The relationship between HPV and genes expression (miRNA-744, BCL-2, CASPASE-3) in epithelial cervical abnormalities

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This study was conducted from April 2017 to February 2018 on a total number of 90 subjects included 70 Iraqi patients with cervical abnormalities 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 and 20 apparently healthy women. The patients and healthy were aged between 25-55 years. The aim of the study is to explore the relationship between HPV, genes expression of the genes miRNA-744, Bcl-2 and caspase-3 and epithelial cervical abnormalities. The results showed that women aged more than 30 yrs are in risk to have cervical abnormalities more than those less in age. The results revealed that the folding values of miRNA-744 were highly significant in patients positive to HPV virus \((2.8 \times 10^{12})\) comparing to patients negative to HPV or control \((9.4 \times 10^{7} \text{ and } 2.11 \times 10^{8})\) respectively. The increased folding value of miRNA-744 in patients positive to HPV was parallel to a significant increase of folding value of caspase-3 and decreased value of Bcl-2. On the other hand, the decreased value of the miRNA-744 in patients negative to HPV showed increased folding value of caspase-3 and Bcl2. These results indicate that HPV virus could be play a role in down regulating of Bcl2 which induce the apoptosis via increase the folding value of caspase-3 and its regulator miRNA744.

**Keywords:** Cervix, caspase-3, Bcl2, miRNA744, HPV, gene expression

**INTRODUCTION**

Changes in cervix cells as abnormal cells as dyskaryosis or dysplasia can be due to inflammation or infection (Jeronimo et al., 2016). Human papilloma virus (HPV) is a common virus that affects both females and males. The HPV virion has a double-stranded, circular DNA genome of approximately 7900bp, with eight overlapping open reading frames. Many cervical precancers changes that could lead to cancer are related to HPV (Jeronimo et al., 2016). Most of these changes occurred in genes related to cells cycle and apoptosis pathways. Programmed cell death (apoptosis), is a highly regulated process that eliminate unwanted or dysfunctional cells (Wu, 2017). Cancer may arise from the dysfunction in the apoptotic pathway this process is regulated by many factors and proteins such as Caspase family and Bcl-2 (Roy and Cardone, 2002). MicroRNAs (miRNAs) are endogenous, small with 18–25 nucleotides, and non-coding RNAs that negatively regulate gene expressions at the post-transcriptional level through binding to the 3 untranslated region (UTR) belonging to a specific messenger RNAs (mRNAs) (Calin and Croce, 2006; Valencia-Sanchez et al., 2006). With sequences partially complementary to their target mRNAs, miRNAs play critical roles in moderating gene expression in a variety of organisms (Kwak et al., 2010; Garzon et al., 2009; Tagawa and Seto, 2005). Studies have confirmed that miRNAs participate in various biological
processes, including cell growth, development, proliferation and metabolism, via inhibition of mRNA translation (Kwak et al., 2010). miR-744 has been suggested to play an inhibited role in many cancers or tumors, including colon cancer and breast cancer as well as gastric cancer (Song et al., 2012). Because modulation of miR-744 is common to a number of cancers, it has been hypothesized that miR-744 may play an essential role in tumor growth, development and tumorigenesis (Tan et al., 2015; Lin et al., 2014; Vislovukh et al., 2013 ; Helwak et al., 2013 ). It is of interest that miR-744 might be a potential target for the inhibition of cervical cancer and could be a target for HPV virus. The study aims to explore the correlation between HPV virus, miRNA-744 and cervical abnormalities including carcinoma.

MATERIALS AND METHODS

This study was conducted from April 2017 to February 2018 on a total number of 90 subjects included 70 Iraqi patients with cervical abnormalities 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 and 20 apparently healthy women. The patients and healthy were aged between 25-55 years. Buccal swab-Pap smear were collected from each patients women and healthy control. The samples were preserved in 0.75 ml TRIzol reagent then 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.

