TIMP-2 gene methylation in cervical precursor and invasive lesions

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A B S T R A C T

Objective: To analyze the presence of HPV-DNA and TIMP-2 gene methylation in cervical precursor and invasive lesions, as well as to study the associations among the latter, the presence of HPV-DNA, and the clinical evolution of such lesions.

Methods: Cross-sectional study that includes 49 biopsy or brush smear samples from women with a normal cervix, LSIL, HSIL, microinvasive carcinoma and invasive carcinoma. The presence of HPV-DNA and specific methylation was analyzed using PCR. Thirty-eight biopsy samples for HSIL, microinvasive carcinoma and frank invasive carcinoma as well as 11 brush smear samples for LSIL and normal cervix were analyzed.

Results: TIMP-2 gene methylation was detected in 86.8% (33/38) of the samples from the group with lesions and 50% (4/8) of the normal samples (p = 0.03). HPV-DNA was detected in 81.6% (31/38) of the samples from the group with lesions and 25% (2/8) of the normal samples (p = 0.003). HPV-DNA was more frequent in the methylated samples (50%), and the group with methylation had a higher risk of unfavorable evolution than the group without methylation; however, such observations were not statistically significant (p = 0.19).

Conclusion: TIMP-2 gene methylation and the presence of HPV-DNA were characteristic of the group with cervical lesions. Methylation was not associated with the presence of HPV-DNA or an unfavorable clinical evolution.

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1. Introduction

Cervical cancer is the second leading cause of death by cancer among women throughout the world (Ferlay et al., 2002). In Brazil, cervical cancer is the third most common tumor in the female population and the fourth leading cause of death in women by cancer (INCA — Instituto Nacional do Câncer, 2013). Diagnosing the tumor at advanced stages is one of the primary causes of high mortality and only allows for palliative radiotherapy and chemotherapy treatment with a great recurrence risk in a short period of time (Thuler, 2008).

Human papillomavirus (HPV) is necessary for the development of precursor and invasive lesions, as has been demonstrated through epidemiologic and molecular studies (Bosch and de Sanjose, 2003). A series of molecular genetic and epigenetic events transpire during the initial progression of HPV-induced lesions through cervical cancer development, which ultimately prevent the cell from generating an adequate immune response and support tissue invasion and metastasis (Stetler-Stevenson et al., 1991).

In mammals, methylation is the most common epigenetic modification and functions as a regulatory mechanism for gene integrity and expression (Furtado et al., 2010). Abnormal CpG island methylation in promoter regions for tumor suppressor genes is related to gene transcription inactivation in cancer and is a molecular biomarker (Strathdee and Brown, 2002). Methylation of genes that express proteolytic enzyme inhibitors, such as matrix metalloproteinases (MMPs), can be an important event in invasion (Ivanova et al., 2004).

Matrix metalloproteinases (MMPs) are zinc- and calcium-dependent lytic enzymes and are secreted as proenzymes (Davidson et al., 1999; Ivanova et al., 2004; Strathdee and Brown, 2002). One specific MMP group that belongs to the Matrixines family has been implicated in the proteolytic system of carcinogenesis because it participates in stroma, lymphatic and blood vessel invasion (Brummer et al., 2002; Ivanova et al., 2004).

MMP activity is controlled by tissue inhibitors of metalloproteinases (TIMPs), which are 22 kDa proteins that form a complex with the enzymes by binding at their active site (Benassi et al., 2001; Branca et al., 2006). Tumor invasion and metastases can be inhibited by TIMP...
overexpression in tumor cells (Branca et al., 2006). Thus, gene inactivation through methylation of the promoter region may support a loss of control over tissue proliferation, which facilitates invasion and metastasis (Benassi et al., 2001; Nuovo et al., 1995).

The objective for the study herein was to analyze the presence of HPV-DNA and TIMP-2 gene methylation in cervical precursor and invasive lesions as well as to study the associations among the latter, the presence of HPV-DNA and clinical evolution of such lesions.

### 2. Methods

#### 2.1. The population used for the study

The work described herein is a cross-sectional study evaluating TIMP-2 gene methylation and the presence of HPV-DNA in cervix samples from 49 women at the Cervical Pathology Outpatient Clinic and General Outpatient Clinic in the Institute of Gynecology for the Federal University of Rio de Janeiro (Universidade Federal do Rio de Janeiro — UFRJ) from July 2005 to July 2011.

