# Expression profile of microRNA-29b and its action on matrix metalloproteinase 2 (MMP-2) in the cervical carcinogenic process

Perfil de expressão do microRNA-29b e sua ação sobre a metaloproteinase de matriz 2 (MMP-2) no processo da carcinogênese cervical

Perfil de expresión del microARN-29b y su acción sobre la metaloproteinasa 2 de la matriz (MMP-2) em el proceso carcinogénico cervical

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**Abstract**
Background: Cervical cancer is the fourth most common cancer among women worldwide. In order to make the diagnosis earlier and more accurate, increase the prospect of using microRNA-29b, which targets matrix metalloproteinase-2 (MMP-2), proteolytic enzymes that play a significant role in the degradation of the important extracellular matrix in progression of cervical carcinogenic lesions. Objectives: The aim of this study was to evaluate the levels of expression of microRNA-29b at all histological levels of carcinogenesis and its relationship with MMP-2 in this type of cancer. Methods: RNA was extracted from eighteen paraffin-embedded biopsy samples diagnosed with carcinoma and 19 considerable healthy samples, after which a real-time PCR technician was performed for expression analysis. Results: Our results show underexpression of microRNA-29b and MMP-2. This decrease in expression was statistically significant and differentiated in the histopathological subtypes. In addition, it has been shown that MMP-2 is directly regulated by microRNA-29b. No other studies were found that performed the same analysis on cervical cancers. Conclusion: Our results indicate a potential diagnosis of microRNA-29b and MMP-2 for lesions and cervical cancer, and show a direct association between these molecules.
**Keywords:** Cervical cancer; microRNA-29b; Biomarker; Matrix metalloproteinase 2.

**Resumo**

Introdução: O câncer cervical é o quarto tipo de câncer mais comum entre as mulheres em todo o mundo. A fim de tornar o diagnóstico mais precoce e preciso, aumenta a perspectiva de utilização de microRNA-29b, que tem como alvo a metaloproteinase-2 da matriz (MMP-2), enzimas proteolíticas que desempenham significativo papel na degradação da matriz extracelular e consequente progressão das lesões cancerígenas cervicais. Objetivos: O objetivo deste estudo foi avaliar os níveis de expressão do microRNA-29b em todos os níveis histológicos da carcinogênese cervical e sua relação com a MMP-2 nesse tipo de câncer. Métodos: o RNA foi extraído de dezoito amostras de biópsia embebidas em parafina com diagnóstico de carcinoma e 19 amostras consideradas saudáveis, foi realizada a técnica de PCR em tempo real para análise de expressão do microRNA-29b. Resultados: Nossos resultados mostram subexpressão de microRNA-29b e MMP-2. Essa diminuição da expressão foi estatisticamente significativa e diferenciada nos subtipos histopatológicos. Além disso, foi demonstrado que a MMP-2 é regulada diretamente pelo microRNA-29b. Não foram encontrados outros estudos que realizassem a mesma análise em cânceres cervicais. Conclusão: Nossos resultados indicam um potencial diagnóstico de microRNA-29b e MMP-2 para lesões e câncer cervical, e mostram uma associação direta entre essas moléculas.

**Palavras-chave:** Câncer cervical; microRNA-29b; Biomarcador; Metaloproteinase de matriz 2.

**Resumen**

Antecedentes: El cáncer de cuello uterino es el cuarto cáncer más común entre las mujeres en todo el mundo. Para hacer el diagnóstico más temprano y más preciso, aumente la posibilidad de utilizar microARN-29b, que se dirige a la metaloproteinasa-2 de la matriz (MMP-2), enzimas proteolíticas que desempeñan un papel importante en la degradación de la matriz extracelular en la progresión de lesiones cancerígenas cervicales. Objetivos: El objetivo de este estudio fue evaluar los niveles de expresión del microARN-29b en todos los niveles histológicos de carcinogénesis y su relación con MMP-2 en este tipo de cáncer. Métodos: Se extrajo ARN de dieciocho muestras de biopsia incluidas en parafina diagnosticadas con carcinoma y 19 muestras sanas considerables, tras lo cual se realizó un técnico de PCR en tiempo real para el análisis de expresión. Resultados: Nuestros resultados muestran una subexpresión de microARN-29b y MMP-2. Esta disminución de la expresión fue estadísticamente significativa y diferenciada en los subtipos histopatológicos. Además, se ha demostrado que MMP-2 está regulada directamente por microARN-29b. No se encontraron otros estudios que realizaran el mismo análisis sobre los cánceres de cuello uterino. Conclusión: Nuestros resultados indican un diagnóstico potencial de microARN-29b y MMP-2 para lesiones y cáncer de cuello uterino, y muestran una asociación directa entre estas moléculas.

