Research Article

Evaluation Microrna-330 Gene Expression In Human Papillomavirus Type 16-Associated Prostate Cancer Patients Using RT-QPCR

Zaid Mahmood Jasim Hussein[1], Fadyia Mahdi Muslim Alameedy[2]

[1,2] Department of Pathological Analysis, Faculty of Science, University of Kufa, Najaf, Iraq.
Email: zaidm.dankji@student.uokufa.edu.iq
Email: fadyiam.alameedy@uokufa.edu.iq

ABSTRACT

Background: Human Papillomavirus (HPV) type 16 is one of the most common sexually transmitted viruses, suggesting a potential long-standing link to prostate cancer. The viral genome has been seen as most demonstrated effect to prostate carcinogenesis and levels of miRNA-330 expression, ignoring the capability of miRNA-330 in tumor-suppression role by regulating cell death and proliferation pathways, Human Papillomavirus type 16 kept the tumor activity. This inapparent functional reversal or equal could lead to different results depending on the potency of the virus to produce the oncogenic proteins, Because of the unknown influence of these factors, given the limited number of studies linking these two factors. **Objective:** the aim of this study is to find the relationship between the Human Papillomavirus type 16 infection and develop of the prostate cancer in the sphere of influence of the microRNA-330. **Methods:** the study has conducted in a cohort of prostate cancer patients after the samples were isolated from the Hospital of the (Middle Euphrates Cancer Center), ranging from 47 to 98 years old (n = 100), and divided into two group (PCa with HPV16 and PCa without HPV16). quantified the levels of viral genome and miRNA-330 were done by RT-qPCR. **Results:** The results showed a positive correlation, with a P-value < 0.05, indicating a clear correlational effect of latent human Papillomavirus type 16 infection. This correlation was further supported by the high gene expression rate of miRNA-330 about 5.27 ± (1.3) in PCa patients with HPV16, while 1 ± (0.22) in PCa patients without HPV16. **Conclusions:** This study provides novel insights into the nature and progression of prostate tumors and the role of miRNA-330 as a biomarker. The study took place in Najaf, Iraq.

Keywords: Human Papillomavirus type 16; Prostate cancer; MicroRNA; miRNA-330; HPV-16 detection; RT-qPCR.

INTRODUCTION

Human Papillomavirus according to the most scientist descriptions, it's recognized as a sexual transmitted infection, that most common in cause cervical cancer [1]. Human Papillomavirus is characterizing with producing a brutal oncogenic protein. Specifically, E6 and E7 [2]. The Basic types of HPV that produces those proteins are called high risk group [3]. Human Papillomavirus type 16 is belonged to this group and it's the almost dangerous type by the causing cervical, prostate, anal and neck cancers additionally to others [4,5]. It belongs to the Human Papillomaviridae family, which is characterized by its small size and lack of envelope [6,7]. The capsid is icosahedral and the genome is double strand DNA with size nearly 8 kilobase [8,9]. Mechanistically, HPV-16 used his oncogenic proteins to inhibition the tumor suppression pathway by interfere with p53 and pRb [10]. It's essential to enable the virus to create a suitable environment for replication and remain dormant for years within patients [11,12].

MicroRNAs (miRNAs) are a small non coding RNA molecule that have a potential role in regulate the post-gene expression and their effect is either up or down-regulation. That's highlighted their present across plants, animals, and viruses. Their structure length is about 22 nucleotides, have emerged as key players in a number of biological processes and disease mechanisms [13]. The pathological disorders as specific case, miRNA can do multiple function in order to achieve balance, whether the medical condition is severe or mild, the productivity or inhibition of stimuli is a priority, especially in cancer where miRNA can function as either tumour suppressor or oncogene. It shows that cancers have been widely linked to the dysregulation of miRNA expression, whether due to genetic mutations, epigenetic modifications, or altered processing [14]. For example, the MicroRNA-330 suppresses the tumour mainly by regulating the expression of genes that promote the development of cancer and by promoting apoptosis to prevent the growth and metastasis of cancer cells [15,16]. Independent PCa studies have demonstrated that microRNA-330 suppresses tumor growth and metastasis by targeting oncogenes such as LEF1 [17]. MicroRNA-330 could theoretically be a critical convergence point for oncogenic development of prostate cancer and human Papillomavirus type 16 infection.

