Does Human Papilloma Virus Have A Role in Urinary Bladder Carcinoma of Egyptian Patients?

Moshira M. Abdelwahed and Mohammad I. Shaban

Department of Pathology, Faculty of Medicine, Menoufiya University,
Mohammadshaban1973@gmail.com

Abstract: ***Background***: Bladder cancer is the second most commonly occuring genitourinary cancer in adult. Egypt has the highest bladder cancer rate in the world with local factors most probably responsible for such prevalence. In recent years, viral infections including human papilloma virus (HPV) have been implicated in bladder carcinogenesis. HPV is a small circular DNA virus that infects stratified squamous epithelium and has an established etiological role in tumors of the urogenital tract and anal region. Several previous studies have looked for an association between HPV and bladder cancer development, however, its possible role is still controversial. ***Objective***: To investigate the possible etiological role of HPV in Egyptian bladder carcinoma. ***Patients & Methods:*** 42 Egyptian patients with bladder carcinoma, 17 cases with cystitis as well as 15 cervical carcinoma cases as a positive control were included in this study. Formalin fixed paraffin embedded tissues were used and stained with; H&E to study histopathologic features, immunohistochemistry for P16 & Ki 67 as well as the tissue processed for PCR for HPV expression. ***Results***: Only one case of bladder carcinoma showed positivity for HPV with complete negativity in the cystitis group. 52% of bladder carcinoma cases showed P16 expression & 21.4% showed over expression. P16 expression was higher in cases associated with bilharziasis and in transitional carcinoma cases associated with squamous differentiation. ***Conclusion***: The low prevalence of HPV in this study does not support an etiologic role of HPV in Egyptian bladder carcinogenesis. However, the over expression of P16 in a subset of bladder carcinoma cases could raise a possibility for other HPV type that is not detected by our probe.

[Moshira M. Abdelwahed and Mohammad I. Shaban. **Does Human Papilloma Virus Have A Role in Urinary Bladder Carcinoma of Egyptian Patients?** *J Am Sci* 2012;8(12):899-905]. (ISSN: 1545-1003). http://www.americanscience.org. 125

Key words: HPV, P16 and bladder carcinoma.

1. Introduction

Bladder cancer is the second most commonly occurring genitourinary cancer in adults ***(Kazuya et al., 2007)***. In western countries, more than 80% of bladder cancers are transitional cell carcinoma with squamous cell carcinomas the second most common morphological type identified **(*Vecchia et al., 2010)***. In Egypt, which is known to have the highest bladder cancer rate in the world, with local factors most probably responsible for such a skewed prevalence ***(Mokhtar et al., 2007)***. Interactions of different carcinogenic and cocarcinogenic agents are responsible greatly for increasing the probability of bladder urothelial carcinoma development. These include alcohol, smoking habits, prolonged use of analgesic and infection with schistosoma hematobium. More recently also viral infections have been implicated in this pathology ***(Cotran et al., 2010).***

In cattle, bovine papilloma virus has been implicated in transitional cell carcinoma formation ***(Syrjanen, 2000)***. Human papilloma virus (HPV) is a small circular DNA virus that infects stratified squamous epithelium. Although, many HPV types are associated with benign lesions, certain types such as HPV16- and HPV-18 have an established etiological role in tumors of the urogenital tract and anal region ***(Beckmann et al., 2009)***. HPV has also been postulated as carcinogen in a range of other epithelial malignancies including cancers of the skin, oral cavity, esophagus and conjunctiva. Indeed it has been estimated that almost 10% of the world wide cancer burden is linked to HPV infection ***(Youshya et al., 2005)***. Several previous studies have looked for an association between HPV infection and bladder TCC development. However, the wide range of frequency detected, varying between zero and 81%, this means that the role of HPV in bladder carcinogenesis remains controversial ***(Bryant et al., 2010).***

HPV- induced cancers are associated with wild type TP ^{53} and KB genes and low levels of' P^{53} and pRb proteins. The P^{16} tumor suppressor genes (CDK N2A) are often up regulated in these cancers because it is negatively regulated by pRb. Overexpression of' P^{16} has been repeatedly reported in HPV associated cancer. In cervical and genital lesions, high levels of P^{16} protein expression are associated with high risk of HPV infection ***(Pao et al., 2010)***.

