EVALUATION OF THE RELATIONSHIP BETWEEN GENETIC POLYMORPHISMS AND GENE EXPRESSION OF THE KLK3 GENE IN SUSCEPTIBILITY TO BENIGN PROSTATIC HYPERPLASIA IN IRAQI PATIENTS

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Abstract
Prostatic hyperplasia is an anatomical enlargement of the prostate gland that affects men with age and leads to more annoying urinary symptoms than a serious disease. The causes of this disease are not yet fully known, but some androgenic hormones may play a role in this. The testosterone hormone is converted into the compound dihydrotestosterone, and the effect of this compound on the prostate leads to The importance of prostate gland hyperplasia lies in its causing an anatomical obstruction in the neck of the bladder, which controls the mechanism of expelling urine out of the body, and if not treated, it leads to urine retention in the bladder and ureters, where in the final stages, hydronephrosis occurs and enlarged kidneys after obstruction of the urethra, and if this hydronephrosis continues for a long time, it leads to functional insufficiency of the kidneys. The current study aimed to evaluate the role of genetic polymorphism and gene expression in the KLK3 gene at the variant locus of rs2271095, rs11573 is a gene located on chromosome 19 that mainly encodes for enzymes that have a role in prostate endothelial hyperplasia by increasing the gene expression of the gene. The results of the current study showed that there is no genetic heterogeneity at the rs2271095 variant site. The TT homozygous genotype and the T allele are considered a causative factor, with an odds ratio of 2.05 and 1.56, respectively. According to Fisher's probability P=0.666, so this genotype C and its allele C is an etiologic factor according to the odds values of 1.00 and 0.64, respectively, noting that the different proportions for genotypes and their alleles due to the small sample size. As for the differential genotype TC, according to Fisher's probability P=0.205, the genotype CT and allele C is a protective factor for the disease according to the odds values of 0.40 and 0.64, respectively. There are no statistically significant differences between the observed and predicted values of the three genotypes and alleles, with Hardy-Weinberg likelihood values of 0.3437 and 0.0154 in the patient and healthy group, respectively. rs11573 heterozygous site according to Fisher's likelihood (P = 0.268) between patients and healthy people. The homozygous TT and T allele genotypes were considered as an etiologic factor with an odds ratio of 1.96 and 1.48, respectively. According to Fisher's probability P=0.666, so this genotype CC and its C allele is a protective factor for the disease according to the likelihood values of 1.00 and 0.68, respectively. According to Fisher's probability P=0.253, so this genotype CT and its C allele is a protective factor for the disease according to the likelihood values of 0.49 and 0.68, respectively. The results showed that the distribution of the three genotypes of TT, CT, CC and the allelic frequency of the KLK3 gene at the rs11573 T/A/C variant site in the study population according to Hardy-Weinberg's law is balanced, as there are statistically significant differences between the observed and expected values of the three genotypes and the nights, as the Hardy-Weinberg probability value was 0.9926 NS and 0.2001 NS in the patient and healthy group, respectively. The results of the current study show high levels of gene expression of the KLK3 gene in BPH patients compared to healthy people, as the real time PCR results showed a difference in the CT values of the patient group compared to the control group and the CT values were lower in patients compared to the control group and the folding level in patients (9.04±3.91) was higher than healthy people (1.00±0.00) with statistically significant differences (P<0.001***) and high statistical significance (P<0.001***).
Keywords: KLK3 , Single nucleotide polymorphisms , Transversion , Gene

