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Molecular Localization of Epstein-Barr Virus and BCL-2 Expression in Tissues from Patients infected with Nasopharyngeal Tumors.

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ABSTRACT

In order to prove the implication of Epstein-Barr virus in nasopharyngeal cancer of Iraqi patients. Seventy (70) formalin-fixed, paraffin embedded nasopharyngeal tissues were obtained in this study ;40 biopsies from nasopharyngeal carcinoma (NPC) and (20) from benign nasopharyngeal tumors as well as (10) apparently normal nasopharyngeal autopsies control group. The age of these individuals (patients and control groups) were ranged between 4 and 75 years. the patients samples were collected from the archives of histopathology laboratories of AL-Shaheed Gazi Al-Hariery Hospital for Specialized Surgery /Baghdad Teaching Hospital in Baghdad Medical City ;Al-Hilla ;AL-Saddar(Al-Najef); Al-Hussein (Kerblla) as well as many private histopathology laboratories that generously helped as and are kindly thanked in the present dedication. We found the percent of EBV-EBERs -ISH in tissues with NPC observed in47.5% (19 out of 40 cases), and in the benign was detected in 20% (4 out of 20 cases) while, in the healthy control group was detected in10% (1 out of 10 cases), in contrast with proto oncogene (BCL-2) we found the positive signals of BCL2-IHC in tissues with NPC observed in 50% (20 out of 40 cases), and in the benign was detected in 40% (8 out of 20 cases), while in the healthy control group was detected in 10% (1 out of 10cases).In addition to this we noticed that the higher percent of EBV-EBERs in according to the sex of patients in males (35%) more than female (27.5%), while was found the percent of EBV-EBERs according to age of patients 15% in age stratum between 31-40 years, and was found that the percent of EBV-EBERs according to grade of nasopharyngeal cancer patients in grade III (22.5%). Our results indicate that the EBV might contribute to the development of subset of nasopharyngeal tumors and the effect of EBV on proto oncogene (BCL2). The differences between the percentages of EBERs detection in tissues NPC & control groups and proto oncogene were statistically highly significant (chi-square & Phi value = < 0.0001).

Keyword: EBV; Nasopharyngeal tumor, BCL2, In Situ Hybridization, IHC.

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is the most common neoplasm to affect the nasopharynx (NP). Arising from the epithelial lining of the nasopharyngeal mucosa, NPC is distinct from squamous cell carcinoma affecting other sites of the pharyngeal space. NPC typically originates in the lateral wall of the nasopharynx and is noted as a locally aggressive neoplasm with a high incidence of metastases to cervical lymph nodes. The primary tumor can extend within the nasopharynx and/or to the base of the skull, palate, nasal cavity or oropharynx. Distant metastases can arise in bone, lung, mediastinum, and, more rarely, the liver (Brennan,2006;Chunfang Hu,2010).

Many DNA and RNA viruses have proved to be oncogene in animals , however only few viruses have been linked with human cancer like EBV,HPV and CMV (Kumar,*et al*.,2003). At least 15% of human tumors world have arrived cause (Kumar,*et al*.,2003;Jawetz,*et al*.,2012).

EBV is atypical virus consisting of a core containing linear, double strand DNA: an icosahedral capsid approximately 100-110nm in diameter, containing 162 capsomeres, an amorphous material that surrounded the capsid, tegument and an envelope containing viral glycoprotein spikes on its surface (Clifford,*et al*.,2003) sequence analysis has defined tow strains of EBV: type I and type II (alternatively named EBV A and B) which differ at the domain that encode EBV latent proteins, namely EBERs, and the nuclear antigens EBNA-LP,2,3A,3B and 3C in latently infected cell (Roizman,1991).

There are two small non-polyadenylated and non-coding RNAs, EBER1 and EBER2, are detected in all forms of viral latency, and are frequently used as a diagnostic marker for EBV infection. The EBERs are transcribed from the EcoRI-J fragment of the EBV genome, and are 167 and 172 nucleotides in length respectively. EBERs are abundantly transcribed in latently infected cells, with up to 107 copies of the EBERs present in each cell (Nanbo & Takada 2002).

BCL-2 is a human proto-oncoprotein located in the membranes of the nuclear envelope, endoplasmic reticulum, and in the outer membrane of mitochondria. Overexpressed BCL-2 protein in NPC has been reported in a higher percentage than other head and neck cancers (Yang,*et al*,.2001).

Upregulation of BCL-2 has been closely related to aggressive traits in NPC including lymph node involvement, metastasis, recurrence, and poor survival rates in NPC patients (Chen,*et al*,.2010; Fendri,*et al*,.2011).

MATERIALS AND METHODS

The study was designed as a retrospective one. It has recruited 70 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.

There are two main method in this study:

***In Situ* Hybridization technique (ISH).**

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 Biotinylated-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) , 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 pepsin solution (ES1) to the slides, then the slides were incubated at 37°C for 20-30 minutes in humidity chamber. Then the slides immersion in distilled water for 5 minutes to remove pepsin solution. Then we add the 10 µl of (EBERs) RNA 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 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 wash buffer 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 wash 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 alcohol, (95%, once for one minute then, 100% twice times for 2 minutes each); cleared by Xylene, then mounted with permanent mounting medium (DPX).

Immunohistochemstry technique (IHC).

Immunohistochemistry / Detection system (Ab80436-Expose mouse and rabbit specific HRP/DAB Detection Immunohistochemistry Detection Kit (Abcam, England)).was used to demonstrate the protein expression of (BCL-2) encoded by Proto Oncogene. 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 primar 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 (El_Sisy,1999)

Chi –square & phi test was used to detect the significance of variables in our study. All the statistical analysis was done by SPSS program (Version– 20) & P value was considered significant when $p < 0.001$.

RESULTS

The Result of EBV-RNA (EBERs) by *In Situ* Hybridization Technique (ISH).

Results of EBERs- ISH Signal Scoring:

The Epstein-Barr Encoded RNAs (EBERs) score signaling was detected in tissue blocks obtained from patients with malignant nasopharyngeal tumor, benign nasopharyngeal tumor, and healthy nasopharyngeal tissue. The score signal of EBV-ISH was detected as blue discoloration at the site of complementary sequences in the nuclear region. Figures (1 , 3) shows the positive result of EBV-ISH detection where 47.5% (19 out of 40 cases) from malignant group showed positive signals included (17.5%: 7 out of 19 cases) in the strong score (score III),followed by (12.5%: 5 out of 19 cases) in the moderate score (score II),and (17.5%: 7 out of 19 cases) in the weak score (score I). The benign group revealed 20% positive signals which represented (4 out of 20 cases) in this group

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included (0.00 0 out of 4 cases) in the strong score (score III), followed by (15%: 3 out of 4 cases) in the moderate score (score II), and (5%: 1 out of 4 cases) in the weak score (score I). The healthy control group revealed 10% positive signals which represented (1 out of 10 cases) in this group included (0 out of 10 cases) in the strong score (score III),followed by (10%: 1 out of 10 cases) in the moderate score (score II), and (0.00: out of 10 cases) in the weak score (score I) . The Statistically analysis showed medium significant differences depending on (Chi-square & Phi test) in p>0.001.

