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Evaluating the Importance of the Concurrent Methylation Pattern of the *DAPK1* Gene and the Quantification of Human Papillomavirus as a Diagnostic Biomarker in Cervical

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ABSTRACT

Background & Objective: Cervical cancer is the second most prevalent cancer in women worldwide and is one of the leading causes of deaths in women with cancer in developing countries. The most well-known cause of this cancer is being infected with human papillomavirus (HPV). This study aimed at evaluating the concurrent methylation pattern of the human papillomavirus genome in studying the *DAPK1* gene as a diagnostic biomarker in cervical cancer.

Materials & Methods: This study was approved by the Ethics Committee of the Islamic Azad University of Tabriz under the following ethical code, i.e., IR.IAU.TABRIZ.REC1398.001.This case-control study was conducted on 150 paraffin-embedded samples (75 marginal samples and 75 cervical cancer tissue samples) taken from women with cervical cancer. After extracting the total DNA, spectrophotometry and electrophoresis were performed on agarose gels to determine the quantity and quality of the extracted DNA. Changes in the *DAPK1* gene methylation pattern were examined using High Resolution Melt Analysis Curve Method.

Results: The changes in the*DAPK1* gene expression in the cervical tumor tissues increased compared to the marginal tissues, indicating a statistically significant difference (P<0/0001).

Conclusion: The results of the current study showed that the *DAPK1* gene can be introduced as a possible prognosis for cervical cancer diagnosis and treatment. However, more extensive studies with more samples are needed to be carried out.

Keywords: Cervical cancer, *DAPK1* gene, Human papillomavirus, Methylation

Introduction

Cervical cancer (CC) is the second most prevalent cancer among women worldwide. This cancer is continually occurring in poor countries, such as Central and South America, Black Africa, and some parts of Oceania and Asia with a prevalence rate of 30 cases per 100,000 women; however, the prevalence rate of this cancer in North America and Europe is reported to be 10 cases per 100,000 women **(1)**. Nearly 1.4 million women suffer from CC worldwide. Identifying and treating women with cervical intraepithelial neoplasia (CIN) or carcinoma in situ (CIS), a precursor lesion for invasive cervical cancer, are important factors in preventing CC. CC originates from the normal epithelium through clearly distinct morphological changes and turns into carcinoma or cancer through a series of well-defined pre-invasive lesions **(2)**. Histologically, CC appears either as squamous cell carcinoma (SCC) or adenocarcinoma (AC), among which SCC is regarded as the dominant type of cancer **(3)**. Approximately 275,000 women annually die from CC worldwide and the highest prevalence of the disease is in women aged 35 to 39 and 60 to 65 years old **(4,5)**. Dangerous HPVs have been identified as causes of CC. These viruses cause infection in the cervical epithelium. Persistent infection of the epithelium promotes precancerous changes, invades the base membrane eventually, and causes CC. HPV can be identified at least in 99.7% of squamous cells and cervical adenocarcinomas **(6)**. Human papillomavirus (HPV) infection is the main etiologic agent with more than 100 identified HPV variants that are divided into two groups. The first group consists of low-risk variants and the main agents are HPV 6 and HPV11. The second group is known as high-risk variants, including HPV16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 52, HPV 56, HPV 58, and HPV 59, which lead to CC **(4,5)**. Natural history studies have demonstrates that CIN of any degree is created by infection with genital HR-HPVs. Furthermore, the HR-HPV types, especially the HPV16 type, become quite dominant when the CIN level rises **(5)**. Laboratory studies have proven that genital HR-HPVs encode two susceptible oncogenes, namely E7 and E6, and disable cell cycle control by P5 and retinoblastoma (Rb) genes. Using optimized test systems and examining CC

samples extracted from several countries around the world, researchers found HPV DNA in 99.7% of CC cases **(7,8)**. The stability of the human papillomavirus (HPV) is the major etiological factor in the development and progression of CC and precursor lesions **(9)**. Other factors, including HPV type, environmental factors, and their levels, play important roles in the progression of CC infection **(10)**. Among the genes involved, the role of death-associated protein kinase 1 (*DAPK1*) gene in CC has been extensively studied. *DAPK1* is a novel 160-kD calmodulin-dependent serine/threonine kinase that acts as a positive mediator of apoptosis, while apoptosis is linked to the growth, progression, and metastasis of human cancer **(11)**. The serine-rich C-terminal tail of the *DAPK1*gene, preserved in proteins containing the death domain, plays a negative regulatory role in inhibiting *DAPK1*, whereas removal of this domain increases the lethal activity **(12)**. *DAPK1* hypermethylation has been repeatedly reported in a variety of cancers including colon, head and neck, bladder, lung, B-cell lymphoma, and ovary cancers. Since*DAPK1* is a positive mediator of apoptosis, it suppresses*DAPK1*-mediated apoptosis with *DAPK1* and may stimulate metastasis in cancer cells **(13)**. This study aimed at evaluating the importance of the concurrent methylation pattern of the *DAPK1* gene and the quantification of HPV as a diagnostic biomarker in cervical cancer.