Therefore, we planed to study the expression of HPV in urinary bladder carcinoma in Egyptian patients trying to know if it has a role in this type of cancer or not by using PCR. Also we will study the

expression of P^{16} in this type of cancer using immunohistochemical method and comparing the studied bladder cases with cervical carcinoma cases as a positive control group.

2. Materials and methods

This study was carried out upon tissue specimens taken from 42 Egyptian patients with bladder carcinoma, 17 cases of cystitis as well as 15 cases of cervical carcinoma as a control group. The specimens were taken during the period between December 2007 to June 2008 from Pathology Department, Faculty of Medicine, Menofiya University. The specimens were fixed in formalin, embedded in paraffin and stained by:

* H&E stain to study histopathological features.
* Immunostain to study P^{16} and Ki ^{67} expression.
* PCR to study HPV expression.

Clinicopathological data of the cases as age, sex, tumour size, tumour type and lymph node metastasis were obtained from the patients files.

1) Histopathological examination:

A Five um thick tissue sections were cut and stained with H & E to know the type of carcinoma, histologic grading of TCC and SCC that was defined according to World Health Organization and International society of urologic pathology classification (***Cotran et al., 2010)***. Tumour staging was designed according to TNM American Joint committee on cancer -union, Internal center to cancer staging system ***(AJCC-UJCC, 1997).*** Bilharziasis was demonstrated as calcified or viable ova in the tissue.

(2) Immunohistochemistry:

Four um thick tissue sections from formalin fixed paraffin embedded tissue mounted on poly -L-lysine coated slides were deparafinized in xylene and rehydrated through serial baths of alcohol and water. The slides were heated in the microwave oven at 700 watt for 5 min. The hydrated sections were then treated in methanol containing 0.3 hydrogen peroxide for 30 min to eliminate endogenous peroxidase activity and were washed in phosphate buffered saline (PBS). The primary monoclonal antibody for ki67 and p16 was added. The slides were rinsed in PBS solution and incubated in biotinylated secondary antibody. The slides were washed in PBS and incubated in an avidin-biotin peroxidase complex (Signet, USA) for 30 min. After washing PBS, a chromogen reaction was developed by incubating the slides with a freshly prepared solution of 3.3 diaminobenzidene tetrahydrochlonde (0.04%) and hydrogen peroxide (0.03%). A positive reaction to P^{16} appeared as cytoplasmic or nucleocytoplasmic brownish colour, whereas in Ki 67 positivity appered as browen colour in the nucleus. The expression of P^{16} and Ki ^{67} were analyzed in the entire sections of microscopic fields.

Scoring of P^{16}

The biopsies were scored as positive when more than 5% of cells (cut off point) stained positively and were separately graded as described by ***Lee et al. (2001)*** as follow:

(1) Negative: less than 5% of cells were positive
(2) Sporadic positivity: 5-10% of cells with weak and scattered positivity.
(3) Focal positivity: 10-30% of cells with strong positivity spreading in one tissue area.
(4) Diffuse positivity: > 30% of cells with strong positivity spreading in several tissue areas.

Biopsies that exhibited a diffuse pattern were considered to have high IHC expression of P16. Focal distribution was interpreted as moderate expression and sporadic as low expression.

For statistical analysis the cases were classified as: Negative (less than 5%), focal (5-30%) and strong (more than 30%).

Scoring of Ki 67

We counted the positive nuclei in at least 1000 cells and divided on 100 to know the index ***(Margulis et al, 2011).***

HPV analysis:

DNA was extracted from paraffin blocks using the method as previously described. Briefly, formaline fixed paraffin embedded tissue sections were digested overnight with 2 mg/ml protein Kinase K *(Biometra Gottingen, Germany)* at 55 ^{o}c. After digestion extraction with phenol (chloroform \isoamyl alcohol 1:24-25) in two steps (chloroform \isoamyl alcohol 1:24) was performed. The liquid phase was precipitated with 2 volumes of 100% ethanol and 1/10 volume of 3 mol/L sodium acetate. Finally the DNA pallet was dissolved in Tris/EDTA buffers and measured with a spectrophotometer. All samples were subjected to polymerase chain reaction amplification using the general consensus primers GP5+ and GP6+ using the primer sequencing and amplicon sizes as described by ***Jacobs et al. (1997)*** using a protocol with minor modifications as published recently ***(Schneider et al., 2007)***.