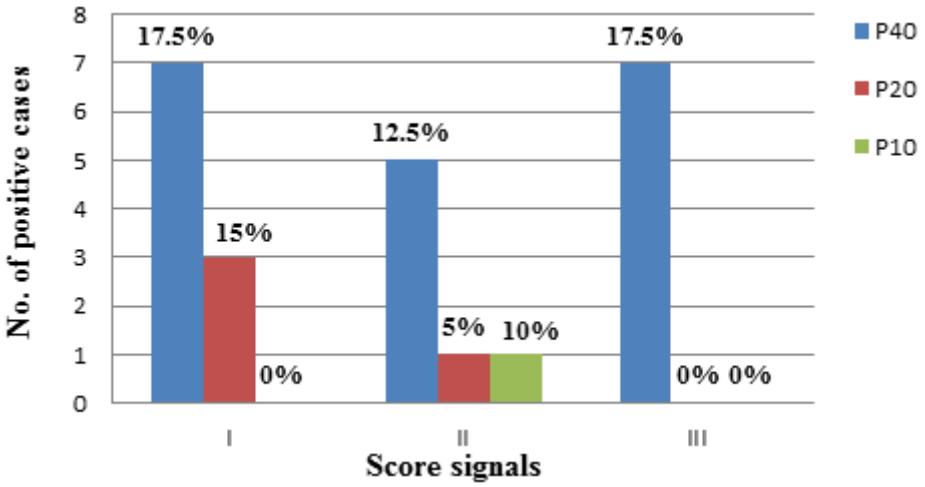

Figure 1: The percentage of EBERs score signaling in malignant, benign nasopharyngeal tumor, and healthy nasopharyngeal tissue. (Chi-calculated = 5.2287, Chi-table = 18.4668 , Phi = 0.637)

P40=malignant cases **P20=benign cases** **P10=control cases**
I=weak score **II=moderate score** **III=strong score**

Results of EBERs- ISH Signal Intensity

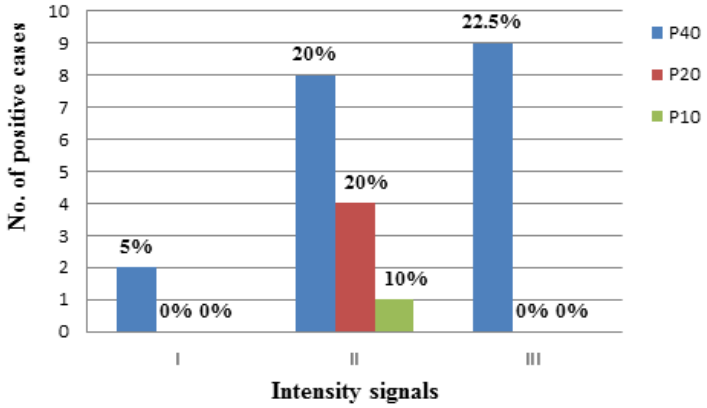

Figure 2: The percentage of EBERs intensity signaling in malignant, benign nasopharyngeal tumor, and healthy nasopharyngeal tissue. (Chi-calculated = 5.3441, Chi-table = 18.4668, Phi = 0.638)

P40=malignant cases **P20=benign cases** **P10=control cases**
I=weak intensity **II=moderate intensity** **III=high intensity**

The Epstein-Barr Encoded RNA (EBERs) intensity signaling was detected in tissue blocks obtained from patients with malignant nasopharyngeal tumor, benign nasopharyngeal tumor, and healthy nasopharyngeal tissue. The intensity signal of EBV-ISH was detected as blue discoloration at the site of complementary sequences in the nuclear region. Figures (2 , 3) shows the positive result of EBV-ISH detection where 47.5% (19 out of 40 cases) from malignant group showed positive signals included 22.5% (9 out of 19 cases) in the high signal intensity (III),

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followed by 20% (8 out of 19 cases) in the moderate signal intensity (II), and 5% (2 out of 19 cases) in the low signal intensity (I) . The benign group revealed 20% positive signals which represented (4 out of 20 cases) in this group included 0% (0 out of 4 cases) in the high signal intensity(III), followed by 20% (4 out of 4 cases) in the moderate signal intensity (II) , and 0% (0 out of 4 cases) in the low signal intensity (I) . The healthy control group revealed 10% positive signals which represented (1 out of 10 cases) included 0% (0 out of 1 cases) in the high signal intensity ,followed by 10% (1 out of 1 cases) in the moderate signal intensity, and 0% (0 out of 1 cases) in the low signal intensity. The Statistically analysis showed, medium significant differences depending on (Chi-square & Phi test) in p>0.001.