Materials and Methods

This study was approved by the Ethics Committee of the Islamic Azad University of Tabriz under the following ethical code, i.e., IR.IAU.TABR-IZ.REC1398.001. This case-control study was performed on samples from 85 patients with cervical cancer (CC) (whose CC was confirmed by pathologic findings confirmed by pathology department of Tabriz Pediatric Hospital and Imam Khameini Hospital in Tehran) and their respective control group, sampled from the margins of their tumors. Patients who were candidates for chemotherapy between 2017 and 2019 were studied after signing written consent and filling out the questionnaire (the questionnaire contained questions about the patients' data, such as their age, gender, family history). In order to target patients, patients with pathologic outcome of cervical cancer were included in the study. Inclusion criteria were a definitive diagnosis of CC with positive pathologic results, mean age ranging from 35 to 53 years, being at one of the four stages of CC, and accurate grading of the disease by a pathologist. And patients with other related diseases such as uterus cancer were excluded. So a part of the tumor and the marginal tissue were prepared by the surgeon to study the methylation pattern of the HPV and the *DAPK1* gene to compare it with the tumor margin. In the control group, the same patients' samples of the marginal site of the tumor (healthy adjacent tissue) were studied. Extracting the samples' DNA was conducted using the salt saturation method as follows: 6cc of lysis buffer was added to the samples and closed with para-film. The tubes were repeatedly turned upside down and placed in a refrigerator for 10-20 minutes. Afterward, they were centrifuged for 15 minutes at 1800rpm. Then, 2cc of SE buffer and 200 µL of 10% SDS (Merck, Germany), and 10 µL proteinase K (Fermentas, France) were added to the resulting sediment, vortexed, and stored at 37°C overnight in a bain-marie. After complete dissolution of proteins, 900 µL of NaCl solution (Merck, Germany) and 1.5 mL of chloroform were added to the solution and centrifuged for 5 minutes at 3800rpm. The supernatant was removed and transferred to another test tube. Cold absolute ethanol was added and shaken gently until DNA strands appeared. They were then placed in a microfuge at 1200rpm for 15 seconds to precipitate the DNA. Ethanol 70 was added to the microtubes at the rate of 200–100 µL. The microtube containing the DNA precipitation was placed under the hood for 10 minutes to completely dry the DNA precipitation. Finally, the 0 precipitation of DNA was dissolved in 200 µL of TE buffer. After extracting the DNA, it was necessary to determine the quality and quantity of the extracted DNA. To evaluate the quantity of the extracted DNA, the NanoDrop spectro-photometer was used such that a certain volume of the extracted DNA was diluted with distilled water or TE buffer and reached a specific volume. After calibration, a specific volume of the diluted DNA sample was poured into the coat. OD=260 and OD=280, respectively, were read on the two devices. To evaluate the quality of the extracted DNA, the DNA samples of varying concentrations were electrophoresed on agarose gel. For this purpose, 1.5% gel was first made. The samples were loaded onto the gel after its closure and electrophoresed at 90°C for 30 minutes. The DNA content of each sample was calculated by comparing the fluorescent intensity of the sample with standard DNA bands. To investigate the methylation of DAPK1 gene, total DNA was isolated from paraffin block according to the instructions of DNA extraction kit (Merck, Germany).To investigate the DNA methylation of the *DAPK1* genome, the cervical tissue samples were used separately. The homogenized tissue was transferred to a 1.5 mL tube in 0.5 mL. 200 µL of Lysis Buffer HL was added, and incubated for 5 min at room temperature. Proteinase K was added in 2 µL and 20 µL of Carrier DNA, and mixed. The internal control was added from the binding buffer HL and mixed. The mixture was transferred to the RTA Spin Filter and incubated at room temperature for 1 minute. It was then centrifuged for 11 minutes at 11,000rpm. The mixture was again transferred to the RTA Spin Filter inside the new RTA receiver. Wash buffer I was added to RTA Spin Filter in the amount of 500 µL. It was centrifuged at 11,000 rpm for 1 minute. The mixture was then transferred to the new RTA Spin Filter. From the wash buffer II, 700 µL was added and centrifuged. The filter was removed and placed on a tube. The RTA Spin Filter was transferred into a new tube. It was centrifuged for 4 minutes to completely remove ethanol at maximum speed and it was transferred to a new filter for 15 minutes. The elution buffer, which was