Fifteen cervical biopsy specimens from Egyptian patients were also included in this study as a positive control.

Statistical analysis:

The chi square test and Fisher's exact test were used to compare the differences. The software used for the calculations was the ***SPSS*** (Statistical Package for the Social Sciences) system. Results were considered statistically significant when the probability of findings occurring by chance was less than 5% (p <0.05%) ***(Dawson and Trapp, 2000)***.

3. Results

This study was carried out on 59 bladder cases that were divided into:

- Malignant group: include 42 cases divided into:
  * Thirty three cases (78. 6%) of TCC
  * Five cases (11.9%) of SCC
  * Four cases (9.5%) of Adenocarcinoma.
- Cystitis group: include 17 cases divided into:
  * Six (35.3) cases were bilharzial
  * Eleven (64.7) were non bilharzial.

Fifteen cases of cervical carcinoma studied as a positive control group.

(I) Histopathological examination:

Clinicopathologic data of the studied cystitis group cases showed that the age of the cases ranges from 21 years to 70 years with a mean± SD of 55± 2.9, the male to female ratio was 7.5:1 as 15 cases (88,2%) were male and only 2 cases were female (11,8%) and 6 cases(35%) were positive for bilharziasis.

Regarding the malignant cases, the clinicopathologic data of the studied cases were summarized in Table (1)

Table-1: Clincopathological data of the studied malignant cases

Variable	Number	%	Mean value and range of the malignant cases
Age group:			
- < 60 Y.	18	44.2%	62.94 ± 10.86 (24-85)
- > 60 Y.	24	55.8%	Median 63 y.
Sex:			
- Male	38	90.5%	
- Female	4	9.5%	
Size of lesion:			
- < 5 cm.	22	53.8%	
- > 5 cm.	20	46.2%	
Histologic type:			
- Urothelial ca.	33	78.5%	
- Squamous ca.	5	11.9%	
-Adenocarcinoma.	4	9.6%	
Bilharziasis:			
- Positive	24	57.7%	
- Negative	18	42.3%	
Grade:			
- Low grade.	20	47.7%	
- High grade.	22	52.3%	
Vascular invasion:			
- Present	2	4.8%	
- Absent	40	95.2%	
Lymph node metastasis:	11	26%	
- Positive.	31	74%	
- Negative.			
Stage:			
- pT1	0	0%	
- pT2	22	52.3%	
- pT3	17	40.4%	
- pT4	3	7.3%	

(II) Immunohistochiemistry:

Ki67 Expression:

Ki67 appear as brown nuclear staining in the positive cells.

Ki67 of bladder cancer range from 0.03 to 0.9 with a mean± SD of 0.39± 0.09. Figures (1&2)

Ki67 in cystitis ranged from 0 to 0.35 with a mean± SD of 0.15± 0.01.

Ki67 expression in cervical carcinoma range from 0.10 to 0.96 with mean± SD of 0.53± 0.02.

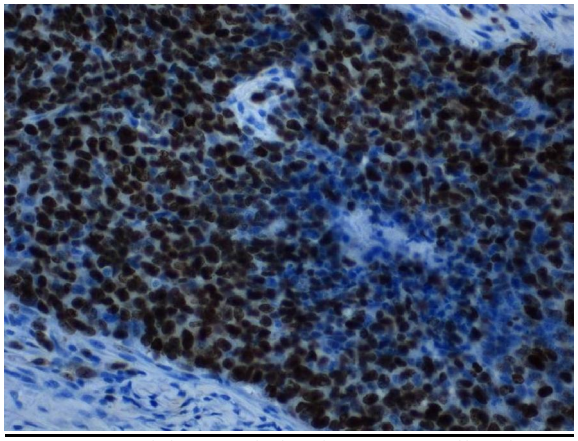

Figure (1): High KI67 index in tumour cells of TCC. (Immunoperoxidase X 100).