Introduction
Kailkarin are a subset of serine proteases that are proteins capable of breaking peptide bonds in proteins that differ greatly in molecular weight, substrate, gene structure, and immunological properties (Guzel et al., 2014). They are found in different tissues and biological fluids (Kornberg et al., 2018). They are divided into two main categories: plasma kallikreins and tissue kallikreins (Wang et al., 2017). Even before the end of the last century, only three human kallikrein genes (KLK1, KLK2 and KLK3) had been characterized, but new developments have led to the identification of 15 different genes with significant similarities. A number of these genes were independently characterized by several researchers, and different experimental names were initially used to describe them (Diamandis et al., 2000). The Human Genome Organization (HUGO) has proposed guidelines for the nomenclature of human genes. Initially, the organization classified some new genes from the family members of the kallikrein family together with other serine proteases under the prefix "PRSS", meaning "protein serine". However, it is clear that this nomenclature does not serve the needs of the future well because the genes of this family are categorized with other genes located in different locations on the genome map (Yousef and Diamandis, 2001). The construction of the first detailed map showing the position of human kallikrein genes allowed for a more rational use of formal gene symbols (Diamandis et al., 2000). Since the polygenic kallikrein family was recognized in rodents and other animals, an agreement was reached in 1992 by a group of researchers on a standardized nomenclature for the three animal and three human kallikrein known at that time (Berg et al., 1992). Based on this model and the HUGO guidelines, an international group of scientists working in this field agreed to adopt more recent nomenclature for human kallikrein, consistent with those previously identified (KLK1-3), as summarized in Table 2-7 (Diamandis et al., 2000). As mentioned earlier, the only hexokinase-activating enzyme among the human kallikrein gene family is hk1 (Fatma et al., 2020). The biological activity of this enzyme with plasma kallikrein is mainly mediated by the release of kinin (Yeon et al., 2020). It binds to specific

receptors on the cell surface associated with the G protein that mediate various biological functions (Aoun et al., 2015). The kallikrein-kinin system is involved in many disease processes including inflammation, hypertension, kidney and pancreatic diseases, and cancers (Madersbacher et al., 2019). The best studied members of the kallikrein family are hk2 and hk3, which are predominantly synthesized by the prostate epithelium but are also present in the endometrium, breast, and vascular glands in lower concentrations and are found in high concentrations in semen with 1% of the concentration of hk3 (Yeon et al., 2020). Over the past decade, hk3 has been used as a key marker for the early detection of prostate and breast cancer but no evidence has been provided about its biological role (Foster et al., 2019). Recent studies have shown that hk2 and hk3 have an important role in differentiating between prostate cancers and BPH (Rafal et al., 2020). KLK3 is a kallikrein family gene that is mainly expressed in the prostate but is also expressed in some other tissues such as breast, thyroid, salivary glands, lung, and bronchus. This gene was discovered in 1980 (Schwartz and Hulka ., 1990). The gene is located on chromosome 19 in region q13.4, it is approximately 5846 base pairs in size and has a coding orientation from the centromere to the telomere region This gene contains five exons and four introns (Rafal et al., 2020). The KLK3 gene encodes PSA, a single chain of 240 glycoprotein amino acids (Pérez-Ibave et al., 2018). The amino acid sequence of PSA is specific and similar to other members of the kallikrein family with 62% identity with hk1 and 77% identity with hk2. PSA is currently the most important serological test in the diagnosis of prostate cancer and benign prostatic hyperplasia (Nasser et al., 2022). KLK3 gene expression is regulated by steroid hormones and has been extensively studied. Two androgen response elements have been identified in the proximal catalytic region at -170 (ARE-1) and -394 (ARE-2), respectively. These elements have been functionally tested and found to be active in prostate cancer cells (LNCaP). Schuur and colleagues (1996) identified different regions of the 5' sequences of the KLK3 gene around (-6 to -4 kDa) and showed a chimeric androgenic response at position 4136 (3-ARE) that affects PSA translation, and that the hormonal regulation of the KLK3 gene is not specific. 6 to -4 kbp) and showed the presence of a chimeric androgenic response at position 4136-(3-ARE) which affects PSA translation and that hormonal regulation of *KLK3* is not tissue-specific as it was found to be regulated by steroid hormones in normal breast tissue and in a breast cancer cell line (Nasser et al., 2022). KLK3 mRNA transcripts were detected in prostate tissues including normal, enlarged prostate and prostate cancer. By in situ hybridization, *KLK3* mRNA truncations were observed in the secretory/glandular epithelium of the prostate, while basal cells, internal epithelium, stroma and smooth muscle cells were negative. In benign specimens, KLK3 mRNA signals were found to be strong and spread in the cytoplasm in the perinuclear region of secretory cells, while in prostate cancer, the pattern of mRNA distribution and density was found to be heterogeneous in the cytoplasm of secretory cells (Cosma et al., 2017). The cellular expression pattern and density of PSA *parallels the mRNA of the KLK3 gene*, and PSA expression in cancerous tissues showed a heterogeneous pattern while it is uniformly distributed in benign tumors (Lilja et al., 2008).

