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Immunohistochemical Assessment of Expressional Protein of Cyclin Dependent Kinase Regulator-2 in Human Cytomegalovirus Infected Tissues with Bladder Transitional Cell Carcinoma

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ABSTRACT

Background: Urinary bladder cancers are mostly affecting the older people, ranking the fifth most common in the western world, where the transitional cell (urothelial) carcinoma is the most common type. Human cytomegalovirus infects and on comodulates tumor cells, thereby increases their malignancy. Cyclin dependent kinase 2 (Cdk2) phosphorylates retinoblastoma protein which is important in transition from G1 to S phase, thus the inhibition of this protein results in apoptosis of the targeted tumorous cells.
Objective: To analyse of the co-expression of Cdk2 along with HCMV infection of a group of bladder transitional cell carcinoma tissues.
Patients and methods: Seventy formalin-fixed, paraffin- embedded bladder biopsies were enrolled in this study; 30 from bladder cancers , 25 from benign tumors and 15 bladder tissues with unremarkable pathological changes (as an apparently healthy control group). *Chromogenic In Situ Hybridization* (CISH) was done for detecting HCMV whereas *immunohistochemistry* (IHC) was used to assess the expression of Cdk2.
Results: CISH reactions for HCMV were detected in 56.7 % (17 out of 30) of bladder cancer biopsies and in 24 % (6 out of 25) of biopsies from benign bladder tumors. No positive– ISH reactions were detected in the control tissues. The positive Cdk2-IHC reactions were detected in 60% (18 out of 30) of bladder cancers and in 45% (13 out of 30) of benign bladder tumors biopsies. No IHC- signals were reported in the control tissues. Statistically, highly significant differences were present between each bladder tumors groups and the control group.
Conclusions: Detection of co-expressions of Cdk2 with HCMV in bladder transitional cell carcinoma could point both for a possible roles in bladder pathogenesis and /or carcinogenesis.

Keywords: Bladder transitional cell carcinoma; HCMV; Cyclin dependent kinase 2; CISH; IHC.

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INTRODUCTION

Bladder cancers are the most common malignancies in urinary system, ranking the ninth in the western countries and the fifth most common cancers worldwide. Transitional cell carcinomas are also called urothelial cancers. Approximately 80% of bladder urothelial neoplasms are superficially exophytic papillary lesions without invasion or metastasis while 20% are highly solid, aggressive, non-papillary cancers [1].

In 2016, it was reported in United States that 76,000 new bladder cancer cases and 16,000 bladder cancer-associated mortalities had been occurred The vast majority (90% - 95%) of bladder cancers are transitional cell carcinoma, while 5% are squamous cell carcinoma and less than 2% are adenocarcinoma or small cell carcinoma. These cancers are affecting older patients (more than 90% are older than 55 years) [2].

Cigarette smoking, occupational risks, water containing chlorination by-products or arsenic, exposure to certain drugs and heavy consumption of phenacetic analgesics are recognized as causes of bladder cancers in the developed countries. Infectious agents, Schistosoma haematobium as well as other viral and bacterial urinary tract infections, are recognized as potential risk factors of bladder carcinogenesis [3].

Human CMV is an endemic infection and can establishe lifetime latency in human hosts. The virus has been implicated in the etiology of several malignancies such as colon cancers, malignant gliomas, neuroblastomas, prostatic adenocarcinomas, Hodgkin's disease, oral cancers, Kaposi's sarcomas, Wilm's tumors, cervical and breast cancers [4].

The virus encodes over 200 proteins where ORF 79 has a role in transformation. This virus also produces V-IL-10 protein which through phosphorylation can activate Stat3, a key factor in progression and evasion of tumors of apoptosis. A broad range of cells bind to HCMV via their cellular surface heparin sulfate, [5].

The in situ hybridization is a molecular method for detecting HCMV- nucleic acids in the frozen and fixed cells and tissues, that use radioactive- and non-radioactive moieties for labeling the probes, such as fluorescein, biotin, digoxigenin, and enzymes [6].

Researchers have demonstrated HCMV- nucleic acids and proteins in cancerous tissues, to postulate a direct contribution of HCMV in cancers. However, viral localization *perse* within such tumor do not a definitive evidence or have a causal linkage between HCMV and human cancers. Direct causative role for HCMV has been abandoned, and thus kept for a restricted role of virus in modulation and modification of tumor growth .Evidences have been accumulated that HCMV protect tumor cells from apoptosis and modulate angiogenesis [7]. Here in, if a link of HCMV with carcinogenesis could be proved, tumor

tissues infected with such virus might respond to either antiviral or antitumor therapies.

