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The Relationship between Human Papilloma virus (HPV) infection and Gene Expression of *miRNA-744*, *BCL-2*, *CASPASE*-3 genes Associated with Cervical Cancer in Iraqi Patients

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Abstract: Most types of HPV are harmless and some types are high risk and can cause cervical cancer or abnormal cells in the lining of the cervix that sometimes turn into cancer. A total number of 90 subjects (70 Iraqi patients with cervical dysplasia (abnormalities) and 20 apparently healthy women) were included in this study. The patients and healthy were aged between 25-55 years. The molecular detection in our study showed that 30 of patients were positive to HPV and 40 patients were negative to virus compared to negative results in healthy women. The most common genotypes is HPV-16. Also multiple infections of HPV-HR were observed in the infected women. The correlation between gene expression, histopathology and HPV virus showed a significant increase of folding values of the genes *mi-RNA744* and *caspase-3* parallel to decrease of folding value of the gene *Bcl-2* which indicated that the apoptosis rate was increased with the histopathology grade where the highest level of expression of the genes was in carcinoma. The results also revealed that *mi-RNA744* could be up regulator of the gene *caspase-3* and down regulator of the gene *Bcl-2*.

Keywords: HPV, cervical carcinoma, *mi-RNA744*, *caspase-3*, *Bcl-2*

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Introduction:

Cervical carcinoma is a malignancy of the female genital system with a relatively high mortality rate of approximately 12% (1). Despite its complicated pathogenesis, studies have demonstrated that viral infection, sexual behavior, and number of births are major cervical carcinoma determinants (2). Recent clinical trials have shown that cervical carcinoma represents a significant threat to women's health (3). HPV types are classified as either high risk or low risk, with high-risk types being associated with cancer formation while low risk types are not. For example, HPV types 6 and 11 are classified as low risk types, and infection with these types results in the proliferation of epithelial cells and manifests as warts or papillomas on the skin. However, these infections are generally self-limiting and do not lead to malignancy. On the other hand, HPV types 16 and 18 are major high-risk genotypes, which together cause up to 75% of cervical cancer cases as well as a significant number of head and neck squamous cell carcinomas. miRNAs represent a category of short, non-coding RNAs with multiple biological functions. For example, miR-218 has been shown to inhibit the growth of cervical carcinoma cells, while miR-34a is associated with tumor metastasis (4,

5, 6). This suggests that miRNAs are involved in the occurrence and development of cervical carcinoma (6). In addition, preliminary experiments have demonstrated that in cancer cells, expression of miR-187 is significantly higher than that in normal tissues (7). Apoptosis is a form of programmed cell death regulated by several proteins with anti-and pro-apoptotic effects. Anti-tumor strategies aim to kill cancer cells while leaving normal cells undamaged. One potential mechanism to achieve this involves inducing apoptosis by regulating the proteins that promote and inhibit this process (5). Bcl-2 is an extensively studied inhibitor of apoptosis, and as such, drugs targeting it have been designed. However, the efficacy of such molecules in inhibiting Bcl-2 is limited (8). Furthermore, recent research has demonstrated that Bcl-2 is regulated by multiple miRNAs (6). The aim of the study is to explore the relationship between HPV, genes expression of the genes miR-744, Bcl-2and caspase-3 and epithelial cervical dysplasia according to grade of Histopathology.

Materials and Methods:

Subjects:

This study was conducted from April 2017 to February 2018 on a total number of 90 subjects included 12 Iraqi patients with cervical carcinoma, 30 patients with atypia stage and 20 apparently healthy women which were referred to the surgical pathology department of the teaching laboratories in the Medical City Teaching Hospital and Al-Elweya Teaching Hospital, Baghdad,Iraq. The patients and healthy were aged between 25-55 years, a structured questionnaire containing different variables was completed for each woman. Buccal swab-Pap smear were collected from each women under supervision of gynecologist. The samples were used for molecular detection of HPV, genotyping using RT-PCR and to detect the expression levels of *miR-744*, *Bcl-2* and *caspase-3* apoptosis regulator genes.

HPV Molecular analysis:

Real Time PCR for qualitative detection and genotyping of Human Papilloma virus High Risk was done using *HPV* HCR genotype-titre-RT PCR kit (AmpliSens® /Russia) and according to the company instruction.

Gene expression analysis:

Gene expression levels of *miR-744*, *Bcl-2* and *caspase-3* genes were detected using Gene Expression Assays Kits (Promega / USA). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a house keeping gene. The primers which were used listed in table(1).

Table (1): The primers which were used.

Primers	*caspase-3* *	*Bcl-2* *	*miR-744* (Lin *et al.*, 2014)
Forward	5GAACCAAAGATCATACATGGAA-3	5TGGATGACTGAGTACCTGAA-3	5AATGCGGGGCTAGGGCTA-3
Reverse	3ACATAAACCCATCTCAGGATAA-5	3GACAGCCAGGAGAAATCAAA-5	3GTGCAGGGTCCGAGGT-5

***New design**

RT-PCR Amplification programs: RT-PCR condition for HPV and genes were listed in tables 2 and 3.

Table (2): RT-PCR program for HR-HPV

step	Temperature C°	Time	Cycles
1	95	15 min	1
2	95	5 s	5
	60	20 s	
	72	15 s	
3	95	5 s	40
	60	20s fluorescence acquiring	
	72	15 s	

Table (3): RT-PCR program for *mi RNA 744, Bcl-2, Caspase-3* and *GAPDH* genes.

