations of vitamin D and Testosterone and prolactin hormones were measured in the sera of the patients and healthy subjects using the AFIAS vit. D, Testosterone and Prolactin kits and AFIAS-6 Compact Benchtop Automated Immuno-Analyzer (Boditech med. Incorporated / Korea), according to the instructions of the manufacturer. The test is a quantitative test based on the competition of the target molecule to bind the fluorescently labeled antibody so the instrument will measure the total target-labeled antibody complexes in the sera samples.

DNA extraction

Genomic DNA was isolated from frozen whole blood samples of the patients and the controls after

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bringing them to room temperature following the instructions of the gSYNC™ DNA extraction kit (Zymo / USA).

RT-PCR assay

The TaqMan RT-PCR[20] reactions were performed using the Sacace instrument/ Italy. The total volume of each component for each assay was 10 µl of 2X TaqMan probe® Master, 0.5 µl of 20X Assay working solution, 3µl of genomic DNA, then 6.5 µl nuclease free D.W. was added to reach the final volume of 20 µl in the sterile tube. The tubes were capped and centrifuged to eliminate the bubbles. The thermal cycling conditions include: enzyme activation at 95 ºC for 10 minutes, denaturation at 95 ºC for 15 sec, then annealing and extension at 60 ºC for one minute by scanning the excitation, the final step repeated 40 times, to detect the SNP ID :2228570. The statistical analysis system- SAS program was used to investigate the effect of different factors on the parameters of the study. The Chi-square test was used to significantly compare the percentage and least significant difference –LSD test (ANOVA) or t-Test was used to significantly compare between means. It is also used to estimate the correlation coefficient between variables in this study [21]. The platform http: www.ommnicalculator.com/biology/allele-frequency was used to assess the genotype and allele frequencies. The Hardy-Weinberg equilibrium was then performed, and the results were examined using a chi-squared test that the software utilized.

Results and Discussion

The frequency of Vit. D receptor *FokI* SNP represented by the frequency of genotypes TT, TC and CC was investigated in Iraqi patients with prostate cancer and BHP compared with healthy controls through direct detection of the genotypes by using the RT-PCR technique. A significant difference ($p \leq 0.05$) was recorded between the homozygous TT genotypes in PCa in 5 (20%) and 19 (76%) healthy controls. The *FokI* TT genotype odd ratio at (95% CI) was 0.08 (0.04-0.16) with a preventive fraction equal to 70%. This fraction refers to the protective effect of the TT genotype. No differences were seen in the frequency of heterozygous genotype TC between 6(24%) of PCa patients and 4(16%) of healthy controls respectively. The *FokI* TC genotype OR at (95% CI) was 0.60 (0.29-1.22). The fisher exact test was 0.163 with a preventive fraction equal to 9.5%, this fraction refers to low protection attribution of the TC genotype. A significant difference was seen in the CC homozygous genotype frequency between PCa 16 (64%) and (0) in the healthy controls. The OR at (95%CI) was undetermined (infinity) (53.64-infinity) with an attribution fraction of 100% with CC genotype as the disease related genotype as shown in Table1.

Table 1. Distribution of VDR gene (*FokI*) rs2228570 polymorphism genotypes in prostate malignant and control samples.

Groups	Study groups		Odds	CI 95%	Fisher's exact	Attributable	prevented
Genotype	PC	Control	Ratio		probability *	fraction	fraction
TT	(5) 20%	(19) 76%	0.08	0.04 - 0.16	0.000*	-------	70.0%
TC	(4)16%	(6)24%	0.60	0.29 - 1.22	0.163 NS	-------	9.5%
CC	(16) 64%	(0) %	infinity.	53.64- infinity	0.000*	100.0%	------
Total	**25**	**25**					
			Alleles distribution				
T	(14)28%	(44)88%	0.05	0.02-0.11	0.000*	- - - - -	83.3%
C	(36)72%	(6)12%	18.86	8.96-40.34	0.000*	68.2%	----

*Significant at ($P \leq 0.05$), NS: Non-Significant.

The frequency of the allele T in the PCa patients and healthy controls was 14(28%) and 44 (88%) respectively, it seems to be the protective allele, while the frequency of the allele C in the PCa patients and healthy controls was 36(72%) and 6(12%) respectively. The OR at (95%CI) was 0.05 at (0.02-0.11) and 18.86 at (8.96-40.34) which may be a conformation of the relation between the C allele and the disease.

The distribution of the polymorphic genotypes of *FokI* in the BPH patients compared with control

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subjects is shown in Table 2. The genotype TT was present in (13) 52 % of the patients compared with (19) 76% of the control subjects. The odd ratio was 0.34 which means this genotype is most likely present in the healthy statues under the CI of 95%. The fisher exact test shows a significant relation with the healthy status. The TT genotype prevents the disease by 50%. The TC genotype is present in (8) 32% of the patients of BPH compared with (6) 24% of control subjects.