Cell cycle regulation includes a sequential phosphorylation and de-phosphorylation of several cyclin-dependent kinases (cdks) for their activation or inactivation, respectively .They form functional complexes with a family of regulatory subunits, called cyclins, where the progression from G1 to S phase is regulated by the accumulated cyclins D, E and A, to activate different cyclin-dependent kinases [8].

The cdk2 action is inhibited by members of Cip/Kip family (p21Cip1, p27Kip1 and p57Kip2), therefore, their absence is necessary for cyclin E-cdk2 activation and progression from G1 to S phase. In turn, Cyclin E-cdk2, phosphorylates and thus targeting, p27Kip1 for degradation. Immune impairment is considered as a risk factor for CMV disease in patients with malignancies, and an incidence of CMV infection in those patients varies among different studies [9].

Several laboratory tests were used to diagnose the virus, among them,, polymerase chain reaction (PCR) is the most sensitive and rapid one [10]. In addition, in situ hybridization was found as a method for localizing and detecting specific mRNA or DNA sequences in morphologically preserved tissues sections as well as cell preparations by hybridizing them to the complementary strand of a nucleotide pro and the virus of interest be and utilizing the value of correlating the detection of viral agent or any molecular markers with histopathological expression of the disease.

This study was designed to analyse of the co-expression of Cdk2 along with HCMV infection of a group of bladder transitional cell carcinoma tissues.

RESULTS

I. Age of the patients with bladder tumors

No significant difference (P>0.05) between the mean age of patients with transitional bladder carcinomas (65.6±6.3 years) and patients with benign bladder tumors (61.8± 14.6 years) (Table 1).

Table 1: Mean age (years) of bladder tumors patients and their healthy control group.

Studied groups	N	Mean	Std. Deviation	Std. Error	Mini.	Maxi.	Comparison of significant	
							P-value	Sig.
Transitional Bladder Cancers	30	65.6	6.3	1.32	57	89	0.187	NS
Benign Bladder Tumors	25	58.9	11.5	2.63	55	83		
Healthy control	15	72.40	4.9	1.66	62	78	-	-
Total	70							

II. Histopathological grade description of transitional bladder cancers

On distributing transitional bladder cancers group according to their grading (Table 2), the present results show that well differentiated bladder cancers (grade I) constituted 30 % (9 out of total 30 cases) ,whereas moderately (grade II) and poorly (grade III) differentiated bladder cancers constituted 43.3 % (13 out of total 30 cases) and 26.7 % (8 out of total 30 cases), respectively.

The statistical analysis reveals no significant differences among the grades of bladder cancers (P > 0.05).

Table 2: Bladder cancers according to their grades.

Grades	No.	%	Comparison of significant	
			P-value	Sig.
Well differentiated	9	30	0.283	NS (P > 0.05)
Moderately differentiated	13	43.3		
Poorly differentiated	8	26.7		
Total	30	100		

III. Human Cytomegalovirus - Associated Bladder Transitional cancers

Seventeen out of thirty 30 tissues with transitional bladder cancers showed positive chromogenic in situ hybridization reactions (constituting 56.7% of the total transitional bladder cancer tissues of this study) (Table 3 and Figure 1). The benign bladder tumors group revealed 24% positive signals which represented 6 out of 25 tissues in this group. None of control tissues group presented positive signals for HCMV-ISH test. However, in comparison to the percentage of HCMV -DNA in healthy control group as well as in the group of benign bladder tumors, the differences between the percentages of HCMV-DNA in tissues of patients with transitional bladder cancers and each of these groups are statistically very highly significant (P value = < 0.0001).

Table 3: Results of in situ hybridization for detecting HCMV – DNA in bladder tissues

Studied groups		HCMV		Total	Comparison of significant	
		Positive	Negative		P-value	Sig.
Transitional Bladder Cancers	N	17	13	30	0.001	Highly Sig. (P<0.01)
	%	56.7	43.3	100		
Benign Bladder Tumors	N	6	19	25		
	%	24	76	100		
Healthy Control Tissues	N	0	15	15		
	%	0	100	100		