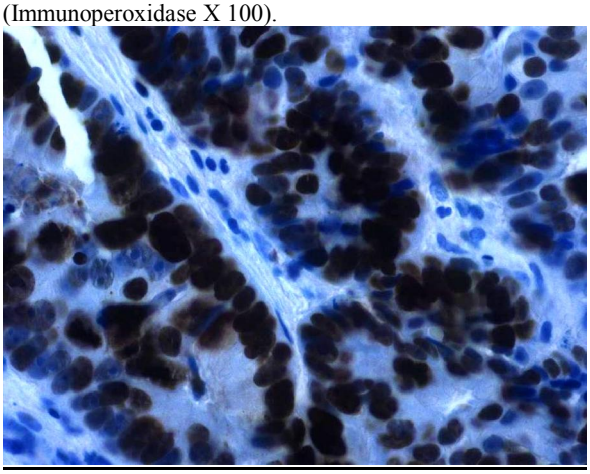

Figure (2): Nuclear localization of Ki67 in tumour cells of papillary TCC. (Immunoperoxidase X 400).

P16 Expression:

Bladder carcinoma:

P16 was positive in 22 (52.4%) cases of bladder carcinoma with nucleocytoplasmic expression. Nine of them showed strong diffuse positivity (21.4%) and 13 cases (30.9%) showed focal positivity.

Correlation between clinicopathological parameters and p16 expression in carcinoma cases showed high expression of p16 in the cases associated with bilharziasis and in transitional cases associated with squamous differentiation. Table (2) & Figures (3-5)

Cystitis:

Nine out of the 17 (52.9%) studied cystitis cases were positive for p16 in the covering urothelium. The expression was focal. Three of the positive cases were associated with bilharziasis (33.3%) and 6 cases were non bilharzial (66.7%). Of the 8 negative cases (47.1%) three cases were bilharzial (37.5%) and 5 cases were non bilharzial (62.5%).

Cervical carcinoma:

All cases were positive for p16 and showed diffuse strong nucleocytoplasmic staining.

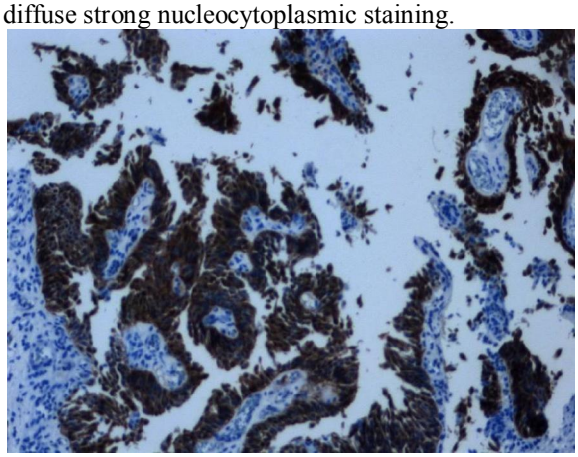

Figure (3): Strong expression of P16 in papillary TCC. (Immunoperoxidase X 100).

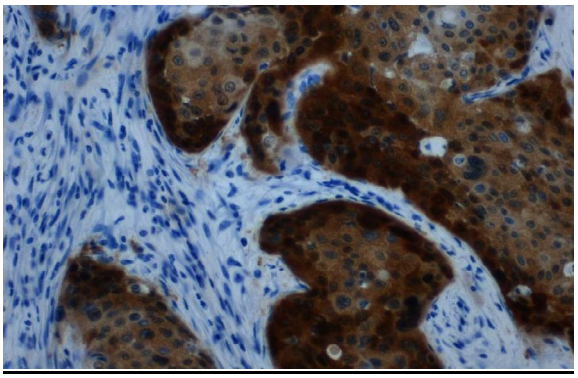

Figure. (4): Strong cytoplasmic expression of P16 in TCC with squamous differentiation. (Immunoperoxidase X 200).

