Original Article

Detection of Human Papilloma Viruses type 16 and type 18 in patients with transitional cell carcinoma of the bladder by *in situ* hybridization

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Summary:

Background: Transitional cell carcinomas (TCC) of the bladder are a major health problem. Recently, some studies link high risk Human papilloma viruses' type 16 and type 18 with bladder carcinoma.

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Materials and methods: Fifty formalin fixed, paraffin embedded tissues with TCC of the bladder from Specialized Surgical Hospital in Baghdad were included in this study. In addition, ten apparently normal bladder autopsies were collected from the Forensic Medicine Institute Archives and used as control group. Tissue blocks were sectioned and sticked on charged slides and used for the detection of HPV-16 and HPV-18.

Results: The expression of HPV-16 and HPV-18 DNA signals in TCC of the bladder tissues in the present study was 36% (18 out of 50) and 14% (7 out of 50) respectively, where strong correlation was found between expression of HPV-16 and TCC of the bladder while no correlation with HPV-18.

Conclusion: HPV-16 and HPV-18 might have an important role in the course of TCC of the bladder.

Key word: Transitional cell carcinomas of the bladder, High risk HPV, Carcinogenesis.

Introduction

Urinary bladder cancer is one of the most common cancers worlds wide, with the highest incidence in industrialized countries [1]. More than 90% of bladder cancers are transitional cell carcinomas (TCC). About 5% of bladder cancers are squamous cell carcinomas (SCC). There are also uncommon bladder cancers, such as adenocarcinoma and small cell carcinoma, which are responsible for less than 2% of all bladder cancers [2].

Transitional cell carcinoma of the urinary bladder is the second most common tumor of the genitourinary tract. It is also the second most common cause of death from these cancers [3]

Many agents including radiation, chemicals and viruses, have been found to induce human cancer [4]. Viral factors are the most important class of the infectious agents associated with human cancers [5]. It was estimated that 17-20 % of world wide incidence of cancers attributable to a viral etiology [6]. Human papilloma viruses (HPVs) are DNA viruses that have specific tropism for squmous epithelia [7]. To date, more than 100 types of HPVs have been reported, which are classified as low-risk and high-risk types according to their associations with malignant tumors. High-risk HPVs encode two oncogenes, E6 and E7, which play important role in carcinogenesis. E6 has two zinc finger domains and interacts with tumor suppressor p53 and degrades it to escape from apoptosis and to disrupt cell cycle checkpoint machinery [8].

It is clear that continued expression of the viral oncogenes is necessary for histopathologic progression and the malignant phenotype of an HPV-associated tumor [9]

So this study aims to detect HPV type 16 and type 18 DNA signals using ISH in TCC of the bladder and study the correlation between HPV-16 and -18 expression and different clinicopathological variables like: age, gender, grade and pattern of growth and presence or absence of muscle invasion in transitional cell carcinoma of the bladder.

Materials and Methods:

Patients and tissue samples. Fifty patients with bladder carcinoma, 35 (males) and 15 (females), with an age ranged from 25 to 70 years, were included in this retrospective study, The patients samples were collected during the period from February 2009 till June 2009 from the archives of histopathology laboratories of Specialized Surgical Hospital in Baghdad, related to the records of March 2008 to June 2009.

The diagnosis of these tissue blocks were primarily based on the obtained histopathological records of bladder biopsy samples in the hospital laboratory. Confirmatory histopathological re-evaluation of each obtained tissue blocks was done by specialist

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pathologist. In addition ten apparently normal bladder autopsies were collected from the Forensic Medicine Institute Archives. They were 5 (males) and 5 (female) and range of the age was the same patients group. Formalin-fixed, paraffin embedded tissue blocks were sectioned (4µm) thickness, one section was stained with Haematoxylin and Eosin, 2 sections were mounted on charged slides to be used for *In situ* hybridization, for the detection DNA of HPV type 16 and -18.

In situ hybridization procedure. The slides were placed in oven at 60˚C over night to deparaffinized tissue sections. The slides were dehydrated by graded alcohol concentration (100%, 95% and 70%) and distal water then treated with proteinase K solution and dehydrated. One drop of the biotinylated long cDNA probe for HPV-16 and HPV-18 was placed on them (Maxim Biotech Cat. No.: IH-60058 and IH-60059). Hybridization/ detection kit were used purchased from Maxim Biotech/USA Cat. Number IH-6001(IHD-0050) was placed on the tissue section in oven at 95˚C for 8-10 minutes to denature the double strands of DNA. The slides were then placed in a humid chamber and incubated over night at 37°C to allow hybridization of the probe with the target nucleic acid. The slides were soaked in protein block at 37˚C until the cover slips falls, and then treated with streptavidin-alkaline phosphatase-conjugate. One to two drops of bromo-chloro-indolyl phosphartel /nitro blue tetrazolium substrate-chromogen solution (BCIP/NBT) were placed on tissue section at room temperature for about 30 minutes, the latter was monitored by viewing the slides under the microscope. A blue colored precipitate will form at the site of the probe in positive cells. Slides were then counterstained using nuclear fast red and sections were mounted with a permanent-mounting medium (DPX). Finally the examination under light microscope by a pathologist at power 400.