**Palabras clave:** Cáncer de cuello uterino; microARN-29b; Biomarcador; Metaloproteinasa de matriz 2.

## 1. Introduction

Cervical cancer is the fourth most common cancer among women worldwide, with an estimated 569,847 new cases and 311,365 deaths in 2018, and with a projection of 776,857 cases for the year 2040 (Globocan, 2019). The carcinogenic process of the uterine cervix occurs due to the infection and persistence of the Papillomavirus human (HPV), which by the action of its oncoproteins deregulate the host cell cycle. The transformed cell gradually occupies the epithelial tissue until it ruptures the basement membrane, and cancer then arises (Zur Hausen, 1999).

Screening for pre-cancerous cervical lesions makes this cancer one of the types with the highest cure rates (World Health Organization, 2014), however, despite progress in screening and diagnosing women with cervical disease, by Pap smear, accompanied by colposcopy and biopsy, a large number of false-negative and false-positive results are observed, thus evidencing the need to identify clinically useful biomarkers to increase the accuracy of the diagnosis and provide information regarding the risk of progression to a more severe injury (Dehn et al. 2007, Hwang & Shroyer 2012, Barut et al. 2015, Laengsri et al. 2018).

About this respect, the prospect of using microRNAs (miRNA) as these biomarker molecules, which are single-stranded RNA molecules composed of 19–25 nucleotides, which do not encode proteins and which act as potent post-transcriptional regulators of gene expression, such as oncogenes or tumor suppressors (Calin et al. 2004, Acunzo et al. 2015).

Among them, microRNA-29b, related to the regulation of cell cycle control molecules such as p53, MDM2 and Mcl-1, and proteins that maintain tissue structure, such as matrix metalloproteinases (MMPs), are shown to express themselves if unregulated in most human cancers (Yan et al. 2015a), with decreased expression in osteosarcoma (Zhang et al, 2014), lung cancer (Wang et al. 2015), breast (Shinden et al. 2015), prostate (Yan et al. 2015b), liver carcinoma (Yan et al. 2015c), head

and neck carcinoma (Chen et al. 2016), endometrium (Wang et al. 2018), and leukemias (Ngankeu et al. 2018). The report of increased expression of this microRNA was described only in cases of luminal breast cancer (Chou et al. 2013), nasopharyngeal carcinoma (Qiu et al. 2015) and acute myeloid leukemia (Mundy-Bosse et al. 2016).

The aim of this study is analyze the expression profile of microRNA-29b, as well as its action in the process of cervical carcinogenesis, through the evaluation of one of its molecular targets, matrix 2 metalloproteinase (MMP-2).

## 2. Methodology

### 2.1 Patients and casuistry

The study was carried out using the observational, cross-sectional analytical method with comparison of groups. This study was approved by the Research Ethics Committee of the Integrated Health Center Amaury de Medeiros, University of Pernambuco, fully obeying the ethical principles established in resolution 466/12 of the National Health Council of Brazil (CNS), under CAAE: 64662116.1. 0000.5192.

Ninety-three paraffinized biopsies of the uterine cervix were selected, 18 samples of cervical intraepithelial neoplasia grade 1 (CIN 1), 20 samples CIN 2, 18 samples CIN 3 and 18 of cancer, following the diagnostic criteria described by Solomon et al. 2002. We used as control samples 19 biopsies considered healthy, that is, without cytological and architectural characteristics compatible with inflammation or pre-neoplastic lesions and cancer.

12 to 15 cuts, 4 µm thick, were made of all paraffinized biopsies, which were conditioned in microtubes and stored in a freezer -80ºC until the moment of RNA extraction.

