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Epstein Barr Virus- Encoded Small Untranslated RNAs (EBERs) in Relation to Translational Expression of P27 Tumor Suppressor Gene in Patients with Bladder Tumors/Mid-Euphrates-Iraq

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Abstract : This study was designed as retrospective research. A total number of (60) formalin-fixed, paraffin embedded bladder tissues were included. Among these cases fifty (50) tissue blocks with bladder neoplasia were furtherly divided into two groups: Thirty (30) tissue blocks with invasive bladder cell carcinoma and twenty (20) tissue blocks from cases with benign bladder tumor. In addition, ten (10) bladder tissue blocks without any significant pathological changes (apparently healthy) were included as a control group for this study.The age of these individuals (patients and control groups) were ranged between 18 and 72 years. Histopathological sections were made for these bladder biopsies and stained by hematoxylin and eosin for final definitive diagnosis. For detection of EBV,ultra sensitive version of In Situ Hybridization(ISH) method was used while the translational expression of P27 gene was immunohistochemically demonstrated. Positive EBV-EBERs -ISH reactions in malignant bladder tissues was detected in 13 out of 30 (43.3%) and in 20% (3 out of 20) of the benignbladder tissues. No positive – ISH reaction for EBV-EBERs was observed in the control bladder tissues.Statistically, the differences between the percentages of positive EBV –EBERs - ISH reactions in bladder cancer group with either benign bladder tumors or control groups are highly significant (P value = < 0.0001).Positive immunohistochemical reactions for P27 protein were observed in 16 cases (53.3 %) of bladder carcinoma and in7 cases (35%) benign bladder tumors while the control groups showed negative P27 immunostaining reactions.
Key word : EBERS-EBV. P27 Bladder Tumor. ISH . IHC.

Introduction

Urinary bladder cancer is the fifth most common cancer in the western world and is responsible for about 3% of all cancer-related deaths. Approximately 55,000 new patients are diagnosed with bladder cancer annually in the United States, and 15,000 of them die of the disease each year[1]. Transitional cell (urothelial) carcinoma (TCC) is the most common urinary bladder neoplasm in the western world. Current pathogenetic

concepts postulate that common urothelial neoplasms of the bladder arise via two distinct but somewhat overlapping pathways: papillary and nonpapillary[2].

Approximately 80% of urothelial tumors of the bladder are superficially growing exophytic papillary lesions that may recur but usually do not invade and metastasize. They originate from hyperplastic urothelial changes. The remaining 20% of urothelial tumors are highly aggressive, solid, nonpapillary carcinomas with a strong propensity to invade an metastasize[2,3].

Epstein Barr Virus is one of the eight known human herpesviruses. Its genome is a linear, double stranded DNA, about 170kb in length. Latently infected cells contain the genome as a circular plasmid in the nucleus. The terminal repeat (TR) sequences are present at both ends of the linear form of the genome and these repeats mediate the circularization in the infected cell, An unusually large tandemly repeated DNA sequence in the genome of EBV is known as the major internal repeat (IR1). The IR1 site divides the EBV genome into long and short unique sequences (UL and US). These sequences are filled with closely packed genes[4].

In addition, the EBV genome contains a viral cytokine, vIL-10, that was pirated from the host genome. This viral cytokine can prevent macrophages and monocytes from activating T-cells are required for EBV-dependent transformation of B-cell[5,6].

The small untranslated RNAs EBER-1 and -2 are accumulated at high levels during all forms of latency and regulate apoptosis through different mechanisms. EBER-1 interacts with the interferon-inducible protein kinase R (PKR), and inhibits its activation by double-stranded RNAs, protecting infected cells from IFN-induced apoptosis[7].

Epstein Barr virus encoded small RNA-2 has however a more prominent role in EBV-mediated growth transformation, as viruses lacking the coding sequence for this RNA were significantly less efficient in generating lymphoplastoid cell lines (LCLs) in vitro, and the cell lines generated proliferated at much lower rates, due to reduced autochrine IL-6 production[8]. These observations have been extended to epithelial cells lines, where EBERs induce the expression of growth factors that promote cell survival[9]. In addition ,a recent study has found that EBV infection may have related to the initial occurrence or further development bladder carcinoma. In analysis of EBV and bladder cancer cases Grinstein et al. [10] observed that 37% (7/19) of bladder cancer cases evaluated displayed EBV by immunohistochemistry and PCR.

