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Research Article

# Molecular Detection of Human Papilloma Virus 31&33 in Prostate Carcinoma and Prostate Benign Tissues from a Group of Iraqi Patients

Noor Sami Al- Lebawy

*College of Science/Al_Muthana University, Iraq*

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**ABSTRACT**

Background: Human papillomavirus (HPV) has been connected to the progress of different types of human cancers. Prostate cancer is the second most common cause of cancer in men that might have such relation with Human papillomavirus (HPV). Objective: To assess the rates of occurrence HPV infection in the samples of prostate tissues with carcinoma and benign. Patients and methods: Seventy blocks of formalin-fixed, paraffin- embedded prostate tissues were used in present study; (30) biopsies from prostate carcinoma, (20) biopsies from prostate benign tissues and (20) from apparently normal prostate tissues as a control group. Detection of HPV-31 &33 was done by ultra-sensitive version of (ISH) whereas immunohistochemistry (IHC) technique was followed to display the *cdk2* gene expression. Results: Detection of HPV-31&33-CISH reactions in the prostate carcinoma tissues was observed in 17 out of 30 (46.67%), while in those tissues with prostate benign was 35% (7 out of 20). No positive HPV-31 &33-CISH reactions in apparently normal prostate tissues of control group were revealed. The positive CDK-2-IHC reactions was detected in 46.67% (14 out of 30 cases) of prostate carcinoma and 30% (6 out of 20 cases) of benign prostate.The statistical difference between the rates of CDK-2 in prostate carcinoma and prostate benign tissues was highly significant (P value = < 0.0001). Conclusions: Our results indicate that the HPV might contribute to the development of a subset of prostate malignant tumors and benign.

**Keyword:** HPV; prostate carcinoma; prostate benign, CDK-2, ISH, IHC.

## INTRODUCTION

Prostate cancer is a disease that is becoming an important public health concern worldwide[1]. According to the World Health Organization, in 2012, prostate cancer was the second most common cause of cancer in men[2] and the fifth leading cause of cancer death among men, with an estimated 1.1 million new cases diagnosed and 307,000 deaths in 2012[3] In 2016, approximately 180,890 cases of PCa were newly diagnosed and 26,120 deaths have been estimated in the United States[4] Since human papillomavirus (HPV) infection was first identified as a risk factor for cervical cancer, several studies have investigated HPV in relation to prostate cancer with mixed results[5]. When Taylor and Mainous combined the results of ten of these studies, they observed a significant positive association between HPV and prostate cancer[6]. HPV types which are associated with these mucocutaneous sites are classified as high risk (HR) or low risk (LR) depending on their ability to cause cancer. HPV16, 18, 31, 33 and 45 are HR types commonly associated with malignancies[7], The E6 and E7 oncoproteins encoded by HR HPV types are responsible to immortalize human keratinocytes through inactivation of p53 and pRB tumour suppressor proteins, respectively[8]. The E6 and E7 proteins are consistently expressed in cancer cells and inhibiting their expression blocks the malignant phenotype[9].

Cyclin-dependent kinases (CDK/Cyclins) form a family of heterodimeric kinases that play central roles in regulation of cell cycle progression, transcription and other major biological processes including neuronal differentiation and metabolism. Constitutive or deregulated hyperactivity of these kinases due to amplification, overexpression or mutation of cyclins or CDK, contributes to proliferation of cancer cells, and aberrant activity of these kinases has been reported in a wide variety of human cancers[10].

Cell proliferation is mediated by several signaling molecules and checkpoints (CDKs) that regulate cell division. The progression through the cell cycle is positively regulated by cyclins (D and E)/cyclin-dependent kinases (CDK4, CDK6, and CDK2) complex[12]. Cyclin dependent kinases (Cdks), a family of serine/threonine kinases, faithfully control the mammalian cell cycle by binding to cyclins[12]. Under normal circumstances, D-type cyclins activate Cdk4 and/or Cdk6 and initiate phosphorylation of Retinoblastoma protein (Rb) family early in the G1 phase[13]. This leads to the release of E2F transcription factors and results in activation of transcription of E2F responsive genes required for cell cycle progression[14]. In the late G1 phase, cyclin E activates Cdk2[15] and completes the phosphorylation of Rb leading to passage through the restriction point at the boundary of the G1/S phase, and to S phase initiation. Later Cdk2 plays an

**Author for Correspondence:noorsamiel15@yahoo.com*

important role in S phase progression by complexing with cyclin A. Finally Cdk1/cyclin B complexes actively participate and complete mitosis[16].