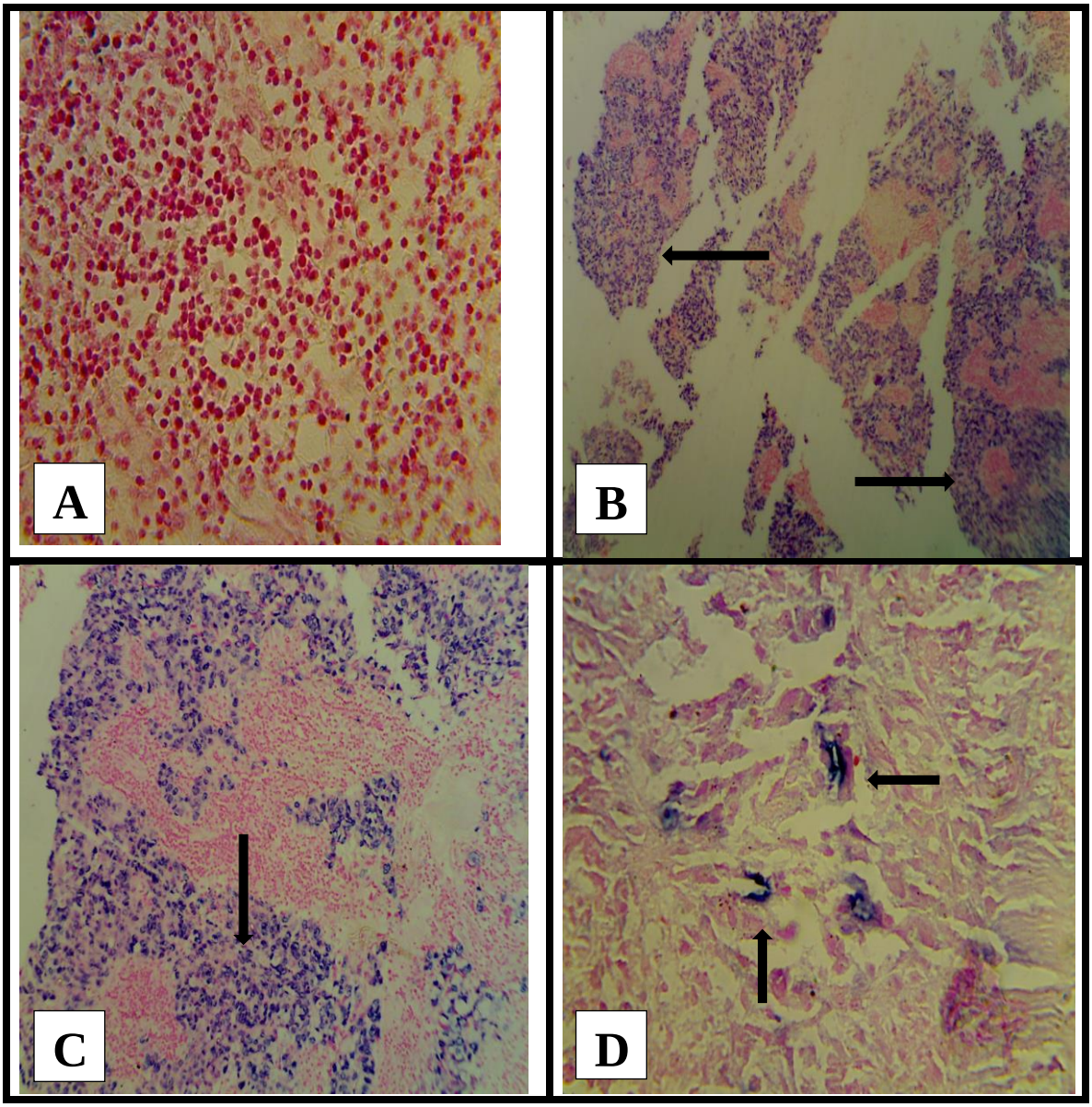

Figure 3*: In Situ* Hybridization (ISH) for EBERs-EBV RNA Detection of Nasopharyngeal Cancer Using Biotinylated-Labeled EBERs-EBV Probe; Stained With NBT/BCIB (Blue) and Counter Stained by Nuclear Fast Red (RED)

A-Nasopharyngeal cancer with negative EBERs-EBV-ISH Reaction (40x)
B-Positive EBERs-EBV-ISH Reaction with Strong signal score and High signal intensity (4x)
C- Positive EBERs-EBV-ISH Reaction with Moderate signal score and Moderate signal intensity (10x)
D- Positive EBERs-EBV-ISH Reaction with Low signal score and Weak signal intensity (40x)

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The Result of EBERs in Malignant, Benign Nasopharyngeal Tumor , and Healthy Nasopharyngeal Tissue According to The Sex of Patients:

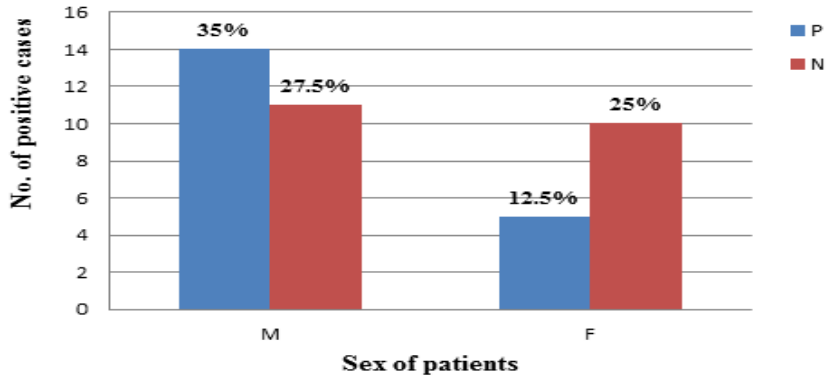

Figure 4 : The positive & negative result of EBERs according to the sex of patients in malignant nasopharyngeal tumor (chi-calculated = 1.199703, chi-table = 10.8275, Phi = 0.173)

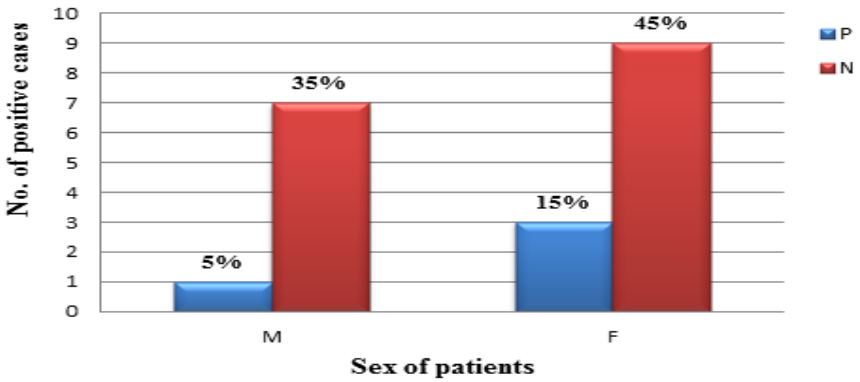

Figure 5 : The positive & negative result of EBERs according to the sex of patients in benign nasopharyngeal tumor (chi-calculated = 0.46875, chi-table = 10.8275, Phi = 0.715)

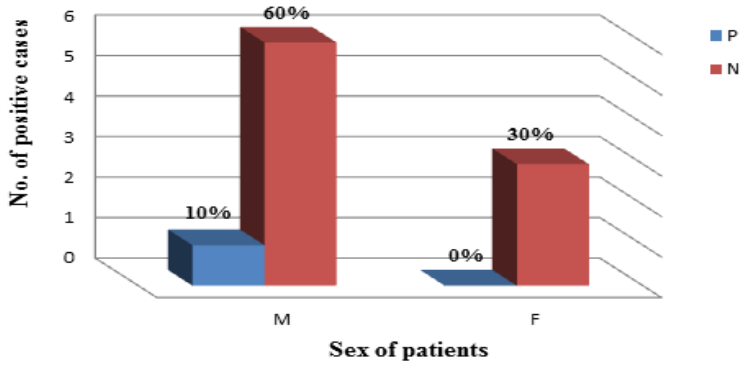

Figure 6 : The positive & negative result of EBERs according to the sex of patients in healthy nasopharyngeal tissue (chi-calculated = 0.47619, chi-table = 10.8275, Phi = 0.724).