Molecular detections of human papilloma virus (HPV) : The molecular detection part of this study was focused on the analysis of extracted DNA form women by using DNA-sorb-AM nucleic acid extraction kit (DNA-sorb-AM nucleic acid extraction kit (AmpliSens® /Russia)). Total cellular DNA was extracted from cervical specimens using the HPV genotyping kit for 14 types (Federal Budget, Russia). Real Time PCR for qualitative detection and genotyping of Human Papilloma virus High Risk was also used using HPV HCR (human papilloma viruses of high carcinogenic risk) genotype-titre-FRT PCR kit (AmpliSens®/Russia) with a condition as follow:95C for 15 min for 1 cycle, 95C for 5s, 60C for 20s,72C for 15s for 5 cycles and 40 cycles for: 95C for 5s, 60C for 20s fluorescence acquiring and 72C for 15s.

Molecular detection of gene expression of miRNA-744, BCL-2, CASPASE-3 cDNAs for miRNA-744, BCL-2, CASPASE-3 and GAPDH (reference gene) genes were produced using TRIzol® LS Reagent , SYBRR Green, RT-qPCR System from Promega-USA, primers (Table-1) and according to the manufacturer’s protocol. Amplification of BCL-2, CASPASE-3 genes was performed as follow: 95C for 15 min for 1 cycle, 40 cycles for: 95C for 5s, 54-60C for 20s and 72C for 15s. While the amplification condition of miRNA-744 and reference genes was as in Lin et al., 2014.

RESULTS AND DISCUSSION

The age of all women was categorized as those who were less than 30 years old versus those equal to and over 30 (Table 2). The results revealed that 26.67 % of the studied women in general were less than 30 years old while 73.33 % were equal or over 30. This may lead to conclude that women aged more than 30 yrs (group 2) are in risk to have cervical abnormalities more than those less in age. They were two subgroups in this study, the first group the women with abnormal pap smear revealed that 21.43% of the women were less than 30 (< 30) years and followed by 78.66% of women whose age about more than 30 years old (>30) years. The second group the apparently healthy women (with normal pap smear) revealed that 45% of the women were about (<30) years and followed by 55% of women whose age about (>30) years. This could be due to long exposing of the aged women to the infectious factors and environmental factors.

The results agreed with Chaithanya (2016) who reported that the age range of patients with epithelial cell abnormality pre-neoplastic and neoplastic lesions was 24-82 years and constituted 2.2% cases of the study which is similar to the study done by Tailor et al., (2016) who found that there is a correlation between age range was 2nd to 8th decade and abnormal PAP smears accounted to 2.61% of total smears taken (Tailor et al., 2016).
Table-1 : Primers used in the study .

<table>
<thead>
<tr>
<th>Primer</th>
<th>Bcl-2 (122 bp)</th>
<th>Caspase -3 (93 bp)</th>
<th>miR -744 (Lin et al., 2014)</th>
<th>GAPDH (reference gene) 258bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>TGGATGACTGAGTACCTGAA</td>
<td>GAAACCAAGATCATCATGGAA</td>
<td>AATGCAGGGCTAGGGGCTA</td>
<td>AGA AGG CTG GGG CTC ATT TG</td>
</tr>
<tr>
<td>Reverse</td>
<td>GACAGCAGGAGAAATCACAA</td>
<td>ACATAAACCATCAGGATAA</td>
<td>GTGCGGGTCCGAGGTG</td>
<td>AGG GGC CAT CCA CAG TCT TC</td>
</tr>
</tbody>
</table>

The results agreed with those reported by Wright (2014) who displayed that the aging is a risk factor for persistent infection. The rate of persistent high-risk infection for women older than age 40 is 50%, compared with a persistence rate of 20% in women younger than age 25 years. Women aged 41–66 years, with old age, HPV infection, cervical inflammation, smoking, and those reporting post menopause have strong risk for cervical lesions (Zhang et al.,2015). According to the study by Ranabhat SK et al (2011), 80% of the abnormal lesions were found in the age group above 40 years as about 20% of the patients above 30 years (Ranabhat et al., 2013).

Real time PCR quantification

Real-time RT-PCR data were quantified as Ct values that are inversely related to the amount of starting template: high Ct values correlate with low levels of gene expression, whereas low Ct values correlate with high levels of gene expression. Amplification plots were created when the fluorescent signal from each sample is plotted against cycle number, therefore, amplification plots represent the accumulation of product over the duration of the real time PCR experiment. Figures (1 to 6) showed amplification Bcl-2, GAPDH and caspase-3 gene expression with melting curve of all genes.

