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Evaluation of P16INK4a Expression in Pre-malignant and Malignant Cervical Tissues

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Abstract

Background: Cervical cancer (CC) is the fourth most prevalent malignancy in women worldwide and a major cause of cancer-related mortality among females in developing countries. P16INK4a is a tumor suppressor protein with an inhibitory role in the proliferation of abnormal cells. The association between the expression of this protein and CC has established it as a diagnostic biomarker for CC.

Objectives: This study evaluated the expression level of P16INK4a in cervical squamous cell carcinoma (CSCC).

Methods: Eighty-three formalin-fixed paraffin-embedded (FFPE) cervical tissue samples were obtained from the pathology department of Kowsar Gynecology Reference Hospital in Qazvin Province, Iran. The expression level of the P16INK4a gene was investigated using relative quantitative real-time PCR (RT-qPCR).

Results: P16INK4a overexpression was observed in 55% (12/22) of cancerous samples, 46% (6/13) of intraepithelial squamous lesions, and 33% (8/24) of tissues with koilocytic changes, with N-fold overexpression of 6.01 (P = 0.001), 3.24 (P = 0.003), and 1.12 (P = 0.007), respectively.

Conclusions: The results of this study showed that the expression level of P16INK4a increased with cancer progression. Therefore, the expression level of P16INK4a can be utilized as a biomarker for diagnosis.

Keywords: P16INK4a, Cervical Cancer, Gene Expression

1. Background

Cervical cancer (CC) is the fourth most common cause of death in women worldwide, and primary screening for precancerous lesions significantly decreases mortality (1). The World Health Organization (WHO) estimated that 604,000 women were diagnosed with CC and 342,000 women died in 2020 (2). The correlation between CC and HPV infection is well-established (3). Approximately 90% of women with CC test positive for human papillomavirus (HPV) infection (4). The CC is caused by persistent HPV infection, particularly HPV-16/18 (5). The progression of CC from HPV infection to high-grade lesions and carcinoma occurs gradually over several years (6). Thus, it is essential to identify an effective, rapid, affordable, and minimally invasive diagnostic method for CC.

Squamous cell carcinoma (SCC) and adenocarcinoma (AC) are the two main histological types of CC (7). Based on global mortality data from 2018, SCC and AC accounted for nearly 80% and 20% of CC deaths, respectively (8). The CC primarily occurs in developing countries, with a high prevalence in Asia and the Sub-Saharan region of Africa due to limited screening for HPV and lack of vaccination programs (2, 9). In contrast, in developed countries, HPV vaccines are widely

available, resulting in a reduced incidence of neoplastic and dysplastic lesions (10, 11).

Currently, the most commonly used CC screening methods include the Pap test (Pap smear), HPV test, and colposcopy. However, the false-negative rate for Pap smears is relatively high. While colposcopy is straightforward, its accuracy is dependent on human factors (12, 13). Therefore, there is a clinical need for diagnostic techniques that are accurate, valid, sensitive, and specific.

Human papillomavirus is a causative factor for CC and mediates oncogenesis via E6 and E7 proteins, which interact with p53 and p16, tumor-suppressor genes and cell cycle regulatory proteins, respectively, resulting in malignant cervical transformation (14). Additionally, these viral oncoproteins E6 and E7 play essential roles in promoting and sustaining cervical carcinogenesis, and both are overexpressed during cervical transformation (15, 16).

P16INK4a is a cyclin-dependent kinase (CDK)2 inhibitor encoded by the CDKN2A gene (17). P16INK4a is overexpressed in HPV-positive CC (18) and in a subset of head and neck squamous cell cancers, playing a critical role in cell cycle regulation (19). Although immunohistochemical (IHC) analyses have shown strong associations between P16INK4a protein expression and CC (20), few studies have quantitatively evaluated P16INK4a mRNA expression.

2. Objectives

This study assessed the expression level of P16INK4a mRNA in cervical squamous cell carcinoma (CSCC) as a biomarker using relative quantitative real-time PCR (RT-qPCR) in Qazvin, Iran.

3. Methods

3.1. Tissue Samples

One hundred archived formalin-fixed paraffin-embedded (FFPE) cervical tissue samples were obtained from the Pathology Department of Kowsar Gynecology Reference Hospital in Qazvin, Iran, between June 2009 and November 2019. All hematoxylin and eosin (HE)-stained slide samples were assessed and confirmed by an expert pathologist, and the intended zone was punched.

