Furtado et al., J Clin Case Rep 2016, 6:4
DOI: 10.4172/2165-7920.1000781

Journal of Clinical Case Reports

Case Report Open Access

# DAPK and P16 Methylation and HPV and EBV Infection in Small Cell Carcinoma of Cervix: A Case Report

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## Abstract

**Purpose**: Small cell carcinoma (SMCC) of the cervix is rare and aggressive tumor, often diagnosed in an advanced stage and their prognosis is generally poor. A case of SMCC showing Human Papillomavirus (HPV) and Epstein barr virus (EBV) infection and Death-associated protein kinase (DAPK) and p16 genes methylation is presented. A 74-year-old woman was diagnosed as having stage IB2 of SMCC.

**Methods**: HPV and EBV infection and DAPK and p16 gene methylation were found using molecular approaches.

**Results:** The sample was positive for HPV and EBV infection and showed DAPK and p16 promoter regions methylated

**Conclusion**: Methylation of DAPK and p16 gene could be used as a molecular marker in cervical cancer.

**Keywords**: DAPK; p16; HPV; EBV; Carcinoma; Cervix

## Introduction

Small cell carcinoma (SMCC) is a rare and aggressive neoplasm of the uterine cervix, accounting for almost 1% to 3% of all uterine cervix cancers [1]. The most tumors are similar to squamous cell carcinoma (SCC) of the cervix in presentation, however they have a poor prognosis. SMCC tends to develop hematologic and limphatic metastasis early and it is associated with high mortality. Common sites of metastases include the lung, liver and bone [1-3]. In 1997, a workgroup sponsored by the National Cancer Institute and the College of American Pathologists recommended uniform terminology for these tumors with 4 categories: typical carcinoid tumor, atypical carcinoid tumor, large cell neuroendocrine carcinoma, and small cell carcinoma [1,2].

Most of what is known regarding the epidemiology of small cell carcinoma is derived from studies of lung cancer. Indeed, of the major lung cancer types, cigarette smoking has been most strongly associated with small cell lung carcinoma (SCLC) [2,3]. Risk factors for extrapulmonary small cell carcinoma are unknown [4].

In concordance with SCC of the uterine cervix, cervical SMCC are associated with human papillomavirus (HPV) in approximately two thirds of cases [3]. It is well accepted that the oncoproteins encoded by E6 and E7 of HPV have the ability to bind host cell regulatory proteins, thus causing consecutive functional alterations, including increased proliferative activity [3,5]. EBV has been suggested as another oncogenic virus in cervical carcinogenesis, based on findings on the clonal nature of EBV in cervical carcinoma cells and the presence of EBV in precancerous lesions of the cervix [6]. The methylation of promoter regions of genes related to cancer leads to the suppression of their expression, being a common epigenetic event in tumor evolution. DAPK is a positive apoptosis mediator that leads to apoptosis cells transformed by oncogenes and p16 is involved with cell cycle controlling cell proliferation [7].

Clinical and pathologic features of SMCC of the uterine cervix have not yet been fully elucidated due to its low incidence. Therefore, we report a case of small cell carcinoma of the uterine cervix focusing on HPV and EBV infection and methylation patterns of two genes usually methylated in cervical carcinoma.

## Case Report

A 74-year-old woman, that became pregnant 20 and gave birth 18, presented intense bleeding transvaginal initiated 3 months before. At the clinical examination the uterus showed increased volume, reduced mobility and tumoral cervical lesion. The cytology suggested cervical adenocarcinoma, however, histology revealed anaplastic SMCC of the cervix FIGO stage IB2. The tumor stained positively for neuron-specific enolase and chromogranin.

Initially, anti-hemorrhagic radiotherapy was carried out (600 cGy), followed by 6 chemotherapy cycles with etoposide 150 mg and carboplatinum 560 mg and concomitant radiotherapy (200 cGy).

One month after finishing chemotherapy, the patient showed lost of weight, anorexia and had abnormal vaginal bleeding, and requiring transfusion. The abdomen-pelvic tomography evidenced, at that time, an expansive heterogeneous cellular mass measuring 16.5 × 16.2 cm, which was not observed at the first tomography 5 months before treatment. The patient died 14 months after diagnosis.

## Molecular Findings

A biopsy fragment was use to DNA extraction for viral infection and methylation status analysis [6].