previously heated to 56°C, was added to 100 µL in RTA Spin Filter and the mixture was incubated at room temperature for 1 minute. Initially, methylation changes were examined on the studied DNAs using the EZ-96 DNA Methylation-GoldTM kit (Zymo Research, Irvine, CA) and according to the manufacturer's instructions. Briefly, concentrations of 1.10, 1.20 and 1.50 µL of altered metabolized DNA were prepared. The concentration of 1.20 was used as a template for real-time PCR. According to the kit's instructions, amplification was performed in a volume of 20 µL, comprising 10 λ of the main solution (Mix Master), 0.4 λ of the forward primer, 0.4 λ of the reverse primer, 5 λ of absulphated DNA, and 4.2 λ of distilled water. After modification, an Eco device manufactured by Biosystems Eco was used to measure gene methylation. The primers for the DAPK1 gene were designed using primer 3 software, synthesized by Bioneer (Bioneer, Germany), and used at a final concentration of 100 nm. The characteristics of the primers used in this study are as follows. The amplification reaction was performed according to the following temperature pattern (Table 1).

Table 1. The characteristics of the primers used in the current study

Sequence (5'->3')	Length	Tm
Forward primer: GGCGAGGGCTTCATTCTTCC	20	61.90
Reverse primer: AAACTGTCACGCCTCACCAA	20	61.34
Product length 140		

The amplification reaction was performed for 40 cycles according to the following temperature pattern:

Hot start activation was performed at 95°C. Initial denaturation and holding were done at 95°C for 10 seconds and annealing was conducted at 57°C for 20 seconds. Elongation was carried out at 72°C for 25 seconds. The light was also absorbed at this temperature. After these steps, the melting step was measured at 55 to 95°C to separate the DNA bands affected by the metabisulfite in terms of the temperature differentiation with high-resolution melting (HRM). The reactions were based on the use of EvaGreen® dye (Fermentas, France). After performing the real-time PCR reactions, the products were electrophoresed on 1.5% agarose gel; then, they were stained with ethidium bromide (Merck, Germany) for DNA staining to visualize the fragments against ultraviolet on the gel. Finally, they were photographed with a Polaroid camera.

Examining the Quantification of HPV

Auxiliary standards included in the kit (HPV S 1-5) were performed by the same sample extraction method and with the same volume.

To produce a standard curve in RotorGene ™ 2000/3000/6000, all 5 standards were defined in the Rotor Gene software edit menu. Standards were defined as IU/µL. The following formula was used to convert the values determined using the standard curve to IU/mL of the samples.

$$\text{Result (IU/ml)} = \frac{\text{Result}\left(\frac{\text{IU}}{\mu\text{l}}\right) \times \text{Elution Volume (µl)}}{\text{Sample Volume (ml)}}$$

The sample size was determined 150 samples using the following formula, with P-value=0.1, 95% confidence interval, and tolerable error of 0.04. After completing the laboratory work, using the Hardy-Weinberg law, the expected and observed frequencies were calculated and entered into SPSS 16 (SPSS Inc. Chicago, Ill., USA). This software was used to find out the frequency of each methylation pattern studied and their frequencies were evaluated. Analysis of variance was used to compare the mean number of altered factors in the study population. The null hypothesis in carrying out the analysis of variance was the equality of the dependent variable's means at all levels of the independent variable. Since the significance level of the test was less than 0.05, the null hypothesis was rejected. In the analysis of variance, the sum of squares, degrees of freedom, mean squares, F statistics, and significance levels of variance were calculated.

Results

In the present study, 85 women with CC aged 35 to and 53 years old, with a mean age of 46.3 years, were studied. Electrophoresis of the samples was performed on 1.5% agarose gel to determine the accuracy of the extracted DNA. The presence of a specific and uniform band indicated the quality of the extracted DNA (Figure 1).

Real-Time PCR Results

Evaluating the methylation pattern of the *DAPK1* gene was performed without considering the variance involved by using the EvaGreen® method and comparing high-resolution melting.

The *DAPK1* gene melting curve was obtained as a single peak, indicating that there was only one PCR product.