As for bladder cancer, [11], detected the EBV genome in 34% of whole bulk tissue samples with polymerase chain reaction (PCR), without specifying the infected cell population. Subsequently,[12] demonstrated EBV-encoded RNA within both carcinoma cells and infiltrating lymphocytes in 21%, only infiltrating lymphocytes in 7%, and only carcinoma cells in 3% of bladder cancers obtained from Taiwanese patients. To date, it remains unclear whether EBV has any significant role for pathogenesis in bladder cancers.

In some bladder cancer cell lines, for example, the combination of cyclin E over expression and p53 loss induces centrosome amplification and chromosome instability[13]. More generally, in tumor cells, cyclin E can be deregulated by a number of mechanisms, including gene amplification, down regulation of p27 or down regulation of the F-box protein Fbw7 (also called hCDC4), which tags phosphorylated cyclin E for proteosomal degradation[14].

Mutations in the gene encoding hCDC4 have been found in breast, ovarian, endometrial[15] and colorectal cancers[16] and are associated with elevated levels of cyclin E protein. More recently, the over expression of miR-27a in pediatric acute lymphoblastic leukemia (ALL) has been shown to suppress Fbw7 expression, leading to improper cell cycle progression and DNA replication stress, consistent with dysregulation of cyclin E expression[17]. They have found that deregulation of cyclin E can also occur through post-translational processing of the full-length cyclin E by an elastase-like protease to generate low molecular weight (LMW) isoforms[18,19]. Expression of these LMW isoforms in tumor cells leads to increased genomic instability[18] due to premature activation of CDC25C14 and shortening of the length of mitosis from nuclear envelope breakdown to prometaphase[20]. Cyclin E levels in tumor tissue associated strongly with disease-specific and overall survival in patients with stage I, II and III disease but had no impact on outcome in patients with stage IV disease. cyclin E expression was found to be deregulated in ovarian cancer[21] as well as melanomas[22]. Invasive transitional-cell carcinomas (TCCs) have greater expression of cyclin E mRNA than do

superficial TCCs or normal bladder cells[23]. Del Pizzoet al., 1999[24] showed that p27(Kip1) expression and cyclin E expression are down regulated as the stage of disease advances.

This study is aiming to analyze the rate of concordance of P27-gene translational expression and Epstein Barr Virus- Encoded Small Untranslated RNAs (EBERs) in bladder tissues from a group of patients with malignant and benign bladder tumors.

Materials and methods

The study was designed as a retrospective one. It has recruited 60 selected formalin fixed, paraffin embedded bladder tissue blocks; among them, [25] tissue biopsies from bladder carcinoma with different grades as well as[20] tissues with benign bladder hyperplasia and apparently normal bladder tissue autopsies which were collected from the archives of Forensic Medicine Institute / Baghdad and used as bladder healthy control groups. The diagnosis of these tissue blocks were based on their accompanied records. A consultant pathologist reexamined all these cases to further confirm the diagnosis following trimming process of these tissue blocks.

In one hand, the detection of EBV by ISH kit (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany) was performed on 4μm paraffin embedded tissue sections usingdigoxigenin-labeled oligo-nucleotidesprobe which targets Epstein-Bar-Virus (EBV) EBER RNA. 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 EBV.

For the In Situ Hybridization procedure,the slides were placed in 60c hot-air oven overnight 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 are:

Incubation of slides for 18 hr at 70°C on hot plate. Rehydration process was done at room temperature which include : Slides were immersed 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 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,(alternatively, dewaxing protocols routinely used in immunohistochemistry procedures, e.g. 2-5 min xylene, 2-5 min 100% ethanol, 2-5 min 96% ethanol, 1-5 min 70% ethanol, can be used . Air drying of sections.

