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COMBINED EFFECTS OF TLR4 AND HIF-1A WITH COX-2 GENES IN CERVICAL SQUAMOUS CELL CARCINOMA ASSOCIATED WITH HPV INFECTION

Molecular techniques are used for the detection of HPV-related cervical cancer. An average of 48 million instances of cervical cancer are reported each year, 80 % of which occur in developing countries. ***Aim.*** *To study the correlation of HPV16, HPV18, and HPV 31 genotypes with cervical cancer tissues, and their effects on the expression levels of TLR4, HIF-1α, and COX-2 genes in infected women.* ***Methods.*** *35 samples were collected from women with cervical squamous cell carcinoma and 30 — from women with normal cervical tissues. Subsequently subjected to HPV genotyping and expression levels of TLR4 and HIF-1α with COX-2 genes were analyzed using a real-time polymerase chain reaction.* ***Results.*** *Molecular detection showed the presence of HPV16, HPV18, and HPV31 genotypes in tested samples. 25 (71.42%) of 35cases containing cervical carcinoma are associated with high-risk HPV, while the other 10 (28.57 %) are associated with low-risk HPV genotypes. The result showed elevation in the levels of gene expression 1.52, 48.0, and 28.8 fold in TLR4 and HIF-1α with COX-2, respectively, in positive HPV-tested cervical cancer samples compared to its values with the housekeeping gene (β actin) to 1 as control characterized by lower expression of target genes.* ***Conclusions.*** *The study indicates that TLR4 and HIF-1α with COX-2 genes are overexpressed in cervical squamous cell carcinoma linked to HPV, which has been reported in all diagnosed cases.*

Keywords*: HPV, COX-2, TLR4, HIF-1α, cervical cancer.*

On average 48 million instances of cervical cancer are reported each year, with 80 % of them occurring in developing nations (Wang X et al., 2022). Human papillomavirus (HPV) represents one of the most prevalent viral infections and fourteen HPV genotypes are known to be linked to cancer. The primary mode of transmission for this virus is sexual contact, and infection

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usually starts shortly after sexual activity. Many oncogenic types of HPV have been reported, and around 70% of cervical cancer worldwide is caused by HPV genotypes 16 and 18 (Kombe et al., 2021). Every HPV is a dsDNA virus without an envelope, over 100 genotypes of HPV have been detected, and relying on their transformation ability in the women's genital tract, they are divided into high and low-risk HPV (Yu et al., 2021). The prevalence of cervical cancer is rising, while the age at which it first appears is dropping. To prevent cervical cancer and create new treatments, it has been suggested that high-risk HPV E6 and E7 oncoproteins associate with the tumor suppressors pRB and p53, respectively (Sen, Ganguly & Ganguly, 2018), and are required for malignant conversion. This is one way that these viral proteins cause tumors (Yim & Park, 2005), so it is essential to understand how it begins and progresses (Wang et al., 2022). The development of invasive cervical cancer has been linked, to varying degrees, to cofactors such as parity, the use of oral contraceptives, tobacco use, and immunosuppression particularly concerning HIV infection with other sexually transmitted diseases, and inadequate nutrition (Shayan, Nazari, & Kiwanuka, 2021). Toll-like receptors (TLRs) are mode recognition receptors that control bacterial infection by determination of conserved compounds from harmful bacteria. TLRs are expressed in immune cells, some tumor cells, and normal epithelial cells (El-Zayat et al., 2019). Inflammation mediated by TLR4, induced by exogenous or endogenous ligands, in addition to enrolled in acute and chronic disease, plays a substantial role as an amplifier to the inflammatory response (Yang et al., 2018). Recent research has demonstrated the significance of TLR4 in identifying chemicals released by necrotic cells and injured tissues. When these molecules attach to TLR4, they are referred to as damage-associated molecular pattern molecules (DAMPs), and they strongly induce an inflammatory response (Yang et al., 2017). The activation of the TLR4 system in tumor cells by endogenous TLR ligands produced by damaged tissues and proinflammatory cytokines may enhance tumor cell proliferation and apoptotic resistance. Another study showed that nuclear factor-κB (NF-κB) activation may contribute to the onset and progression of cancer. TLR4-activated cells cause inflammation and tumors via MyD88 and NF-κB-associated signaling pathways (Chen et al., 2022). Women who are HPV-positive exhibit a more diverse vaginal microbiota along with a higher pH and a decreased relative abundance of *Lactobacillus* species. There is a strong correlation between the development of cervical dysplasia and new bacteria, as well as with the deregulation of pathways and hyperexpression of cytokines that cause chronic inflammation (Mitra et al., 2016)