In Figure 2, the first 7 cycles do not show a noticeable change in the number of amplified products; however, from cycle 8, the reaction enters an incremental phase, called the exponential phase. The higher the amount of the primary pattern for amplification, the lower the cycle the real-time or PCR reaction enters the exponential phase. The lower the threshold cycle (ct) that enters step 2, the greater the gene expression and vice versa.

After obtaining the *DAPK1* gene expression changes in all the studied cases, the patients were divided into two groups and the HPV viral load was studied using Genome Diagnostic Kit Catalog No. 9111004. The standard curve corresponding to the standards 1 to 8 inside the kit was drawn and reported according to the kit instructions (Figure 3).

After obtaining the standard curve and calculating R2 and reaction accuracy, the extracted viral samples were studied. The PCR amplification curve for different viral loads was as follows (Figure 4).

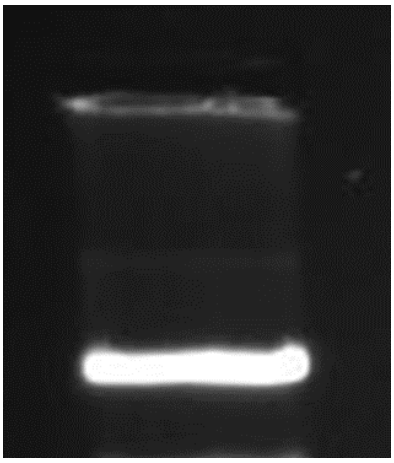

Figure 1. The distribution of means and standard deviations of the scores of fatigue and mental well-being in terms of fear and sever fear in the subjects.

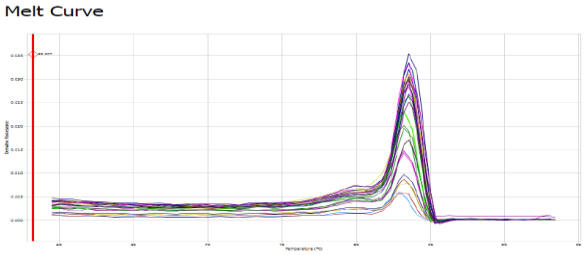

Figure 2. The correlation matrices of the subscales of the Persian version of the Wijma questionnaire with the overall fatigue index and mental well-being of the subjects

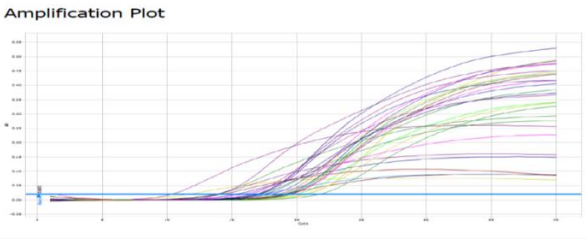

Figure 3. The PCR amplification curve for the *DAPK1* gene of several positive CC samples

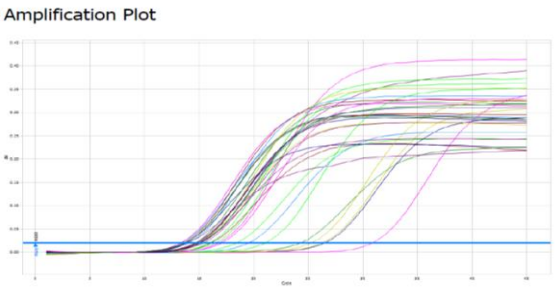

Figure 4. The PCR amplification curve for some positive samples with both HPV and CC

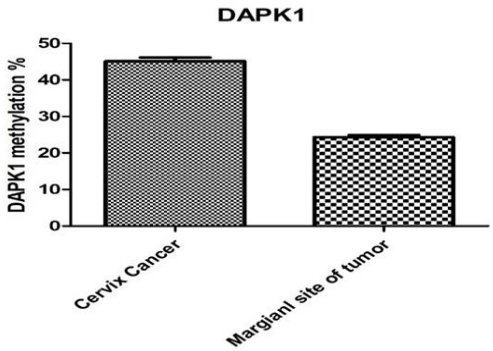

Figure 5. Changes in the methylation pattern based on methylation percentage in the patients with CC and their marginal site of the tumors

Figure 5 shows the difference in the *DAPK1* gene methylation pattern between the two groups, i.e., the marginal and CC groups. Changes in the *DAPK1* gene methylation pattern increased in the CC group.

There was a significant difference in the *DAPK1* gene expression (P<0/0001). Moreover, the means were significant. This is a two-way test. However, R=~ 1 indicates a more significant relationship in changing our gene methylation pattern.