METHODOLOGY

The study is a cross-section study. The study population is divided into two groups, namely prostate cancer patients infected with Human Papillomavirus type 16 and prostate cancer patients not infected with Human Papillomavirus type 16. The study is based on 100 whole blood and serum samples taken from patients with prostate cancer during November 2025 to January 2026, from males with ages varying from 47 to 98 years. Clinical samples were collected from different places in Al-Najaf province, including the Middle Euphrates Cancer Centre. Samples were taken after verbal consent was obtained from all groups. Blood samples were obtained within two minutes from each subject. Two tubes were collected; one EDTA tube and one Gel tube. The contents of each tube were then divided into three equal pieces and transferred into miniature Eppendorf tubes. The EDTA samples were divided into three micro-EDTA tubes and the Gel tube samples were spun to separate the serum. The isolated serum samples were then aliquot into three separate Eppendorf tubes which were coded and labelled carefully to make sure good traceability. After preparation, the samples were put into appropriate containers and transported directly to a refrigerated container filled with ice to ensure their safety throughout travel. These were then carefully transferred to the laboratory and stored at –80°C till needed for Real-Time PCR. One hundred samples of patients with prostate cancer infected with Human Papillomavirus type 16 and a group of patients with prostate cancer not infected with the virus were subjected to a range of procedures.

Real-time quantitative polymerase chain reaction (RTqPCR) Technique

Viral DNA was extracted by using viral nucleic acid extraction kit (gSYNC TM DNA extraction kit, Genaid, Lot No. FA30411-GS, USA). This extraction process was performed at the College of Science, Department of Pathological Analysis, University of Kufa. Real time PCR technique was used to detect the gene of Human Papillomavirus type 16 and molecular expression of microRNA-330 in prostate cancer patients, (GoTaq® qPCR and RT-qPCR Systems, Appendix.2; AddSYBRMaster (2xcon.), AddBio, cat.No.70201, Korea; One Step RT-qPCR SYBR Kit (PCK59, Tinzyme Co., Limited, China) were used for RT-qPCR analysis. The last volume of the mixture was 20 µl. This technique was hanged in Alamin center for advanced research and biotechnology in Al-Najaf province, by using (Analytik Jena\Qtower3G) and in the Najaf Specialized Laboratory, affiliated with Dr. Sabah Al-Fatlawi, by using (Bioeer) device.

Human Papillomavirus type 16 DNA Extraction

Viral DNA was extracted by using viral nucleic acid extraction kit (gSYNC TM DNA extraction kit, Genaid, Lot No. FA30411-GS, USA) following the manufacturer's protocol (see Appendix 1). The procedure was performed according to the following steps:

1. A total of 20 µl of Proteinase K was added to the bottom of a 1.5 ml microcentrifuge tube, followed by the addition of 200 µl of the serum sample.
2. 200 µl of GSB buffer was added into the mixture and shaken vigorously to mix.
3. Heat the mixture to 60°C for 5 min, flipping the tubes over every 2 min to improve the efficiency of Proteinase K digestion and cell lysis.
4. Add 200 µl of absolute ethanol immediately and vortex or shake vigorously for 10 seconds. A precipitate formed was carefully removed by pipette.
5. The entire lysate including insoluble material was loaded on a GS column in a 2 ml collection tube. The sample was centrifuged at 14,000–16,000 × g for 1 min to allow DNA to bind to the column membrane. If the mixture did not pass completely through the membrane, the centrifugation time was extended until complete passage was achieved.
6. Discard flow-through in collection tube and place GS column in a new collection tube (2 ml).
7. Add 400 µl of W1 buffer to the GS column and centrifuge at 14,000–16,000 × g for 30 s. We drained the flow through
8. The GS column was placed into a 2ml collection tube. Then 600 µl of wash buffer (prepared in absolute ethanol) was added. The column was then centrifuged for 30 sec at 14,000–16,000 × g and the flow through discarded.
9. Place the GS column in the 2 ml collection tube and centrifuge for 3 min at 14,000-16,000 × g to dry the column matrix and remove residual ethanol.
10. The dried GS column was transferred to a new 1.5 ml microcentrifuge tube and 100 µl of TE buffer was added directly to the middle of the column matrix. The column was allowed to stand for at least 3 minutes to permit full

absorption of the elution buffer, followed by centrifugation at 14,000–16,000 × g for 30 seconds to elute the purified DNA.
This extraction process was performed at the College of Science, Department of Pathological Analysis, University of Kufa.