The calculation of gene expression fold change was made using relative quantification. Relative quantification, 2−ΔΔCt algorithm, is the fold expression relative to a calibrator with Housekeeping gene (patients and control), (Monica et al., 2011).

The slopes of target genes (miR-744) which obtained from standard curve equations, were (11 × 10⁹ to 3 × 10⁶ copy μl⁻¹). Figures 7 to 9 revealed Slop mi-RNA744 ,amplification mi-RNA744 and melting curve. The calculation results of Rsq (R²) for those genes were 0.99 and the calculation results of real time PCR assays efficiency were 1.22%.
Figure 1: Amplification plot for Bcl-2 gene expression

Figure 2: Bcl-2 expression, Melting on Green temperature ranged from 72°C to 95°C.

Figure 3: Amplification plot for Housekeeping (GAPDH) gene expression

Figure 4: GAPDH expression, Melting on Green temperature ranged from 72°C to 95°C.
Figure 5: GAPDH expression, Melting on Green temperature ranged from 72°C to 95°C.

Figure 6: Amplification plot for Caspase-3 gene expression.

Figure 7: Absolute quantification by the standard curve of miRNA744.
The standard curve method employs a dilution series of known template copy number in the qPCR assay. Linear regression of log concentration (copy μl⁻¹) versus CT gives the standard curve, and this is then used to calculate template concentration (copy μl⁻¹) of the sample.

The correlation between HPV virus, caspase-3, BCL-2, miRNA744 gene expression and epithelial cervical abnormalities.

The results listed in table-3 revealed that the folding values of miRNA744 were highly significant in patients positive to HPV virus (2.8 × 10^{12}) comparing to patients negative to HPV or control (9.4 × 10^{7} and 2.11 × 10^{8}) respectively. The increased folding value of miRNA744 in patients positive to HPV was parallel to a significant increase of folding value of caspase-3 and decreased value of Bcl-2. On the other hand, the decreased value of the miRNA744 in patients negative to HPV showed increased folding value of caspase-3 and Bcl2. These results indicate that HPV virus could be play a role in down regulating of Bcl2 which induce the apoptosis via increase the folding value of caspase-3 and its regulator miRNA744. Patients negative to HPV virus also have significant folding value of Caspase-3 but with increased level of Bcl-2 gene expression. The high folding in patients with negative HPV virus suggest that the Bcl-2 could be inactive mutated gene.
Table 3: The relationship between HPV and genes expression of caspase-3, BCL-2 and miR-744 in epithelial cervical abnormalities.

<table>
<thead>
<tr>
<th>The Group</th>
<th>Caspase-3</th>
<th>BCL-2</th>
<th>miRNA744</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.7 ± 0.05 b</td>
<td>1.3 ± 0.03 b</td>
<td>2.11 x 10^6 b</td>
</tr>
<tr>
<td>HPV +</td>
<td>2.3 ± 0.08 b</td>
<td>0.94 ± 0.03 b</td>
<td>2.8 x 10^12 a</td>
</tr>
<tr>
<td>HPV -</td>
<td>4.5 ± 0.14 a</td>
<td>2.66 ± 0.08 a</td>
<td>9.4 x 10^7 c</td>
</tr>
<tr>
<td>LSD value</td>
<td>0.861 **</td>
<td>0.958 **</td>
<td>1.822 **</td>
</tr>
<tr>
<td>P-value</td>
<td>0.0075</td>
<td>0.0026</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Means having with the different letters in same column differed significantly. ** (P<0.01).

The results also showed that HPV virus could influence the up expression of miRNA744 which down regulate the expression of Bcl-2 and up regulate the caspase-3 which promote the apoptosis and effect the cell cycle.

Cell apoptosis can be initiated by a mitochondria-dependent or -independent apoptosis pathway (Dlamini et al., 2004). Both pathways trigger apoptosis by activating different caspase cascades and converging on caspase-3, the executor of apoptosis (Zhang et al., 2015). Apoptosis through the activation of caspase-3 by both caspase-8- and caspase-9-dependent pathways was also demonstrated by others (Alikhani et al., 2003, 2004). Both pathways trigger apoptosis by activating different caspase cascades and converging on caspase-3, the executor of apoptosis (Zhang et al., 2015). Bcl-2 inhibits apoptosis by preventing oligomerisation and insertion of proapoptotic Bcl-2 family members (Bax, Bak) into the outer mitochondria membrane (Konopleva et al., 2016). This prevents the release of cytochrome c from mitochondria, thus inhibiting the cytochrome c dependent cleavage of pro-caspase into active enzymes (Kaufmann and Hengartner, 2001).