## MATERIALS AND METHODS

The study was designed as a retrospective one. It has recruited 70 selected formalin fixed, paraffin embedded prostate tissue blocks; among them, (30) tissue biopsies from prostate carcinoma with different grades as well as (20) benign prostate and 15 apparently healthy prostate tissues as a negative control group for this study. The diagnosis of these tissue blocks were based on their accompanied records.

Following trimming process, a consultant pathologist reexamined all these prostate tissues to further confirm the diagnosis. One section was mounted on ordinary glass slide and stained with hematoxyline and eosin, while another slide was mounted on charged slide to be used for ISH for detection of HPV31&33.

The detection of HPV31&33 by CISH kit (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany) was performed on 4µm paraffin embedded tissue sections using digoxigenin-labeled oligo-nucleotides probe which targets HPV31&33Virus- DNA. For the *in situ* hybridization procedure, the slides were placed in 60c hot-air oven over night then the tissue sections were de-paraffinized and then treated by graded alcohols according to the standard methods and the details of processes for performing ISH reaction with this probe were applied according the instructions of the manufacturing company (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany). The main steps for ISH procedure were: incubation of slides for 18 hr at 70°C on hot plate, then rehydration process was done at room temperature which include: slides immersion in two changes of absolute ethanol for one minute each, then immersion in ethanol (95%) for one minute each, after that immersed in ethanol (70%) for one minute each, finally immersion in a distilled water for 5 minutes to remove residual alcohol. After that, slides were allowed to dry completely by incubating them at 37°C for 5 minutes.

Then a routine dewaxing protocols were used; 2-5 min xylene, 2-5 min 100% ethanol, 2-5 min 96% ethanol, 1-5 min 70% ethanol. Air drying of sections. Then pepsin solution application to the tissue sections and incubated for 20-30 min at 37°C in a humidity chamber. After that immersion slides in distilled water and drain off the water, air dried sections. Then addition of the probe and denaturation of the slides at 75°C for 5 min on hot plate, then transferred the slides to a humidity chamber and hybridize for over night at 37°C and then post-hybridization and detection processes that included removing the cover slip by submerging in 1x wash buffer TBS, then washed for 5 min in 1x wash buffer TBS at 55°C. Then application of Rabbit-anti-DIG - antibodies to the slides and incubate for 90 min at 37°C in a humidity chamber.T hen slides were rinsed in detergent wash buffer for 5 minutes (twice times) and then drained. Then application of Anti-Rabbit-AP-Polymer drop- wisely to the slides and incubate for 90 min at 37°C in a humidity chamber. Then slides were rinsed in detergent wash buffer for 5 minutes (twice times) and then drained. After that one to two drops of 5-bromo3-chloro3-indoly/phosphate/nitro blue tertrazolium substrate-chromogen solution (BCIP/NBT) were placed on tissue sections. Slides were incubated at 37°C for 2 hours or until color was developed completely. Color development was monitored by viewing the slides under the microscope. A dark blue colored precipitate forms at the complementary site of the probe in positive cells. Then the slides were rinsed in distilled water for 5 minutes, then counter staining process by immersion of the slides in Nuclear Fast Red stain for 90-120 seconds, then washing process was followed by immersion the slides for 1 minute in tap water. Sections were dehydrated by ethyl alcohol, (95%, once for one minute then, 100% twice times for 2 minutes each); cleared by Xylene, then mounted with permanent mounting medium (DPX).

Chi –square test and T- test were used to detect the significance between variables of our study. All the statistical analysis was done by SPSS program (Version–17) & P value was considered significant when $p < 0.05$.