Table 2. Distribution of VDR gene (*FokI*) rs2228570 polymorphism genotypes in BPH patients and control samples.

Groups Genotype	Study groups BPH	Control	Odds Ratio	CI 95%	Fisher's exact probability *	Attributable fraction	prevented fraction
TT	(13)52%	(19)76%	0.34	0.19 - 0.63	0.000*	-------	50.0%
TC	(8)32%	(6)24%	1.49	0.80 - 2.80	0.213	10.5%	
CC	(4)16%	0	infinity .	5.56-infinity	0.000	100.0%	--------
Total	**25**	**25**					
	Alleles distribution						
T	(34)68%	(44)88%	0.29	0.14 -0.60	0.001	----------	62.5%
C	(16)32%	(6)12%	3.45	1.66-7.39	0.001	22.7%	------

*Significant at (P≤0.05), NS: Non-Significant.

Table 3 shows the distribution of the three genotypes TT, TC and CC VDR of (*FokI*) polymorphism respectively, in PCa and benign prostate hyperplasia patients. The TT significantly appeared in 13 (52%) BPH, Odd ratio (0.23), CI at 95% (0.12-0.43) and a preventable fraction at 76.9%, while it appeared only in 5(20%) in PCa patients. The TC genotype was significantly found in 8 (32%) of benign prostate hyperplasia with an Odd ratio (0.4), CI at 95% of 0.2-0.8 and prevented fraction of 59.5%, but it appeared in only 4(16%) of PCa patients. The CC genotype significantly appeared in 16 (64%) of PCa patients with an Odd ratio of 9.33, CI at 95% of (4.76-18.49) with an attributable fraction of 89.3%. The allele frequency of T was highly significant in 34 (68%) of BHP patients with an Odd ratio of 0.18, CI at 955 of (10-0.34) and a preventive fraction of 81.7%, while the frequency of C alleles was highly significant in 36 (72%) PCa patients with Odd ratio 5.46, CI at 95% (2.9-10.05) with attributable fractioned 81.7%.

Table 3. Distribution of VDR gene (*FokI*) rs2228570 polymorphism genotypes in PC patients and BPH patient's samples.

Groups Genotype	Study groups PC	BPH	Odds Ratio	CI 95%	Fisher's exact probability *	Attributable fraction	prevented fraction
TT	(5)20%	(13)52%	0.23	0.12 -0.43	0.000*	-----	76.9%
TC	(4)16%	(8)32%	0.40	0.20 0.80	0.009*	-----	59.5%
CC	(16)64%	(4)16%	9.33	4.76-18.49	0.000*	89.3%	-----
Total	**25**	**25**					
	Alleles distribution						
T	(14)28%	(34)68%	0.18	10 - 0.34	0.000*	--------	81.7%
C	(36)72%	(16)32%	5.46	2.97-10.05	0.000*	81.7%	-----

*Significant at (P≤0.05), NS: Non-Significant.

Table 4 shows the expected and observed frequencies of the VDR gene (*FokI*) genotypes by Hardy-Weinberg equilibrium equation. The only significant differences (X^2 >3.84) between observed and expected frequencies for PCa, compared to BPH patients and the control group were seen in the distribution of the genotypes in PCa patients at *P*≤0.05.

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Table 4. Expected Frequencies of VDR gene (*FokI)* rs2228570 Genotypes Using Hardy-Weinberg Equilibrium

Groups		TT	TC	CC	X^2	P
PCa Genotypes	Observed no.	5	4	16	9.0*	0.002
	Expected no.	2	10.1	13		
BPH Genotype	Observed no.	13	8	4	1.75 ^{NS}	0.18
	Expected no.	11.6	10.9	2.6		
Control Genotypes	Observed no.	19	6	0	0.46 ^{NS}	0.49
	Expected no.	19.4	5.3	0.4		
Total observed		37	18	20		

*If $P \leq 0.05$ is not consistent with HWE. Significant differences ($X^2 > 3.84$) between observed and expected frequencies for all PCa, BPH patients and the control group. NS: non-significant.

The effects of the genotypes on the levels of testosterone and prolactin as well as Vit. D levels were detected in patient groups (PCa and BPH) compared with their levels in the healthy control group. Table 5 shows the effects of the genotypes in PCa patients. There are no significant differences at $P \geq 0.05$ in the levels of the testosterone and prolactin hormones in the three genotypes. Importantly, the genotype of the patient had an impact on the level of Vit. D in the sera. There is a significant difference at $P \geq 0.05$ in was seen in Vit D. concentration in the sera of the patients with TT genotype (10.88 ±1.89 ng/ml) and CC genotype (8.87 ±0.66 ng/ml) respectively. As well as, a significant difference at $P \geq 0.05$ was seen in the Vit. D concertation in the sera of patients with genotype TC (12.75 ±1.31 ng/ml) and CC (8.87 ±0.66 ng/ml) respectively. In the same time, the concentration of Vit. D in the sera of the patients with the genotype TT (10.88 ±1.89 ng/ml) did not statistically differ from its concentration in patients' sera with TC genotype (12.75 ±1.31 ng/ml).