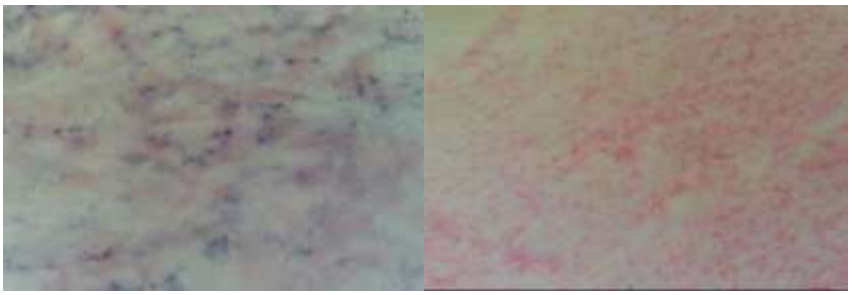

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Figure 1: In situ hybridization results for HCMV-DNA detection in bladder tumors; BCIP/NBT stained and counter stained by nuclear fast red; A. Transitional bladder cancer tissue with negative HCMV-ISH reaction (20X).B. Transitional bladder cancer tissue with positive HCMV-ISH reaction (20X).

IV. The grading of transitional bladder cancers according to the CISH results for HCMV-DNA detection

The HCMV-DNA positive C1SH results were detected in 61.5% (8 out of 13) of tissue with transitional bladder cancers showing moderate differentiated grade, followed by (5 out of 9) tissues showing well differentiated grade where it comprised 55.6% of the total number of this grade, and lastly by (4 out of 8) tissues with poor differentiated grade where it constituted 50% of total number of this grade.

Statistically, the distribution of CISH results for detection of HCMV DNA according to grading of transitional bladder cancers shows significant differences (P>0.05) (Table 4).

Table 4: Results of CISH for HCMV – DNA detection according to the grading of Transitional bladder carcinoma

Grade		HCMV– CISH Reactions		Total	Comparison of significant	
		Positive	Negative		P-value	Sig.
Well Differentiated	N	5	4	9	0.02	Sig. (P>0.05)
	%	55.6	44.4	100		
Moderately Differentiated	N	8	5	13		
	%	61.5	38.5	100		
Poorly Differentiated	N	4	4	8		
	%	50	50	100		
Total	N	17	13	30		
	%	56.7	43.3	100		

V. Results of CDK2- IHC Signal Scoring:

The expression of CDK2 protein was detected as a brownish discoloration at nuclear localizations (Figure 2). Group showed CDK2- expression. The expression of CDK2 was detected in 53.3% (16 out of 30) tissues with transitional bladder cancers and in 44% (11 out of 25) tissues with benign bladder tumor. A high percentage (56.2%: 9 out of 16 cases) was involving tissues with malignant transitional bladder tumor that have moderate score (score II), while, in benign bladder tumor tissues, 45.5% (5 out of 11) were

found to have either weak score while none of control (score I) or moderate score (score II). Statistically, significant differences (p <0.05) were found on comparing the results (according to score) when each group of transitional bladder cancers or benign bladder tumors were compared to control tissues group , but the difference between the group of malignant and benign bladder tumors was statistically not significant(Table 5).

Table 5: Immunohistochemistry results of CDK2 protein according to the IHC- signal scoring.

CDK2 Protein expression		Healthy Bladder Tissues (n=15)		Benign Bladder Tumor		Transitional Bladder Cancers (n=30)		P
		N	%	N	%	N	%	
Negative		15/15	100.0	14/25	56.0	14/30	46.7	
Positive		0	0.00	11/25	44.0	16/30	53.3	
Scoring	I	0	0.0	5/11	45.5	3/16	18.8	P< 0.004 significant
	II	0	0.0	5/11	45.5	9/16	56.2	
	III	0	0.0	1/11	9.00	4/16	25	
Mean Rank		100.4		88.4		93.1		

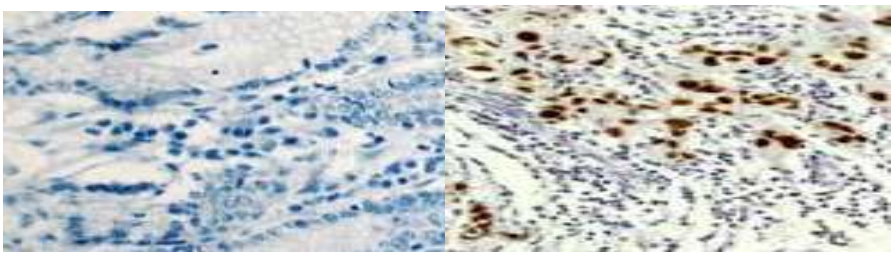

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Figure 2: Immunohistochemical results for CDK2 expression of transitional bladder cancers; DAB chromogen stained (brown) and counter stained by Mayer's hematoxyline (blue); A. Transitional bladder cancers with negative CDK2- IHC reaction (20X).B. Transitional bladder cancers with positive CDK2- IHC reaction (40X).