## RESULTS

*Distribution of patients with prostate carcinoma, prostate benign and control according to their age*

The archival specimens collected in this study were related to prostate cancer patients whom ages were ranged from 17 -58 years and the mean age of those prostate cancer patients was (40.9 ± 11.03 ) years.the mean age of patients with prostate benign was (41.2± 9.16) years and whom age ranged

Table 1: Distribution of Study Groups According to the Mean and Range of their Age (Years).

| Studied groups | No | Mean Age / Year | Std. Deviation | Std. Error | Range | | T. test | LSD test (P-value) |
|---|---|---|---|---|---|---|---|---|
| | | | | | Min. | Max. | | |
| Prostate Carcinoma | 30 | 40.90 | 11.034 | 2.015 | 17 | 58 | 5.698 | $P^1$=0.00 HS |
| Prostate Benign | 20 | 41.25 | 9.164 | 2.049 | 28 | 57 | 4.675 | $P^2$=0.00 HS |
| Control | 20 | 60.45 | 11.628 | 2.600 | 37 | 75 | -0.105- | $P^3$= 0.917 NS |
| Total | 70 | | | | | | | |

Note: $P^1$= Control Vs Prostate Carcinoma, $P^2$= Control Vs Prostate Benign, $P^3$= Prostate Carcinoma Vs Prostate Benign.

Table 2: Statistical Analysis for the Distribution of Age Strata According to the Histopathological Diagnosis of Studied Groups.

| Age groups /Year | | Studied groups: prostate carcinoma | Studied groups: Prostate Benign | Pearson Chi-Square (P-value) |
|---|---|---|---|---|
| ≤ 20 | No. | 1 | 0 | |
| | % | 3.3% | 0% | |
| 21 – 40 | No. | 15 | 9 | P=0.359 |
| | % | 50% | 45% | Non sign. |
| 41 – 60 | No. | 14 | 11 | (P>0.05) |
| | % | 46.7% | 55% | |
| Total | No. | 30 | 20 | |
| | % | 100% | 100% | |

* Non-Significant differences using Pearson Chi- square test at P>0.05 level.

Table 3: Results of *In Situ* Hybridization for Detecting HPV31&33 –CISH in Tissues with Prostate Carcinoma and Benign

| HPV 31&33 | | Studied groups: Prostate Carcinoma | Studied groups: Prostate Benign | Pearson Chi-Square (P-value) |
|---|---|---|---|---|
| Negative | No | 13 | 13 | |
| | % | 43.33% | 65% | P=0.155 |
| Positive | No. | 17 | 7 | Non sign. |
| | % | 46.67% | 35% | (P>0.05) |
| Total | No. | 30 | 20 | |
| | % | 100% | 100% | |

* Non-Significant differences using Pearson Chi- square test at P>0.05 level.

Table 4: Distribution of HPV31&33 Signal Scoring Associated with Prostate Carcinoma and Benign by using CISH Technique

| HPV 31&33 Scoring | | Studied groups: Prostate Carcinoma | Studied groups: Prostate Benign | Pearson Chi-Square (P-value) |
|---|---|---|---|---|
| Negative | No | 13 | 13 | |
| | % | 43.33% | 65% | |
| Low | No. | 6 | 5 | P=0.155 |
| | % | 20% | 25% | Non |
| Moderate | No. | 7 | 1 | sign. |
| | % | 23.34% | 5% | (P>0.05) |
| High | No. | 4 | 1 | |
| | % | 13.33% | 5% | |
| Total | No. | 30 | 20 | |
| | % | 100% | 100% | |

from28 - 57 years,while the mean age of appearently healthy individuals (control) was (60.45±11.62) years and their mean age was ranged from 23 - 68 years and the statistical analysis shows highly significant differences (P<0.01) between age strata distribution of

Table (5): Distribution of HPV31&33 Signal Intensity Associated with Prostate Carcinoma and Benign by using CISH Technique

| HPV 31&33 Intensity | | Studied groups: Prostate Carcinoma | Studied groups: Prostate Benign | Pearson Chi-Square (P-value) |
|---|---|---|---|---|
| Negative | No. | 13 | 13 | |
| | % | 43.33% | 65% | |
| Weak | No. | 3 | 1 | P=0.467 |
| | % | 10% | 5% | Non |
| Moderate | No. | 5 | 6 | sign. |
| | % | 16.67% | 30% | (P>0.05) |
| Strong | No. | 9 | 0 | |
| | % | 30% | 0% | |
| Total | No. | 30 | 20 | |
| | % | 100.0% | 100.0% | |