The samples were collected from outpatient cervical biopsies generated using a surgical high-frequency ultrasound device (Wavetronic 5000 Loktal — Brazil) or through conventional conization at a surgical center. The negative controls were cervix fragments from hysterectomized specimens (derived from surgeries for benign uterine corpus pathologies) or cervical brush smears collected at the General Outpatient Clinic in the above institution. Samples from women with a low-grade squamous intraepithelial lesion (LSIL) diagnosed through cytology and colposcopy were also collected using a cervical brush smear from the Cervical Pathology Outpatient Clinic.

The population used for this study included seven women with LSIL, 13 with high-grade squamous intraepithelial lesions (HSIL), 13 with microinvasive carcinoma (FIGO IA1) (Pecorelli et al., 2009) and seven with frank invasive squamous carcinoma. The negative control group included nine women; four samples were collected from biopsies and five from brush smears.

Clinical evolution was “favorable” for women with no recurrence or cervical lesion persistency upon treatment, whereas the evolution was “unfavorable” for women with disease recurrence or persistency upon treatment. For the LSIL cases that only included a follow-up without treatment for two years, evolution was “unfavorable” when the woman exhibited lesion persistency or cytological progression.

A small fragment of the surgical specimen was removed for molecular analysis and frozen at $-20^\circ C$ in Eppendorf tubes for DNA extraction. Subsequently, the presence of HPV-DNA was analyzed through polymerase chain reaction (PCR), and the presence of TIMP-2 gene promoter region methylation was analyzed through methylation-specific PCR (MSP). The remaining surgical specimen was fixed in 10% buffered formalin, dehydrated, embedded in paraffin, cut into 5 μm sections and stained with Hematoxylin–Eosin for histological analyses at the Laboratory of Pathology in the Institute of Gynecology at the UFRJ.

The specimens collected through brush smears were also stored in Eppendorf tubes at $-20^\circ C$.

These methods were performed at the Laboratory of Control of Gene Expression in the Institute of Biophysics Carlos Chagas Filho for the UFRJ and the Laboratory of Virology at the Department of Microbiology and Parasitology for the Fluminense Federal University (Universidade Federal Fluminense — UFF).

The project was approved by the Research Ethics Committee at the UFRJ Maternity-School using the protocol number 25/2009 on December 11th, 2009.

#### 2.2. DNA extraction

The materials collected from the patients (biopsy fragments and cervical brush smears) were fully digested using proteinase K (10 mg/mL) for 16 h at 55 °C. Subsequently, the mixture was treated twice with phenol/chloroform (1:1) and precipitated with ethanol p. a. for 16 h at $-20^\circ C$. The samples were washed with 80% ethanol, resuspended in 20 μl water and frozen at $-20^\circ C$ until use. To confirm the extracted DNA integrity, PCR was performed using primers for p53 exon 5, which generated a 274-bp product, as described by Pestaner et al. (1994).

#### 2.3. HPV-DNA detection through PCR

The presence of HPV-DNA was assessed through PCR using the primers MY09/MY11, which amplify a 450-bp DNA fragment, and the primers GP5+/GP6+, which amplify a 140-bp DNA fragment. The reaction was performed in a thermocycler using the following protocol: 5 min denaturation at 95 °C, 35 cycles of 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and elongation at 72 °C for an additional 10 min. The HeLa cell line was used in the positive control reaction to detect HPV. In the negative control reaction, DNA was substituted with distilled water. The PCR results were visualized using 10% polyacrylamide gel electrophoresis, followed by silver nitrate staining. The approximate size of the amplified fragment was calculated using the molecular weight marker Base Pair Ladder (Pharmacia Biotech).

