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RESEARCH ARTICLE

Role of Human Papillomavirus in Hypoxia Microenvironment

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ABSTRACT

Background: Human papillomavirus is a double-stranded DNA and globally, there are approximately 300,000 deaths annually due to cervical cancer in women and 500,000 new infections with this virus. Toll-like receptor 4 (TLR4) is a receptor that triggers the innate immune response against the invading pathogen. Hypoxia in the solid tumor microenvironment participates in tumor development as a result of persistent infection.

Materials and Methods: Samples of 48 tissue biopsies and Pap smear samples were taken from women's cervix from Al-Mwani Teaching Hospital, Basrah Women and Children Hospital, and Al-Fayhaa Teaching Hospital. PCR was used to detect the presence of HPV in 89.6% of cases. Extraction of RNA and gene expression was performed for toll-like receptor4, hypoxia-induced factor-1α, and B-actin.

Results: The current study shows higher gene expression levels for all target genes in all positive samples compared to negative samples. The expression level of TLR4 of the positive samples was more than one times that of the negative samples. The gene expression of the hypoxia-induced factor-1α in positive samples was approximately 36-fold greater than in control samples.

Conclusion: The current study indicates that there is a real increase in the level of gene expression for TLR4 and HIF-1α and that a higher level of expression of target genes in positive samples indicates a relationship with HPV infection compared to negative samples.

INTRODUCTION

Human papillomavirus is one of the most common viruses in the world. There are at least 14 genotypes that can cause cancer. This virus is mainly transmitted through sexual contact and infection begins soon after sexual activity begins. Types 16 and 18 are considered the most dangerous genetic types, as the incidence of cervical cancer in women reaches 70%.[1] HPV has circular dsDNA with an icosahedral symmetry. There are more than 100 genotypes, such as HPV18, HPV16, HPV31, HPV32, HPV52 and HPV58 can affect the genital canal. HPV can be divided into groups based on its ability to transform normal cells into cancerous, low and high-risk genotypes of HPV.[2] He found of about 40 strains of HPV that can cause genital infections.[3] HPV structure shown in Figure (1).

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TLR4 is the natural immune receptor that has the main role in a cellular signal transduction system and regulates of acute inflammatory response and apoptosis. TLR4 mediates the innate immune response in eliminating pathogens.[4,5] Hypoxia has been detected in many solid tumors and is a type of cellular stress that plays an important role in the development of transformed cells and their resistance to available therapies. Hypoxia-inducible factor 1α (HIF-1α), a transcription factor, is associated with uncontrolled cervical cancer cell growth.[6] HIF-1α is considered a catalyst factor for hypoxia, as this factor has a main role in the emergence of different types of malignancies.[7]

The current study aims to detect viral DNA in the cervix and genotype the high-risk genotypes of HPV, in addition to evaluating the expression of the TLR4 and HIF-1α levels *in-vivo*. The gene expression of the previous genes was associated with an alteration in HIF-1α in a microenvironment and all that lead to converting normal cells into cancerous cells was also investigated.

MATERIALS AND METHODS

Samples collection

A 48 samples were collected from Al-Fayhaa Teaching Hospital, Basrah Teaching Hospital, and Basrah Women and Children's Hospital in Iraq from September to December 2019. About 43 cervical tissue samples and 5 Pap smears were collected. Samples were classified as positive based on the presence of HPV DNA by conventional PCR.

DNA and RNA extraction

DNA was extracted from the samples to detect the HPV DNA by conventional PCR using a total DNA extraction kit according to ReliaPrep™ FFPE gDNA Miniprep System/ Promega and following the manufacturer's instructions. Also, RNA was extracted to determine the HPV16, 18, and 31 genotypes depend on E6 and E7 specific primers and gene expression evaluation of TLR4, HIF-1α, and β-actin of infected samples compared to the control samples using total RNA extraction kit according to ReliaPrep™ FFPE Total RNA Miniprep System/ Promega following the manufacturer's instructions.

Reverse transcription to synthesize complementary DNA

Extracted RNA samples were converted to complementary DNA (cDNA) using Fast HisenScript™ RH(-) RT PreMix Kit (CAT: 25087) from iNtRON Biotechnology. Template RNA was mixed with the reaction components and the total volume was 20 µL. The reaction was achieved under the conditions as follows: RT step for 45 minutes, at 45°C and RTase inactivation step at 85°C for 10 minutes.