Statistical analysis was done using Chi-Square test for tables with frequencies percentages, range, mean and standard deviation. Values were considered statistically significant when $p<0.05$.

Results:

Histopathpological classification. Fifty formalin-fixed, paraffin embedded tissue blocks were collected from bladder carcinoma patients and histopathologial re-examination with hematoxylin and eosin stain was done.

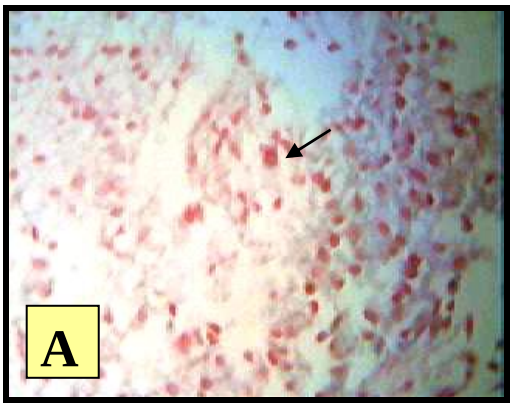
The specimens were graded into 3 grades (grade I to III) according to world health organization classification [10]; Grade I: well differentiated transitional cell carcinoma of the bladder (n=4), Grade II: moderately differentiated transitional cell carcinoma of the bladder (n=31) and Grade III: poorly differentiated transitional cell carcinoma of the bladder (n=15).

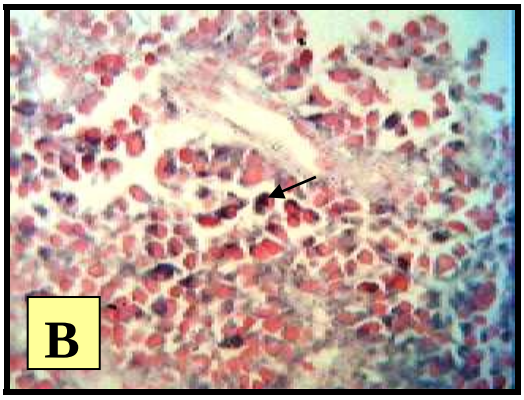
Each carcinoma was also distributed according to the pattern of growth, as follows: papillary type (n=28; 56%) and solid type (n=22; 44%). More ever, muscle invasion was seen in 26 cases (52%) while non-invasion was seen in the rest 24 cases (48%).

In situ hybridization results: The results of ISH have demonstrated that 18 out of 50 (36%) with transitional cell carcinoma of the bladder cases were positive for HPV-16 DNA while 7 out of 50 (14%) with transitional cell carcinoma of the bladder cases were positive for HPV-18 DNA. Human papillomavirus16 and 18 DNA was not detected in the healthy control group. The statistical analysis of the distribution of positive results (Figure 1) demonstrated significant differences and as shown in (Table 1).

Table (1): The percentage of HPV16 and 18 DNA ISH-detection tests in the studied groups.

HPV DNA-ISH reaction results		Studied groups			Comparison of Significance	
		Transitional cell carcinoma of the bladder		Healthy Control	p-value	Sig.
Type 16HPV	Positive	N %	18 (36 %)	0	0.00	Highly Sig. (P<0.01)
	Negative	N %	32(64 %)	10		
	Total	N %	50 (100%)	10 (100%)		
Type 18 HPV	Positive	N %	7 (14 %)	0	0.00	Highly Sig. (P<0.01)
	Negative	N %	43 (86 %)	10		
	Total	N %	50 (100 %)	10 (100%)		





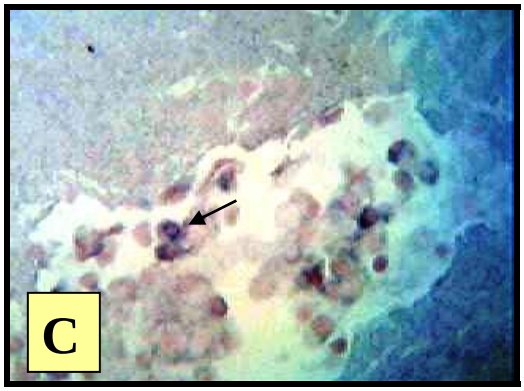


Figure (1): *In situ* hybridization for HPV 16 and -18 DNA in bladder tumor section, stained by BCIP/NBT-Chromogen and counter stained with nuclear fast red (NFR) is shown as dark-blue of the nuclei in positive cases (magnification power, 400). A-HPV-DNA signal negative expression, B-HPV16 DNA signals positive expression, C-HPV18 DNA positive expression.

Table (2) and (3) demonstrated the correlation between expression of HPV-16 and -18 with different variables. Our results showed that there were no significant differences between *in situ* hybridization expression of both HPV-16 and HPV-18 with age, gender, grade, pattern of growth, and muscle invasive. Based on Chi-square test of analysis and Fischer exact test.