VI. The CDK2-tumor suppressor gene expression according to the histopathological grading of transitional bladder cancers

Table (6) shows the relation of CDK2-IHC expression to the grade of transitional bladder cancers in this study. The percentage of positive - IHC reactions in the well differentiated grade was (55.5%; 5 cases out of 9) while the percentage of those tissues with moderately differentiated grade that showed positive CDK2-IHC reactions was (61.5%; 8 cases out of 13) and lastly the percentage of these cancers with poorly differentiated grade that showed such positive IHC reactions was (37.5%; 3 cases out of 8). Statistically, there are no-significant differences regarding the distribution of CDK2-IHC reactions according to tissue differentiation of transitional bladder cancers in the present study (P> 0.05).

Table 6: The expression of CDK2-IHC reactions according to the differentiation of transitional bladder cancers.

Cancer Grade		IHC Expression of CDK2 Protein		Total
		Positive	Negative	
Well	Count	5	4	9
	% within grade	55.5%	44.5%	100.0%
	% within P21	31.2%	28.6%	29.6%
Moderate	Count	8	5	13
	% within grade	61.5%	38.5%	100.0%
	% within P21	50%	35.7%	44.4%
Poor	Count	3	5	8
	% within Grade	37.5%	62.5%	100.0%
	% within P21	18.8%	35.7%	25.9%
Total	Count	16	14	30
	% within grade	53.3%	46.7%	100.0%
	% within P21	100.0%	100.0%	100.0%

VII. Co-existed expression of HCMV-CISH and CDK2 – IHC in tissues with transitional bladder tumors

The positive CDK2-IHC expression that associated with positive HCMV-ISH reaction constituted (64.7%: 11 out of 17 tissues) in transitional bladder cancers group while in transitional bladder cancers tissues that showed HCMV-negative reaction by ISH technique, the percentage of positive CDK2 expression was (35.3% :6 out of 17 tissues). Also, in benign bladder tumors the percentage of positive CDK2-tumor suppressor gene expression that showed also positive HCMV-ISH reaction was constituted (56.2%: 9 out of 16 tissues), while the percentage of positive CDK2 expression in transitional bladder cancers tissues that showed HCMV negative reaction was (43.8% : 7 out of 16 tissues) (Table 7).

Table 7: Co-existence of HCMV and CDK2 – expressions in tissues with bladder tumors.

Studied groups				HCMV- ISH Reactions		Total
				Positive	Negative	
Transitional Bladder Cancers	CDK2 IHC Reactions	Positive	N	11	6	17
			%	64.7	35.3	100
		Negative	N	6	7	13
			%	46.2	53.8	100
		Total	N	17	13	30
			%	56.7	43.3	100
Benign Bladder Tumors	CDK2 IHC Reactions	Positive	N	9	7	16
			%	56.2	43.8	100
		Negative	N	7	7	14
			%	50	50	100
		Total	N	11	14	25
			%	44	56	100
Healthy Control Tissues	CDK2 IHC Reactions	Positive	N	0	0	0
			%	0	0	0
		Negative	N	0	15	15
			%	0	100	100
		Total	N	0	15	15
			%	0	100	100

DISCUSSION

The incidence of bladder cancer is increasing, estimated to be the ninth most common cancer worldwide and the 13th cause of cancer related death. Also, this cancer is the 5th most commonly diagnosed non-cutaneous solid malignancy. The interaction of different carcinogenic and co-carcinogenic agents are responsible for urothelial bladder carcinoma, such as alcohol and smoking habits, Schistosoma haematobium infection, exposition to chemicals such as benzidine and bnaphtilamine, analgesic (phenacetin) and antineoplastic drugs (ciclofosfamide, c1ornapazine) prolonged use, but these agents are surely not the unique responsible ones [11].

Bladder cancer is common prevalence is in older population with age over 60 years of old. We findings are consistent with to these data where present studied patients mean age 65.6 ranged between 58-82 years and are also consistent with [12] with age ranged from 36-83 years of their patients and mean age 66 years.

Previous studies reported that more than 90% of all bladder cancers are transitional cell carcinoma which is the most common kind of bladder malignancies [13].

Regarding histological grade of the cancers of this study, the biopsies were collected from cases consisted of 13 moderate differentiated grade 9 well grade and 8 were poorly differentiated carcinoma .Our findings are also compatible with Al Bazzaz ,[14] who reported the highly incidence in the moderate and the lowest occurrence with poorly differentiated carcinoma samples.