Hypoxia is a kind of cellular stress recognized as crucial for the tumor's development in addition to its invasion, growth, metastasis, and resistance to medications (Gandhi et al., 2017). TLR4 expression and HIF-1α expression show a positive correlation in cervical cancer cells, and the bacterial LPS-induced accumulation of HIF-1 in immune cells like macrophages and monocytes depends on TLR4 (Xiong et al., 2022; Peng et al., 2020), HIF-1α is considered a hypoxia trigger since it plays a crucial function in the formation of different tumors (Peng et al., 2020). TLR4 is also involved in sustaining elevated HIF-1α activity, which increases tumor invasiveness, promotes tumor angiogenesis, and elevates resistance to chemo and radiotherapy by its participation in the transcriptional regulation of several genes, including the COX gene (Priego-Hernández et al., 2023).

Arachidonic acid is converted by COX into the precursors of prostaglandins, prostacyclins, and thromboxanes. Two isoforms of COX are known as COX-1 and COX-2 (Williams et al., 1999). Several inflammatory mediators, including prostaglandins (PG), thromboxane, and leukotrienes, have been linked to the pathological

processes that lead to cancer. The first stage of proteinoids' production is catalyzed by COX-2 enzyme, which is involved in several physiological and pathological processes in humans and animals (Szweda et al., 2019). Proliferation, angiogenesis, invasiveness, and apoptosis are stimulated by increased COX-2 activity and prostaglandin production (de Moraes et al., 2007). The expression of COX-2 rapidly increases when pathogenic processes are accompanied by pain, inflammation, and fever. Since COX-2 was shown to be overexpressed in tumor tissue, a poor response to chemotherapy is linked to elevated COX-2 expression; this enzyme possibly contributes to the development of cancer (Gandhi et al., 2017). The study aimed to investigate the correlation of HPV16, HPV18, and HPV 31 genotypes with cervical cancer tissues and their effects on expression levels of TLR4, HIF-1α, and COX-2 genes in infected women.

Materials and Methods. ***Sample collection.*** 65 cervical tissue samples were collected from women aged between 30 to 40 years at the Maternity and Child Hospital located in the Maysan province, Iraq. Cervical biopsies were available for cases suffering from carcinoma and clinical cervical and uterine abnormalities. All cases were tested for HPV infection. 30 samples were taken from women who had hysterectomy procedures performed for non-neoplastic reasons, such as abnormal uterine bleeding or uterine fibroids, which histopathology verified as non-neoplastic, and 35 samples were taken from cervical cancer patients.

Preparation of tissue samples to histopathological study. All tissue samples were prepared in the same way in the histopathology laboratory, where the tissue was processed by dehydration in an ascending series of ethanol alcohol, cleared by xylene, and embedded in paraffin. Paraffin blocks were made and the sections were prepared by sectioning into thin 5 μm slices by a microtome and stained by H&E stains. All slices were reviewed by pathologists based on the histopathological criteria for cervical squamous cell carcinoma, including nuclear hyperchromasia, high N/C ratio, mitotic figures, hyperkeratosis, breakage of the basement membrane, and invasion of the underlying tissue.

DNA extraction and HPV genotyping. The available extraction kit (Roche, Germany) was used to extract DNA from tissue samples in accordance with the manufacturer's instructions. The iNtRON Biotechnology RealMODTM Green SF 2X qPCR mix kit was used in the present study for real-time PCR to identify HPV genotypes. 2 μL of DNA template, 10 μL of Master Mix, 1 μL of each of the forward and reverse primers, and 6 μL of Free DEPEC-D.W. were combined to create the 20 μL total volume of the reaction. For the real-time PCR, flow cycling conditions were as follows: 10 min of primary denaturation at 95 °C, 40 cycles of 15 sec at 95 °C, 30 sec at 60 °C, and 30 sec at 72 °C. The last extension was carried out for 5 min at 72 °C. To ensure primer specificity, the reaction was subject to the melting curve analysis. The primers used to detect HPV are presented in Table 1.