Materials and Methods

The research investigation was carried out at the University of Diyala in Iraq, in the Molecular Genetics Lab of the Faculty of Education for Pure Science The current study was conducted on a group of patients and healthy people visiting Baquba Teaching Hospital / Consulting Clinics, as blood samples were taken from healthy people and patients with BPH from October 2022 until February 2023, and the number of study samples amounted to 80 samples divided into 50 men with BPH and 30 healthy men. DNA was extracted using the System gDNA Miniprep Blood ReliaPr extraction kit, which was supplied by Bioneers in South Korea. To amplify the *KLK3* gene at the location of variants rs2271095 , mixture for the polymerase chain reaction 1.5 µl forward primer 5"-TGGTTCAGGTCACATGGGGA -"3 , 1.5 microliters of the reverse primer and 5"-CTACCCATGCGTGTGCTCAG-3",andrs11573forward primer 5"TAGCACCGCTTATCCCCTC"-3" and reverse primer 5"-CCAGAACTTTCCCTCTCTCCC -3", 3 µl DNA, 5 µl master mix, and 14 µl free nuclease water. For every sample, the reaction product had a total volume of 25 microliters. The reaction mixture for the samples of healthy people and diabetes patients was then added to the polymerase chain reaction device. The following reaction conditions were programmed into the apparatus: five minutes at 94°C for initial denaturation, thirty seconds at 94°C for denaturation, thirty seconds at 63°C for primer annealing, five minutes at 72°C for extension, and five minutes at 72°C for final extension. This was carried out due to a total of 35 cycles involving primer annealing, denaturation, and extension. After the data from the polymerase chain reaction were collected, the samples were electrophoresed for 1.5 hours at 90 volts on a 1% agarose gel. The amplification product was shipped to Microgen Company in South Korea, where it enabled Sanger nucleotide sequencing of the *KLK3* gene. The Hardy-Weinberg equation was used to ascertain which genotype was a causative factor and which genotype was a protective factor based on the analysis of the nucleotide sequencing data using the Genius application.Quantitative real-time polymerase chain reaction KLK3 genes' relative expression level was measured by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). Beta-actin was used as the reference gene. All primers were designed by Third Author according to the NCBI-primer blast. The sequences of the designed primers are as *KLK3* gene foreword 5"-GAGAGCTGTGTCACCATGTGG -3" and Reverse5"-CCAGGGTTGGGAATGCTTCTC -3"and B-Actin gene Forward 5"-CCCATCACCATCTTCCAGGAGGG-3" and Reverse 5"-CATGCCAGTGAGCTTCCCGTTCA-3". Beta-actin with an amplicon size of 90 bp and annealing temperature 60°C. KLK2: with an amplicon size of 196 bp and annealing temperature 60°C. real-time PCR reactions were performed in 20µl reaction volume containing(0.5µl) of each primer (100pmol/µl),10 µl NEB luna universal qPCR Master Mix, 3 µl cDNA and 6 µl Water RNase-Free. Standard cycling protocol was applied to perform Real-time PCR. Amplification condition included: 10 min at 95°C for Initial Denaturation, 15 s at 95°C for denaturation, 60 s for annealing at the specific temperature for each gene, followed by 15 s at 72°C for extension, with forty cycles. Gene expression assessment was performed based on the $2^{-\Delta\Delta CT}$ method.[15] .