Then application (dropwise) Pepsin Solution (ES1) to the tissue/cell section and incubate for 20-30 min at 37°C in a humidity chamber. After that we immersed slides in distilled water and drain off the water, air dried sections. Then add the probe to the center of a cover slip and place cover slip upside down on target area). Denaturation of the slides at 75°C for 5 min on hot plate, then we add the 20 μl of cDNA probe added to each section and slides were covered by cover slips be careful to avoid trapping any air bubbles. After that probe and target DNA were denaturized by placing in pre-warmed oven at 75°C for 8-10 minutes, slides were transferred to a pre-warmed humid hybridization chamber and incubated at 37°C for overnight. Then the slides were allowed not dry out at any time during the hybridization and staining. All reagents used during hybridization and detection were warmed to room temperature. At the next day, slides were soaked in pre-warmed protein block at 37°C until the cover slips fell off and should be careful not to tear the tissue, and then the slides were allowed to remain in the buffer for 3 minutes, at 37°C after cover slips were removed. Then application of AP-Streptavidin(AB9) drop wise (3-4 drops per slide) to the slides and incubate for 30 min at 37°C in a humidity chamber. Then washed in wash buffer TBS (prepared by usingWB5) and then twice times for 1 min in distilled water and application of 5-bromo3-chloro3-indoly/phosphate/nitro blue tertrazolium substrate-chromogen solution NBT/BCIP(SB4) drop wise (4 drops per slide) to the slides and incubated for 40 min at 37°C in humidity chamber. Slides were incubated at 37°C for 30 minutes or until color development was developed completed. 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 30 seconds, then washing process was followed by immersion the slides for 1 minute in distilled water. After that 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). Then final evaluation by light microscope.

Immunohistochemistry / Detection system (Abcam . England) was used to demonstrate the P27 tumor suppressor genes. This technique is based on the detection of the product of gene expression (protein) in malignant and normal cells using a specific monoclonal antibodies, i.e. Primary antibody for specific epitope (usually mouse antihuman monoclonal antibody), which binds to nuclear targeted protein .

The bound primary antibody is then detected by secondary antibody (usually rabbit or goat anti mouse), which contains specific label (in this context we used peroxidase labeled polymer conjugated to goat anti mouse immunoglobulin). The substrate is DAB in chromogen solution, positive reaction will result in a browning color precipitate at the antigen site in tested tissues.

Statistical analysis

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

Results

The studied groups

The total number of Sixty studied bladder tissues that were included in this research work was distributed on two groups:-

1. Thirty tissues collected from cases with different grades of bladder carcinomas.
2. Twenty tissues collected from cases of bladder benign.
3. Ten apparently healthy bladder tissues were used as control group. These were obtained and selected as an apparently healthy bladder tissues according to the histopathological examinations.

Techniques:

All these tissues were submitted for in situ hybridization (ISH) technique. Using standard histopathological criteria, these tumors were re-examined for final classification into benign tumor and malignant tumors.

Distribution of patients with bladder tumor and healthy control group according to their Age

The archival specimens collected in this study were related to bladder tumor patients whom ages were ranged from fifteen years to seventy five years. The mean age of the patients with bladder carcinoma (41.7 $\pm$ 0.9 years) was higher than the mean age in the group of healthy control (35.8$\pm$ 0.74 years). There are significant statistical differences ($p < 0.05$) between different groups according to age (Table 1)

Table (1): Distribution of bladder tumor patients according to their age .

Maximum	Minimum	S.E	S.D	Mean Age	N	The Patients
72	25.0	0.24	0.9	41.7	30	Malignant Bladder Tumors
68	34	0.23	0.8	40.5	20	Benign Bladder Tumors
63	18.0	0.13	0.74	35.6	10	Healthy B. Tissues Control
(P <0.05) = 0.009						Statistical Analysis

I.EBV-EBERs -Associated Bladder Tumors

The results of EBV- ISH among study groups

It was found after application and analysis of (ISH) results of EBV--EBERs in the tissues obtained from patients with bladder cancer as well as benign bladder tumors that[13] out of thirty patients with carcinoma of bladder showed positive in Situ hybridization reaction where they constituted 43.3% of the total bladder cancer cases of this study (Table 2, Figure 1). In the benign group, 15% has revealed positive signals, which represented 3 out of 20 cases in this group, whereas none of control group presented with positive signals for EBV-EBERs-ISH test. However, in comparison to the percentage of EBV -EBERs in healthy control group as well as in the group of benign bladder tumors, the differences between the percentages of EBV-EBERs in bladder cancers and each of these groups are statistically very highly significant (P value = < 0,001).

Table (2): Results of in situ hybridization for detecting EBV in tissues with bladder tumors.

Studied groups		EBV-EBERS -ISH		Total	Comparison of significance	
		Positive	Negative		P-value	Significance.
bladder Cancer	N	13	17	30	0.0001	Highly* Significant. (P<0.001)
	%	43.3	56.7	100		
Benign bladder	N	3	17	20		
	%	15	85	100		
The Control	N	0	10	10		
	%	0	100	100		

- The difference in signal scoring of positive reactions for EBV- EBERs between benign bladder tumors and bladder cancer groups (healthy controls are not part in this comparison, since all of them were negative) was statistically highly significant [HS] (P Kruskal-Wallis = 0.001).