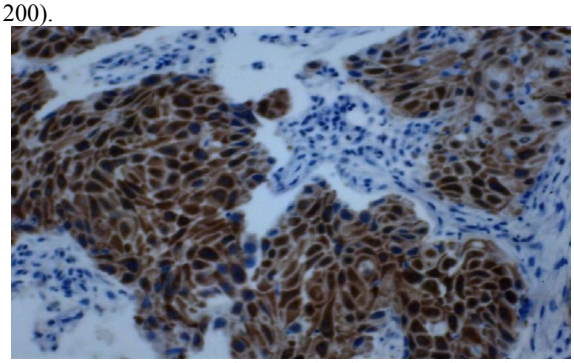

Figure (5): Strong nucleocytoplamic expression of P16 in papillary TCC. (Immunoperoxidase X 400).

Table-2: Comparison between p16 positive and negative cases regarding clincopathological data.

Variables	P16 negative cases	P16 positive cases		*p*-value
		Focal (N= 13)	Diffuse (N= 9)	
Age				0.91
- Median	75	62	57	
- Range	48-75	41-81	37-75	
- Mean± SD	60±8.6	61±13.7	59±14.1	
Sex				0.96
- Male	18(47%)	12(32%)	8(21%)	
- Female	2(50%)	1(25%)	1(25%)	
Types of biopsy				0.15
- cold cup	5(62%)	1(12.5%)	2(25%)	
- Radical	6(67%)	1(11%)	2(22%)	
- TURT	9(36%)	11(44%)	5(20%)	
Histological type				0.11
- TCC	16(46%)	10(29%)	9(26%)	
- SCC	3(100%)	0(0%)	0(0%)	
- Adenocarcinoma	1(25%)	3(75%)	0(0%)	
Grade				o.7
- Low	10(46%)	8(36%)	4(18%)	
- High	10(50%)	5(25%)	5(25%)	
Associated sqaumous diff. (in TCC)				0.04*
- Present	7(88%)	1(12%)	0(0%)	
- Absent	13(38%)	12(35%)	9(26%)	
Bilharziasis				0.02*
- Negative	9(32%)	11(39%)	8(29%)	
- Positive	11(79%)	2(14%)	1(7%)	
Ki67				0.22
- Median	0.36	0.45	0.36	
- Range	0.09-0.82	0.08-0.9	0.03-0.6	
- Mean±SD	0.38±0.19	0.49±0.26	0.31±0.26	

PCR for HPV:

Bladder carcinoma:

Only one case of the studied malignant cases was positive for HPV by GP&EIA PCR, this positive case showed strong diffuse positivity of p16 and high expression of Ki67, Figures (6 &7). This positive case was female patient aged 40 years with low grade invasive papillary TCC. She did not have immunodeficiency or genital wart.

Cystitis:

All the studied cases (17) were negative for HPV by either GP PCR or EIA PCR.

Cervical carcinoma:

Ten cases from the studied 15 cases of cervical carcinoma were positive for GP PCR, while 12 cases were positive by EIA PCR for HR HPV.

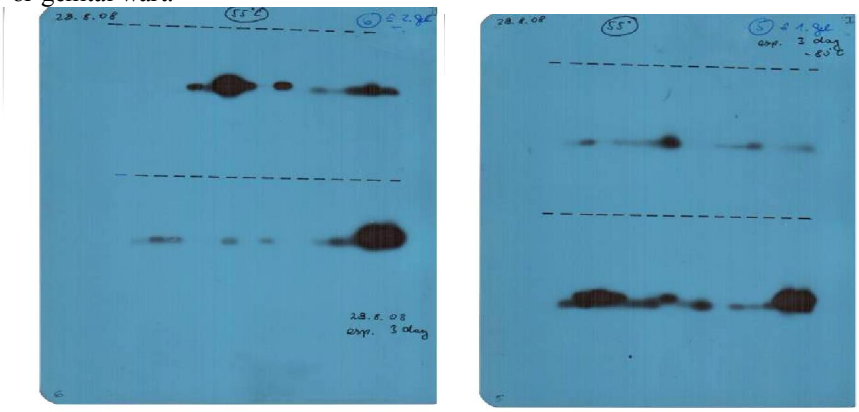

Figure (6): PCR photo showed DNA of HPV in only one case of bladder carcinoma.