The Epstein-Barr Encoded RNAs (EBERs) was detected in tissue blocks obtained from patients with malignant nasopharyngeal tumor, benign nasopharyngeal tumor , and healthy nasopharyngeal tissue . The signal of EBV- ISH was detected as blue discoloration at the site of complementary sequences in the nuclear region. Figures (4 , 5 , 6) shows the positive results of EBV RNA-ISH detection ,where 47.5% (19 of total 40) from malignant group showed positive signals included 35% (14 out of 25 cases) for male and 12.5% (5 out of 15 cases) for female . The benign group revealed 20 % positive signals which represented (4 out of 20 cases) in this group showed positive signals included 5% (1 out of 8 cases) for male and 15% (3 out of 12 cases) for female . The

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control group revealed 10 % positive signals which represented (1 out of 10 cases) in this group for EBV-ISH test included 10% (1 out of 7 cases) for male and 0% (0 out of 3 cases) for female. The statically analysis showed weak significant difference in malignant cases , and high significant differences in the benign and control cases depending on (Chi-square & Phi test) in p>0.001.

The Result of EBERs in Malignant, Benign Nasopharyngeal Tumor , and Healthy Nasopharyngeal Tissue According to the Age of Patients:

The Epstein-Barr Encoded RNAs (EBERs) was detected in tissue blocks obtained from patients with malignant nasopharyngeal tumor, benign nasopharyngeal tumor, and healthy nasopharyngeal tissue . The signal of EBV- ISH was detected as blue discoloration at the site of complementary sequences in the nuclear region . Figures (7 , 8, 9) shows the positive results of EBV RNA-ISH detection ,where 47.5% (19 of total 40) from malignant group showed positive signals included 5% (2 out of 5 cases) in age between (11-20), 5% (2 out of 2 cases) in age between (21-30), 15% (6 out of 7 cases) in age between (31-40), 10% (4 out of 8 cases) in age between (41-50), 12.5% (5 out of 15 cases) in age between (51-60), 0% (0 out of 3 cases) in age between (61-70). The benign group revealed 20% positive signals 20% which represented (4 out of 20 cases) in this group included 5% (1 out of 2 cases) in age between (0-10), 0% (0 out of 4 cases) in age between (11-20), 5% (1 out of 3 cases) in age between (21-30), 10% (2 out of 7 cases) in age between (31-40), 0% (0 out of 3 cases) in age between (41-50), 0% (0 out of 1 cases) in age between (71-80). The control group revealed 10% positive signals which represented (1 out of 10 cases) in this group included 0% (0 out of 2 cases) in age between (11-20), 10% (1 out of 4 cases) in age between (21-30), 0% (0 out of 2 cases) in age between (31-40), 0% (0 out of 1 cases) in age between (41-50), 0% (0 out of 1 cases) in age between (51-60). The statically analysis showed medium significant difference in malignant cases , and high significant differences in the benign and control cases depending on (Chi-square & Phi test) in p>0.001.

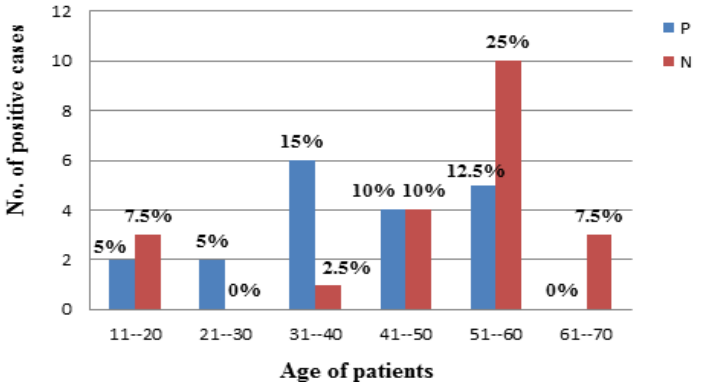

Figure 7 : The positive & negative result of EBERs according to the age of patients in malignant nasopharyngeal tumor (Chi-calculated = 11.12782, Chi-table = 20.51501, Phi = 0.527).

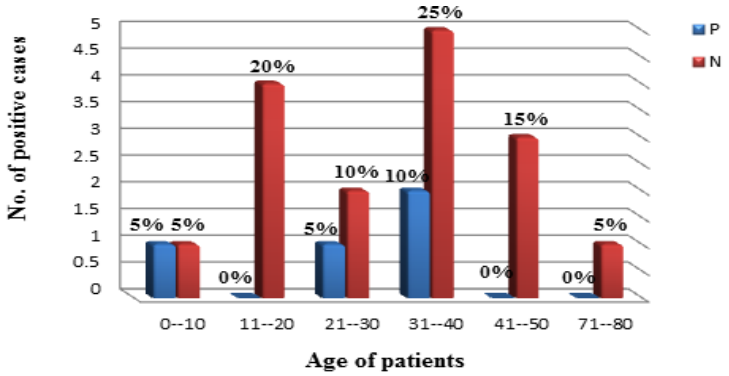

Figure 8 : The positive & negative result of EBERs according to the age of patients in benign nasopharyngeal tumor (Chi-calculated = 3.779762, Chi-table = 20.51501, Phi = 0.771)

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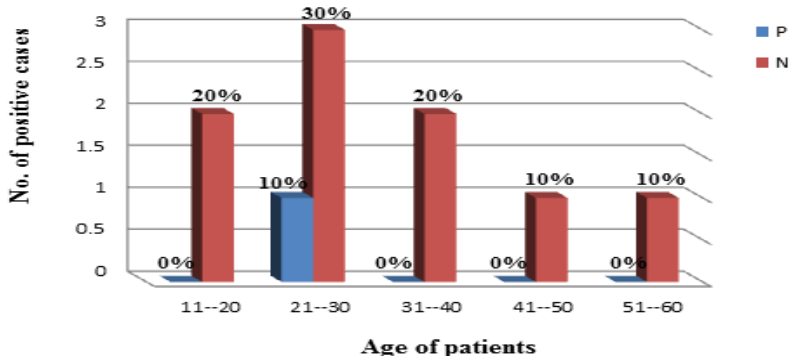

Figure 9 : The positive & negative result of EBERs according to the age of patients in healthy nasopharyngeal tissue (Chi-calculated = 1.666667, Chi-table = 18.467, Phi = 0.764).

The Result of EBERs and grading of nasopharyngeal carcinoma:

The Result of EBERs in Malignant Nasopharyngeal Tumor According to the Grades of Patients:

The Epstein-Barr Encoded RNAs (EBERs) was detected in tissue blocks obtained from patients with malignant nasopharyngeal tumor . The signal of EBV- ISH was detected as blue discoloration at the site of complementary sequences in the nuclear region. Figure (10) shows the positive results of EBV RNA-ISH detection ,where 47.5% (19 of total 40) from malignant group showed positive signals included 17.5% (7 out of 15 cases) in squamous cell carcinoma grade, followed by 7.5% (3 out of 6 cases) in non-keratinizing carcinoma grade, and 22.5% (9 out of 19 cases) in undifferentiated carcinoma grade. The statically analysis showed weak significant difference between grade of nasopharyngeal tumor depending on (Chi-square & Phi test) in p>0.001.

