Journal of Natural Sciences Research
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.3, No.8, 2013
www.iiste.org

Molecular Localization of Epstein Barr Virus and Rb Tumor Suppressor Gene Expression in Tissues from Prostatic Adenocarcinoma and Benign Prostatic Hyperplasia

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Summary
Background Epstein- Barr virus (EBV) is a ubiquitous in that infecting more than 90% of adult population worldwide. Recently, EBV has been linked to the development of variety of human malignancies including prostate tissues that range from benign prostatic hyperplasia (BPH) to prostatic adenocarcinoma (PAC). Somatic point mutations in *Rb gene* have been detected in prostate cancer and are involved in progression steps of prostate carcinogenesis.
Objective: To analyze the distribution and impact of concordant Rb expression and latent EBV infection on a group of prostate adenocarcinoma and benign prostatic hyperplasia**.**
Patients and methods: Seventy- two (72) formalin-fixed, paraffin- embedded prostatic tissues were obtained in this study; (40) biopsies from prostatic carcinoma and (20) from benign prostate hyperplasia as well as (12) apparently normal prostatic autopsies control group. Detection of EBV-EBERs was done by ultra sensitive version of in situ hybridization method where as immunohistochemistry detection system was used to demonstrate the expression of Rb gene.
Results: Detection of EBV-EBERs -ISH reactions in tissues with PAC was observed in 19 out of 40 (47.5%), while in the tissues from BPH was detected in 10% (2 out of 20). No EBV-EBERs positive – ISH reaction was detected in healthy prostate tissues in the control group. The differences between the percentages of EBERs detection in tissues PAC and each of BPH & control groups were statistically highly significant (P value = < 0.0001).
Positive Rb immunohistochemical (IHC) reactions were observed in 19 PAC cases (47.5%) and in 2 BPH cases (10%).
Conclusions: Our results indicate that the EBV might contribute to the development of subset of prostate tumors. In addition, the significant percentage of expression of possible Rb gene as well as EBV in prostate adenocarcinoma could indicate for an important role of these molecular and viral factors in prostatic carcinogenesis.
Key word: EBV; prostate adenocarcinoma, benign prostatic hyperplasia, in situ hybridization.

Introduction

Most common neoplasms of the male genital tract involve the prostate gland (1). Prostate cancer is the fifth common cancer world-wide and second in cancer mortality exceeded only by lung cancer (2, 3).
Viral factors are the most important class of infectious agents associated with human cancers (4). It was estimated that 17-20% of all worldwide incidence of cancers are attributable to a viral etiology (5).
EBV is a typical virus consisting of a core containing a linear, double stranded DNA; an icosahedral capsid, approximately 120-200 nm in diameter, containing 162 capsomeres; an amorphous material that surrounded the capsid,(tegument)and an envelope containing viral glycoprotein spikes on its surface(6). Sequence analysis has defined two strains of EBV : type I and type II (alternatively named EBV A and B) which differ at the domains that encode EBV latent proteins, namely EBERs, and the nuclear antigens EBNA-LP,1,2,3A,3B and 3C in latently infected cell(7).
EBV has been classified as a group 1 carcinogen associated with a variety of lymphoid and epithelial malignancies by the international agency for research of cancer {IARC}(8). Evidence of EBV being a monogenic virus is drives from its ability to infect and transform normal human B cells in vitro, resulting in immortalization of these cells and leading to continuous growth of lymphoblastoid cell lines. Moreover, EBV can transform human squamous epithelial cells in vitro. The virus is involved in the development of several human cancers such as nasopharyngeal carcinoma and various lymphomas (9).
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 (PKRO, and inhibits its activation by double-stranded RNAs, protecting infected cells from INF-induced apoptosis (10).
EBV encoded small RNAs have 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 lymphoblastoid cell lines(LCLs) in vitro, and the cell lines generated proliferation at much lower rates, due to reduced autocrine IL-6 production (11). These observations have been extended to epithelial cells lines, where EBERs induced the expression of growth factors that promote cell survival(12).