Results:

Amplification product of the *KLK3*gene for the coding segment that includes both and rs2271095 and rs11573 variants in Benign prostatic hyperplasia and healthy controls.The results of *KLK3* gene amplification from mitochondrial DNA Benign prostatic hyperplasia and healthy individuals are displayed in Figure 1. In the event that the amplification findings demonstrate that the variations rs2271095 and rs11573 have a molecular weight of 465 bp at the location of the resultant bands in all patients and healthy individual samples.

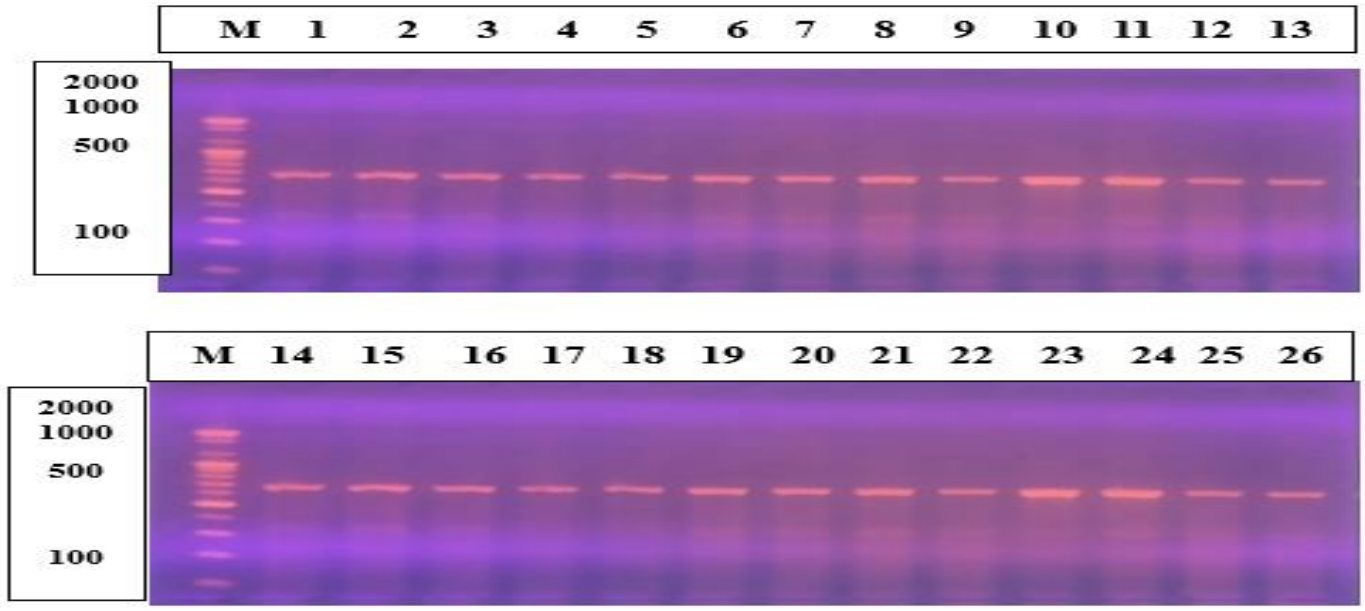

Figure 1: The result of amplification of part of the *KLK3* gene for the coding segment that includes the variants rs2271095 and rs11573 in Benign prostatic hyperplasia of the Dayala population, transferredon agarose gel at a concentration of 1.5% for an hour and a half, at an electrical potential of 90 volts, stained with ethidium bromide dye, and photographed under ultraviolet radiation. The numbers from 1 to 13 represent patient samples, and 14 to 26 represent healthy samples.