RNA Extraction

Total RNA was extracted using TransZol reagent (Cat. No. ET101, TransGen Biotech, China) according to the manufacturer's instructions:

1. 200µl of TransZol was added to 200µl of whole blood sample to homogenized and lysed the sample, after vortex for 3 seconds the sample was incubated for 5 minutes at 25 °C.
2. 40 µl of chloroform was added, and after vortex for 3 seconds the mixture incubated for 3 minutes at 25 °C. and then frozen it for 15 minutes.
3. The samples then centrifuged at no more than 12,000 x g for 15 minutes. The mixture separated into a lower phenol-chloroform phase, an interphase, and an upper aqueous phase. RNA remained entirely in the aqueous phase.
4. The aqueous phase was transferred to a fresh tube, and discarded the lower organic phase. The RNA precipitated from the aqueous phase by added 100 µl of isopropanol. The sample was incubated at room temperature for 10 minutes after mixed it with hand.
5. After centrifuged at no more than 12,000 x g for 10 minutes, the RNA pellet was washed. Then discarded the supernatant and washed the RNA pellet once with 200µl 75% ethanol. The sample mixed by vortex and centrifuged at no more than 7,500 x g for 5 minutes.
6. Carefully aspirated and discarded the ethanol and briefly the air dried the RNA pellet for 5~10 minutes at room temperature. Dissolved RNA in RNase-free water (50µl) and incubated in water bath for 5 minutes, finally stored the samples at -70 °C.
7. The pellet resuspended in 1 mL of 80% ethanol / DEPC Water.
8. Vortexed the sample then centrifuged for 5 minutes at 12,000 × g at 4°C.
9. The supernatant then discarded & air dried the RNA pellet for 5–10 minutes.
10. The pellet resuspended in 50–100 µl of RNase-free water & Stored RNA at -70ºC.

This extraction process was performed at the College of Science, Department of Pathological Analysis, University of Kufa.

Primers Preparation and Design

The primer used in this study was prepared according to the recommendations of the manufactured by dissolving a lyophilized sequences in appropriate volume of nuclease free water to yield 100 pmol/ µl as a stock solution. A working solution was prepared with the final concentration 10 pmol/ µl by dilution methods. These primers were designed based on the NCBI which is for mixed gen for E6-HPV16, microRNA-330 and GAPDH primer (immunity primers) were obtained from OLIGO (Macrogen) company. , as shown in Table (1).