RNA extraction and cDNA synthesis. Approximately 20 mg of cervical tissue samples were used for RNA extraction by the ReliaPrep™ Miniprep RNA System (Promega, Italia) following the manufacturer's instructions. Complementary DNA (cDNA) was produced from extracted RNA samples using the iNtRON Biotechnology's Fast HisenScriptTM RH (-) RT PreMix Kit (CAT: 25087). After the reaction ingredients were mixed with the template RNA, 20 μL of reaction was produced. Under these conditions, the following reaction was attained: 45 min of reverse transcription at 45 °C and 10 min of RTase inactivation at 85 °C.

TLR4, HIF-1α, COX-2, and β-actin gene expression. Using 10 μL of SYBR Green Master, 4 μL of cDNA, 1 μL of each of the forward and reverse primers for TLR4, HIF-1α, COX-2, and β-actin, and 4 μL of distilled water, 20 μL of reaction volume was created. The cycling conditions

of qPCR were set for each gene as follows: primary denaturation for 10 min at 95 °C, followed by 45 cycles at 95 °C for 15 sec, 59 °C for 30 sec, and 72 °C for 30 sec, with a final extension lasting 5 min at 72 °C. The melting curve analysis was utilized to ensure that the primers used in the reaction were specific. Each qPCR assay was conducted in triplicate. Once the mRNA expression levels were normalized against β-actin, they were expressed as ΔΔCT. Table 1 is a list of primers used in real-time PCR.

Statistical Analysis. The Student t-test (Oncomine and UALCAN) and the Wilcoxon test (TIMER2.0) were used to compare the differences between the groups. The difference was statistically significant when the P value was less than 0.05. The results are displayed as mean ± SD.

Results. ***Histopathological analysis and molecular detection of HPV genotypes.*** 30 cases with uterine problems were negative for condyloma, and the cervix had no criteria for any stage of carcinoma — all were tested negative for HPV. Histopathological inspection and molecular results showed that all 35 cases contained cervical carcinoma and were positive for HPV; 10 (28.57 %) of 35 cases contain CIN, cervical epithelium exhibits hyperchromasia, high N/C ratio, prominent mitotic figures, and hyperkeratosis but no breakage of the basement membrane, and all these cases are associated with low-risk HPV types. 19 cases (54.28%) exhibited well-differentiated squamous cell carcinoma where the cells appear hyperchromatic, high N/C ratio, and prominent mitotic figures; tumor cells break the basement membrane and invade the underlying structures forming clusters of cells, which form prominent cell nests where the cells are arranged in whorls like the pattern with central keratin, and the others form cellular clusters without central keratin. 6 cases (17.14%) showed poorly differentiated squamous cell carcinoma, where the tumor cells were arranged in undefined patterns: some of them formed clusters., some formed sheets, and the others were located individually, all growth patterns failed to produce keratin. All 25 cases were associated with high-risk HPV type as shown by the molecular study.

TLR4 gene expression. The findings showed that after deducting the signal from the housekeeping gene (β-actin), the gene expression of TLR4 was elevated in positive samples by more than two CTs prior to the negative sample signal. Here, the housekeeping gene was taken to apply the ΔΔ CTs analysis (Fig. 1). Moreover, TLR4 values in samples that were positive were compared to that of 1, which was roughly 1.56 times greater than that of negative samples' values, after normalizing to 1.

In positive cases, TLR4 gene expression rose approximately by 1.56 times as compared to 1. ΔΔ CTs were used to analyze the data, and the

Table 1. **Primer sequences used in the study**

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
HPV16 E7	GAACCGGACAGAGCCCATTA	ACACTTGCAACAAAAGGTTACA
HPV18E7	CAACGTCACACAATGTTGTGA	TCAATTCTGGCTTC ACACTTAC
HPV31E6	GGTCAGTTAACAGAAACAGAGG	TGGTGTGTCGTCCC TATATACT
β-actin	GATTACTGCTCTGGCTCCTAGC	GACTCATCGTACTCCTGCTTGC
TLR4	CCCTGAGGCATTTAGGCAGCTA	AGGTAGAGAGGTGGCTTAGGCT
HIF1α	TATGAGCCAGAAGAA CTTTTAGGC	CCCAGGTCCTCGCTTATGATCT
COX-2	ACACCTC TGTAGGTCACTGTTG	CAAGACAGATCATAAG CGAGGA