#### 2.4. Analyzing methylation at the TIMP-2 gene promoter region using MSP

TIMP-2 gene methylation was evaluated in two phases using a method from Herman et al. (1996) and Rosas et al. (2001). The first

### Table 1

<table>
<thead>
<tr>
<th>LSIL n(%)</th>
<th>HSIL n(%)</th>
<th>Microinvasive carcinoma n(%)</th>
<th>Invasive carcinoma n(%)</th>
<th>Control n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated</td>
<td>6(100)</td>
<td>11(91.6)</td>
<td>11(84.6)</td>
<td>5(71.4)</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>0</td>
<td>1(8.3)</td>
<td>2(15.3)</td>
<td>2(28.5)</td>
</tr>
<tr>
<td>Total</td>
<td>6(100)</td>
<td>12(100)</td>
<td>13(100)</td>
<td>7(100)</td>
</tr>
</tbody>
</table>

$p = 0.03$.}

![Fig. 1. Polyacrylamide gel electrophoresis showing PCR products of methylation. The numbers correspond to the identification of patients with cervical lesions, M: methylated, U: unmethylated, LMW: Base Pair Ladder and bp: base pairs.](image-url)
phage included DNA chemical modification through the sodium bisulfite method. This technique transforms unmethylated cytosine into uracil, but it does not alter methylated cytosine. Thus, approximately 5 μL of DNA was diluted with 50 μL distilled water and denatured in 0.2 M NaOH for 10 min at 37 °C. The denatured DNA was diluted using 550 μL of a solution containing 10 mM hydroquinone (Sigma, St. Louis, MO) and 3 M sodium bisulfite pH 5.0 (Sigma). The solution was covered with mineral oil and incubated for 16 h at 50 °C. Next, the solution was desalted using the Wizard DNA Clean-up System (Promega, Madison, WI), treated with 0.3 M NaOH for 5 min at room temperature and precipitated with ethanol. The modified DNA was re-suspended in 28 μL water and stored at −20 °C. In the second phase, PCR was performed using the modified DNA and specific primers for the methylated and unmethylated TIMP-2 genes in accordance with Galm et al. (2005). Specifically, 2−3 μL of bisulfite-modified DNA was added to a final volume of 50 μL in a PCR mixture with 1× PCR buffer, deoxyribonucleic triphosphate (1.25 mM each), primers (300 ng for each reaction), 3 Mm MgCl₂ and 1.25 units of Taq polymerase. The PCR amplifications were performed using 40 cycles. The amplification conditions were as described above. The PCR product was analyzed using polyacrylamide gel electrophoresis.

### 2.5. MSP primers

Specific sequence for unmethylated TIMP-2:

Forward 5′−GTA ATA AAA TTG TGG TGT TTA AGT TT-3′  
Reverse 5′−TTC TCT CTT CCT TAT CTC AAA AAC ACA-3′.

Specific sequence for methylated TIMP-2:

Forward 5′−AAT AAA ATT CCC GTG TGT CTC AAG TTC-3′  
Reverse 5′−CTT TCC TCT TTA TCT CGA AAA CCC G-3′.

These primers amplified a DNA fragment approximately 200-bp long.

### 2.6. Statistical analysis

A two-tailed Fisher’s exact test was performed to assess the association between methylation and the presence of HPV-DNA with clinical evolution for such lesions. The significance was 0.05.

### 3. Results

The average age of the population used for the study was 40.6 years (ranging between 23 and 67 years).

#### Table 2

<table>
<thead>
<tr>
<th>DNA-HPV</th>
<th>Normal cervix n(%)</th>
<th>LSIL n(%)</th>
<th>HSIL n(%)</th>
<th>Microinvasive carcinoma n(%)</th>
<th>Invasive carcinoma n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>2(25)</td>
<td>5(83.3)</td>
<td>9(75)</td>
<td>11(80)</td>
<td>6(85.7)</td>
</tr>
<tr>
<td>Negative</td>
<td>6(75)</td>
<td>1(16.6)</td>
<td>3(25)</td>
<td>2(15.3)</td>
<td>1(14.2)</td>
</tr>
<tr>
<td>Total</td>
<td>8(100)</td>
<td>6(100)</td>
<td>12(100)</td>
<td>13(100)</td>
<td>7(100)</td>
</tr>
</tbody>
</table>

*p = 0.003.