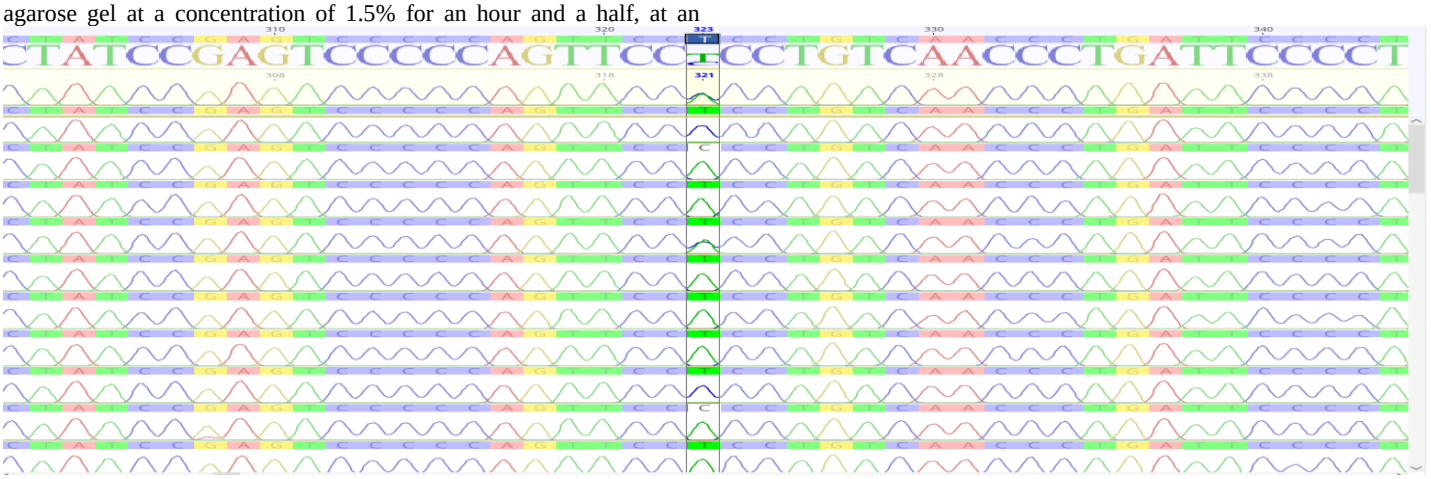

Figure 2: The position of the rs2271095 T/A/C variant and the kind of mutation are displayed by comparing the alignment of the nitrogenous bases of a portion of the *KLK3* gene between samples from Benign prostatic hyperplasia, healthy controls, and the GenBank sample (NCBI, 2023).