The EBV latent proteins expression contribute to most, if not all, of the transforming and immortalizing properties of this prototype DNA oncogenic viral agent. In addition to EBNA1 and the EBERs, human cancer cells , that are latently infected with this virus express the most powerful oncogenic proteins, LMP-1 and LMP-2(A and B) (10).

Besides chromosomal loss and mutation , there are various other mechanisms for Rb inactivation. Also, Rb can be inactivated in tumors by the loss of one allele and hypermethylation of the other alleles(13). Interestingly, a recent survey of Rb status in metastatic breast cancer revealed two cases with duplication of the entire gene(14). This may be related to a phenomena observed in colorectal carcinoma, where high expression of pRb was shown ,paradoxically ,to protect from E2F-induced apoptosis (14,15).In addition ,expression of constitutively active phosph-mutant Rb transgenes in mouse mammary epithelium induces adenocarcinoma(16). Thus, both activation and inactivation of pRb can be oncogenic in the mammary gland (16). Rb inactivation was observed to increase the proliferative potential of the cells which was associated with over expression of cyclin dependent kinase (17). The deregulation of the Rb pathway is the primary function of each of the DNA tumor virus oncoproteins that promote cellular proliferation, this includes the adenovirus E1 A protein, polyoma virus ,SV40 T antigen and HPV E7 protein(18). Rb is functionally inactivated in 25-30% of prostate cancers; furthermore, Rb loss is correlated with increasing tumor stage and grade. The clinical consequences of Rb loss are unknown. It was shown previously that Rb loss results in a castrate resistant phenotype. The hypothesized that Rb loss would down regulate the G1-S cell cycle arrest normally induced by irradiation, inhibit DNA repair, and subsequently sensitize cells to mitotic catastrophe (19).

Materials and methods:

The study was designed as a retrospective one. It has recruited 72 selected formalin fixed, paraffin embedded prostatic tissue blocks among them; (40) tissue biopsies from prostatic carcinoma with different grades and (20) benign prostate hyperplastic tissue blocks as well as (12) apparently normal prostate tissue autopsies which were collected from the archives of Forensic Medicine Institute / Baghdad and used as prostate healthy tissues 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.

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 .The detection of EBV-EBERs by ISH kit (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany) was performed on 4µm paraffin embedded tissue sections using digoxigenin-labeled oligo-nucleotides probe which targets Epstein-Bar-Virus (EBV) EBER RNA.

For the in situ hybridization procedure, the slides were placed in 60 C˚ hot-air oven over night then the tissue sections were de-paraffinized and via then incubation of slides for 15 min (twice time) in xylene then treatment by graded alcohols via incubation for 5 min in 100% ethanol(twice time) .The same dewaxing were protocols routinely used for immunohistochemistry procedures, e.g. 15 min xylene (twice time), 5 min 100% ethanol(twice time), 5 min 96% ethanol(one time), 5 min 70% ethanol(one time), were used, 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. Then we done digestion process by add proteinase K to the slides, then the slides were incubated at 37°C for 15 minutes. Then the slides were dehydrated by immersing them sequentially in the following solution at room temperature for the indicated times, distilled water for 1 minute, 70% ethanol for 1 minute, 95% ethanol for 1 minute and 100% by incubating them at 37% for 5 minutes. 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 denaturated by placing the cover slipped-slides in pre-warmed oven at 95°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, then the slides were allowed to remain in the buffer for 3 minutes, at 37°C after cover slips were removed. After that we add streptavidin-alkaline phosphatase conjugate reagent were added to tissue sections. Then slides were kept in a humid chamber at 37°C for 20 minutes. Then one to two drops of Slides were rinsed in detergent wash buffer for 5 minutes and then drained. After that One to two drops of 5-bromo3-chloro3-indoly/phosphate/nitro blue tertrazolium substrate-chromogen solution(BCIP/MBT) were placed on tissue section. 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 form 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 alchol, (95%, once for one minute then, 100% twice times for 2 minutes each); cleared by Xylen, then mounted with permanent mounting medium (DPX).