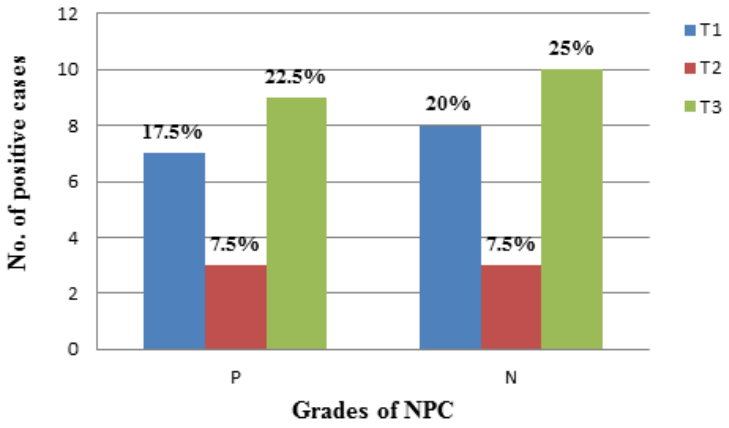

Figure 10 : The positive & negative result of EBERs in patients according to the grades of malignant nasopharyngeal tumor (Chi-calculated = 0.687684, Chi-table = 13.8155, Phi = 0.133)

T1=squamous cell grade T2=Non-keratinizing grade T3=Undifferentiated grade

The Result of Proto Oncogene (BCL-2) by Immunohistochemistry Technique (IHC).

Results of BCL2- IHC Signal Scoring:

The Proto Oncogene (BCL2) score signaling was detected in tissue blocks obtained from patients with malignant nasopharyngeal tumor, benign nasopharyngeal tumor, and healthy nasopharyngeal tissue. The score signal of BCL2-IHC was detected as brown discoloration at the site of complementary sequences in the cytoplasmic region. Figures (11 , 13) shows the positive result of BCL2-IHC detection where 50% (20 out of 40 cases) from malignant group showed positive signals included 5% (2 out of 40 cases) with in strong score (score

III), followed by 27.5% (11 out of 40 cases) in the moderate score (score II), and 17.5% (7 out of 40cases) in the weak score (score I). The benign group revealed 40% positive signals which represented (8 out of 20 cases) in this group included 15% (3 out of 20 cases) with in strong score (score III), followed by 10% (2 out of 20 cases) with in the moderate score (score II), and 15% (3 out of 20 cases) in the weak score (score I). The healthy control group revealed 10% positive signals which represented (1 out of 10 cases) in this group included 0% (0 out of 10 cases) with in strong score (score III), followed by 10% (1 out of 10 cases) in the moderate score (score II), and 0% (0 out of 10 cases) in the weak score (score I). The statically analysis showed medium significant difference depending on (Chi-square & Phi test) in p>0.001.

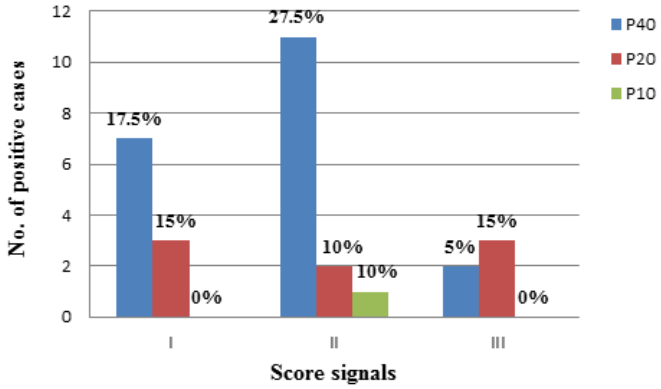

Figure 11: The percentage of BCL2 score signaling in malignant, benign nasopharyngeal tumor, and healthy nasopharyngeal tissue (Chi-calculated = 4.6917, Chi-table = 18.4668, Phi = 0.402).

P40=malignant cases P20=benign cases P10=control cases
I=weak score II=moderate score III=strong score

Results of BCL2- IHC Signal Intensity:

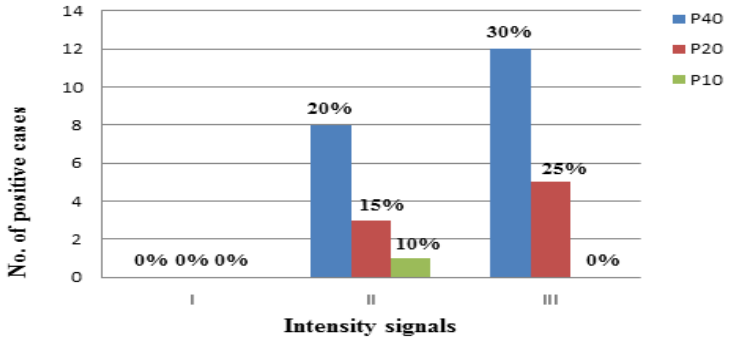

Figure 12: The percentage of BCL2 intensity signaling in malignant, benign nasopharyngeal tumor, and healthy nasopharyngeal tissue (Chi-calculated = 1.4819, Chi-table = 13.8155, Phi = 0.226).

P40=malignant cases P20=benign cases P10=control cases
I=weak intensity II=moderate intensity III=high intensity

The Proto Oncogene (BCL2) score signaling was detected in tissue blocks obtained from patients with malignant nasopharyngeal tumor, benign nasopharyngeal tumor, and healthy nasopharyngeal tissue. The intensity signal of BCL2-IHC was detected as brown discoloration at the site of complementary sequences in the cytoplasmic region. Figures (12 , 13) shows the positive result of BCL2-IHC detection where 50% (20 out of 40 cases) from malignant group showed positive signals included 30% (12 out of 40 cases) with in high intensity (III), followed by 20% (8 out of 40 cases) in the moderate intensity (II), and 0% (0 out of 40 cases) in the weak intensity (I). The benign group revealed 40% positive signals which represented (8 out of 20 cases) in this group included 25% (5 out of 20 cases) with in high intensity (III), followed by 15% (3 out of 20 cases) in the moderate intensity (II), and 0% (0 out of 20 cases) in the weak intensity (I). The healthy control group revealed 10% positive signals

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which represented (1 out of 10 cases) in this group included 0% (0 out of 10 cases) with in high intensity (III), followed by 10% (1 out of 10 cases) in the moderate intensity (II), and 0% (0 out of 10 cases) in the weak intensity (I). The statically analysis showed weak significant difference depending on (Chi-square & Phi test) in p>0.001.