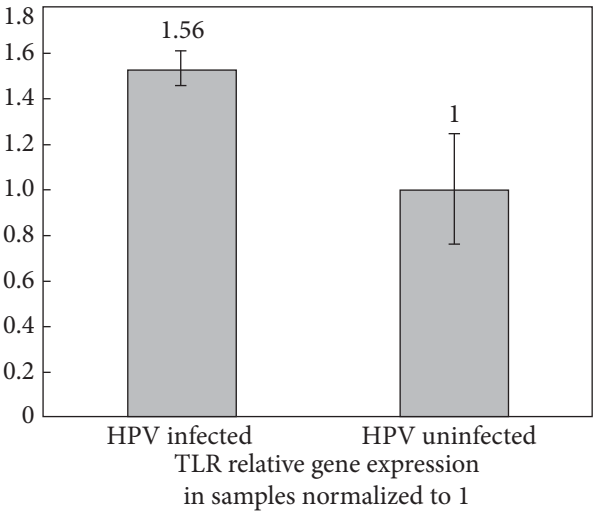

Fig. 1. The relative expression of TLR4 gene in both positive and negative samples

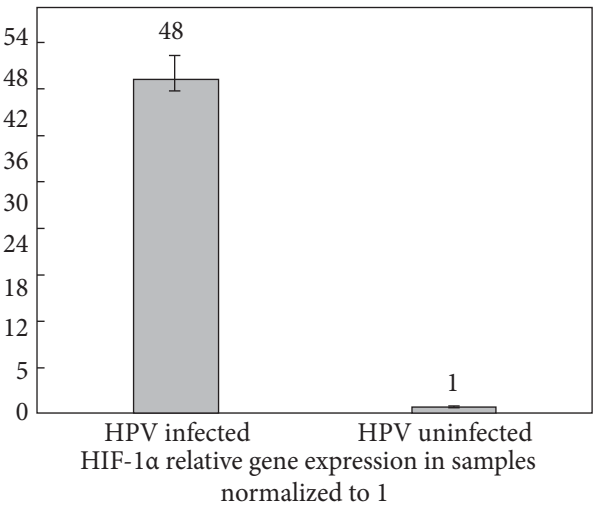

Fig. 2. The relative expression of HIF-1α gene in both positive and negative samples

housekeeping gene (β actin) was normalized to 1. P = 0.0031 in the t-test.

HIF-1α gene expression. The results of HIF-1α gene expression showed a significant increase in positively tested tissue samples compared to the negative samples. After deducting the β-actin signal, the HIF-1α fluorescence signal for the negative sample lagged behind the positive sample signal by over 14 CTs. After normalizing the negative control samples to 1, the data were examined to determine the difference in folds of expression. Fig. 2 shows that the expression in the positive samples was roughly 48 times higher than in the negative control.

In comparison with the first sample, HIF-1α gene expression rose by about 48 times in positive samples. ΔΔ CTs were used to analyze the data, and the house-keeping gene (β actin) was normalized to 1. P= 0.0038 significant for the t-test.

COX-2 gene expression. When comparing positively tested tissue samples to negative samples, the COX-2 gene expression results revealed a significantly higher level in the positive samples. In positive samples, the COX-2 gene expression fluorescence signal was approximately 60% of the controls. Positive samples were compared to a normalized value of 1, which was applied to the

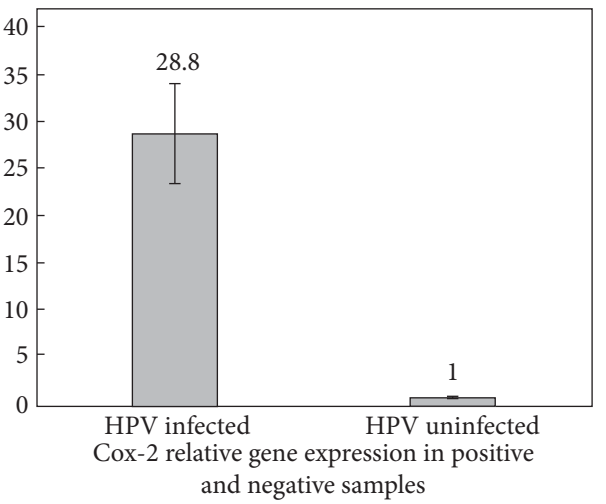

Fig. 3. The relative expression of COX-2 gene in both positive and negative samples

negative samples. Cervical cancer caused a 29-fold increase in COX-2 expression in positive samples compared to negative samples, as shown in Fig, 3.