PCR for detection of HPV and EBV. The presence of HPV and EBV DNA was investigated in the DNA from the cervical samples using PCR with the primers and cycles described below. DNA-HPV detection was carried out using the MY09 and MY11 consensus primers [8], which produce a 450 bp product. The mixture was denatured at 95_C for 5 min, followed by 35 PCR amplification cycles. Each cycle consisted

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**Received** March 05, 2016; **Accepted** April 21, 2016; **Published** April 26, 2016

**Citation:** Furtado YL, Lattario F, Silveira FA, Carvalho MGC, Almeida G (2016) DAPK and P16 Methylation and HPV and EBV Infection in Small Cell Carcinoma of Cervix: A Case Report. J Clin Case Rep 6: 781. doi:10.4172/2165-7920.1000781

of heating at 95_C for 1 min, 60_C for 1 min, and 72_C for 1 min. The final elongation step was prolonged for 10 min to ensure complete extension of the amplified products. The detection for EBV DNA used primers TC67 and TC69. The former amplifies a portion of the Bam M region of EBV, which is reiterated ten times in the genomic DNA and produces a 288 bp product [9]. Forty PCR cycles were effected. Each cycle consisted of heating at 94_C for 30 s, 55_C for 10 s, and 72_C for 30s. The final elongation step was prolonged for 7 min. The HeLa and Raji cell lines were used as positive controls for HPV and EBV, respectively. Samples containing distilled water were used as negative controls.

Sequencing of EBV. DNA sequencing was carried out to confirm EBV infection in cervical cells. DNA was purified through a column (GFX PCR and Gel Band Purification Kit; Amersham Biosciences, Amersham, UK). After that, sequence reactions on PCR products were performed by using a BigDye terminator cycle sequencing readyreaction kit (Applied Biosystems) and an ABI Prism 3100 autosequencer (Applied Biosystems, Foster City, CA). The sequences of the three groups were aligned using the Clustalw 1.81 program [10].

Bisulfite treatment. Genomic DNA was treated with sodium bisulfite as described by Rosas et al. [11]. Briefly, lg of genomic DNA was resuspended in 50 lL of water and denatured in 0.2 M NaOH for 10 min at 37_C. The denatured DNA was then diluted in 550 mL of freshly prepared solution containing 10 mM hydroquinone (Sigma, St. Louis, MO) and 3 M of sodium bisulfite (Sigma) at pH 5.0 and incubated for 16 h at 50_C. After 16 h, the DNA sample was desalinated through a column (Wizard DNA Clean Up System; Promega, Madison, WI), treated with 0.3 M NaOH for 5 min at room temperature, and precipitated by ethanol. The bisulfite-modified genomic DNA was resuspended in 28 lL of water and used immediately or stored at 220_C.

## PCR amplification of bisulfite-treated DNA

The modified DNA was used as a template for PCR amplification using specific primers for either methylated or modified unmethylated DNA. Because the bisulfite treatment converts unmethylated cytosines to uracil but leaves methylation cytosines intact, specific primers were designed to accommodate these changes. The primers used for unmethylated reaction were 59-GGAGGATAGTTGGATTGAGTTA-ATGTT-39 (sense) and 59-CAAATCCCTCCCAAACACCAA-39 (antisense). The primers for methylated reaction were 59-GGATAGTC-GGATCGAGTTAACGTC-39 (sense) and 59-CCCTCCCAAACGC-CGA-39 (antisense). For PCR amplification, 2 lL of bisulfite-modified DNA were added to a final volume of 50 lL PCR mix containing 1 3 PCR buffer, deoxyribonucleotide triphosphates (each at 1.25 mM), primers (300 ng each per reaction), 3 mM $MgCl_2$, and 1.25 units of Taq polymerase. All PCR amplifications were performed for 40 cycles. Each cycle consisted of heating at 95_C for 1 min, 60_C for 1 min, and 72_C for 1 min. The final elongation step was prolonged for 10 min to ensure complete extension of the amplified products.

## Detection

The amplified PCR products were analyzed by 10% polyacrylamide gel electrophoresis in a 1 3 Trisborate-EDTA buffer (pH 8.0) at 80 V for 3 h and silver stained as described by Rosenbauer and Riesner [12]. Approximate fragment sizes were estimated by coelectrophoresis with the 100 Base Pair Ladder (Amersham Biosciences).

## Results

The sample was positive for HPV and EBV infection and showed DAPK and p16 promoter regions methylated.

## Discussion

Owing to the low incidence of SMCC, it is difficult to collect data on enough cases for analysis. Previous studies have not reached a consensus with regard to the prognostic factors of SMCC. Some studies have considered the International Federation of Gynecology and Obstetrics (FIGO) stage as an important prognostic factor [13]. Although HPV is considered the primary factor for cervical cancer development, it has benn suggested that other viruses such as EBV may be related to carcinogenesis [6].

Hypermethylation of the promoter region is one of the mechanisms by which genes related to cancer, such as DAPK and p16, are activated. This epigenetic alteration has been found to be present in most cervical intraepithelial neoplasias and in case of invasive cancer [14]. This study showed methylation in both DAPK and p16 gene, what may have contributed to the prognosis of the patient and lack response to chemotherapy treatment as already been described in some tumors with aberrant methylation status of some genes [15].

In conclusion, our case provides an example of DAPK and p16 genes methylation interrelationship within prognosis and chemotherapy treatment response. This finding is extremely important since these genes could be exploited as molecular markers for prognosis and treatment. This approach could be an interesting alternative tool for cancer patient characterization, but further studies remain to be done before it acquires routine clinical status.

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