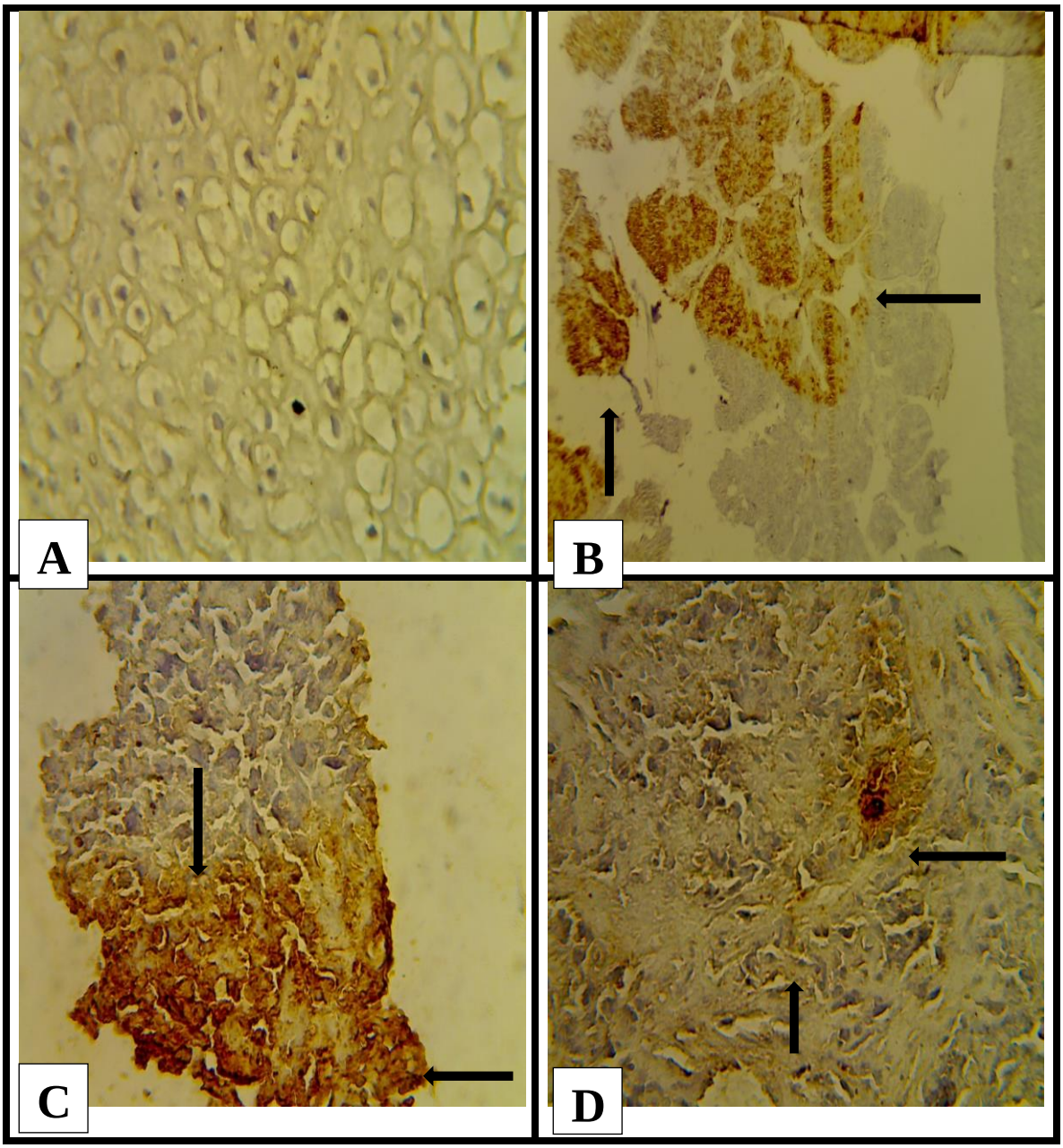

Figure 13: Infiltrative Nasopharyngeal Carcinoma Sharing The Result of Immunohistochemistry Staining Protein Over Expression Using Biotinylated Anti-BCL2 Protein Antibody; Stained By DAB-Chromogen (Brown) and Counter Stained By Mayer's Heamatoxylin (Blue).
A-Nasopharyngeal Cancer with negative .
B-IHC-reaction with Strong signal score and High signal intensity (4x)
C- IHC-reaction with Moderate signal score and Moderate signal intensity (10x)
D- IHC-reaction with Low signal score and Weak signal intensity (40x)

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The Correlation Between Epstein Barr Encoded RNAs (EBERs) and Proto Oncogene (BCL2)

In the present study we found the positive result of EBERs 47.5%(19 out of 40 cases) in malignant nasopharyngeal tumor, followed by 20% (4 out of 20 cases) in benign nasopharyngeal tumor, and 10% (1out of 10 cases) in healthy nasopharyngeal tissue.

In contrast we found the positive result of BCL2 50% (20 out of 40 cases) in malignant nasopharyngeal tumor, followed by 40% (8 out of 20 cases) in benign nasopharyngeal tumor, and 10% (1out of 10 cases) in healthy nasopharyngeal tissue (Table 1). The statically analysis showed high significant differences between EBERs and the proto oncogene (BCL2) depending on (Phi test) in $p>0.001$.

Table 1: The Correlation Between EBERs and Proto Oncogene(BCL2).

EBV antigen	Proto Oncogene
EBERs	BCL2
Phi value	0.798

DISCUSSION

Nasopharyngeal carcinoma is a highly malignant tumor arising from the epithelia lining of the nasopharynx and is distinguished from other cancers of the head and neck by its distinct histopathology, racial and geographical distribution, clinical characteristics and treatment**.** (Chunfang ,2010).

Epstein–Barr virus (EBV) is the causative agent of mononucleosis and is also associated with several malignancies, including Burkitt' lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma (Alaina,*et al*.,2013).

In current study we found the percentage of infection of NPC in Iraq is 47.5% (Figure : 1-1) this result is agreement with the Iraqi cancer registry (2012) where recorded in 2009 the percent of nasopharyngeal cancer in Iraq is 48%.

The percentage of high EBV-RNA in malignant nasopharyngeal tumors was found to be higher in comparison to the benign group . This finding reflects a possible role of the EBV-infection in the carcinogenesis of nasopharyngeal malignant tumors group. majority of these patients' tumors are EBV-positive.

Epstein-Barr virus (EBV) is causatively associated with the development of nasopharyngeal carcinoma (NPC) as almost all undifferentiated NPC is comprised of monoclonal expansions of cells latently infected with EBV (Raab-Traub, 2002; Tao & Chan, 2007).

In the present study we noticed the EBER-ISH technique is much more sensitivity and specifity for detecting latent EBV infection in NPC samples because there is the most abundant EBER transcription (up to 10^7 copies per cell) during latent EBV infection, EBERs have a stable secondary structure, and the majority of EBERs are located within the cell nucleus where they are complexed with the cellular protein (Han,*et al*.,2000). This finding agreement with (Bar,*et al*.,2004) where found the EBER ISH is more sensitive than the polymerase chain reaction for detecting EBV latency in tissues and enables the localization of the EBV signal in the tumor cells.