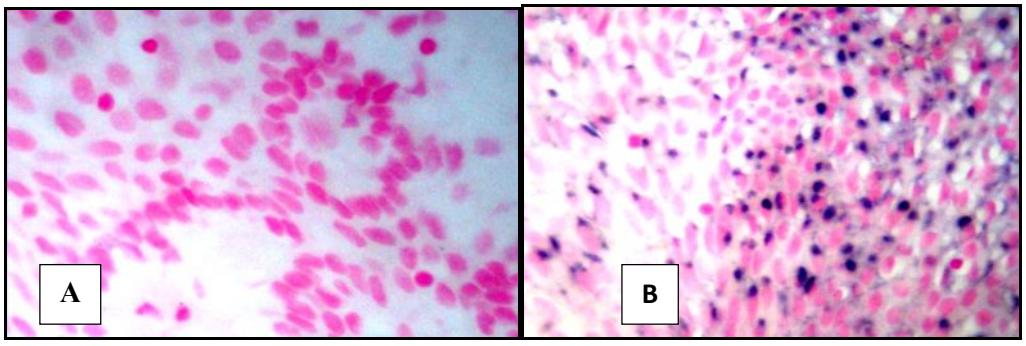

Fig.(1) :In Situ Hybridization (ISH) for EBV-EBERs -ISH Deduction Bladder Cancers Using Biotinylated -Labeled EBV-EBERs Probe ;Stained with NBT/ BCIP (Blue)and Counter Stained by Nuclear Fast Red(Red).A. Bladder Cancer with negative EBV-EBERs -ISH reactions (40X). B.Positive -EBV-EBERs -ISH reaction with strong score and high signal intensity(40X).

Co-existence of EBV-EBERs-ISH and P27 –IHC expression in tissues with bladder cancers.

The percentage of positive **P27**-tumor suppressor gene expression that associated with positive EBV-EBERs ISH reaction was constituted (53.3%:16 out of 30 cases) in bladder cancer group. Also, in benign bladder tumors the percentage of positive P27-Tumor suppressor gene expression was constituted (35%: 7 out of 20 cases) (table 3 and Fig. 2). The statistical analysis showed significant association ($p<0.05$) on comparing the results (according to score) when group of bladder cancer was compared to benign and control group.

Table (3): Co-localization of EBERs along with P27 gene expression in tissues with bladder cancers.

Studied groups				EBV- EBERS-ISH		Total
				Positive	Negative	
Bladder Cancer	P27 IHC Reaction	Positive	N	7	6	13
			%	53.8	46.2	100
		Negative	N	9	8	17
			%	52.9	47.1	100
		Total	N	16	14	30
			%	53.3	46.7	100
Benign Bladder Tumors	Rb IHC Reaction	Positive	N	3	17	20
			%	15	85	100
		Negative	N	4	16	18
			%	28.6	71.4	100
		Total	N	7	13	20
			%	30	70	100
The Control	Rb IHC Reaction	Positive	N	0	0	0
			%	0	0	0
		Negative	N	0	12	12
			%	0	100	100
		Total	N	0	12	12
			%	0	100	100

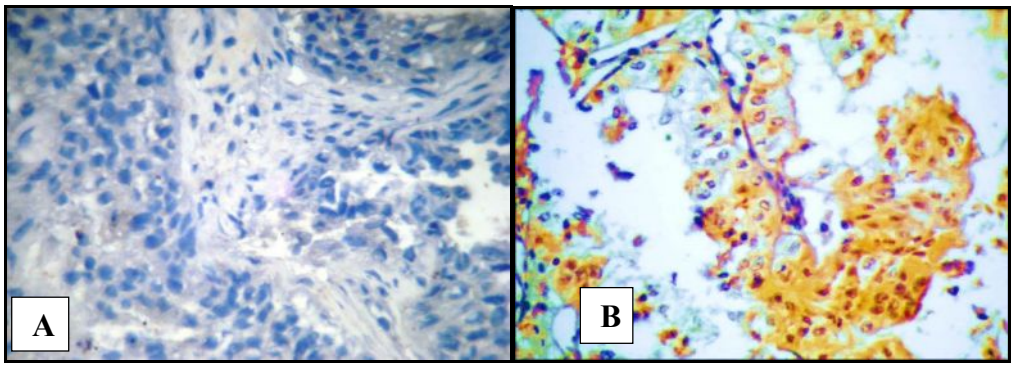

Fig.2 : Immunohistochemical results for P27 expression detection in bladder tumor; DAB chromogen stained (brown) and counter stained by Mayer's hematoxyline (blue); A. Bladder cancer with negative P27IHC reaction (20X).B. Bladder Carcinoma with positive P27- IHC reaction (20X).