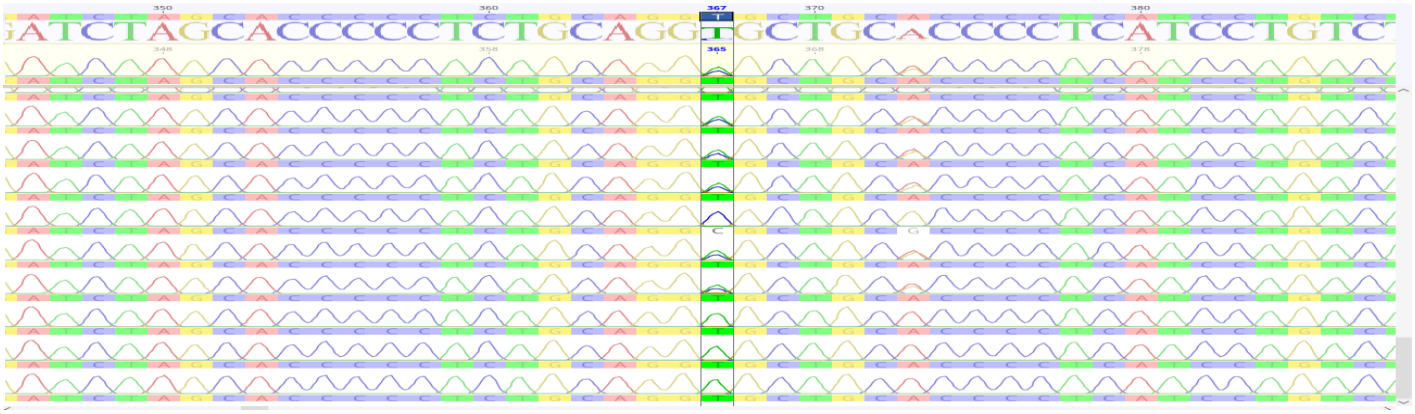

Figure 3: The position of the rs11573 T/A/C variant and the kind of mutation are displayed by comparing the alignment of the nitrogenous bases of a portion of the *KLK3* gene between samples from Benign prostatic hyperplasia, healthy controls, and the GenBank sample (NCBI, 2023).

Table 1. Expected frequencies of genotype and alleles of the coding region rs2271095 T/A/C for *KLK3* by using Hardy-Weinberg equilibrium

Hardy P-values	Allele frequencies		Genotype// rs2271095 T/A/C					Groupe
	C	T	CC	TC	TT	No.	Observed	24 Patients
0.0154**	10	38	3	4	17			
	20.83	79.17	12.5	16.66	70.83	%		
	Not diagnosed		1.04	7.92	15.04	No.	Expected	
			4.34	32.99	62.67	%		
0.3437 NS	14	34	3	8	13	No.	Observed	24 Control
	29.17	70.83	12.5	33.33	54.16	%		
	Not diagnosed		2.04	9.92	12.04	No.	Expected	
			8.51	41.32	50.17	%		

Table 2. Genotype distribution and allele frequency of *KLK3* rs2271095 T/A/C SNPs

N	Patients No. (%)	Control No. (%)	Fisher's/P-value	O.R. (C.I.)
TT	17 (70.83%)	13 (54.16%)	0.253 NS	2.05 (0.54 - 8.06)
TC	4 (16.66%)	8 (33.33%)	0.205 NS	0.40 (0.09 - 1.60)
CC	3 (12.5%)	3 (12.5%)	0.666 NS	1.00 (0.16 - 6.42)
Total	24 (100%)	24 (100%)		
Allele	*Frequency*			
T	38 (79.17%)	34 (70.83%)	O.R. (C.I.) = 1.56 (0.61 - 4.09)	
C	10 (20.83%)	14 (29.17%)	O.R. (C.I.) = 0.64 (0.24 - 1.64)	
*(P≤0.05), NS: Non-Significant.				

Table 3. Expected frequencies of genotype and alleles of the coding region rs11573 T/A/C for *KLK3* by using Hardy-Weinberg equilibrium

Hardy P-values	Allele frequencies		Genotype// rs11573 T/A/C					Groupe
	C	T	CC	TC	TT	No.	Observed	24 Patients
0.2001 NS	13	35	3	7	14			
	27.08	72.92	12.5	29.16	58.33	%		
	Not diagnosed		1.76	9.48	12.76	No.	Expected	
			7.34	39.5	53.17	%		
0.9926 NS	17	31	3	11	10	No.	Observed	24 Control
	35.42	64.58	12.5	45.83	41.66	%		
	Not diagnosed		3.01	10.98	10.01	No.	Expected	
			12.54	45.75	41.71	%		

Table 4. Genotype distribution and allele frequency of KLK3 rs11573 T/A/C SNPs

Genotype// rs2271095 T/A/C	Patients No. (%)	Control No. (%)	Fisher's/P-value	O.R. (C.I.)
TT	14 (58.33%)	10 (41.66%)	0.268 NS	1.96 (0.61 - 6.33)
TC	7 (29.16%)	11 (45.83%)	0.253 NS	0.49 (0.14 - 1.64)
CC	3 (12.5%)	3 (12.5%)	0.666 NS	1.00 (0.16 - 6.42)
Total	24 (100%)	24 (100%)		
Allele	Frequency			
T	35 (72.92%)	31 (64.58%)	O.R. (C.I.) = 1.48 (0.61 - 3.58)	
C	13 (27.08%)	17 (35.42%)	O.R. (C.I.) = 0.68 (0.28 - 1.63)	
*(P≤0.05), NS: Non-Significant.				