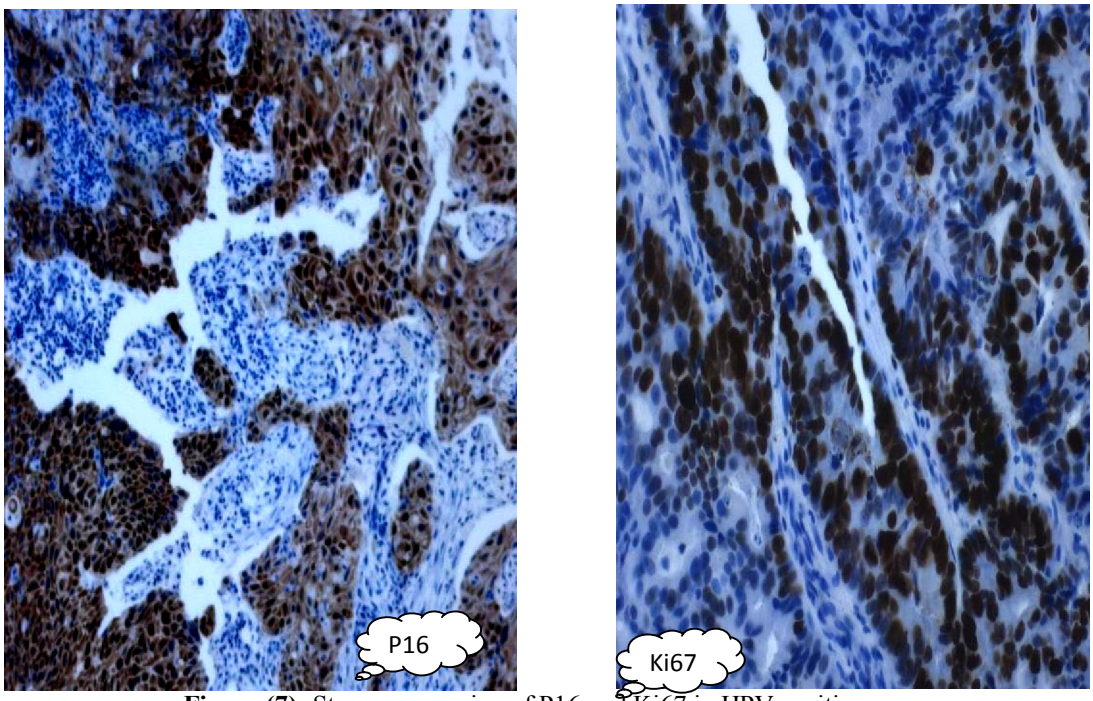

Figure (7): Strong expression of P16 and Ki67 in HPV positive case.

4. Discussion

Bladder cancer is the second most commonly occuring genitourinary cancer in adults (Kazuya et al., 2007). The increased incidence of bladder carcinoma observed in the past three decades has stimulated researches into the identification of possible etiological agents. The possible role of viruses in this respect is still highly controversial. The significant association of HPV infection and genital cancer in both sexes has promoted attempts to

identify HPV in bladder cancer as Well as in various benign lesions of urinary tracts (Melchers et al., 2009).

More than ninety types of HPV were identified and more than thirty five of them have specific tropism for male and female genitourinary tract (Shimada et al., 2008).

The bovine type of HPV is known to cause bladder cancer in cattle. It is founded that HPV infection is more common in smokers for non causal socioeconomic reasons. There are only a few previous reports suggesting some correlation between the HPV infection and nonneoplastic or neoplastic lesions of urinary bladder (Bryant et al., 2010).

Therefore this study tried to investigate the expression of HPV in Egyptian bladder carcinoma cases to evaluate their possible role as a co-carcinogenic factor.