3.1. Detecting TIMP-2 gene methylation

Six percent of cases (3/49; one case of LSIL, one of HSIL and one from the control group) showed no gene amplification. Methylation was detected in 80.4% (37/46) of the cases analyzed. Where the TIMP-2 gene was amplified, 86.8% (33/38) of the samples from the women with cervical lesions and 50% (4/8) of the samples from women with a normal cervix exhibited TIMP-2 methylation. A statistically significant difference (p = 0.03) was observed for the proportion of methylation in the group with cervical lesions and the control group (Table 1, Fig. 1).

3.2. HPV-DNA detection

HPV-DNA was detected in 71.7% (33/46) of the samples analyzed. Specifically, 81.6% (31/38) of the samples from women with cervical lesions were positive for HPV-DNA compared with only 25% (2/8) in the samples from the control group. The difference between these groups was statistically significant (p = 0.003) (Table 2, Fig. 2).

3.3. Association between the presence of HPV-DNA and TIMP-2 gene methylation in the group with cyto/histological lesions

The association between the presence of HPV-DNA and TIMP-2 gene methylation in the group of women with cervical lesions was not statistically significant (p = 1.00) (Table 3).

3.4. Association between TIMP-2 gene methylation and clinical evolution of the disease

The group with TIMP-2 gene methylation had a 2.3-fold (CI 95% 0.23−22.82) higher risk of unfavorable evolution (recurrence, persistency or progression) than the group without methylation. Where TIMP-2 gene methylation was detected, 36% (12/33) of the women exhibited unfavorable evolution, whereas this percentage was only 20% (1/5) without methylation. Although methylation and unfavorable clinical evolution were associated, the association was not statistically significant (p = 0.19) (Table 4).

4. Discussion

Detecting methylated genes implicated in tumor suppression in cervix specimens (both biopsy fragments and exfoliated cells) (Furtado et al., 2010; Ivanova et al., 2004) is technically viable and a significant source for potential biomarkers in cervical carcinogenesis. Further, evidence shows that tumor suppressor gene methylation in women infected with HPV may indicate a precursor lesion with a higher risk of histological progression (Duenas-Gonzalez et al., 2005; Esteller, 2000; Lattario et al., 2008a).

Only two studies have focused on TIMP-2 gene methylation in the cervix. In one such study, Ivanova et al. (2004) reported TIMP-2 gene methylation in 47% of cervical cancer cases and concluded that this epigenetic alteration might be a significant biomarker for progression of this cancer and it can be used to evaluate a prognosis during the initial phase of tumor development. Consistent with such studies, TIMP-2 gene methylation was detected more frequently in LSIL, HSIL and microinvasive carcinoma herein. Methylation was also more frequent for the group with unfavorable clinical evolution. Although this analysis was not statistically significant (p = 0.19), we can infer that TIMP-2 gene methylation may be a prognostic biomarker for unfavorable lesion.
evolution independent of the evolution and treatment phase. We have not found published studies demonstrating this type of association to date.

The other referenced study was by Parashar and Capalash (2012), which reported no clear relationship between TIMP-2 gene methylation and proteinase expression in cervical cancer cell lines. Notably, results from studies using cancer cell lines are inconsistent with studies performed using fresh material removed from a biopsy or cervical brush smear. The authors hypothesized that only one promoter region was methylated for this gene or such methylation did not occur in the promoter region proximal to the transcription site, which might promote partial gene inactivation. We did not assess proteinase expression because our sole objective was to study TIMP-2 gene methylation as a biomarker. However, under the hypothesis wherein methylation inactivates only certain regions in the TIMP-2 gene, protein expression may be reduced, which would impair tumor suppression by TIMP-2.

Interestingly, methylation was also detected in samples from a normal cervix herein. Methylation in the negative controls has been previously described for tumor suppressor genes (Ivanova et al., 2004). Lattario et al. (2008b) reported DAPK methylation in cervix samples and concluded that it might be associated with transformation in cervical carcinogenesis. Two HPV-DNA-positive control samples were used herein. Although the cellular morphology was not altered, TIMP-2 gene methylation was detected in these samples. Thus, this may be a predictive factor for cervical epithelial lesion progression, which justifies the suggestion by certain authors to group such characteristics and to include a methylation screen in standard cervical cancer detection methods (Sun et al., 2012).