Discussion

The nucleotide sequence of the KLK3 gene was analyzed and the results revealed a point mutation of the translocation type for rs2271095 and rs11573 as shown in Figures 2 and 3, respectively, when compared to the original variant locus sequence. The results of the present study showed an association between KLK3 gene polymorphism and the risk of BPH in men from Diyala governorate. The number of patients carrying the TT and T allele genotype was found to be significantly higher in patients compared to the control group. The number of patients carrying the TT homozygous genotype was (17) and the T allele was (38) in the BPH patient group was significantly higher, and the percentages were 70.83 and 79.17, respectively. 83 and 79.17, respectively, compared to the control group (healthy people) with 54.16 and 70.83 and Fisher's exact probability (P = 0.253) between patients and healthy people. The homozygous TT and T allele genotypes were considered as an etiologic factor with an odds ratio of 2.05 and 1.56, respectively. The CC homozygous genotype and C allele showed a significant reduction in the BPH patient group with Odd ratio values of 3, 12.5, and 38, respectively, compared to the control group with Odd ratio values of 3, 12.5, 14, and 12. 17 According to Fisher's probability, the probability value was P=0.666, so this genotype C and its C allele is considered an etiologic factor for the disease according to the odds values of 1.00 and 0.64, respectively, noting that the different proportions for genotypes and their alleles due to the small sample size. The TC and C allele genotypes showed significant reductions in patients with values of (4) 16.66 and (10) 20.83, respectively, compared to the control group with values of (8) 33.33 and (14) 29.17, respectively, according to Fisher probability 0.205 P=, so the CT and C allele genotypes are a protective factor for the disease according to the odds values of 0.40 and 0.64, respectively.The results showed that the distribution of the three genotypes of TT, CT, CC and the allelic frequency of the KLK3 gene at the rs2271095 T/A/C variant site in the study population according to Hardy-Weinberg law is balanced, as there are no statistically significant differences between the observed and expected values of the three genotypes and alleles, as the Hardy-Weinberg probability value was 0.3437 and 0.0154 in patients and healthy people, respectively. In this regard, a 2017 study by Santiago Rodriguez et al.Genetic polymorphisms of several variants of the KLK3 gene, including rs2271095, rs2735839 and rs6998, were significantly associated with PSA concentrations in the blood, and this is consistent with a study by Hui-Yi Li et al. 2021, which showed that polymorphisms of some variants of the same gene, including rs2271095, directly affect PSA values by affecting androgen receptor pathways, and thus is a useful tool for early prediction of prostate tumor. The rs11573 T/A/C heterozygote showed that the observed number of patients carrying the TT homozygote was (14) and the T allele was (53) significantly increased in the BPH patient group according to the mentioned ratios 58.33 and 72.92, respectively, compared to the control group (healthy people), as the values (10) and 41.66 and (31) and 64.58, according to Fisher's probability (P = 0.268) between patients and healthy people. The homozygous TT and T allele genotypes are considered an etiologic factor, with an odds ratio of 1.96 and 1.48, respectively. The homozygous CC and C genotype also showed a slight decrease in the BPH patient group with values of (3) and (12.5) and (13) and 27.08, respectively, compared to the control group with values of (3) and (12.5) and (17) and (35). 42 According to Fisher's probability, the probability value was P = 0.666, so this genotype CC and allele C is a protective factor for the disease according to the likelihood values of 1.00 and 0.68, respectively, and the difference in values between genotypes and alleles due to the small sample size.While the TC and C genotypes showed a significant decrease in patients according to the values (7) and 29.16 and (13) and 27.08, respectively, compared to the control group, the values (11) and 45.83 and (17) and 35.42, respectively, according to the Fisher probability of 0.253 P=, so the CT and C genotypes are a protective factor for the disease according to the likelihood values of 0.49 and 0.68, respectively.The results showed that the distribution of the three genotypes TT, CT, CC and the allelic frequency of the KLK3 gene at the rs11573 T/A/C variant site in the study population according to Hardy-Weinberg's law is balanced, as there are Statistically significant differences between the observed and predicted values of the three genotypes and nights, with a Hardy-Weinberg probability value of 0.9926 NS and 0.2001 NS in the patient and healthy group, respectively. In the same regard, a 2013 study conducted by Santiago Rodriguez et al. in patients with very low PSA showed that genetic polymorphisms of the rs11573 and rs7252245 variants of the KLK3 gene did not significantly differ in the values of the two variants and therefore there was no effect of both variants on low PSA values. Another study by (Nishi Gupta1 et al., 2017) The rs11573 variant was observed in men suffering from infertility. It is known that PSA is the main enforcer of the process of liquefaction of semen, which releases sperm to start their movement towards the egg, and the complete or partial failure of semen liquefaction leads to loss of sperm movement, which causes or contributes to infertility, and the study showed that in addition to semen, there is a protein called sarmentogenin that has a role in fertilizing sperm even if the dilution of semen is normal. A previous study by Yoshida et al (2008) reported a strong association between the KLK7 gene polymorphism and semen viscosity. As for gene expression, the results of the current study show higher levels of gene expression of the KLK3 gene in BPH patients compared to healthy people, as the real time PCR results showed a difference in the CT values of the patient group compared to the control group, and the CT values were lower in patients compared to the control group, and the level of folding in patients (9.04±3.91) is higher than healthy people (1.00±0.00) with significant differences with high statistical significance (P<0.001***). Jasmin et al. 2019 study of prostate cancer patients in which the KLK3 gene was expressed in more than half of the study samples, in the same regard Francesco et al. 2021 study found an inverse relationship between KLK3 gene expression and lymph node activity as well as a decrease in gene expression in patients with myeloid leukemia and advanced prostate cancer patients. Another study showed an inverse relationship between KLK3 gene expression and COL1A1, a gene that encodes collagen, which plays a role in building connective tissue and provides a substrate for prostate tissue (Koo et al. 2015). A decrease in KLK3 gene expression was associated with an increase in the concentration of TGF-beta, a transforming growth factor that secretes a protein that controls cellular proliferation and differentiation and plays an important role in diseases such as lung fibrosis and prostate cancer (Spratt et al., 2019). Another study showed that PSA inhibits the invasive capabilities of tumor cells by supporting the extracellular matrix (ECM) and inhibiting the migration of tumor