Table 6: The Percentage of CDK2-IHC Score Signaling in Prostate Carcinoma

| CDK-2 signal scoring | | Prostate Carcinoma: No. | Prostate Carcinoma: % | P-value |
|---|---|---|---|---|
| Negative | | 16 | 53.33 | $\chi^2$test |
| Positive | | 14 | 46.67 | P=0.00 |
| Scoring | Low | 5 | 35.71 | Highly |
| | Moderate | 5 | 35.71 | sign. |
| | High | 4 | 28.58 | (P<0.01) |

Table 7: The Percentage of CDK2-IHC Intensity Signaling in Prostate Carcinoma

| CDK-2 signal intensity | | Prostate Carcinoma: No. | Prostate Carcinoma: % | P-value |
|---|---|---|---|---|
| Negative | | 16 | 53.33 | $\chi^2$test |
| Positive | | 14 | 46.67 | P=0.00 |
| Intensity | Weak | 2 | 14.29 | Highly |
| | Moderate | 5 | 35.71 | sign. (P<0.01) |
| | Strong | 7 | 50.00 | |

control and prostate carcinoma; and control and prostate benign. However there was non-significant difference between prostate carcinoma and prostate benign as shown in Table (1). In prostate carcinoma, the most affected age stratum 21 - 40 was constituting (50%:15) followed by the age stratum of 41 - 60years (46.7%:14) and lowest affected group was the age stratum of less than 20 years which constituting (3.3%:1).while in prostate benign the most affected age stratum of 41 - 60 was constituting (55%:11) followed by the age stratum of 21 - 40 years (45%:9). The statistical analysis shows non-significant differences (P>0.05) among age strata distribution of those studied groups prostate carcinoma and prostate benign as shown in the Table (2).

Table 8: The Percentage of CDK2-IHC Score Signaling in Benign Prostate

| CDK-2 signal scoring | | Benign Prostate No. | Benign Prostate % | P-value |
|---|---|---|---|---|
| Negative | | 14 | 70 | $\chi^2$test P=0.00 Highly sign. (P<0.01) |
| Positive | | 6 | 30 | |
| Scoring | Low | 2 | 33 | |
| | Moderate | 3 | 50 | |
| | Strong | 1 | 17 | |

Table 9: The Percentage of CDK2-IHC Intensity Signaling in Benign Prostate

| CDK-2 signal intensity | | Benign Prostate No. | Benign Prostate % | P-value |
|---|---|---|---|---|
| Negative | | 14 | 70 | |
| Positive | | 6 | 30 | $\chi^2$test P=0.00 Highly sign. (P<0.01) |
| Intensity | Weak | 2 | 33 | |
| | Moderate | 3 | 50 | |
| | Strong | 1 | 17 | |

*Histological grading of prostate carcinoma.*

The most elevated percentage of histological grades of the studied prostate carcinoma was grade I (50%: 15), followed by grade II (33%:10), while the less percentage was grade III (17%:5). A significant differences (P<0.05) was seen as in Figure (1).

*Screening- HPV31&33-CISH Test*

It was found after application and analysis of (ISH) for detection of HPV31&33 DNA in the tissues obtained from patients with Prostate Carcinoma as well as benign prostate hyperplasia that seventy (17) out of thirty (30) patients with carcinoma of prostate showed positive *in situ* hybrization reaction where it constituted 46.67% (Table 3 and Figure 2). In prostate benign tumors, the percentage of positive tissues was 35% (7 out of 20) for screening HPV31&33-CISH test. None of control group presented positive signals for HPV31&33-ISH test However, in comparison to the percentage of HPV31&33 in healthy control group as well as in the group of benign Prostate hyperplasia, the differences between the percentages of HPV31 & 33 in tissues of patients with Prostate cancers and each of these above mentioned groups are statistically nonsignificant (P value >0.05).

*Positive HPV31&33 - CISH Signal Scoring:*

The percentage of Prostate Carcinoma and Benign with high score signaling HPV31&33-CISH test were (13.33%:4) and (5%: 1), respectively .While, prostate carcinoma and benign with a moderate score were (23.34%:7) and (5%: 1), respectively, whereas the low score were (20% :6) and (25%: 5) in prostate carcinoma and benign, respectively.Statistically, the differences between the viral DNA-CISH score in prostate carcinoma and prostate benign were non significant at the 5 percent level (P>0.05) as shown in Table (4) & Fig. (2).