Regarding HPV expression, all the studied cystitis cases were negative. However, only one case of the bladder carcinoma was positive for HPV. This result was similar to that of Tekin et al. (2008) who detected only two out of forty two cases of bladder carcinoma positive for HPV. On the other hand, others reported strong correlation between HPV and bladder carcinoma (Anwar et al., 2009; Mistro et al., 2009; Mevorach et al., 2010 and Ferr et al., 2010).

In this study P16 was positive in 22(52.4%) cases of bladder carcinoma with nucleocytoplasmic expression. Nine of them showed strong diffuse positivity (21.4%) and 13cases (30.9%) showed focal positivity. In cystitis 9/17 (52.9) cases showed focal positivity for P16 in the covering urothelium. This result was in agreement with that of Lee et al. (2010) who found high P16 expression in bladder tumors.

Overexpression of P16 has been repeatedly reported in HPV associated cancer. In cervical and genital lesions, high levels of P16 protein expression is associated with high risk HPV infection (Nicholas et al., 2009). In the present study all the positive HPV cervical carcinoma cases showed overexpression of P16. This goes with previous studies who reported that P16 is expressed in most cervical lesions and the status of immunoreactivity allows differentiation between infection with low risk HPVs and high or intermediate risk HPVs. This might be attributable to differences in functional inactivation of RB protein by HPVs (Lee et al., 2010). Therefore, determination of P16 expression status by IHC could serve as a reasonable surrogate marker for biologically relevant high risk HPV infection. In the present study, the positive HPV case showed P16 overexpression. However, P16 was also over expressed in a subset (21.4%) of the studied carcinoma cases that raise a possibility for presence of other high risk HPVs that could not be detected by our probe in this study.

Correlation between clinicopathological parameters and P16 expression in carcinoma cases studied showed high expression of P16 in cases associated with bilharziasis and in transitional carcinoma cases associated with squamous differentiation, this result was similar to that obtained by Abdulamir et al., 2009 who stated that P16 expression was higher in the schistotomal bladder tumors than non- schistosomal tumors.

Overexpression of P16 observed in this study with bilharzial infection as well as in the positive HPV infected case could raise a question if there is some sort of relation between these two possible etiological agents or not . This need further extended study.

Corresponding author
Mohammad I. Shaban
Department of Pathology, Faculty of Medicine, Menoufiya University,
Mohammadshaban1973@gmail.com

References

Abdulamir A, Rand R, Haider S and Fatimah A (2009): Tumor markers of bladder cancer: the schistosomal bladder tumors versus non-schistosomal bladder tumors. *Journal of Experimental & Clinical Cancer Research*. (28):1756-66.

Anwar K, Inuzuka M, Shiraishi T and Nakakuki K (2009): Detection of HPV DNA in neoplastic and non-neoplastic cervical specimens from Pakistan and Japan by non-isotopic in situ hybridization.*Int* J *Cancer* ; 47675-80.

Beckmann AM, Daling JR, Sherman KJ, Maden C, Miller BA, Coates RJ (2009):. Human papillomavirus infection and anal canal. *Int Cancer* ; 43:1042-49.

Bryant P, Davies P, Wilson D (2010): Detection of human papillomavirus DNA in cancer of the urinary bladder by *in* situ hybridization.*Br* J *Urol* ; 68:53-61.

Bullwinkel J, Baron-Lühr B, Lüdemann A, Wohlenberg C, Gerdes J and Scholzen T (2006): "Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells". J. Cell. Physiol. 206 (3): 624–35.

Cotran RS, Kummar V and Robbins SL (2010): The urinary bladder. In: Robbins pathological basis of disease, 7th edition. WB sauders Co., Pdladelphia USA, pp 974-981.

Dawson SB. and Trapp RB. (2000): Basic & clinical biostatistics. 3rd ed.: Appleton & Lange.

Ferre F, Garduno F and Lewis F (2010): Detection of human papillomavirus types 6/11, 16 and 18 using the polymerase chain reaction in bladder

carcinoma. In: Furth M, Greaves M, editors. Cancer cells. vol. 5. New York: Cold Spring Harbor Laboratory, :215-8.