Immunohistochemistry / Detection system (US Biological Inc . USA) was used to demonstrate the Rb protein encoded by Rb tumor suppressor gene. This technique is based on the detection of the product of gene expression (protein) in malignant and normal cells using a specific primary monoclonal mouse antihuman antibodies for specific epitope on that nuclear targeted protein . The bound primary antibody is then detected by secondary antibody (goat anti mouse) , which contains specific label (used peroxidase labeled polymer conjugated to goat anti mouse immunoglobulin) . The substrate is DAB in chromogen solution and the positive reaction has resulted in a brown color precipitate at the antigen site in tested tissues (20).

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

Results

The distribution of Gleason's grading of prostate carcinomaccording to the ISH results for EBV-EBERs detection.

The EBV-EBERs positive results of 1SH were detected in 50% (8 out of 16) of tissues with prostatic cancers showing Gleason's grade (8-10) (poorly differentiated grade), followed by the tissues showing Gleason's grade (5-7) (moderate differentiated grade) (i.e. 6 out of 13) where it comprised 46.2% of the total number of this grade , and lastly by tissues with Gleason's grade (2-4) (well differentiated grade) where it constituted 45.5% of total number of this grade (i.e. 5 out of 11).

Statistically, the distribution of ISH results for detection of EBV-EBERs according to the Gleason's grading of prostate carcinoma shows non-significant differences (P>0,05) (table 1).

Table (1): Distribution of ISH results for EBV-EBERs according to Gleason's grading of prostatic carcinoma

Gleason's Grade		EBV-EBERS-ISH		Total	Comparison of Significance	
		Positive ISH	Negative ISH		P-value	Significance
2-4	N	5	6	11	0.15	Non* Significant (P>0.05)
	%	45.5	54.5	100		
5-7	N	6	7	13		
	%	46.2	53.8	100		
8-10	N	8	8	16		
	%	50	50	100		
Total	N	19	21	40		
	%	47.5	52.5	100		

*The difference in signal scoring results for detection of EBV-EBERs-ISH according to the Gleason's grading of prostate carcinoma shows non-significant differences (P>0.05) [NS] (P Kruskal-Wallis = 0.15).

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 prostatic cancer as well as benign prostatic hyperplasia that (19) out of (40) patients with carcinoma of prostate showed positive in situ hybridization reaction where it constituted 47.5% of the total prostatic cancer cases of this study (table 2 and figure 1). In the benign group, 10% has revealed positive signals, which represented 2 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 prostatic hyperplasia, the differences between the percentages of EBV-EBERs in prostatic 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 prostatic tumors.

Studied groups		EBV-EBERS -ISH		Total	Comparison of significance	
		Positive	Negative		P-value	Significance.
Prostatic Cancer	N	19	21	40	0.0001	Highly* Significant. (P<0.001)
	%	47.5	52.5	100		
Benign Prostatic Hyperplasia	N	2	18	20		
	%	10	90	100		
The Control	N	0	12	12		
	%	0	100	100		

* The difference in signal scoring of positive reactions for EBV- EBERs between benign prostatic hyperplasia and prostatic 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).

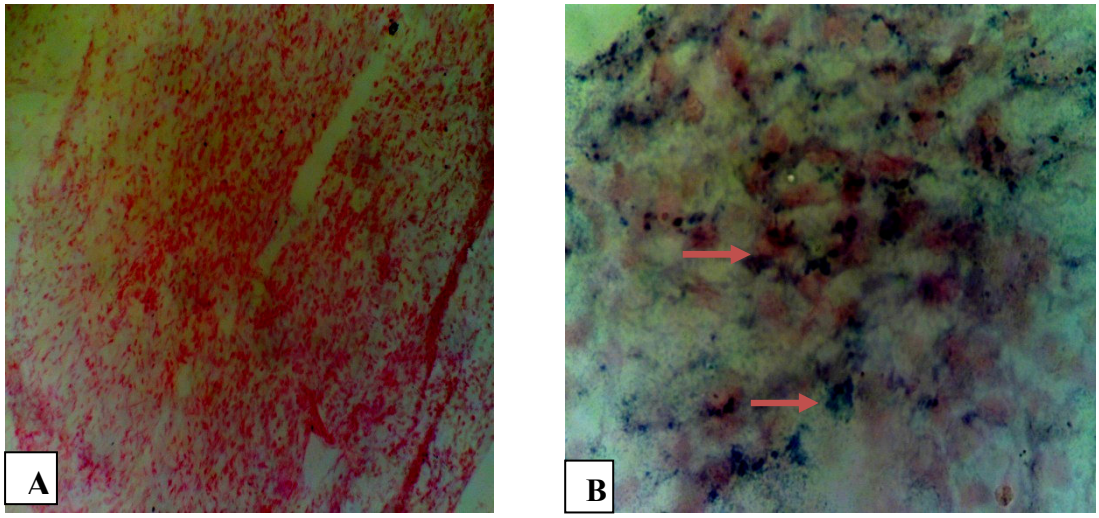


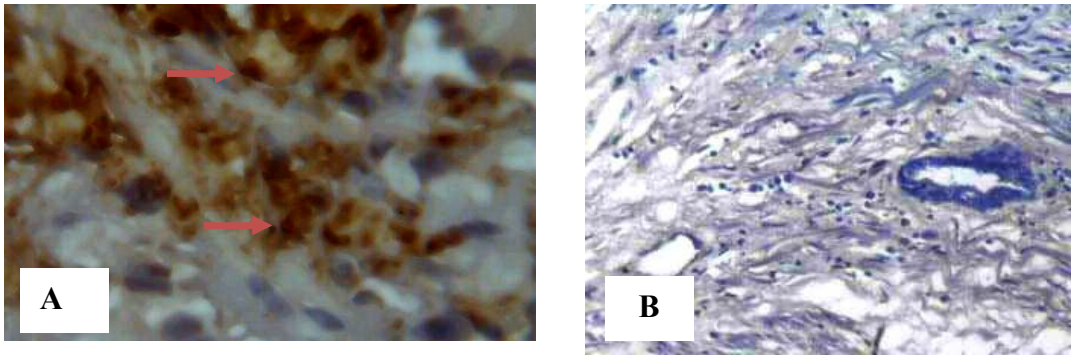
Figure (1): In situ hybridization results for EBV-DNA detection in prostate tumors; BCIP/NBT stained and counter stained by nuclear fast red; A. Healthy Prostatic tissues with negative ISH reaction for EBV (10X).B. prostate cancer with positive ISH reaction for EBV-DNA (40X).

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

The percentage of positive Rb-tumor suppressor gene expression that associated with positive EBV-EBERs ISH reaction was constituted (63.2%:12 out of 19 cases) in prostatic cancer group, while the percentage of positive Rb expression was (36.8% :7 out of 19 cases) in prostatic cancerous tissues that showed EBV-EBERs-negative reaction by ISH technique. Also , in BPH the percentage of positive Rb-Tumor suppressor gene expression that showed also positive EBV- EBERS reaction was constituted (10%: 2 out of 20 cases) in prostatic cancer group, while the percentage of positive Rb expression in prostatic cancerous tissues that showed EBV-EBERS negative reaction was (28.6% : 4 out of 20 cases) (table 3 and figure 2). The statistical analysis showed significant association ($p<0.05$) on comparing the results (according to score) when group of prostate cancer was compared to control group , but the statistical difference between benign breast tumor and control groups was not significant.

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

Studied groups				EBV- EBERS-ISH		Total
				Positive	Negative	
Prostatic Cancer	Rb IHC Reaction	Positive	N	11	8	19
			%	63.2	36.8	100
		Negative	N	5	16	21
			%	23.8	76.2	100
		Total	N	16	24	40
			%	40	60	100
Benign Prostatic Hyperplasia	Rb IHC Reaction	Positive	N	2	18	20
			%	10	90	100
		Negative	N	4	14	18
			%	28.6	71.4	100
		Total	N	6	14	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



(Figure 2): Immunohistochemical results for Rb expression detection in prostate tumor; DAB chromogen stained (brown) and counter stained by Mayer's hematoxyline (blue); A. Prostate cancer with positive IHC reaction (100X).B. Benign prostatic hyperplasia with negative IHC reaction (10X).

Discussion:

Significantly high percentage of EBERs detection in PAC group (47.5%) was observed on comparison to BPH and control groups. These results are consistent to those reported by (21) who identified EBV in 37% (7 out of 18 cases) of prostate adenocarcinoma in US males by immunohistochemistry and PCR techniques and to those obtained by (22) who identified EBV in 40% (4 out of 10 cases) of malignant prostate tissue in Australia. However, (23) identified EBV in 8.8% (31 out of 352 cases) in benign and malignant prostate tissues in Sweden while (24) identified EBV in 8% (16 out of 200 cases) of malignant prostate tissues. The small sample size enrolled in the studied groups has compromised the statistical power of this study to detect the effects of these factors under consideration. In addition, the lack of detailed clinical information attached to those prostate tissue samples that were included in this study has also deprived the present study to reach to a solid impression for the real role of those mixed viral infections in prostate carcinogenesis and in turn raised a suggestion to compel an integrate team-work study, at molecular and virological levels to elucidate the role of these factors and many other agents in prostate carcinogenesis in this country. Also in the future, it will be interesting to design experimental studies to understand the synergistic effect of HPV with EBV and /or HSV mixed infections in prostatic carcinogenesis.

The reason for EBV to exert its oncogenic influences in a particular patients is unknown but is probably associated with co-factors. The findings in the research by (25) have supported hypothesis that the prostate is a habitat for multiple viral and other infectious agents ,some of which have oncogenic potential. In addition ,a

study has found that EBV infection may have related to the initial occurrence or further development prostate carcinoma. It is possible that EBV exerts its oncogenic influences in concert with co-factors including a possible collaboration with EBV (22).

Among the examined tissues with Gleason's grades 6-8 ,1-5 ,and 9-10 that were collected from patients with prostatic cancer , 35.7% , 40.0% and 46.2% of them respectively have showed positive – in situ hybridization reactions for EBV-EBERs whereas the rest of the evaluated tissues denied to show any reaction for such viral EBERs. It is noteworthy in this study that an increasing trend of association of EBV infection to accompany the deterioration in the histopathological features of the examined prostatic cancer tissues, that is an increasing percentages of detection of EBV EBERS with the advancing of Gleason's grading of cancerous tissues of this study. This could also means, in turn, that there are an additional possible effects of EBV infection, along with other factors, in deterioration of the histopathology of prostatic cancerous tissues obtained from those Iraqi studied patients.

Structural alterations in the entire coding regions (exons 1 to 27) of the retinoblastoma (Rb) gene in primary human prostate cancers were investigated, using polymerase chain reaction and single strand conformational polymorphism analysis of RNA. Of 25 samples obtained from patients, four (16.4%) were found to have Rb alterations. DNA sequencing of the PCR products revealed point mutations resulting in single amino-acid substitutions of exons 6 and 19 in two cases, and base deletions of exons 8 and 17 in two cases(26). Two of four cases with Rb mutations were moderately differentiated localized tumors and other two with Rb mutations were poorly differentiated tumors with metastases. Our results could suggest that RB gene mutation is involved in progression steps of prostate carcinogenesis.

EBV encodes six nuclear proteins, designated EBNA 1-6. The EBNA-5 protein of EBV is also able to bind RB in vitro. In addition Rb can interact with several cellular proteins, including the transcription factor E2F (19). (16) found a striking co-localization between the EBNA-5 (alternatively designated EBNA-LP)and Rb proteins in the lymphoblastoid cell line The researchers.(19) have found the COOH- terminal region of EBNA-5 is not required for complex formation with Rb, forms a complex with E2F during S phase. The latter complex contains cyclin A and cdk2 as well. The Rb has been shown to directly repress c-myc promoter activity in keratinocytes through an element upstream of the P1 transcription initiation site in the c-myc promoter(16).

Conclusions

The high percentage of EBV-associated PAC and BPH in our results might indicate for the oncogenic potential of EBV in these cases as well as pointing for its crucial role in development, transformation and /or progression of a subset of prostate cancers and benign prostatic hyperplasia.

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