*Positive HPV31&33 - CISH Signal Intensity*

The percentage of prostate carcinoma and benign with strong intensity signaling HPV31&33-CISH test were (30%:9) and (0%: 0), respectively .While, prostate carcinoma and benign with a moderate intensity were (16.67%:5) and (30%: 6), respectively, whereas the weak Intensity were (10% :3) and (5%: 1) in prostate carcinoma and benign, respectively.Statistically, the differences between the viral DNA-CISH intensity in prostate carcinoma and prostate benign were non significant at the 5 percent level (p>0.05) as shown in Table (5) & Fig. (2).

*The Results of CDK2-IHC Score Signal in Prostate Carcinoma.*

Table (6) display the positive result of cdk2-IHC detection where was 46.67 % (14 out of 30 cases) from prostate carcinoma group showed positive signals including 35.71 % (5 out of 14 cases) in the low and moderate score followed by 28.58 % (4 out of 14 cases) in the high score Fig. (2).Statistically, highly significant differences between negative, low, moderate and high scoring cases at 1 percent level (P<0.01).

*The Results of CDK2-IHC Intensity Signal in Prostate Carcinoma.*

Table (7) presents the positive result of cdk-2-IHC detection where as 46.67 % (14 out of 30) of prostate carcinoma exhibits positive signals including 50% (5 out of 14 cases) in the strong intensity, followed by 35.71% (5 out of 14 cases) in the moderate intensity and 14.29% (2 out of 14 cases) in the weak intensity Fig.(3).statistically, highly significant differences between negative, weak, moderate and strong intensity cases at 1 percent level (p<0.01) in prostate carcinoma group.

*The Results of CDK2-IHC Score Signal in Benign Prostate.*

Table (8) display the positive result of CDK2-IHC detection where was 30 % (6 out of 20 cases) from benign prostate group showed positive signals including 50 % (3 out of 6 cases) in the moderate score followed by 33% (2 out of 6 cases) in the low score, while 17 % (1 out of 6 cases) in the strong score Fig.(3).Statistically, highly significant differences between negative, low, moderate and high scoring cases at 1 percent level (P<0.01).

*The Results of CDK2-IHC Intensity Signal in Benign Prostate.*

Table (9) presents the positive result of cdk-2-IHC detection where as 30 % (6 out of 20) of benign prostate exhibits positive signals including 50% (3 out of 6 cases) in the moderate intensity, followed by 33% (2 out of 6 cases) in the weak intensity and 17% (1 out of 6 cases) in the strong intensity Fig.(3).statistically, highly significant differences between negative, weak, moderate and strong intensity cases at 1 percent level (p<0.01) in benign prostate group.

## DISCUSSION

Prostate carcinoma is the most commonly diagnosed cancer in men. However, the etiology and molecular pathobiology of PCa is still not clear. The viral etiology of prostate carcinogenesis, which includes environmental,

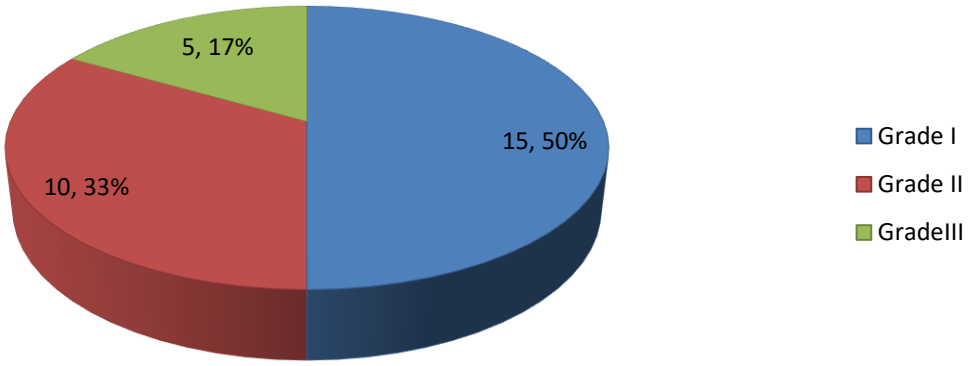


Fig 1: Frequency of histological grades of the studied malignant prostate tumors.