Detection of HPV by PCR

HPV detection of 48 samples was performed by conventional PCR using the Maxime PCR PreMix kit (i-Taq for 20 mL rant) according to the instructions recommended by the manufacturer (iNtRON Biotechnology), and the PCR reaction mixture was as follows. GP5+(5'TTTGTTACTGTGGTAGATACTAC-3') and GP6+ (5'GAAAAAARAAACTGTAATCARATTC3) were used for molecular identification using conventional PCR. Primers amplify 150 bp of the conserved L1 region. The PCR condition was set by the following steps of: denaturation for 15 minutes at 95°C, then 42 cycles of denaturation step for 30 sec at 95°C, annealing step for 40 seconds at 63°C, and an extension step at 72°C for 50 seconds. Finally, the final extension step at 72°C for 5 minutes and storage at 10°C for 5 minutes. PCR products were visualized under a UV source on a 2% agarose gel.

Genotypes identification

Using qPCR, high-risk HPV genotypes were detected. According to the Go Taq R G2 Green Master Mix instructions, the total volume of the reaction includes 20 µL, which consisted of 10 µL of Master Mix (SYBR Green dye) with template cDNA (3 µL), 1-µL of each forward and reverse primers for HPV18 E7, HPV31 E6, and HPV16 E7, and Nuclease-free water (5 µL).

The conditions of Real-time PCR were set as follows: the initial denaturation step of the reaction is for 5 minutes at 95°C, then the denaturation step (15 seconds at 95°C), annealing step (60°C for 30 seconds), and extension step (30 seconds at 72°C) for 44 cycles. The last step of the reaction was a extension for 5 minutes at 72°C. The melting curve confirmed the specificity of the primers.

qPCR detection of target genes

According to the manufacturer's instructions, Go Taq R G2 Green Master Mix was prepared for real-time PCR. Mix Forward prepared a 20 µL total reaction volume and reverse primers (1-µL for each one), 10 µL of SYBR Green dye, 3 µL of cDNA template for detection of target genes (Toll Like receptor4, hypoxia-induced factor-1α, and house-keeping gene (β-actin), and 4 µL of nuclease-free water.

Each of the following genes TLR4, HIF-1α, was detected by real-time PCR under conditions as follows: the initial denaturation step of reaction at 95°C for 10 minutes. Then 44 cycles of 3 steps: the first denaturation step for 15 sec at 95°C, the second annealing step for 30 seconds at 60°C, and the last extension step at 72°C for 30 seconds. Finally, in addition to the final extension step at 72°C for 5 minutes and storage at 10°C for 5 minutes. The melting curve confirmed the specificity of the primers.

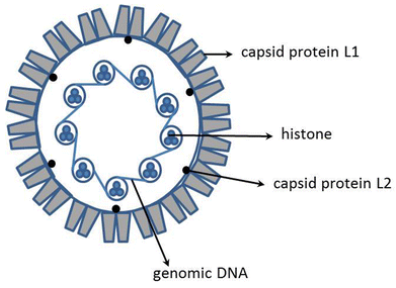

Figure 1: Structure of human papillomavirus

RESULTS

PCR detection

Molecular detection of HPV using GP5+/GP6+ primers which amplify 150 bp of the conservative L1 region and bands, were visualized under UV source in agarose electrophoresis. The results proved the presence of the virus in 43 samples out of 48 in a ratio of 89.6% (Figure 2).

Genotypes identification

By using specific primers to detect high-risk genotypes 18, 16 and 31 using qPCR, 28 (82%), 15 (44%) and 7 (20.5) samples were found positive for genotypes 16, 18 and 31, respectively.

Gene expression analyses

Expression of Toll Like Receptor 4

The gene expression of the TLR4 was detected by qPCR by mixing specialized primers with SYBR green dye and DNA templates, and the reaction conditions were optimized to obtain the best results and ideal amplification. It was found that samples infected with HPV had a high level of TLR4 gene expression of more than 2 ct compared to negative samples after subtraction of the housekeeping gene signal. The analysis of ΔΔ CTs was used after subtracting the housekeeping gene.

Expression of hypoxia induced factor-1α

The HIF-1α plays an important role in the occurrence of uncontrolled cell growth in cancerous tissues. To evaluate the level of gene expression for HIF-1α, reaction conditions were improved in the presence of a DNA template with specialized primers and SYPER green dye in quantitative PCR. It was found that the hypoxia-inducing factor 1α had high gene expression in samples infected with HPV, where the gene expression was 36 times higher than the gene expression level in the negative samples after subtracting them from the housekeeping gene signal.