Table (2): *In situ* hybridization expression of HPV type-16 DNA as related to clinicpathological profile of patients with TCC.

Variables		HPV-16 positive	**HPV-16 negative**	Comparison of Significance	
				Chi^2-value	Sig.
Age	25-39	2 (11.1%)	2 (6.3 %)	0. 35	Non Sig. (P>0.05)
	40-54	2 (11.1%)	9(28.1%)		
	55-70	14(77.8%)	21(65.6 %)		
Gender	Male	12(66.7%)	23(71.9%)	0. 70	Non Sig. (P>0.05)
	Female	6(33.3 %)	9(28.1%)		
Tumor grade	I	2(11.1%)	2(6.3 %)	0. 42	Non Sig. (P>0.05)
	II	9(50%)	22(68.8%)		
	III	7(38.9%)	8(25 %)		
Pattern of growth	Papillary	8(44.4 %)	20(62.5%)	0.21	Non Sig. (P<0.05)
	Solid	10(55.6 %)	12(37.5 %)		
Muscle invasion	Invasive	11(61.1%)	13(40.6%)	0. 16	Non Sig. (P>0.05)
	Non invasive	7(38.9%)	19(59.4%)		

Table (3): *In situ* hybridization expression of HPV type-18 DNA as related to clinicpathological profile of patients with TCC.

Variables		HPV-18 positive	HPV-18 negative	Comparison of Significance	
				Chi^2-value	Sig.
Age	25-39	1 (14.3 %)	3(7%)	0.68	Non Sig. (P>0.05)
	40-54	2 (28.6 %)	9(20.9 %)		
	55-70	4(57.1%)	31(72.1%)		
Gender	Male	6(85.7 %)	29(67.4%)	0.32	Non Sig. (P>0.05)
	Female	1(14.3 %)	14(32.6%)		
Tumor grade	I	0	4(9.3%)	0.56	Non Sig. (P>0.05)
	II	4(57.1 %)	27(62.8%)		
	III	3(42.9 %)	12(27.9 %)		
Pattern of growth	Papillary	3(42.9%)	25(58.1%)	0.45	Non Sig. (P<0.05)
	Solid	4(57.1%)	18(41.9 %)		
Muscle invasion	Invasive	3(42.9 %)	21(48.8 %)	0.76	Non Sig. (P>0.05)
	Non invasive	4(57.1%)	22(51.2%)		

Discussion:

Human papilloma virus is a group of genetically related viruses that commonly infect stratified squamous epithelium. Unlike many other viruses that infect epithelium and induce lysis of the cells, HPVs induce proliferative changes that result in both benign and malignant tumors. HPV infections are known to affect predominantly adult, sexually active age groups [11]. Human Papilloma virus infects epithelial cells and causes a variety of lesions ranging from common warts /verrucae to cervical neoplasia and cancer [12]. High-risk human papillomvirus usually HPV type 16 (HPV-16) and HPV-18 had been theorized that integration of HPV DNA into the human genome [13].

Current study demonstrated that the prevalence of HPV-16 and -18 DNA was found in 32% and 14%, respectively, of patients with transitional cell carcinoma of the bladder. This result was in agreement with the findings of El Mawla *et al.*, [14] who reported that bladder carcinogenesis is probably related to bacterial and Human papilloma virus. In the present study the percent of HPV-16 was higher than HPV-18. Human papilloma virus-16 is the most prevalent genotype in cervical carcinoma, and is also the most frequently detected HPV type in oropharyngeal and tonsillor SCC [15]. It is found up to 90% of HPV-positive cases [16].

Similar study carried out by Khaled *et al.*, (2001). Who reported that HPV 16/18 DNA was detected by ISH in 46% of Egyptian bladder carcinomas (23/50 cases). positivity was 47.8% for squamous cell carcinoma and 36.4% for transitional cell carcinoma. In this study, (75%) of the HPV-16 DNA positive tumors occurred in male this may be related to the

higher incidence rate of transitional cell carcinoma of the bladder in males than females. There was no significant association between HPV-16 and 18 presence and gender. A previous study has also found no association between HPV-16 presence and gender [18]. Regarding comparison of HPV16 and 18 DNA expression results according to grade, pattern of growth and muscle invasion of patient with transitional cell carcinoma of the bladder revealed that no significant correlation among them. Studies from the general population showed a variable incidence of high risk HPV DNA which ranged from 2.5% to 81%, with HPV-16 DNA occurring more frequently. HPV was detected in both papillary and invasive cancers [19].

According to the results of Iraqi cancer registration of period 1999-2004 bladder carcinoma is the fifth among the commonest tenth cancers being third in males and eighth in female [20].

The results of this study are consistent with results of previous studies [21] [22]. Suggesting that HPV-16 and HPV-18 may play an important role in TCC of the bladder or could facilitate its progression. Histopathological, epidemiological and molecular studies are necessary to confirm our observation and to evaluate the prevalence of HPV-16 and HPV-18 infection in relation to transitional cell carcinoma of the bladder in Iraqi populations.

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