Discussion

In the current study, we found new evidence that there is an association between EBV-EBERs and bladder carcinoma and indicate the important role of EBV in bladder tumors. The positive EBV-EBERs -ISH reactions in malignant bladder tissues in the present study were documented in 43.3% while in the benign bladder tissues was detected in 20%. No positive – ISH reaction for EBV-EBERs was observed in the control bladder tissues. These result were in consistent with the finding of[11] who detected EBV genome in 34% of whole bulk tissue samples using PCR and[25] who demonstrated EBV-encoded RNA within both carcinoma cell and infiltrating lymphocytes in 21%, only infiltrating lymphocytes in 7%, and only carcinoma cells in 3% of bladder cancers obtained from Taiwanese population.[26] reported that EBER-expressing lymphocytes were detected in the bladder carcinomas in 26 out of 39 cases (66.7%) while all normal urinary bladder specimens showed negative results. The frequency of the infiltrating EBV-positive lymphocytes in the stromal region of bladder carcinoma was significantly higher in advanced T-stages than in earlier stages[26].

These differences might be related to the geographic variation, the sensitivity of the probe used for ISH, or differences between the subjects studied, yet a definitive reason is not apparent.

The reason for EBV to exert its oncogenic influences in a particular patient is unknown but is probably associated with co-factors. Again it is possible that HPV exerts its oncogenic influences in concert with co-factors including a possible collaboration with EBV[27,28]. reported that co-cultivation with viral producers resulted in an approximately 800-fold higher efficiency of infection than use of a cell-free system, indicating the importance of cell-to-cell contact in EBV infection. Although the theory that an immunosuppressive environment in the tumor stroma allows virus persistence and replication is reasonable for our results, this is a highly selective look and further examinations are needed to support this theory.

In the present study the immunohistochemical positive results for P27 protein were observed in 16/30 cases (53.3 %) of bladder carcinoma and in 7/20 cases (35%) benign bladder tumors while the control groups showed negative P27 immunostaining reactions.

Steeg and Abrams[29] reported that p27 and cyclin E play a central role in the transition from late G1 to S phase. Therefore, it is possible to assess the role of p27 and cyclin E in tumor recurrence and progression and therefore their value in predicting survival in patients with bladder cancer.

By analogy of the current results with other types of cancers, a significant inverse correlation of p27 protein levels with grade and stage was found. Low p27 protein levels are associated with poorer survival in breast[30], gastric[31], prostate[32], upper uninary tract[16], and bladder cancers[24]; [33]. In addition, it has revealed that a low protein level of p27 was correlated with a negative prognosis for patients with lung[34], colorectal[35], and ovarian cancers[36]. In the present study, low protein levels of p27 were associated with poorly differentiated grade bladder cancers and as such patients with these cancers could have unfavorable prognosis. It is likely that p27 affects differentiation pathways and acts as a tumor suppressor gene in different human tumors and therefore the evaluation of p27 protein levels may indicate the biological behavior of human tumors. Since p27 levels are mainly regulated by ubiquitin mediated proteasome degradation, which is targeted by cyclin ECdk2 phophorylation of p27[37,38], it is likely that loss of p27 expression and high expression of cyclin E are associated with cancer progression and unfavorable prognosis. In this respect, low p27 protein levels and increased protein levels of cyclin E are correlated, for example, with shortened survival in breast cancer[38-42]. There may be different regulatory mechanisms in the expression of these genes between breast and bladder cancer. [24] suggested that cellular down-regulation of cyclin E may be an attempt to offset loss of p27 expression during tumor growth via a feedback inhibitory loop.

Low p27 was correlated with poorly differentiated grade, muscle invasion, lymph node involvement and poor survival in bladder cancer patients[24] and so could the low staining results of p27 in current study are possibly to be correlated with such sequels[38].

Conclusions

The highly significant translational expression of P27 gene as well as high rate of occurrence of EBV in bladder carcinomain our results could indicate for an important role of these molecular and viral factors in the bladder carcinogenesis of subset of bladder malignant tumors.

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Compliance with Ethical Standards:

Conflict of Interest: The authors declare no competing interests.

Ethical approval: This article does not contain any studies with animals performed by any of the authors.

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