The Bcl-2 protein family includes major regulators of cell survival, involving Bax and Bad, which promotes or suppresses apoptosis progression. And Caspases signaling pathway was partly regulated by Bcl-2 (Basu et al., 2014). The data above indicated that restoration of miR-744 could suppress the growth and proliferation of cervical cancer cells and Bcl-2 might be a potential target of miR-744. Similarly, the mRNA level of Bcl-2 was decreased for miR-744 up-regulation in both two cervical cancer cells (Wang and Wang, 2011). In the absence of Bcl-2, apoptotic signals provoke cytochrome c release from the mitochondria into the cytoplasm where it associates with Apaf-1 (apoptosis activating factor 1) in the presence of dATP. Apaf-1 recognizes the inactive pro-caspase 9 and forms the apoptosome, which triggers autocatalitic processing of pro-caspase 9. In turn, active caspase 9 activates downstream executioner caspases(Pižem et al.,2001). Caspase 3 is the ultimate executioner caspase that is essential for the nuclear changes associated with apoptosis, including chromatin condensation. As revealed previously, caspases including caspase 3 are synthesized as «uncleaved« proenzymes without activity. Therefore, apoptotic cell death mediated by caspase 3 is induced only after the inactive 32 kDa caspase 3 is cleaved into active fragments of 17 kDa and 12 kDa (Strasser et al., 2000).

As replication of HPV occurs in differentiated keratinocytes, in the upper layers of the epidermis, E6 can partially prevent apoptosis by down regulation of the proapoptotic members of the Bcl-2 family (Sharon Shnitman et al., 2007). miR-744 promoted apoptosis in cervical cancer cells via Caspase-3 activation and could target Bcl-2 and then activate Caspase-3 activity, resulting in apoptosis in cervical cancer cells (Xiao-Fang and Yun , 2016 ). In addition, Caspase-3 expressed highly with miR-744. The data illustrated that miR-744 had a possible effects on accelerating apoptosis in cervical cancer growth. Expression of miR-744 is over exhibited an enhancement on expression in miR-744 . Further, the predicted target transcripts are
involved in signaling pathways regulating apoptosis and cell cycle progression in cervical cancer (Phuah et al., 2013).

Numerous miRNAs act as either tumor suppressors or oncogenes, and the aberrant miRNAs expression is included in the initiation and progression of human tumors or cancers. MiR-744 is identified as a tumor related gene recently, and there are only a few researches on its function. Presently, it is difficult to determine miR-744 as a tumor suppressor or oncogene. For instance, miR-744 is over-expressed in head and neck cancer tissues (Song et al., 2012). And markedly up-regulated serum expression of miR-744 has been also suggested in gastric cancer (Lin et al., 2011). Thus, it was necessary to further clarify the role of miR-744 in cervical cancer progression. More experiments demonstrated that accumulation of miR-744 decreased the proliferation of cell lines significantly. The decreased proliferation of the cervical cancer cell lines resulted from suppressed cell colony formation, invasion and migration in vitro. Caspase-3 is an essential indicator of apoptosis, and the activation of the enzyme presents the apoptosis capacity in a numeric form in order to conduct quantitative research (Basu et al., 2014).

CONCLUSION
The results showed that women aged more than 30 yrs are in risk to have cervical abnormalities more than those less in age. The results also indicate that HPV virus could be play a role in down regulating of Bcl2 which induce the apoptosis via increase the folding value of caspase-3 and its regulator miRNA744.

CONFLICT OF INTEREST
The authors declared that present study was performed in absence of any conflict of interest

ACKNOWLEGEMENT
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AUTHOR CONTRIBUTIONS
Human samples were taken under the scientific ethics of the MHO and the institute of Genetic Engineering and Biotechnology-University of Baghdad.

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