According to cytological and histological diagnoses, all samples were divided into four groups: (1) Samples with normal reports (without neoplastic or dysplastic changes); (2) samples with cervicitis and koilocytic changes; (3) specimens with squamous intraepithelial lesions, including low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL); and (4) samples with cases of cancer (SCC and AC).

3.2. RNA Extraction and cDNA Synthesis

The FFPE samples were cut from each block for RNA extraction. RNA was extracted using a purification kit (Roche High Pure FFPE DNA isolation kit, Germany) according to the manufacturer's instructions. The quality and quantity of the extracted RNA were assessed using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The RNA extracted from each sample was transcribed into cDNA using the Bioneer AccuPower CycleScript RT Premix (dN6) (Bioneer, Korea) according to the manufacturer's instructions.

3.3. cDNA Quality Control

The quality of the synthesized cDNA was verified by running it on a 2% agarose gel. A SYBR green-based real-time PCR was performed, with β-actin (ACTB) used as an internal control.

Briefly, for each reaction in a 0.1 mL reaction tube, the mixture included 2 µL of cDNA template, 10 µL of 2X TAKARA SYBR Premix ExTaq II SYBR Green master mix (TAKARA BIO INC, Japan), 0.8 µL each of forward and reverse primers, 0.4 µL of ROX reference dye, and 6 µL of sterile dH2O, bringing the total volume to 20 µL per reaction.

The temperature protocol consisted of an initial denaturation at 95°C for 2 minutes, followed by 40 cycles at 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 30 seconds, without a final extension step. All experiments were conducted using the step one plus real-time system (Applied Biosystems, CA, USA). Only samples positive for ACTB were included in the study.

3.4. Quantitative Real-time PCR

SYBR green-based real-time PCR was conducted to analyze the expression of the P16INK4a gene relative to ACTB, used as the reference gene for normalization. The primers used for real-time PCR are listed in Table 1.

Table 1. Primers Used to Amplify Fragments of the P16INK4a and ACTB Genes

Primers	Sequence (5 - 3)	Ref.
P16INK4a		(21)
Forward	GGGGGCACCAGAGGCAGT	
Reverse	GGTTGTGGCGGGGGCAGTT	
ACTB		(22)
Forward	CTGGAACGGTGAAGGTGACA	
Reverse	AAGGGACTTCCTGTAACAATGCA	

For each reaction in a 0.1 mL reaction tube, the mixture included 2 µL of cDNA template, 10 µL of 2X TAKARA SYBR Premix ExTaq II SYBR Green master mix (TAKARA BIO INC, Japan), 0.8 µL each of forward and reverse primers, 0.4 µL of ROX reference dye, and 6 µL of sterile dH2O, making a total volume of 20 µL per reaction.

The temperature protocol began with an initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 30 seconds, without a final extension step. Melting curves were generated between 50°C and 95°C with a temperature increment of 0.2°C.

Each run included negative controls for both P16INK4a and ACTB. The PCR products were electrophoresed on a 2% agarose gel to verify correct amplification and were sequenced to confirm PCR specificity.

3.5. Statistical Analysis

After data collection, the findings were presented as statistical tables and numerical indices. Statistical analysis was performed using SPSS for Windows version 21.0 (IBM, US). The chi-square test or Fisher's exact test was applied for data analysis. A P-value of less than 0.05 was considered statistically significant.

4. Results

Out of 100 FFPE cervical tissue samples, real-time PCR for P16INK4a gene expression was performed on 83 samples (17 samples were excluded as they did not meet the inclusion criteria). Based on histological reports, the 83 included samples were categorized as follows: Normal samples without neoplastic or dysplastic changes (n = 24), pre-cancerous samples with koilocytic changes (n = 24), samples with high and low intra-epithelial neoplasia (n = 13), and samples with CC (n = 22). Positive P16INK4a expression with variable mRNA levels was observed across the groups, including the normal sample group 3/24 (12.5%), the pre-cancerous sample group 14/37 (38%), and the cancerous sample group 12/22 (55%) (Table 2).

Table 2. P16INK4a Positive and Negative Samples According to Pathology Group [a]

Group	Positive	Negative	P-Value [b]
Normal (n = 24)	3 (12.5)	21 (87.5)	-
Koilocytic changes (n = 24)	8 (33)	16 (67)	0.007
Squamous intraepithelial lesions (n = 13)	6 (46)	7 54 ()	0.003
Cancer (n = 22)	12 (55)	10 (45)	0.001

[a] Values are presented as No. (%).