Table (1) the primer design of Human Papillomavirus type 16

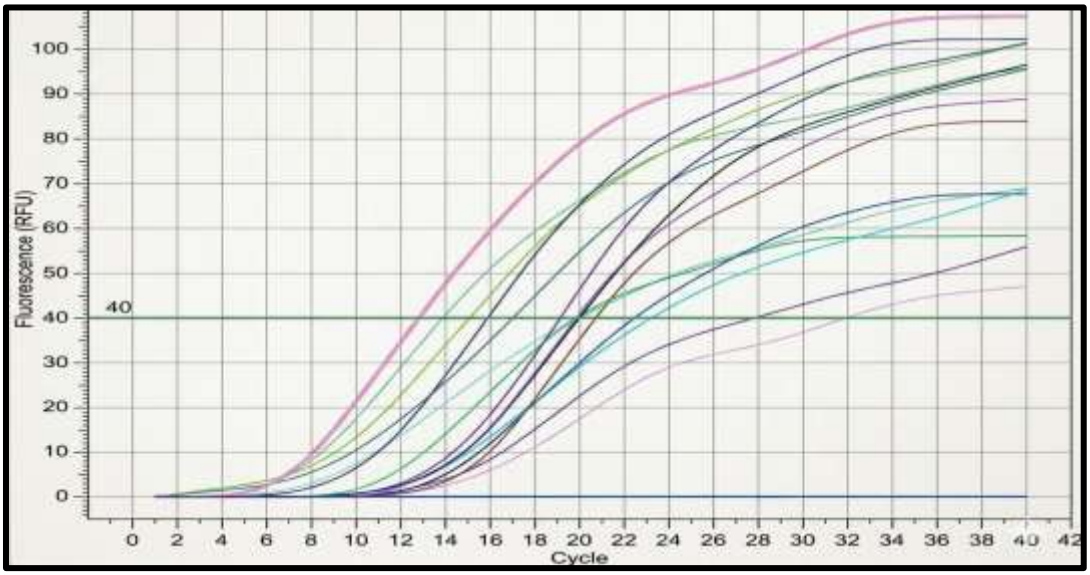
Oligo\Name	Sequence	Length	PCR product size
E6-HPV-16-F	GGCCACTGAGCATCCAAGAA	20 bp	150bp
E6-HPV-16-R	ACTCAAACCTCGCAGACACG	20 bp	
GAPDH-F	CTTTGCAGCAATGCCTCCTG	20 bp	130bp
GAPDH-R	GAAAGGTGGGAGCCTCAGTC	20 bp	
Micro-RNA-330-3p-F	GTCGTATCCAGTGCAGGGT	19 bp	
Micro-RNA-330-3p-R	AACAGAGCAAAGCACACGG	19 bp	
Micro-RNA-330-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTTTTTTTTTTVN	55 bp	

Statistical analysis

The unpaired T-test was used to compare the two groups of patients with prostate cancer (HPV-16 positive and negative). Normality was assessed with the Shapiro-Wilk test. Ct values were not normally distributed, and nonparametric methods were used to analyze differences between groups using the Mann–Whitney U test. The association of miR-330 expression was analyzed in patients with HPV-16-associated prostate cancer by Spearman's rank correlation coefficient. All analyses and visualizations were performed with GraphPad Prism version 10.

Results

The results of RT-qPCR readings detected that (19%) of total patients were infected with Human Papillomavirus type 16, while (81%) weren't infected with the virus.



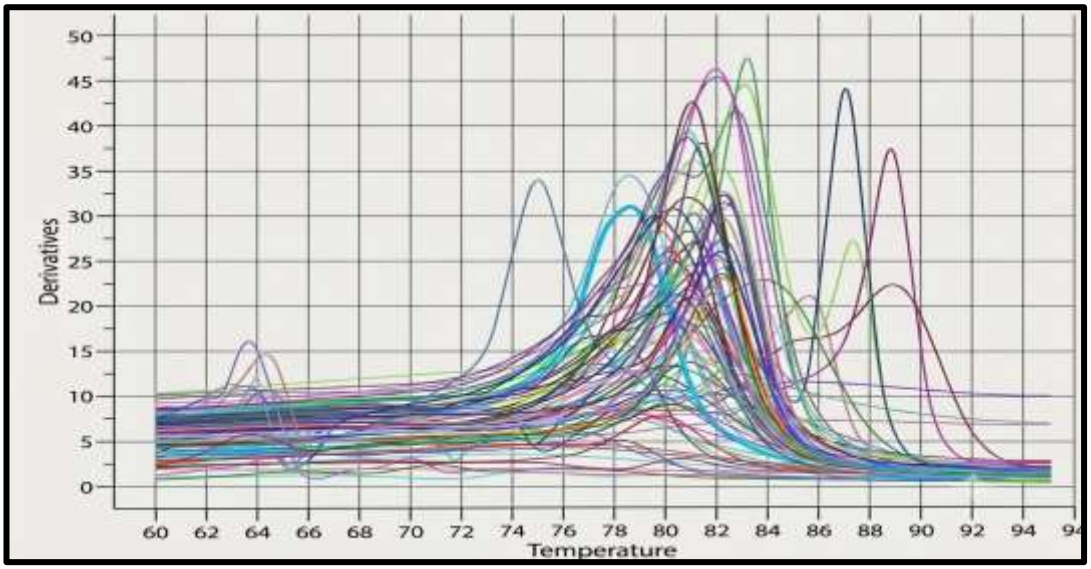


Figure (1) A- The amplification curve

Figure (1) B- The melting curve

Figure (1) Detection of Human Papillomavirus type 16 by RT-qPCR with prostate cancer.