Discussion

Cervical cancer (CC) is a malignancy in cervical tissues that can be seen in both epidermal and adenocarcinoma. CC is the third most common cancer of the female reproductive system. In developing countries, CC is more prevalent than breast cancer. CC is rarely seen in women who have never had sexual intercourse, and it is generally very dangerous and can

be life-threatening. In this type of cancer, cancer cells invade and damage adjacent tissues and organs **(14)**. In a study, Inbal *et al.* reported that methylation of the DAP-kinase promoter region is associated with all the features of the advanced stage and the tumor size, lymph node involvement, and DAP-kinase that link suppression of apoptosis to metastasis **(15)**. Mouse lung tumors with highly metastatic behavior did not express DAP-kinase unlike their low metastatic potential counterparts. The introduction of DAP-kinase into highly metastatic Lewis carcinogenic cell lines reduced their metastatic potential. Thus, experimental studies have provided evidence that loss of DAP-kinase expression may facilitate tumor metastasis, which is inconsistent with the findings of the present study. Death-associated protein kinase (DAP-kinase) contains a *death* domain and is a positive mediator between interferon-γ-induced programmed cell death and DAP-kinase **(16,17)**. It suppresses tumor growth by increasing apoptosis in the body, and DAP-kinase also increases the sensitivity of laboratory cells to apoptotic signals **(18)**, which is inconsistent with the findings of this study. Previous studies have demonstrated that hypermethylation at CpG sites of DAP-kinase can suppress gene expression. Accordingly, the DAP-kinase expression is most likely suppressed in tumor cells containing a hypermethylated promoter. In fact, methylation status of DAP-kinase is closely related to gene expression in lung cancer cell lines and demethylation repair of DAP-kinase gene expression **(15,19)**. In another study, Kissil *et al.* indicated that the association between hypermethylation of DAP-kinase and poor survival rate suggests the important role that DAP-kinase plays in tumor invasion and metastasis of lung cancer **(17)**. Cohen *et al.* found that DAP-kinase is also implicated in tumor necrosis factor-α and Fas-induced apoptosis. Besides, the apoptotic function of DAP-kinase can be blocked by Bcl-2 as well as caspase inhibitor P35; since Bcl-2 is often expressed in lung cancer **(16)**. In another study, Cohen *et al.* reported that the overexpression of DAP-kinase causes apoptosis death and morphological changes of apoptosis, such as cell-rounding and cell-shrinking in several cellular systems. In addition to apoptotic enhancer activities, several lines of evidence suggested that DAP-kinase plays a key role in tumor suppression. DAP-kinase expression is often disrupted in different tumor cell lines and tissues **(20)**. Overexpression of DAP-kinase in several cell lines leads to cell death, and this mortality feature strongly depends on the intrinsic kinase activity. In 1999, Cohen *et al.* stated that Bcl-2 prevents cell death induced by ΔCaM-DAPk mutation. A possibility is that DAP-kinase may be involved in one of these mitochondrial pathways **(20)**. Raveh *et al.* reported that DAP-kinase induces apoptosis through a p53-dependent mechanism **(21)**, which is inconsistent with the findings of the current study. In 2012, in a study entitled *Human Papillomavirus DNA Methylation as a Potential Biomarker for Cervical Cancer*, Clarke *et al.* mentioned that identifying methylated viral DNA may distinguish women with intraepithelial cervical neoplasia Grade 2 + (CIN2+) from women with HPV type carcinogenic infection who do not show signs of CIN2+ **(22)**. In 2016, Mersakova *et al.*, in their study entitled *DNA Methylation and Detection of Cervical Cancer and Precancerous Lesions Using Molecular Methods*, emphasized on the importance of the association of HR-HPV testing with methylation analysis in patients with intraepithelial cervical neoplasia. They found that being aware of the genetic and epigenetic changes associated with CC could be useful for screening, diagnosis, and employing new treatments for CC precursor lesions **(23)**.

In a study by Jiang et al., the researchers concluded that the odds of surviving those who expressed higher levels of the molecule were significantly higher in chemotherapy treatments than those who expressed lower levels of the protein **(24)**.

Gade and his colleagues concluded that the *DAPK1* molecule plays an important role in the autophagy signaling pathway and apoptosis through Factor 6 molecules and CCAAT-binding enhancers associated with protein B in chronic lymphatic leukemia **(25)**.

Conclusion

The results of this study showed that the *DAPK1* gene can be a potential prognosis for the diagnosis and treatment of CC. However, more extensive studies with more samples are needed to be carried out.

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Conflict of Interest

Authors declared no conflict of interests.

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