Yip, *et al*.,(2006) reported that the high expression of EBER in patients with NPC 82% with strong cell staining (>50% positive tumor nuclei) where detected the positive signals of EBER-ISH in patients with NPC according to the score signals in strong score (III) 33.7% (27 out of 80 cases)more than moderate (II) 26.2% (21 out of 80 cases) and weak score (I) 20% (16 out of 80 cases) this result is consistent with our study where found the strong score (III) and moderate score (II) in 17.5% (7 out of 40 cases)more than moderate and weak score (I) 12.5% (5 out of 40 cases) (Figure: 1-1 , 2-2).

By analogy (Saad & Shakir.,2013) found the positive signals of EBER-ISH in patients with Prostatic Adenocarcinoma is 19 out of 40 (47.5%), Also (AL-Khalidy *et al*.,(2010) and Areej,(2013) were detected the EBER-ISH (12%)and (50%) in patients with breast cancer, respectively.

In the present we detected the percentage of EBV infection by EBER-ISH in 47.5% (19 out of 40 cases) for both male and female included 35% (14 out of 40 cases) for male 12.5% (5 out of 40 cases) for female (Figure: 3-1) this result is inconsistent with (Ahmed,*et al*,2015) where found the percent of infection 92/150 (61.3%) samples Out of the 92 infected samples, 58/97 (60%) for males and 34/53 (64%) among females . We can explain this finding according to genetic variability and the etiological factor that different from one region to another.

Abdulamir,*et al*., (2008) found the number of males with NPC was higher than females this result is consistent with our study this predominance of male in NPC belongs largely to the nature of the risk factors in the Middle East society which affects males more than females

In current study we noticed that the highest percentage EBV-infection according to EBERs –ISH in the age between (31-40) (Figure: 4-1) this result is disagreement with the study that make on patients with NPC by (Ismail,*et al*., 2012; Krikelis,*et al*., 2013)

Likewise, Fulya *et al*.,(2004) found the percent of Grade in patients with NPC (8 out of 81 cases) in grade I , followed by (3 out of 81 cases) in grade II, and (70 out of 81 cases) in grade III this result is consistent with our results where found the percent of grade III more than grade I & II in patients with NPC.

The expression of BCL-2 has been consistently detected in NPC tumors(Sheu *et al*., 1997) and is associated with poor prognosis of patients (Chen *et al*., 2009). An induction of cell apoptosis or a regression of tumour growth can be achieved in NPC tumors by blocking the antiapoptotic function of BCL-2 by a BCL-2 family inhibitor or antisense oligonucleotides. Especially, a significant effect has been observed in those NPC cells with high expression of BCL-2 (Yip,*et al*., 2005; Hu *et al*., 2008).

Fu,*et al*., (2013) reported that the results of some studies suggest that elucidation of the relation between EBV and the Bcl-2 family is important in understanding the pathogenesis of EBV-associated diseases include the interactions between EBV-encoded products and Bcl-2 family members in the lytic and latent phases, and the details of the apoptotic pathway in EBV-infected cells, which contribute into the establishment of EBV latency and carcinogenicity.

Overexpression is most likely EBV associated because EBV-positive NPC cells have greater bcl-2 expression than EBV-negative NPC cells (Yang,*et al*,.2001).

In our result shows the highly correlation between the EBERs and BCL2 where noticed that the positive signals of EBERs 47.5% is near or equal to positive signals of BCL2 50% in patients with NPC . This result is compatible with the study by Liu *et al*.,(2008) where found the EBERs detected in 100% (43 out of 43 cases) while BCL2 detected in 88.4% (38 out of 43 cases) in patients with NPC this result make us to suggesting a close correlation of EBERs with proliferation and apoptosis in NPC tissues.

We noticed in our study that the highest percent for score signals of BCL2-IHC in patients with NPC (27.5%) in moderate score (II) followed by (17.5%) in weak score (I) and finally (5%) in strong score (III) (Figure: 11 , 13). This result incompatible with Vera-Sempere,*et al*.,(1997) and John Jenn, et al., (2002) who found the high percent for score signals of BCL2-IHC in patients with NPC according to the score system in the (4.16 %) in strong score ,followed by (16.6%) in the moderate score (II), and (45.7%) in weak score.

CONCLUSIONS

Our results indicate that the EBV might contribute to the development of subset of nasopharyngeal tumors and the EBV have strong correlation with Proto oncogene (BCL2) by inhibition the apoptosis process.

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REFERENCES

[1] Fendri, C. K. Kontos, A. Khabir, R.Mokdad-Gargouri, and A. Scorilas, "BCL2L12 is a novel biomarker for the prediction of short-term relapse in nasopharyngeal carcinoma," Molecular Medicine, vol. 17, no. 3-4, pp. 163–171, 2011.

[2] Alaina A. Brinley,1,2 Corey A. Theriot,1,2 Mayra Nelman-Gonzalez, et al., (2013). Characterization of Epstein–Barr Virus Reactivation in a Modeled Spaceflight System, Journal of Cellular Biochemistry 114:616–624 (2013).

[3] Areej A. Hussein, (2013). Molecular detection of Epstein Barr Virus in Women with Breast cancer, J Fac Med Baghdad, Vol.55, No.2, 2013.

[4] AS Abdulamir, RR Hafidh, N Abdulmuhaimen, F Abubakar, and KA Abbas, (2008). The distinctive profile of risk factors of nasopharyngeal carcinoma in comparison with other head and neck cancer types, BMC Public Health 2008, 8:400 doi:10.1186/1471-2458-8-400

[5] Brennan B (2006). Nasopharyngeal carcinoma. Orphanet Journal or Rare Diseases. 1:23. K. J. Marcus and R. B. Tishler, "Head and neck carcinomas across the age spectrum: epidemiology, therapy, and late effects," Seminars in Radiation Oncology, vol. 20, no. 1, pp. 52–57, 2010.

[6] Chen MK, Yang SF, Lai JC, Yeh KT, Yang JS, Chen LS & Chen HC (2009) Expression of bcl-2 correlates with poor prognosis and modulates migration of nasopharyngeal carcinoma cells. Clin Chim Acta 411, 400-405.

[7] Chunfang Hu PH.D thesis (2010) GENETIC AND GENE EXPRESSION ANALYSIS OF NASOPHARYNGEAL CARCINOMA (NPC) School of Cancer Sciences College of Medical and Dental Sciences The University of Birmingham November 2010.

[8] Clifford M, Smith S, and Aguado-Franceschi S. Comparison of HPV type distribution in high-grade cervical lesion and cervical cancer a meta-analysis. Br. J. Cancer 2003;89:10.