[b] P < 0.05 was considered statistically significant.

The samples reported as positive for P16INK4a gene expression proceeded to the quantitative calculation stage to determine the relative expression level of this gene. The analysis compared the increase in P16INK4a gene expression relative to ACTB as a housekeeping gene in the premalignant and malignant groups against the normal samples as the control group. The final relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Table 3).

Table 3. Quantitative Up Expression Relative to Normal Group (N-fold)

Variables	N-fold Up Expression	P-Value
Koilocytic changes	1.12	0.007
Squamous intraepithelial lesions	3.42	0.003
Cancer	6.01	0.001

Regarding the pathology reports as the gold standard for diagnosis, the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the P16INK4a gene expression assessment in this study were evaluated as a laboratory test compared to the gold standard provided by the pathological group (Table 4).

Table 4. Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value and Accuracy of Our Test Compared to Pathology Reports [a]

Variables	Koilocytic	LSIL	HSIL	Cancer
Sensitivity	33.3	40	66.7	54.5
Specificity	87.5	87.5	87.5	87.5
Positive predictive value	72.7	57.1	40	80
Negative predictive value	56.8	77.8	95.5	67.7
Accuracy	60	73.5	85.2	71.7

Abbreviations: LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions.

[a] Values are presented as percentage.

5. Discussion

Cervical cancer, a largely preventable illness, is one of the most prevalent cancers in low- and middle-income countries affecting women (23). Nearly 85% of deaths worldwide due to CC occur in developing and underdeveloped countries, with the mortality rate being almost 20 times higher in low- and middle-income countries compared to first-world countries (24). Biomarkers have been emphasized in cancer prevention research due to their role in identifying neoplastic transformation processes in HPV-infected epithelial cells. Based on a deep molecular understanding, the cyclin-dependent kinase inhibitor P16INK4a is remarkably overexpressed in all HPV-transformed cells (25). Although the P16INK4a protein is deactivated in most cancers due to mutations, gene deletion, and hypermethylation, an increase in the expression level of P16INK4a occurs in CC (26).

In the current study, P16INK4a overexpression was observed in 3 out of 24 normal samples without neoplastic or dysplastic changes. While most studies report zero or negligible levels of P16INK4a in normal samples (27, 28), some studies, in agreement with our findings, have reported the expression of P16INK4a in normal samples (29, 30). It is noteworthy that the presence and increased expression level of P16INK4a can also be associated with aging and cellular stress; therefore, such changes in non-cancerous cases are not unexpected (31, 32).

In our study, the expression of P16INK4a was significantly increased in the pre-cancerous group, including tissues with koilocytic changes and intraepithelial squamous lesions, compared to the normal group (P-value = 0.007 and 0.003, respectively). A study conducted by Farzanehpour et al. similarly showed a remarkable increase in the expression level of the P16INK4a gene in the pre-cancerous group compared to normal groups (P-value = 0.0013) (33). Furthermore, the results of this study demonstrated a substantially higher expression of P16INK4a in cancerous samples compared to pre-cancerous samples (P-value = 0.001). This considerable increase in P16INK4a expression during the transformation from normal to cancerous tissues has been corroborated in several types of cancer (33-37).

Therefore, based on the analysis of normal, pre-cancerous, and CC samples, the high-level expression of P16INK4a can be regarded as an indicative marker for the early detection of CSCC.

5.1. Conclusions

Although the expression of the P16INK4a gene steadily increases as the disease progresses to cancer, with the highest expression observed in the cancer stage, the low sensitivity of this test makes it unsuitable for screening abnormal cervical conditions. Therefore, it appears that P16INK4a would be more appropriate as a confirmatory complementary test for follow-up treatment.

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Footnotes

Authors' Contribution: The authors contributed to the research conception and design. All authors studied this draft and contributed to and confirmed the final manuscript.

Conflict of Interests Statement: The authors declare that they have no conflicts of interest.

Data Availability: The dataset presented in the study is available on request from the corresponding author

during submission or after publication.

Ethical Approval: The research was approved by the Qazvin University of Medical Sciences Ethics in Research Committee (Ethics code: IR.QUMS.REC.1394.145).

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