This investigation was carried out using serum samples collected from patients diagnosed with prostate cancer on the basis of a CBC, PSA test and CT scan at the time of collection of the sample.

The gene expression of microRNA-330 was analyzed at the molecular level in order to understand host-gene-virus interaction and molecular regulation. This was achieved by the detection of the regulatory biomarker microRNA-330-3p by reverse transcription quantitative polymerase chain reaction (RT-qPCR). The GAPDH gene was used as the primary normalizing gene for comparison analysis. Fold changes in cytokine expression were determined by the $2^{-\Delta\Delta C_t}$ method, and gene expression levels between the two groups were compared using an unpaired T-test. The results are shown as Standard Error of the Mean (SEM). Statistical analysis methods like the Pearson correlation test were employed for the correlation analysis. [18].

Figure (2), (3) and table (2) illustrate an increase in microRNA-330 expression in HPV-16-positive prostate cancer patients compared to the negative control group. This suggests that regulatory biomarker signaling pathways are depressed in the presence of HPV-16 infection. This indicates that HPV-16 may modulate host immune regulatory responses by altering microRNA expression, potentially contributing to viral persistence or influencing tumor progression. Thus, the virus plays a role in shaping the tumor microenvironment in HPV-16-associated prostate cancers.

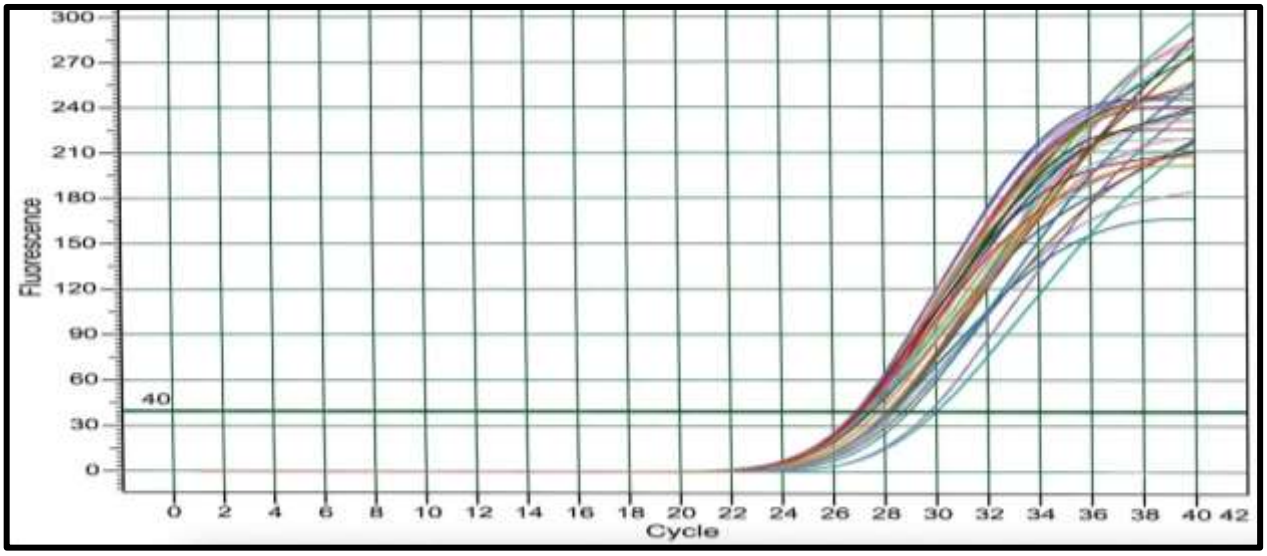


Figure (2) B- The amplification curve

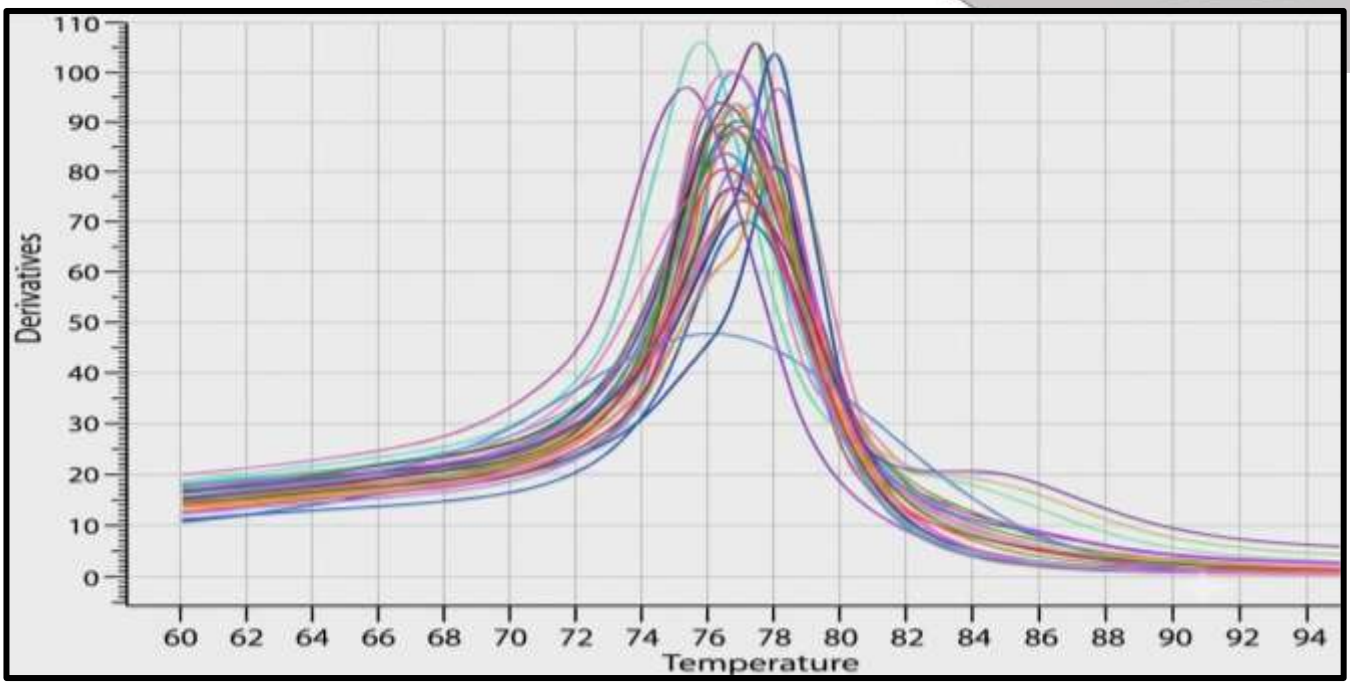


Figure (2) B- The melting curve

Figure (2): Gene expression of MicroRNA-330 and Housekeeping (GAPDH) expression for prostate cancer patients with HPV-16 and prostate cancer patients without HPV-16 by RT-qPCR.

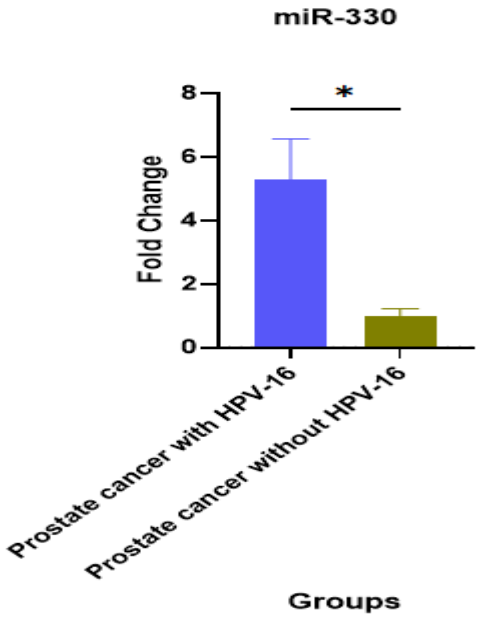


Figure (3): Comparing level of MicroRNA-330 gene expression of prostate cancer patients with HPV-16 and prostate

cancer patients without HPV-16 by RT-qPCR.