Steps	Temperature	Time	cycle
RT. Enzyme Activation	37	15:00	1
Hold	95°C	5m	1
Denaturation	95°C	15s	40-60
Annealing	54°- 60°C	30s	
Extension	72°C	45s	

Statistical Analysis:

The Statistical Analysis System-SAS (2012) program was used to effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage and Least significant difference –LSD test (ANOVA) was used to significant compare between means in this study.

Results and Discussion:

The detections of all HPV genotypes were shown in (Table 4). The results showed that the most common high-risk HPV types among them is the genotype 16 (26.67%). The other high-risk HPV types were HPV- 59 and 56 with the total rate of 13.33%, and 13.33%, respectively followed by HPV types 66, 51, 58 and 68 with the total rate of 10%, 10%, 10% and 6.67%, respectively. The less frequent genotypes were HPV-18, 39, 45 (3.33%). Our results revealed that HPV genotype 16 could be play a role in cervical cancer development since it exist in CIN I, CIN II and carcinoma.

In Iraq, several studies declared an association of HPV with cervical carcinoma. The first was done by Mohammed-Ali (2001) (9) who found that 25% of cervical carcinoma was positive for HPV. Al-Azzawi, (2006) and Al-Shwaikh, (2006) (10,11) were also found that 28.4% and 33% respectively of the cervical neoplasia were HPV-positive.

A multicenter, prospective study to evaluate the prevalence of HPV types in Asian women with invasive cervical cancer showed that HPV-16, -18, -52, -45, -58, -33, or -31 were strongly associated with cervical cancer (12,13, 14).

The correlation between the expression of the genes *caspase-3, BCL-2 and mi-RNA744,* histopathology and HPV virus:

The results listed in table 5 revealed that the folding values of mi-RNA744 were highly significant in carcinoma patients positive to HPV virus (3.8 X 10^{12}) comparing to patients with Atypia (4.5 **X** 10^{8}) or control (2.11 X 10^{8}).

Table (4): Positive results of HR- HPV genotyping real time PCR assay.

Genotype of HPV	Number	Percentage (%)	Genotype of HPV	Number	Percentage (%)
16	8	26.67	56	4	13.33
18	1	3.33	51	3	10.00
59	4	13.33	68	2	6.67
39	1	3.33	45	1	3.33
66	3	10.00	58	3	10.00
Total	30	100%			
Chi-square value	---	9.026 **			
P-value	---	0.0002	** (P<0.01)		

Table (5): Expression of *caspase-3, BCL-2 and mi-RNA744* according to grade of Histopathology in HPV positive patients

Histology Grads	*Casp-3*	*Bcl-2*	*mi-RNA744*	Genotype HPV(+)
Carcinoma (12)	2.59 ± 0.07a	0.73 ± 0.05 b	3.8×10^{12} a	16,18, 45,58
Atypia (2)	1.83 ± 0.05 b	1. 40 ± 0.05 a	4.5×10^{8} b	68 ,51,56,66,59,58
Normal (20)	1.3 ± 0.03 c	1.7 ± 0.05 a	2.11×10^{8} b	NOTE: Similar letters are with NS values
LSD value	0.621 **	0.574 **	1.157 **	
P-value	0.0036	0.0071	0.0001	

The results revealed that the folding values of *caspase-3*, *Bcl-2* and copy number of *mi-RNA744* were highly significant (P<0.01) in patients positive to HPV virus. These results indicated that a correlation between the virus HPV and the regulation of *caspase-3* and *Bcl-2* via increasing level of mi-RNA 744. Previous studies have shown Bcl-2 to be involved in other cancers, enhancing cancer cell proliferation and exacerbating lesions (15, 16, 17). However, none of these have addressed the relationship between *Bcl-2* and cervical cancer. He and Yang (2017) (18) showed that miR-187 induces apoptosis of cervical cancer cells via down regulation of *Bcl-2*. *Bcl-2* is a potential drug target for treatment of this disease, and its inhibition could improve the efficacy of clinical therapy. Because HPV, especially type 16, is reported as an important risk factor for development of cervical dysplasia and cancer, 99.7% of the cervical cancers can be show an association to HPV. One of the main goals of preventing cervical cancer is screening for HPV (19). Over expression of the major oncogenes, E6 and E7, during cell transformation very often results in resistance of transformed cells to apoptosis, this ability to avoid apoptosis is one of the major hallmarks of carcinogenesis (20). Even in countries with established screening programmers, women still die from rapidly progressing cancers that escape periodic examination. Given that HPV16, 18 and 45 appear to have greater progressive potential, and in the event that future cervical screening programs include HPV typing, women infected with HPV16, 18 and 45 may require closer surveillance than women infected with other HR HPV types (21). As a membrane protein, *Bcl-2* is over expressed in cervical cancer tissue. It can prevent apoptosis and prolong the cell life cycle by inhibiting Ca2+ increases in the cell, closing cell nuclear transportation, and acting as an

antioxidant (22). In this study, the expression rate of *Bcl-2* in cervical cancer was significantly higher than in the normal cervix, chronic cervicitis, or CIN tissues. Bcl-2 protein, mainly located in the rough endoplasmic reticulum and the mitochondrial membrane, can enhance resistance to apoptosis factors. *Bcl-2* over expression can inhibit cell apoptosis, and cervical cancer can develop under the regulation of growth suppressor and proliferation genes (23, 24, 25). We conclude that HPV play a role in the transition of disease to carcinoma via inducing the expression of *mi-RNA744* that down regulate the Bcl-2 expression and up regulate the caspase-3 expression which induce apoptosis.

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