Table 5. Effect of rs2228570 genotype on hormones level and Vit. D3 in PCa Malignant group.

Genotype of rs13333226	Mean ±SE		
	Testosterone (ng/ml)	**Prolactin (ng/ml)**	**Vit. D3 (ng/ml)**
TT	9.42 ±0.17	39.58 ±1.30	10.88 ±1.89 ab
TC	8.87 ±0.07	40.02 ±1.40	12.75 ±1.31 a
CC	9.83 ±0.35	39.58 ±0.74	8.87 ±0.66 b
LSD value	1.445 NS	3.571 NS	3.632

The letters a and b refer to the least significant differences at ($P \leq 0.05$), NS: Non-Significant. The normal range for Testosterone is (2-8 ng/ml). Normal range for Prolactin (3-35 ng /ml). Normal range for Vit D (30-120 ng/ml).

There were no significant differences found in the hormones and Vit D concentration in the sera of the BPH patients and in the healthy control group carrying the TT, TC and CC genotypes as shown in Tables 6 and 7 respectively.

Table 6. Effect of rs2228570 genotype on hormone level and Vit. D3 in the BPH group

Genotype of rs13333226	Mean ±SE		
	Testosterone (ng/ml)	**Prolactin (ng/ml)**	**Vit. D3 (ng/ml)**
TT	1.338 ±0.07	37.14 ±0.76	11.84 ±1.33
TC	1.19 ±0.17	36.88 ±0.66	13.12 ±2.36
CC	1.47 ±0.17	36.70 ±1.19	11.00 ±1.73
LSD value	0.426 ^{NS}	2.809 ^{NS}	6.074 ^{NS}

NS: Non-Significant, normal range for Testosterone is (2-8 ng/ml). The normal range for Prolactin is (3-35 ng/ml). Normal range for Vit D (30-120 ng/ml).

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Table 7. Effect of rs2228570 genotype on hormone level and Vit. D3 in healthy control group

Genotype of rs13333226	Mean ±SE		
	Testosterone (ng/ml)	Prolactin (ng/ml)	Vit. D3 (ng/ml)
TT	5.07 ±0.25	15.01 ±0.78	16.05 ±1.19
TC	5.23 ±0.62	14.63 ±1.88	20.00 ±2.46
LSD value	1.178 ^{NS}	3.608 ^{NS}	5.224 ^{NS}

NS: Non-Significant. Normal range for Testosterone (2-8 ng/ml). Normal range for Prolactin (3-35 ng/ml). Normal range for Vit. D3 (30-120 ng/ml).

In this case –control study, the polymorphism in *Fok*I or rs 2228570 typically appeared in 3 genotypes, the dominant TT, TC and CC which represent the dominant, heterozygous and recessive alleles respectively. The dominant genotype TT was significantly appearing in the healthy subjects with a protective role against the recessive CC genotype, which significantly appeared in PC patients. At the same time, there were no significant differences in the distribution of the genotypes between healthy and BPH subjects. The frequency of the protective T allele significantly appeared in the healthy and BHP subjects compared with disease associated allele C which significantly appeared in prostate cancer patients. The rs 2228570 *Fok*I (T/C) substation was classified as one of the significant polymorphisms that are associated with multiple disease conditions including cancers[22]. The polymorphism at rs 2228570 (*Fok*I T/C) substation is the most important polymorphism that alerts the VDR expression and it was found related to several inflammatory metabolic diseases and is related to poor prognosis in head and neck carcinoma [23], breast cancer [24], and papillary thyroid cancer [25] in different ethnic populations.
The VDR *FokI* polymorphism is associated with an increased risk of benign prostate hyperplasia[26] and prostate cancer in the Caucasian population [27,28]. The *FokI*, C allele was found to be a risk factor for breast cancer of Iraqi females[29].