Jacobs M, Snijders P, van den Brule A, Helmerhorst A, Meijer J and Walboomers M (1997) : A general primer GP5+/GP6(+)-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. *J Clin Microbiol*. March; 35(3): 791–795.

Kazuya N, Manabu I and Silverberg E (2007): Statistical and epidemiologic data on urologic cancer. Cancer ; 60:692-717.

Lee K, Jung ES, Choi YJ, Lee KY and Lee A (2010): Expression of pRb, p53, p16 and cyclin D1 and their clinical implications in urothelial carcinoma.J Korean Med Sci. Oct;25(10):1449-55.

Margulis V, Shahrokh F, Shariat A, Raheela A, Arthur I Sagalowsky I and Yair L (2011): Ki-67 Is an Independent Predictor of Bladder Cancer Outcome in Patients Treated with Radical Cystectomy for Organ-Confined Disease. Int. Jor. of Urology. (16) 88-94.

Melchers G, Schift R, Stolz E, Lindeman J and Quint W (2009): Human papillomavirus detection in urine samples from male patients by the polymerase chain reaction. J *Clin Microbiol*; 28:409-12.

Mevorach RA, Cos LR, Santagnese PA, Stoler FA (2010): M. Human papillomavirus type 6 in grade I transitional cell carcinoma of the urethra. J *Urol*; 143:126-8.

Mistro AD, Braunstein JD, Halwer M and Koss LG (2009): Identification of human papillomavirus types in bladder carcinoma. Hum Pathol ; 18:936-40.

Mokhtar N, Adel I and Gouda I (2007): Cancer pathology registry, NCI : Epidemiology of cancer bladder in Egypt, 1st edition, chapter 1,2&3 pg13,28&33, Karger BD. Preparation and use of biotinylated oligonucleotide probes. Focus ; 11:57-8.

Nicholas A, Patricia L, Janice B, Constance M, Nancy B Kiviat M and Laura A (2009): p16INK4a Expression Correlates with Degree of Cervical Neoplasia: A Comparison with Ki-67 Expression and Detection of High-Risk HPV Types. Mod Pathol; 16(7):665–673.

Pao CC, Lin CY, Mass JS, Lai C and Soong K (2010): Detection of human papillomaviruses in cervicovaginal cells using polymerase chain reaction. Hum Pathol; 36; 741-746.

Rahmanzadeh R, Hüttmann G, Gerdes J and Scholzen T (2007): Chromophore-assisted light inactivation of pKi-67 leads to inhibition of ribosomal RNA synthesis". Cell Prolif. 40 (3): 422–30.

Schneider PM, Prinz M., Carracedo A., Mayr WR., Morling N., Parsons TJ., Sajantila A., Scheithauer R. and Schmitter H. (2007): DNA Commission of the International Society for Forensic Genetics (ISFG): Recommendations regarding the role of forensic genetics for disaster victim identification (DVI) *Forensic Sci Int. Genet.* 1(1), 3-12.

Syrjanen K. (2000): Epidemiology of human papillomavirus (HPV) infections and their associations with genital squamous cell cancer. Acta Pathol Microbial lmmunol Scand. 9: 97957-70.

Shimada M, Fukushima M, Mukai H, Kato I, Nishikawa A and Fujinaga K (2008): Amplification and specific detection of transforming gene region of human papillomavirus 16, 18 and 33 in cervical carcinoma by means of the polymerase chain reaction. J *Cancer Res* ; 81:l-5.

Tekin M, Serdar T, Fazil T, Cenk B and Haluk O (2008): Human papillomavirus associated with bladder carcinoma? Analysis by polymerase chain reaction. Int. Jor. of Urology. (6) 184-186.

Vecchia C, Negri E, Avanzo B, Savoldelli A and Franceschi S (2010): Genital and urinary tract diseases and bladder cancer. *Cancer Res* ; 51:629-31.

Youshya R, Bevan I, Wells M and Young L (2005): Demonstration of multiple HPV types in normal cervix and in cervical squamous cell carcinoma using the polymerase chain reaction on paraffin wax embedded material. *J Clin Pathol* ; 43:52-6.

08/12/2012