### 2.2 RNA extraction

The RNA extraction was performed using the protocol adapted from the method established by Piotr & Nicoletta 1987, Kobler et al. 2003 and Ma 2012.

To start the extraction process, the biopsies were deparaffinized using 1 mL of xylol per tube, followed by agitation and water bath at 55ºC for 20 minutes, the tube was centrifuged at 9500 rpm for 5 min and the supernatant was discarded. The dewaxing process was repeated. To wash the pellet, 1 mL of 100% ethanol was added, vortexed and centrifuged at 9500 rpm for 5 min, the washing process was repeated. After centrifugation, the supernatant was discarded and the microtube remained open for total ethanol evaporation.

To the dry pellet, 400 µL of lysis solution (proteinase K 10 U / mL, proteinase K 5x solution, 20% SDS and Milli-Q water) were added and incubated for 3 hours at 55ºC in a water bath. After incubation, 1 mL of TRIzol® was added per tube, vortexed and centrifuged at 12,000 g for 10 minutes at 4º C. The supernatant was transferred to a new tube and incubated at room temperature for 5 minutes. 400 µL of chloroform was added, vortexing briefly, incubating at room temperature for 3 minutes and centrifuged at 12000 g for 15 minutes at 4º C.

The upper phase was transferred to an Eppendorf® RNAse free microtube to which 500 µL of isopropanol was added, gently homogenized, incubated at room temperature for 10 minutes and centrifuged at 12000 g for 10 minutes at 4º C. The supernatant was carefully discarded and pellet was added 1 mL of 75% ethanol, vortexed and followed by washing the pellet for 10 minutes under constant agitation. The tubes were centrifuged at 7500 g for 5 minutes at 4º C and the supernatant was discarded. The tubes were placed in speed vacuum concentrator, at room temperature. To the dry pellet, 25 µL of Milli-Q water was added and the RNA was stored at -80ºC.

The RNA quantification was performed in a spectrophotometer (NanoDrop® ND-1000) at wavelengths from 260 to 280 nm. RNAs with an absorbance ratio of 260/280 nm with values between 1.7 and 2.0 were considered of good quality.

**2.3 Real-Time Polymerase Chain Reaction**

Initially, the construction of the cDNA of the samples was performed using the SuperScriptTM II Reverse Transcriptase kit (Invitrogen) and specific primers for the RNAs of interest.

For the RT-qPCR, the SYBR Green PCR Kit (Qiagen) was used following the preparation: 10 µL of SYBR Green, 0.5 µL of the forward primer (10 mM), 0.5 µL of the reverse primer (10 mM), 8 µL of Milli-Q water and 1 µL of the cDNA (~20 ng / dL), with a final reaction of 20 µL.

The amplification conditions were: 95° C for 15 min, followed by 40 cycles at 94° C for 15 seconds, 55° C for 30 seconds and 70° C for 30 seconds, with final extension at 72° C for 1 min.

At the end of the reactions, the Ct values of each sample for the microRNA-29b were obtained. The Ct data were normalized by the level of expression of the small U6 nuclear RNA. To obtain the relative quantification, the $2\text{-}\Delta\Delta CT$ analysis method was used Livak & Schmittgen 2001 and Schmittgen & Livak 2008.

**2.4 Immunohistochemistry**

Immunohistochemistry was performed using the method described by Hosoya et al. 2008, with some modifications, using silanized slides containing 4 µm thick sections of representative portions of the lesions and paraffin tumor. Antigenic recovery was performed by immersing the slides in a container with pH 6.0 citrate buffer solution, placed in a pressure cooker for 2 min.

The slides were cooled to room temperature and immersed in a 3% hydrogen peroxide ($H_2O_2$) and methanol (1:1) solution, followed by the application of bovine serum albumin (BSA) diluted in 1% PBS for 1h at room temperature for 30 min to block endogenous peroxidase. This was followed by incubation with the primary anti-MMP2 monoclonal antibody (sc-53630; dilution 1: 400) Santa Cruz Biotechnology (Santa Cruz, CA, USA) for 2h in a humid chamber at 37° C.

Subsequently, incubations were performed with secondary (ADVANCETM HRP Link; Dako®, Glostrup, