Impress of EBV RNA (EBERs) and P16 on Grades of Prostate Carcinoma

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Abstract

Prostate carcinoma (PCa) is second most common cancer in men in the world and first most common cancer in American and European men. Epstein Barr virus (EBV) is directly infects B cells and lead to transform of these cells into apoptotic-resistant B cells, EBV is an etiological agent in carcinomas of the breast, lung, colon and prostate, in addition to nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC). PCa varies in their proliferative rate, mostly grow slowly and many of patient die with small undiagnosed PCa and only small proportion of this cancer shows rapid proliferation rate and spread to outside of prostate with preference to the bones and lymph nodes. The aim of this study is detection of EB encoded RNAs (EBERs) and tumor suppressor protein (P16) in Prostate carcinoma as well as their association with its grades. To achieve this purpose In situ hybridization (CISH) was used to detect the presence of EBERs and Immunohistochemistry (IHC) to evaluate the over expression of mutated p16 tumor suppressor gene in tissue after collected thirty 30 formalin-fixed Paraffin embedded tissue blocks were obtained from prostate carcinoma biopsies Transurethral Resection of Prostate (TURP) during the period from May 2016 to December 2016.

The grade of cancer evaluated as following:

{Grade I=≤6, Grade II=(3+4)=7, Grade III=(4+3)=7, Grade IV=(4+4)=8, Grade V=9+10}.

From this study we can conclude the presence of EBERs and P16markers in all PCa grades but highly percentage in grade III because the high percentage of samples in this study from this grades, and presence of EBV increase the proliferation, may be inhibit tumor suppressor protein and killing immune cells.

Introduction

The most commonly used system for classifying histologic characteristics of Prostate carcinoma (PCa) is the Gleason score described by Gleason et al. in 1974 [1,2]. It is based on the glandular style of the tumor. Both the predominant and the second most prevalent architectural pattern are assigned as grade from 1 to 5 (1 being the most differentiated and 5 being the least differentiated). The outcomes of men with moderately differentiated (Gleason scores 5-7) and poorly differentiated (Gleason scores 8-10) cancers succeed without treatment (watchful waiting) [3]. The death rate from PCa at 10 years for men aged (65-74) years with moderately differentiated cancers (Gleason score 5-7) diagnosed with checking in the PSA era and pre PSA era (without screening) was 2-6% and 15-23% respectively. The 10-year cancer death average in PSA and pre PSA eras was 25-38% and 50-66% respectively. For men with poorly differentiated cancers, in other study of men from the prePSA era succeeded with watchful waiting (56% over age 70 years). Progression to remote metastasis or PCa cancer death was 13.9% and 12.3% respectively for Gleason score 6 or below, but it was considerably higher at 18.2 and 22.7%, 30% and 20%, 44.4% and 55.6% for Gleason 3+4, 4+3, and 8-10 respectively [4].

EBV is very prevalent in United States (about 90%) of adults are positive for this virus at four decade of life [5]. Many diseases affect to the prostate gland include infections and neoplasms, prostate cancer (PCa) is the commonest cancer in males with ratio of one in every six men [6]. EBV-encoded RNA 1 (EBER1) and EBER2 are translated RNAs and are the most numerous viral transcripts in latent cells infected with EBV, which play a great role in the effective growth transformation of primary EBV-induced B cells [7].

The tumor suppressor gene (p16) is considered as a cycline-dependent kinase inhibitor and a serious negative cell cycle regulator. One of the common events in PCa is the inactivation

of (p16) gene. Prostate tumor cell growth is inhibited by replacement of p16. The mechanism of PCa suppression by P16 depends on the pRb functional status of the cells, while (p16) causes pRb+ cells to subject inhibition by senescence, whereas pRb- cells are also inhibited, but not by senescence [8].

Materials and Methods

Sample size

Thirty 30 formalin-fixed Paraffin embedded tissue blocks were obtained from prostate carcinoma biopsies Transurethral Resection of Prostate (TURP), The age of the patients ranged between 40-80 years.

Study sites

The samples were collected from archives of laboratory in Ghazy Al-Hariri hospital for surgical specialties and from private histopathology laboratories in Baghdad/Iraq.

Materials

In situ **hybridization detection kit:** Kit contents: In situ hybridization detection kit from abcam lot-N63-922091071.

In situ **hybridization EBV probe:** The Probe (Biotin-labeled) was produced by Zytofast/Germany/Cat Numbers (T-1014-40). The probe contains biotin-labeled oligonucleotides which target EBV EBER RNA.

Immunohistochemistry detection kit of Anti-p16: Anti-p16 ARC antibody [EP1551Y] ab51243 Abcam/United kingdom (UK)

Monoclonal antibody for p16: (ab51243) clone number EP1551Y.

Methods

In situ **hybridization for detection of EBV by EBERs:** Principle of the test: The presence of certain nucleic acid sequences in cells or tissue can be detected with in situ hybridization using labeled RNA Probes. The hybridization results in duplex formation of sequences present in the test object and the specific gene probe. It is indirectly detected using an enzyme-conjugated antibody targeting the tags: the enzymatic reaction of chromogenic substrates leads to the formation of a color precipitate that is visualized by light microscopy at 10-20X (strong blue-violet signals). Slides preparation: Serial thin sectioning of (4 μm) thickness was done" for each paraffin-embedded tissue block and sticking the sections on charged slides". CISH signals were determined for at least 10 high power fields. Nuclear fast red staining was considered a positive result for EBERs. Positive CISH signal patterns were classified as follows: (1) diffuse (D), when nuclei were fully stained; (2) punctate, when distinct dot-like intranuclear signals were noted; (3) mixed, diffuses, and punctate (D/P) if both patterns are noted [9].

Detection of p16 by immunohistochemistry: Principle of the test: This detection system detects a specific antibody bound to an antigen in tissue sections. The specific antibody is located by a biotin-conjugated secondary antibody. After that, add streptavidin-enzyme conjugate that binds to the biotin present on the secondary antibody. The complex (specific antibody, secondary antibody, and streptavidin-enzyme) can visualize with an appropriate substrate/chromogen.

Gleason score: A Gleason score is given to PCa based upon its microscopic appearance [10]. Cancers with a higher Gleason score are more aggressive. Pathological scores range from 2 through 10, with higher number indicating greater risks and higher mortality. A total score is calculated based on how cells look under a microscope, with half the score based on the appearance of the most common cell morphology (scored 1-5), while the other half based off the appearance of the second most common cell morphology (scored 1-5). These two numbers are then combined to produce a total score for the cancer [11].

Results

Distribution grades of malignancy group

Table 1 shows redistribution grades of malignancy group, frequencies, and percentages with comparisons significant.
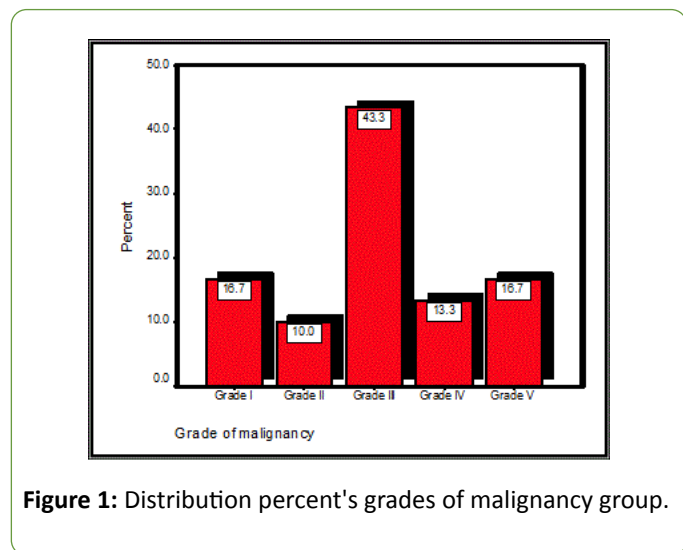
Table 1: Distribution grades of malignancy group (Frequencies and Percent's) with comparison significant.

Grade	No.	Percent	C.S. P-value
I	5	16.67	$\chi^2=6.800$
II	3	10.00	P=0.031
III	13	43.33	(S)
IV	4	13.33	
V	5	16.67	
Total	30	100	
S: Sig. at P<0.05; Testing are based on "Chi-Square (χ^2)" test.			

Results in **Table 1 and Figure 1** shows that majority of percent's grades distribution was found in grade III, and they were accounted 13 (43.33%), while grade II were accounted lowest grade with 3 (10%), as well as significant difference at P<0.05 among frequencies of grades distribution in light of their an expected outcomes distribution.

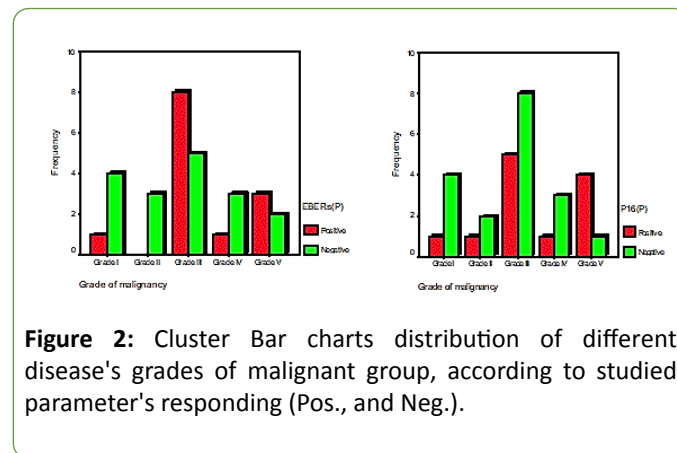**Figure 1:** Distribution percent's grades of malignancy group.

Results of *In situ* hybridization (ISH) for EBV-encoded RNAs (EBERs) and (P16) parameter by IHC with malignant group

Figure 2 shows 8 from 13 (61.53%) cases of the malignant group had a positive EBERs marker in grade III then 3 (23.07%) in grade IV, only 1 (7.69%) in grade I and IV. While, P16 marker had positive reaction in 5 (41.66%) from 12 cases of the

malignant group in grade III, 4 (33.33%) in grade V, and only 1 (8.33%) the same percentage in grade I, II, and IV.

Figure 2: Cluster Bar charts distribution of different disease's grades of malignant group, according to studied parameter's responding (Pos., and Neg.).

Table 2 shows not significant relationship between EBERs and P16 are accounted 0.176.

Discussion and Conclusion

Relationships between grades of malignancy distribution and EBERs, P16 markers

Primarily, 8 from 13 (61.53%) cases of the malignant group had a positive Epstein Barr Encoded RNAs (EBERs) marker in grade III then 3 (23.07%) in grade IV, only 1 (7.69%) in grade I and IV that mean presence of Epstein Barr Virus (EBV) almost in all PCa grades, but highly percentage in grade III may be because most of samples from this grade, and that's mean this virus have essential role of cancer development.

Table 2: Distribution of the studied (EBERs) parameter responding with p16 in malignant group.

Parameter	Response	No. and Percent	EBERs		Total	C.S. (*) P-value
			Positive	Negative		
P16	Positive	No.	7	5	12	C.C.=0.240
		% EBERs	23.3%	16.6%	40.0%	P=0.176 (NS)
	Negative	No.	6	12	18	OR=2.800
		% EBERs	20.0%	40.0%	60.00%	(0.619-12.664)
*S: Sig. at P<0.05; NS: Non Sig. at P>0.05; [C.C.: Testing based on a Contingency Coefficient test], and OR: Odds Ratio.						

Hadi et al. study which done in 2015 was found that (22.5%) of EBV-EBERs in grade III in nasopharyngeal cancer (NPC) patients [12] such results agree with that of present study in relation of virus with high grade cancers, while Tumor suppressor protein (P16) marker had positive reaction in all grades as follow: 5 (41.66%) from 12 cases of the malignant group in grade III, 4 (33.33%) in grade V, and only 1case (8.33%) in the rest grades (I, II, and IV), but high level in grade III which may be indicate to decrease the regulation of tumor suppressor protein and continuous cell division by inhibit p16 role [13].

From the above we can conclude the presence of EBERs and P16markers in all PCa grades but highly percentage in grade III because the high percentage of samples in this study from this grades, and presence of EBV increase the proliferation, may be inhibit tumor suppressor protein and killing immune cells. Emtithal Ibrahim Abdalla Abody study agree with our study because was found significant correlation between expression of p16 and grade of cancer (P=0.00) [14].

Over 107 copies of the EBERs present in each cell, which are abundantly transcribed in latently infected cells [15]. In all EBV associated tumors, EBERs transcripts are expressed, so the

detection of EBERs by ISH method is considered the best way to prove localizing EBV in latent phase in this tissue samples [16].

Results obtained are nearly compatible to Saul Grinstein et al. study which showed strong EBV reactions in 36.8% of neoplastic nuclei from Seven out of 19PCa when exam by (ISH) method. These cases included all grades of the Gleason classification ranging from well-differentiated adenocarcinomas to hypernephroid. The presence of EBV in dysplastic and precancerous proliferations of the prostate may indeed have an optional role in the development of carcinomas of these sites.

Other study suggest: EBERs when released from infected cells, is responsible for immune activation by inducing proinflammatory cytokines and type I interferon [17].

On other hand Ali, et al. found that 19 of 40 (47.5%) PCa cases positive to EBERs when detection by ISH, that's mean this result agree with current study [18].

On the other hand a study which has been done of nasopharyngeal carcinoma showed the EBERs detected by ISH associated with 100% occurrence and its location mainly in the cellular nucleus. Jiang Li, et al. that proved the presence of this virus in cancer tissues [19]. Other study reported the percent of EBV-EBERs -ISH in tissues with NPC observed in 47.5% (19 out of 40 cases), [12] this study compatible with current study also; AL-Khalidy et al., study reported (12%) from breast cancer (BC) positive to EBERs-ISH [20] and Areej A. Hussein study was detected the EBERs-ISH (50%) in patients with (BC) [21].

The percentage of EBV-RNA in PCa was found to be reflects a possible role of the EBV-infection in the carcinogenesis of PCa.

Upstream of Cycline-dependent kinase (CDKs) or CDK inhibitors such as p16 or at the level of retinoblastoma protein (pRB) itself can cause pRB pathway deregulation which commonly in all human tumor types, the primary function of this pathway is to lack uncontrolled cellular proliferation by regulating the G1/S cycle checkpoint, and regulation of apoptosis and transcriptional control [22].

This study was found that the highest positive results of p16-IHC reaction in PCa tissues (12 cases: 40%).

Correlation between EBERs and p16 in malignant group

The relationship between EBERs and P16 are accounted 0.176 in PCa group which is not significant, however the association between EBERs and P16 failed to reach the level of statistical significance, possibly because of small sample size, large studies are needed to determine the prognostic value and role of deletion of p16 in PCa. No any study found to compare between the presence of EBERs and p16 in PC. But in NPC, these two markers were detected in (81%) EBER signals in 67 of 83 specimens while (70%) p16 expression was in 59 of 84. That's indicate to a weak correlation between EBERs

presence and loss of p16 (P=0.1) [23] these results corresponding with current study, so in NPC cases, used the therapeutic strategies targeting the p16 pathway may be consider as a biologically rational approach. But other study was detected an essential association between EBV infection and abnormally regulated cell cycle pathway, but the exact mechanism needs further study [24].

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