[9] Dimitrios Krikelis , Mattheos Bobos2 , Georgia Karayannopoulou , Liliana Resiga, et al., (2013) Expression profiling of 21 biomolecules in locally advanced nasopharyngeal carcinomas of Caucasian patients .BMC Clinical Pathology 2013, 13:1 /1472-6890/13/1

[10] El_Sisy NA. Imunohistochemical detection of P53 in an amelobleastoma . J. of oral pathology 1999; 5:478-489.

[11] F.J. vera-Semperel, J.S. ~urgoslM, .S. Botella' and C. Morera, (1997). lmmunohistochemical expression of Bcl-2 oncoprotein in EBV-associated nasopharyngeal carcinoma correlated to histological type and survival, Histol Histopathol (1997) 12: 9-18

[12] Fu J, Zhou JY, Chong VF and Khoo JB. Indication of lower neck irradiation in nasopharyngeal carcinoma without nodal metastasis: the potential impact of tumor volume. Chin Med J (Engl) 2013; 126: 2120-2124.

[13] FULYA YAMAN AGAOGLU, YAVUZ DIZDAR, ONER DOGAN, CANAN ALATLI, INCI AYAN, et al., (2004), p53 Overexpression in Nasopharyngeal Carcinoma J.in vivo 18: 555-560 (2004)

[14] G Bar-Sela, A Kuten, I Minkov, E Gov-Ari, O Ben-Izhak, (2004). Prevalence and relevance of EBV latency in nasopharyngeal carcinoma in Israel, J Clin Pathol 2004;57:290–293. doi: 10.1136/jcp.2003.013094

[15] Haan, K. M., Kwok, W. W., Longnecker, R. & Speck, P. (2000). Epstein–Barr virus entry utilizing HLA-DP or HLA-DQ as a coreceptor. J Virol 74, 2451–2454.

[16] Hu ZY, Zhu XF, Zhong ZD, Sun J, Wang J, Yang D & Zeng YX (2008) ApoG2, a novel inhibitor of antiapoptotic Bcl-2 family proteins, induces apoptosis and suppresses tumor growth in nasopharyngeal carcinoma xenografts. Int J Cancer 123, 2418-2429.

[17] Hussain Gadelkarim Ahmed, Rania Saad Abdul Gader Suliman, Mohammed Siddig Abd El Aziz and Fawaz D Alshammari , (2015). Molecular screening for Epstein Barr virus (EBV) among Sudanese patients with nasopharyngeal carcinoma (NPC) Infectious Agents and Cancer (2015) 10:6 DOI 10.1186/s13027-015-0002-4

[18] IRAQI CANCER REGISTRY Center (2009) IRAQI CANCER BOARD Iraqi_cancerboard@yahoo.com BAGHDAD – Iraq

[19] Ismail Matalka , Mohammad Al Hamad , Maysa Al-Hussaini , Firas Q. Alzoubi, (2012). The incidence of Epstein–Barr virus in nasopharyngeal carcinoma of Jordanian patients, Eur Arch Otorhinolaryngol (2012) 269:229–234 DOI 10.1007/s00405-011-1562-6.

[20] Jawetz; Carroli, K. C. ; Butel, J. S. and Morse, S. A. (2012)Brooks, Melnick and Adelberg,S.Medical Microbiology . 25 ed.McGraw Hill Companies.USA.

[21] KennethW. Yip, Wei Shi, Melania Pintilie, JosephD.Martin, (2006). Prognostic Significance of the Epstein-BarrVirus, p53, Bcl-2, and Survivin in Nasopharyngeal Cancer, Clin Cancer Res 2006;12(19) October 1, 2006

[22] Kumar, V.; Cotran, R.; And Robbins, S. 2003. Neoplasia. Ch.6 In: basic pathology, 7th edition.

[23] M. K. Chen, S. F. Yang, J. C. Lai et al., "Expression of bcl-2 correlates with poor prognosis and modulates migration of nasopharyngeal carcinoma cells," Clinica Chimica Acta, vol. 411, no. 5-6, pp. 400–405, 2010.

[24] Nanbo, A., and K. Takada. (2002). The role of Epstein-Barr virus-encoded small RNAs (EBERs) in oncogenesis. Rev Med Virol 12:321-6.

[25] Qiu-Yu Liu, An-Jia Han, Shu-Yuan You, et al., (2008). Correlation of Epstein-Barr virus-encoded latent membrane protein 1 (LMP1) to fascin and phosphorylated Stat3 in nasopharyngeal Carcinoma, Chinese Journal of Cancer 27:10, 337-342; October 2008

[26] Raab-Traub, N. (2002). Epstein-Barr virus in the pathogenesis of NPC. Semin Cancer Biol 12(6), 431-41.

[27] Roizman R. Herpesviridae; A brief introduction in virology, 2nd Ed.1991; 1787-1793

[28] Saad Hasan Mohammed Ali , Shakir H. Mohammed Al-Alwany,(2013). Molecular Localization of Epstein Barr Virus and Rb Tumor Suppressor Gene Expression in Tissues from Prostatic Adenocarcinoma and Benign Prostatic Hyperplasia, Journal of Natural Sciences Research ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online) Vol.3, No.8, 2013

[29] Saja J. AL-Khalidy, Ban A. Abdul-Majeed , Faiza A. Mukhlis, Layla M. AL-Omar, (2010). DETECTION OF EPISTEIN BARR VIRUS IN BREAST CANCER USING THE POLYMERASE CHAIN REACTION AND IN SITU HYBRIDIZATION, Iraqi J. Biotech. 9(2): 248-257(2010)

[30] Sheu LF, Chen A, Meng CL, Ho KC, Lin FG & Lee WH (1997) Analysis of bcl-2 expression in normal, inflamed, dysplastic nasopharyngeal epithelia, and nasopharyngeal carcinoma: association with p53 expression. Hum Pathol 28, 556-562.

[31] Tao, Q., and Chan, A. T. (2007). Nasopharyngeal carcinoma: molecular pathogenesis and therapeutic developments. Expert Rev Mol Med 9(12), 1-24.

[32] Yang HJ, Cho YJ, Kim HS, Chang MS, Sung MW KimWH. Association of p53 and BCL-2 expression with Epstein-Barr virus infection in the cancers of head and neck. Head Neck 2001;23:629-636.

[33] Yang HJ, Cho YJ, Kim HS, Chang MS, Sung MW, Kim WH. Association of p53 and BCL-2 expression with Epstein-Barr virus infection in the cancers of head and neck. Head Neck 2001;23:629–636.

[34] Yip KW, Mocanu JD, Au PY, Sleep GT, Huang D, Busson P, Yeh WC, Gilbert R, O'Sullivan B, Gullane P, Bastianutto C & Liu FF (2005) Combination bcl-2 antisense and radiation therapy for nasopharyngeal cancer. Clin Cancer Res 11, 8131-8144.