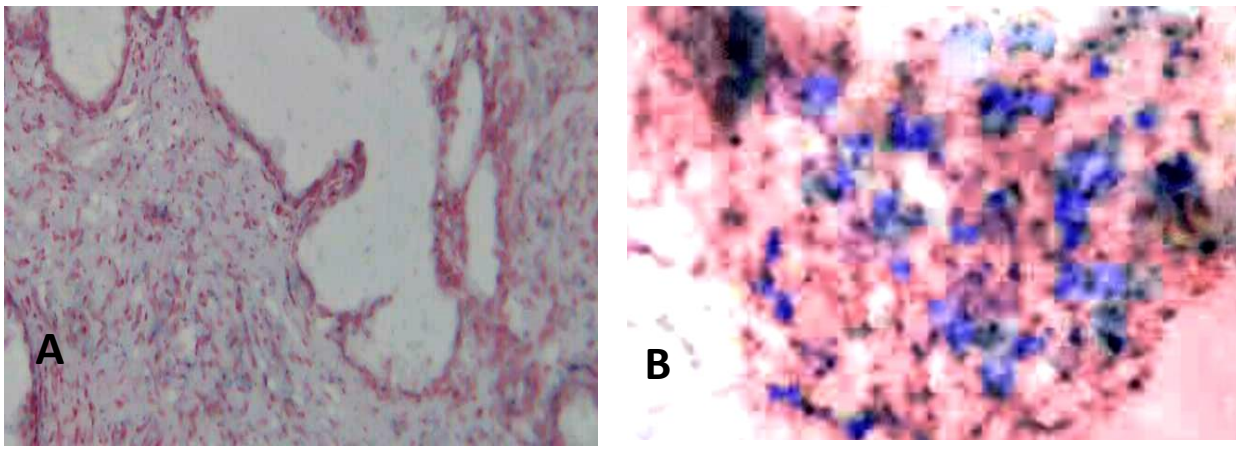


Fig 2: In situ hybridization results for Human Papilloma virus 31-33 (HPV31\33) DNA- detection in prostate tumors; BCIP/NBT stained and counter stained by nuclear fast red; A. prostate cancer with Negative ISH reaction for HPV-331\33 (40X); B. prostate cancer with positive ISH reaction for HPV-331\33 (40X)

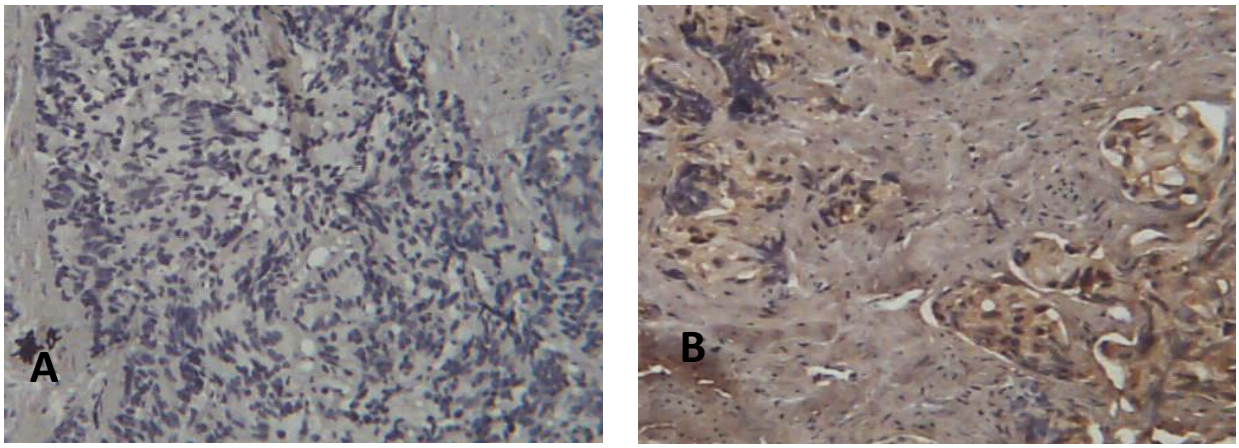


Fig. 3: Immunohistochemical results for CDK2 gene expression detection in prostate tumor; DAB chromogen stained (brown) and counter stained by Mayer's hematoxyline(blue); A.Prostate cancer with positive IHC reaction (10X); B. Prostate cancer with positive IHC reaction (40X).

endogenous and genetic risk factors as well as HPV, is controversial[16].