When compared to 1, the COX-2 gene expression in positive samples increased by about 29 times. ΔΔ CTs were used to analyze the data, and the house-keeping gene (β actin) was normalized to 1**.** P= 0.0038 in the t-test.

Discussion. Molecular detection showed the presence of HPV16, HPV18, and HPV31 geno-

types in tested samples. 25 (71.42 %) of 35 cases containing cervical carcinoma are associated with high-risk HPV, while the other 10 (28.57 %) are associated with low-risk HPV genotypes. These results refer to an elevation in the incidence of HPV, especially in high-risk genotypes 16 and 18. Cervical co-infection with one more HPV genotype was also reported in the current study. These results are consistent with (Mu et al., 2023), who stated the presence of multiple HPV genotypes in the same infections reached six. Also, the presence of more HPV genotypes in the same infected sample is not seldom (Yang et al., 2018). The current results revealed that the gene expression of TLR4 was high in infected samples, which is in line with previous results that mentioned a clear link between the expression of the TLR4 gene at a high level and the tumors out coming from the transformation of squamous cells of the cervix into immortal cells by the HPV oncogenic proteins compared to tested negative samples of cervical tissue (Williams et al., 1999, Szweda et al., 2019). TLRs, especially 4, 5, and 9, are closely linked to HPV infection, and cervical cancer may rapidly grow when TLR4 is activated. *In vitro* studies, the relationship between TLR4 and cervical cancer cells was well investigated, and the results revealed that HPV-related cervical cancer cells proliferate and resist apoptosis by the TLR4 supporting (de Moraes et al., 2007). High-level TLR4 gene expression is associated with HPV infection. In addition, proinflammatory cytokines are produced in large quantities in HPV-related cervical cancer cells when the TLR4 pathway is activated (Gandhi et al., 2017). According to Nikolalic et al. (2023), the endogenous TLR4 activator S100A8/S100A9 may promote the growth of tumors. TLR binding to HPV infection may be caused by the E6 regulatory protein (Feng et al., 2012). Many transcriptional pathways that affect the ability of the immune system to determine the pathogens enable HPV to evade immune monitoring (Jiang et al., 2020). According to this, different high-risk HPVs have different regulatory strategies for managing TLRs (Morale et al., 2022). Many tumors have low O_2 concentrations (Zhao et al., 2021). Hypoxia is less than 1.5% oxygen concentration in tissues, which plays a crucial role in leading to tumor growth (Feng et al., 2012). Increased resistance to radio and chemotherapy may be attributed to hypoxia and represents a negative predictive sign of a tumor, involving the HPV-related tumor. Availability of oxygen is well known to affect the biology of cancer cells. Cancers in the cervix often exhibit low levels of oxygen concentrations, with a heterogeneous distribution of oxygenated positions with a mean of 1.2% oxygen concentration (Deguchi et al., 2023). Previous studies stated that solid tumor tissue has hypoxia microenvironments (Cruz-Gregorio & Aranda-Rivera, 2021). High levels of HIF-1α are observed in these microenvironments, and there is a strong correlation between the highly activated HIF-1α and the unchecked growth of cancer cells as well as the prevalence and progression of cervical cancer in the tissues (Deguchi et al., 2023). Our research revealed that HPV-infected cases had higher levels of HIF-1α expression than uninfected cases, and that TLR4 and HIF-1α expressions in infected cases of cervical cancer are correlated. These findings are consistent with those of Haręża et al. (2022). HIF-1α and TLR4 have a close relationship in the tissue of cervical cancer that is caused by HPV. Since the RNA contents of HIF-1α and TLR4 are similar, these findings suggest a positive correlation between the two genes' expressions (Steinbach & Riemer, 2018). The findings of this investigation demonstrate that elevated Cox-2 expression through TLR4 signaling leads to a rise in Cox-2 and PGE2 synthesis (Vaupel et al., 2021). We can hypothesize that sustained, long-term TLR4 signaling may support cervical cancer cells. Hematological cancer models including RAJI (Burkitt's lymphoma) and U937 (acute promonocytic leukemia) have been shown to overproduce COX-2