According to research by Krasniqi *et al.*, inadequate sunlight exposure to the cutaneous synthesis of vitamin D3 (calcitriol) effectively lowers vitamin D's protective role[22]. This results in an increased prevalence of numerous cancer types [30]. The anticancer effects of vitamin D can be summed up as follows: 1) its antiproliferative properties and induction of G0/G1 cell arrest in the P53-dependent pathway[31,32]. 2) Vitamin D induces apoptosis in prostate cancer through direct activation of caspases [33]. 3) Decreasing the inflammatory response by the regulation of the expression of inflammation leading to carcinogenesis regulated by the $NF_{\kappa}B$ transcription family [34]. 4) Blocking the mitogenic effects of transcriptional factors and protein kinases [35,36] .5) inhibition of tissue invasion through inhibition of matrix metalloprotein's system[37]. 6) controlling the prostaglandin metabolism in the PC [37]. On the other hand, the lack of vitamin D was associated with a high risk of prostate cancer in men [38] and breast cancer in women [39] as well as colorectal cancer[40]. Also, De Flavia *et al*. found that the expression of VDR in prostate epithelial cells declines after 60 years old, leading to intracellular deficiency of Vit. D [41]. Both vitamin D level and *FokI* polymorphism were investigated in several studies and meta-analysis in prostate cancer and showed a contradicting result in the association between vitamin D level and *FokI* polymorphism in prostate cancer patients [42- 46] they did not find a significant association between patients and healthy controls for those parameters together. From another point of view, Yang, *et.al*. 2013, found that the VDR function is disrupted by specific microRNA[46], as well as several mediators that act as coactivates or corepressors or chromatin modulators to regulate the gene expression of VDR targeting genes[47] as well as different cancers [48,49].

This phenomenon could be explained through two main points: the first point: the collaboration of several factors at the same time may induce tumor initiation within the microenvironment that surrounds the prostate epithelial cells. This study, clarified that the low level of Vit. D and high levels of testosterone may promote the transformation of the prostatic cells into a cancerous condition, as the protective role of vitamin D is lost and the cells respond to high signaling stress of testosterone. Second point: the CC genotype of VDR, that results from substation of C instead of T at FokI or rs 2228570, maybe that the receptor responds to testosterone as an alternative ligand which leads to increase cell proliferation as the VDR has the affinity to several steroid and retinoic acid ligands specifically steroid hormones so it responds and

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affects the genes/ pathways those are activated by VDR.

Conclusion

It could be concluded that the vitamin D receptor *FokI* polymorphism is associated with Iraqi prostate cancer patients more than with benign prostate hyperplasia with Vitamin D deficiency.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Authors sign on ethical consideration's approval.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

Authors' Contribution Statement

The authors had cooperated to complete this research. The research was the idea of A. A.A., and she was the one who collected the samples, perform the molecular genetic investigation. L. H. A. A. O. write the original manuscript reviewing, editing and the corresponding author. A. M. A. performed the hormonal tests and vitamin D concentration measurement.

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تأثير تعدد الطرز الوراثية لمستقبل فيتامين دال- *FOK1* في المرضى العراقيين المصابين بسرطان البروستات وتضخم البروستات الحميد

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الخلاصة

استخدم تعدد الطرز الوراثية لمورث مستقبل فيتامين د عند الموقع FokI لتقييم تاثيرتعدد الطرزالروائية على مستويات فيتامين د وهرمون الذكورة وهرمون الحليب في امصال مرضى سرطان البروستات وتضخم البروستات الحميد مقارنة بالأفراد الأصحاء. تم تضخيم موقع الحصر *FOKI* لمورث مستقبل فيتامين د باستخدام تقنية TaqMan RT-PCR وجد أن الطراز الوراثيTT له تأثير حماية من الاصابة بسرطان البروستات وتضخم البروستات الحميد بنسبة 70% و50 % على التوالي، في حين كان الطراز الوراثي CC مرتبطا 100% بكل من سرطان البروستات و تضخم البروستات الحميد و لم يكن توزيع الطرز الوراثية TT و TC و CC متسقًا مع معادلة هاردي واينبرغ في مرضى سرطان البروستات حيث ظهر فرق معنوي بين القيم الملاحظة والمتوقعة باختبارمربع كاي عند مستوى معنوية P ≥0.05. ، و لم تظهر هذه الاختلافات في المرضى الذين يعانون من تضخم البروستات الحميد أو مجموعة السيطرة. بينم اتأثر مستوى فيتامين د بالطراز الوراثي CC لمستقبل فيتامين د - *FOKI* بشكل ملحوظ في مرضى سرطان البروستات مقارنة بمستوياته في الطرز الوراثية TT وTC . ولم يكن هناك اختلاف في مستوى فيتامين د بين الطرز الوراثية الثلاثة في مرضى BPH ومجموعة السيطرة الاصحاء. لم تظهر الطرز الوراثية تأثيرا على مستويات هرموني الذكورة والحليب بين المجموعات المدروسة. ويمكن الاستنتاج أن تأثيرتعدد الطرز الوراثية لمستقبل فيتامين د- *FOKI* مرتبط بمرضى سرطان البروستات العراقيين أكثر من تضخم البروستات الحميد مع نقص فيتامين د في مصل الدم.

الكلمات المفتاحية: تضخم البروستات الحميد، سرطان البروستات، هرمون الحليب هرمون الذكورة، ، تعدد الطرز الوراثية لمستقبل فيتامين د-*FOK1*