cells that derive from the ECM (Cazzaniga W et al., 2016). Considering the complexity of ECM formation and the multiplicity of signals originating from the tumor microenvironment, COL1A1 is an important mediator of ECM signaling that modulates the invasive capacity of cancer cells by activating downstream signaling pathways such as those regulated by FAK, AKT, and PSA (Li M et al., 2020). The ability may hinder the attachment of prostate tumor cells to the extracellular interstitial and thus activate cell adhesion and movement through the extracellular interstitial itself, which explains the high PSA level in both BPH and cancer (Liu X et al., 2015). In line with this study, a 2018 study by Mahal B.A et al. observed a decrease in PSA expression after surgical removal of the tumor. However, the results of our study are not immediately translatable as they are based on semi-quantitative assessments. Future studies should aim to define specific clinicopathological criteria. Nevertheless, our results are of great importance as they clarify at least one of the mechanisms underlying the relationship between PSA levels and extracellular matrix and gene expression.

Conclusions

The risk of benign prostatic hyperplasia in men is associated with genetic polymorphisms of the KLK3 gene at rs2271095 and rs11573. For rs2271095, the TT genotype and T allele is a causative factor and the TC genotype and C allele is a protective factor. For rs11573, the TT genotype and T allele is a causative factor and the CC and TC genotype and C allele is a protective factor.

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