Epidemiological and biological studies have now conclusively proved that a variety of infectious agents are the major causes of cancers worldwide[18]. In the last two decades, at least six different viruses have been linked to the development of specific types of human cancers. HPV, one of the most important infectious agents, has been shown to be linked to prostate cancer, arousing research interests in male genital and urinary systems. The inflammation resulting from sexually transmitted

infections in the course of carcinogenesis has been speculated[19]

Table (1, 2) showed that in both prostate carcinoma and benign, tumors have increased with the proceeding of age of patients and our results are closely agreed with the results obtained by Zhou[3], they revealed that the most common age of diagnosis in PC is between 24 and 43 years old. The present study is in agreement with study conducted by Johansson *et al*.,[20] and Singh *et al*.,[21], who found that the incidence of prostate carcinoma increased as the age of men increased.

The highest percentage and number of prostate carcinoma patients was seen in grade I (50%:15) followed by gradeII (33%:10) and the lowest was in gradeIII (17%:5). The present results could mark for the occurness of low grade of prostate carcinoma in Iraqi patients at earlier age than that expected worldwide. This discrepancy could be attributed or as the result of small sampling in the present study, as compared to other abroad studies. These results also call for more research works into the reasons for the prevalence of this low grade prostate carcinoma in our country. The present results are in agreement with the results by Johansson *et al*. in Sweden, who had documented a majority of prostate carcinoma in this country of low-grade typed (40%) (20). Also, the results of current study is in broadly agreement with the results of study conducted by Ankerst *et al*.[22] who found that 78.76% of prostate carcinoma patients in low grade and 21.23% of them in high grade. In addition, the patients often present themselves to the medical care system at much later stages of the diseases where the low grade prostate carcinoma have evolved into secondary type of high grade once.

The percentages of positive cases of HPV-31 and 33 in prostate carcinoma and benign patients' tissues in present study were 56.76% and 35% respectively as showed in (Table.3). The present study may broadly constitute with the results obtained by several researchers. Singh *et. al.*[21] reported that the percentages of positive cases of HPV in prostate carcinoma and benign patients tissues in were 41% and 20% respectively. In addition, Martinez-Fierro *et al*.,[23] evaluated the presence of viral HPV DNA in prostatic cancer patient's tissues and found a significant positive association between HPV and risk of prostate cancer. Also, The results of study conducted by Adami *et.al*.[24] showed an association between serological evidence for HPV 33 infection and risk for prostate cancer. The risk was significantly elevated for subjects with high antibody levels against HPV 33. The detection rate of DNA of HPV in benign prostatic hyperplasia (BPH) is comparable to that in prostate carcinoma. While there are many explications for the presence of HPV DNA in prostate tissues, the HPV infection etiological role in prostate cancer remains to be recognized[25]. Moreover, the results of study carried out by Glenn *et. Al*[26] confirmed that high risk HPVs are present in benign prostate tissues prior to the development of HPV positive prostate cancer in the same patients. However, in contrast to the results of this study Korodi *et. al.,*[27] revealed that there is no significant an association between serologic marker of HPV-33 infections and risk of prostate cancer. Similarly, Sutcliffe *et. al.*[5] revealed that HPV 31 does not appear to be connected with prostate carcinoma risk, at least by mechanisms anticipated to date, and using laboratory techniques and epidemiologic designs currently available.

The percentage of positive CDK2 in prostate cancer and prostate benign patient's tissues as shown table (6 and 8) indicates a close relationship between HPV31and33 and CDK2 and this due to Cyclin E/CDK2 expression enhanced HPV replication[28]. In addition, E7 oncoproteins which produced by HPV induced CDK2 kinase activity[29]. Lu, *et.al*.[30] revealed that increased CDK2 kinase activity upon androgen stimulation lead to Malignantly stimulates proliferation of prostatic epithelial cells and constitutes one possible mechanism of androgen-dependent tumorigenesis. Also, the increased CDK2 activity elevates poor outcome two- to fivefold in specific tumors, including prostate cancer[31].

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