Table (2): Alterations in the levels of miR-330 of prostate cancer patients with HPV-16 and prostate cancer patients without HPV-16

microRNA	prostate cancer (positive for HPV-16) mean (SEM)	prostate cancer (negative for HPV-16) mean (SEM)	P value
miR-330	5.27 ± (1.3)	1 ± (0.22)	P< 0.05

The relative expression of miR-330 in HPV-16 positive prostate cancer patients was considerably higher (mean 5.27 ± 1.30 (SEM)) than in HPV-16 negative prostate cancer patients (mean 1.00 ± 0.22 (SEM)). There was a statistically significant difference ($P < 0.05$) and miR-330 expression was significantly increased in the presence of HPV-16 infection.

Discussion

The results obtained in the current study for HPV16 DNA detection were partial agreement with the results of Nellessen and Tsydenova [19,20], however, the authors used prostate tissue samples and polymerase chain reaction (PCR) to detect human Papillomavirus type 16 (HPV-16) unlike the present study. The results in Figure (1) indicate the cycle threshold (Ct) values which reflect the relative viral load in each sample.

The incidence of HPV-16 in this study is likely to be heterogeneous for several tangible reasons, or for more existential reasons, such as regional differences determined by the ethics and behaviors of different countries, cancer etiology, diagnostic methods, viral transmission, or the limited time frame. Also, there are sample-related factors, such as sample type, number and size of participants, and finally, sample preservation and transportation methods, not to mention methodological factors in extraction. Therefore, expanding the regions and increasing the duration of sample collection are likely to increase the prevalence rate, depending primarily on the sample type and collection procedures.

Quantitative RT-PCR analysis for certain study reveals that the expression levels of miR-330 are downregulated in prostate cancer (PCa) tissues, especially in high-grade PCa tissues, compared to normal cells [21]. These results are generally counter with the present study, which a major finding in our results was that expression of miR-330 was significantly higher in HPV16-positive prostate cancers than in HPV16-negative prostate cancers. This suggest that miR-330 could be used as a marker of disease progression

Based on the study's findings, which demonstrated that miR-330 was upregulated in HPV16-positive, the research's perspective suggests that HPV-16 infection may further intensify this elevation. This amplification is likely mediated through the immune viral oncoproteins (E6 and E7) response, which are a mechanic defence against the viral protein which known to disrupt host gene regulation. Consequently, miR-330 is increasing, assessing its expression remains valuable for understanding the specific regulatory influences and diagnostic distinctions associated with HPV16 infection.

Mechanistically, miR-330 functions as a tumour suppressor, likely increasing the inhibition of BMI-1 or ELK1 [22]. Decreased ELK1 inhibits downstream signalling cascades, namely the PI3K-AKT and MAPK pathways, which are key promoters of prostate cancer growth [23,24]. According to [25] a lower BMI-1 also inhibits tumour invasion and growth, among other harmful activities.

Conclusions

The significant upregulation of miR-330 expression in HPV-16 positive prostate cancer patients indicates that HPV-16 might influence the expression of microRNAs in the host. This study found that expression of miR-330 was upregulated by ~5.3-fold, indicating a strong association between HPV-16 infection and dysregulation of miRNA expression in prostate cancer.

The Ethical Committee Approval

The study protocol was evaluated and approved by the Ethics Committee of the Faculty of Science, University of Kufa, Iraq. The investigation and the collection of clinical samples were also approved by the Al-Najaf Health Directorate. The study was conducted according to the ethical standards of the Declaration of Helsinki 2013 for research involving human participants. Informed verbal agreement was obtained from all participants before samples were collected, and all personal information was kept confidential during the study.

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