In a study using cell culture, Cardeal et al. (2012) concluded that combined expression of the HPV 16 oncoproteins E6 and E7 reduces TIMP-2 gene expression, which promotes increased expression of MMPs inhibited by TIMP-2. Szmálás and Kónya (2009) related HPV oncoprotein E7 expression to host-DNA methylation, and Burgers et al. (2007) demonstrated that increased DNA methyltransferase 1 (DNMT1) expression was related to E7 expression both in vitro and in vivo. Herein, HPV-DNA was more frequently detected in women with cervical lesions than without such lesions. These observations were statistically significant ($p = 0.003$), whereas the association between methylation and the presence of HPV-DNA was not statistically significant ($p = 1.00$).

Sun et al. (2012) proposed using liquid–based cytology to detect tumor suppressor gene methylation for early HSIL diagnosis. The authors detected more frequent methylation associated with altered cytologies, whereas methylation was a rare molecular event in normal cytologies, which is consistent with the results herein.

In a systematic review of the literature on gene methylation in cervical precursor and invasive lesions, Wentzensen et al. (2009) found 51 published studies. The most frequently cited genes were the following: death-associated protein kinase (DAPK), cell adhesion molecules (CADM), retinoic acid receptor, beta (RARβ), Ras association domain-containing protein 1 (RASSF1), cyclin-dependent kinase inhibitor 2A (p16INK4A), glutathione S-transferase pi (GSTP) and telomerase reverse transcriptase (TERT). The authors suggest that a panel of two to three methylated genes could yield sensitivity at 74% and specificity at 95% in a cervical cancer screening, which demonstrates the significance of methylation as a biomarker.

The applied screening methods do not detect lesions with histological evolution towards cancer nor women with a recurrence of precursor or invasive disease up to 20 years after treatment. These questions must be answered, which is reflected in the published studies that have attempted to determine the most suitable biomarker for cervical cancer.

DNA methylation is a promising molecular marker, and a panel of methylated tumor suppressor in combination with such screening methods may improve sensitivity. Using methylation as a screen for precursor lesion histological classification to determine a treatment and monitor treatment responses may slowly become reality (Harada et al., 2013).

In conclusion, the work herein shows that TIMP-2 gene methylation, which has been poorly studied until now, was characteristic for women with cervical lesions and the presence of HPV-DNA. A statistically significant relationship was not detected for TIMP-2 gene methylation and the presence of HPV–DNA; however, this combination was more frequently observed with an unfavorable evolution. Further studies are necessary to support the use of TIMP-2 methylation as a cervical lesion biomarker.

### Conflict of interest statement

The authors declare that there are no conflicts of interest.

### Acknowledgments

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National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq).

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### Table 3

Association between the presence of HPV DNA and TIMP-2 gene methylation in the cases of cyto/histologic alterations.

<table>
<thead>
<tr>
<th>DNA-HPV/</th>
<th>Methylated n(%)</th>
<th>Unmethylated n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>27(81.8)</td>
<td>4(80.0)</td>
</tr>
<tr>
<td>Negative</td>
<td>6(18.2)</td>
<td>1(20.0)</td>
</tr>
<tr>
<td>Total</td>
<td>33(100.0)</td>
<td>5(100.0)</td>
</tr>
</tbody>
</table>

$p = 1.00$.

### Table 4

Association between TIMP-2 gene methylation and unfavorable clinical evolution.

<table>
<thead>
<tr>
<th></th>
<th>Methylated n(%)</th>
<th>Unmethylated n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfavorable evolution</td>
<td>12(36.3)</td>
<td>1(20.0)</td>
</tr>
<tr>
<td>Favorable evolution</td>
<td>21(66.6)</td>
<td>4(80.0)</td>
</tr>
<tr>
<td>Total</td>
<td>33(100)</td>
<td>5(100)</td>
</tr>
</tbody>
</table>

$p = 0.19$. 

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**References**


Branca, M., Ciotti, M., Giorgi, C., Santini, D., Bonito, D.L., Costa, S., et al., 2006. Matrix metalloproteinase-2 (MMP-2) and its tissue inhibitor (TIMP-2) are prognostic factors in cervical cancer, related to invasive disease but not to high-risk human papillomavirus (HPV) or virus or virus persistence after treatment of CIN. Anticancer Res. 26, 1543–1556.