(Harris et al., 2022). Another study has suggested that PGE2 formed from necrotic cells is an inhibitory DAMP that reduces the immunostimulatory impact of dead cells and that PGE2 released by dying cancer cells via caspase 3 is responsible for the repopulation of cancer cells after cytotoxic therapy (Zhang et al., 2023). The elevated levels of systemic PGE2 and increased cervical COX-2 are linked to papillomavirus infection (Bell et al., 2022). In women infected with papillomaviruses, medications that block the production of PGE2 may help lower the risk of cervical cancer or systemic inflammation (Zhao et al., 2021).

Conclusions. The study indicates that the TLR4 and HIF-1α with COX-2 genes are overexpressed in cervical squamous cell carcinoma linked to HPV, which was revealed in all diagnosed cases. The results of this study also demonstrate that papillomavirus infection is linked to elevated systemic PGE2 levels and increased COX-2 in the cervix. These substances localize autocrine and paracrine effects, triggering a range of intracellular processes that lead to angiogenesis, antiapoptotic activity, cellular growth, and enhanced metastasis.

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Conflict of interest. The research was conducted without any conflict of interest.

Ethics. This study was approved by the Committee of University of Misan, child Hospital, in the Maysan province, Iraq, on January 03, 2022 with NO. 1024, 0412, and signed informed consent was waived**.**

Author contributions. Dr. Mustafa Adnan: research design, analysis of experimental data. Mustafa Adnan and Qayssar Ali Kraidi: manuscript revision and molecular implementation. Dr. Mukhallad Abdul Kareem Ramadhan: histopathological analysis.

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КОМБІНОВАНІ ЕФЕКТИ TLR4 ТА HIF-1α З ГЕНАМИ COX-2
ПРИ ПЛОСКОКЛІТИННІЙ КАРЦИНОМІ ШИЙКИ МАТКИ, АСОЦІЙОВАНІЙ
З ПАПІЛОМАВІРУСНОЮ ІНФЕКЦІЄЮ ЛЮДИНИ

Молекулярні методи використовуються для виявлення ВПЛ-асоційованого раку шийки матки. Щорічно реєструється в середньому 48 мільйонів випадків раку шийки матки, причому 80 % цих випадків припадає на країни, що розвиваються. **Мета.** Вивчити кореляцію генотипів ВПЛ16, ВПЛ18 і ВПЛ31 з тканинами раку шийки матки та їх вплив на рівні експресії генів TLR4, HIF-1α і COX-2 в інфікованих жінок. **Методи.** Було відібрано 35 зразків у жінок з плоскоклітинною карциномою шийки матки та 30 зразків у жінок з нормальними тканинами шийки матки. Згодом було проведено генотипування ВПЛ та проаналізовано рівні експресії генів TLR4 і HIF-1α з генами COX-2 за допомогою полімеразної ланцюгової реакції в реальному часі. **Результати.** Молекулярне дослідження показало наявність генотипів ВПЛ16, ВПЛ18 та ВПЛ31 у досліджуваних зразках. 25 (71.42 %) із 35 випадків, що містять карциному шийки матки, асоційовані з ВПЛ високого ризику, тоді як інші 10 (28.57 %) асоційовані з генотипами ВПЛ низького ризику. Виявлено підвищення рівня експресії генів TLR4 та HIF-1α з COX-2 відповідно в 1.52, 48 та 28.8 разів у позитивних зразках ВПЛ-тестованого раку шийки матки порівняно з його значеннями при збереженні гена (β актину) до 1 в якості контролю, який характеризується нижчою експресією генів-мішеней. **Висновки.** Дослідження показало, що при плоскоклітинному раку шийки матки, асоційованому з ВПЛ, спостерігається гіперекспресія генів TLR4 та HIF-1α з генами COX-2, що було зафіксовано у всіх діагностованих випадках.

Ключові слова: *ВПЛ, COX-2, TLR4, HIF-1α, рак шийки матки.*