Viral infections have been associated to this pathology. Previous studies shown an association of some genotypes of HPV, implicated in the pathogenesis of several human cancer, with bladder neoplasias. Previous studies suggested that cytomegalovirus acts as a cofactor in the pathogenesis of some human cancers [15].

Seventeen out of thirty 30 tissues with transitional bladder cancers showed positive chromogenic in situ hybridization reactions (constituting 56.7% of the total transitional bladder cancer tissues of this study) (Table 3). This finding consistent to Badawi H *et al*., [16] who detected CMV in 48% of bladder cancer patients.

By using polymerase chain reaction, other reporters detected HCMV in 30% of bladder cancer by Gazzaniga *et al*.,[17] also documented that 11% of their bladder cancer patients examined to detect HCMV by using PCR.

One report by Badawi H *et al*.,[18] found a highly significant rate (at $p<0.009$) of HCMV infection in bladder cancer tissue(43%) in group of schistosomal patients , whereas, in non schistosomal patients was detected in 12% both of them. It suggested that HCMV implicated in the development of bladder carcinoma and play a major role in the etiology of bladder cancer. Bedwani *et al.*, [18] showed that schistosomiasis was associated with an increased risk

of bladder cancer and accounted for 16% of bladder cancer cases in that Egyptian population.

The HCMV-DNA positive C1SH results were detected in 61.5% (8 out of 13) of tissue with transitional bladder cancers showing moderate differentiated grade, followed by (5 out of 9) tissues showing well differentiated grade where it comprised 55.6% of the total number of this grade , and lastly by (4 out of 8) tissues with poor differentiated grade where it constituted 50% of total number of this grade .It suggested that HCMV make a great role in the etiology of immunodeficient than in immunocompromont bladder cancer patients[19].

The highly significant correlation of HCMV with bladder cancer in this study could explain the role of viral agent is the most important infection factors associated with human malignancies [20].

Urinary bladder cancer was also shown to be associated with viruses. For example, a meta-analysis , has revealed that infections with high-risk HPV (HR-HPV) types, especially HPV16, might play a significant role in bladder carcinogenesis. HPV16 and HPV18 DNA by using CISH &PCR were detected in nearly 50% of urinary bladder dysplasia and carcinoma specimens when examined using *in situ* hybridization [21]. In another study, HPV16 was found in 40% of transitional bladder cancers [22]. Despite these results, the IARC concluded that still an "inadequate evidence" present to conclude a role of HPV in bladder carcinogenesis.

In the current study the expression of CDK2 was detected in 53.3% (16 out of 30) tissues with transitional bladder cancers and in 44% (11 out of 25) tissues with benign bladder tumor.Constitutive expression of CDKs and the control of various cyclins enables the regulation of specific cell cycle phases by distinct cyclin-CDK complexes [23]. CDK activity is negatively regulated by several inhibitory proteins. Several ATP-competitive small molecule CDK inhibitors have been developed [24].

Particular cdks and particular cyclins are implicated as key molecules which are responsible for progression of cell cycle and cellular division and modulation cdk-cyclin expression has led to an aberrant cancerous cell growth. The cdk2-cyclin A axis promotes G1/S phase transition; this might explain why cdk2/cyclin A down-modulation was accompanied by a G0/G1 phase arrest in the examined cells. Investigation of sarcomatous tissues found that decrease in cdk2 combined with loss of p27 affected the invasion and metastatic spread [25].

The cdk2-cyclin A in many solid tumors are altered, yet, information about cdk2-cyclin A in tissues with bladder cancer are sparse. Studies of bladder carcinoma tissues have revealed that cdk2 closely associated with development and progress of bladder tumors. Cdk2 / Cyclin A has been correlated with tumor grade and poor survival [26] where experiments of knocking down cdk2 or cyclin A have led to a substantial reduction of the tumor cell number, indicating their clinical relevance in bladder cancer.

Cdk2 accumulates at the G1/S phase, whereas cdk1 drives cells into mitosis but no clear reason for this switch over [28]. It was reported that an unfavorable effect of p27 loss associated with bladder carcinoma [29].Experiments have found an indeterminate role of p27 in as much as suppression of this protein elevated growth but simultaneously reduced invasion [30].

Finally, we concluded from this results, positive detection of co-expressions of Cdk2 with HCMV in bladder transitional cell carcinoma could point both for a possible roles in bladder pathogenesis and /or carcinogenesis.

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