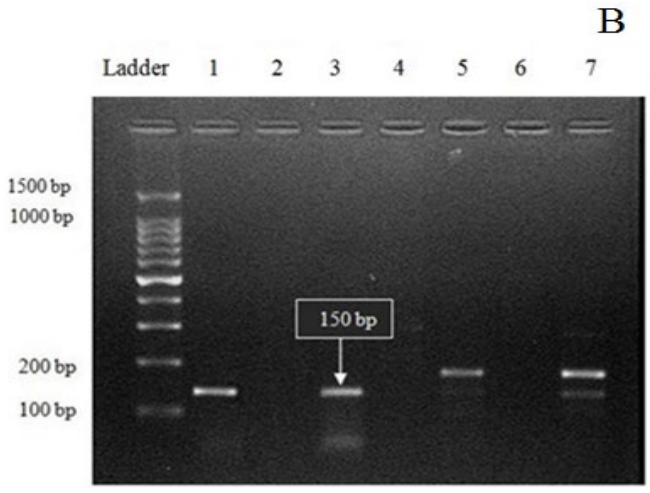

Figure 2: 2% agarose gel electrophoresis of PCR amplified products of HPV, lane 1,3,5,7 = positive samples (150 bp), lane 2, 4, 6 = negative samples, left lane = DNA ladder (100–1500 bp)

DISCUSSION

Using conventional PCR in the current study, a 43 samples out of the total 48 samples were infected with human papillomavirus, which represents about 89.6% of the samples. The result agrees with the Muñoz *et al*., (2003) since HPV DNA was detected in 90.7% of the total tested samples.[8] A slight higher percentage was detected by Bosch *et al*., (1995), which detected HPV DNA in 93% and this might due to the large number of the tested cohort.[9]

The samples were examined using qPCR to detect the number of samples for each genotype HPV18, HPV16, and HPV31, where it was found that 44.1, 82.3, and 20.5% were for genotypes 18, 16, and 31, respectively. These results demonstrate the high prevalence of high-risk genotypes among women with cervical cancer.

Through molecular examination of samples taken from women, it was found that there were multiple infections in the same patient, and it was found that 16 patients had a single infection, which represented 47%. Infection with more than one genotype was reported in 53%. These results were consistent with Nishiwaki *et al*., (2008). It has been found that there are multiple infections with up to 5 genotypes. However, It is common for women to be infected with more than one type of HPV.[10]

TLR4 plays a crucial role in the body's immunity against the virus's carcinogenic proteins in the cervical lining, and through the current study of samples infected with HPV, a high level of TLR4 was found.

Hypoxia microenvironments are present in the solid tumor tissue.[6] In the current study, it was found that samples infected with the virus had a high level of gene expression for the HIF-1α, and it was noted that there was a relationship between the level of gene expression for both TLR4 and HIF-1α. The results of this study were similar to what researcher Cheng *et al*. (2013) found in the cervix when infected with human papillomavirus, a close association was found between the elevation of HIF-1α with TLR4.

Based on the level of TLR4 and HIF-1α genes in the target cells, the expression of which increased in HPV- infected samples compared to the control samples, we propose a mechanism in the context of hypoxic environment results from HPV infection. When hypoxia is increased, the type I interferon mRNA is down-regulated at the signaling level.[11] The HIF-1α high expression might make the nuclear receptor 3 group B1 (NR3B1) involved in mediating the hypoxia condition and hence the NR3B1 prevents the phosphorylation of IRF-3.[12,13] So, despite the increased level of IRF-3 during HPV infection, its activity might be abolished at a later stage when its phosphorylation is blocked.

Although the TLR4 high expression increases the expression of IRF-3 to mount an antiviral response, which was the case in this study, the interferon response is low during HPV infection. This might be attributed to the fact that *E6* hydrophobic pocket interacts with IRF-3 to prevent the phosphorylation of Ser-patch, which is essential for the IRF-3 activation and interferon response.[14]

Taken together, despite the high expression of HIF-1α and TLR4 being accompanied by high expression of IRF-3, the activity of IRF-3 in upregulating the interferon response might be abolished phosphorylation stage. So, the interferon response might be induced through different signaling pathways, which were upregulated in this study and it works through TLR3 phosphorylation.

CONCLUSION

The present study indicates that there is a true increase in the gene expression level of TLR4 and HIF-1α and that the increased expression level of target genes in positive samples indicates an association with HPV infection compared to negative samples.

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AVAILABILITY OF DATA

The data are available for any further communication.

AUTHOR CONTRIBUTION

We have generated all the data by the team at the Genetic Engineering Laboratory in College of Sciences, University of Basrah, Basrah, Iraq.

ETHICAL APPROVAL

Since our paper based on using biopsy samples from human subjects, so we followed the ethical protocols to obtain the agreements from the general manager of the health office and the research unit in the same office to start the work.

COMPETING INTEREST

There are no known conflicts of interest associated with this publication

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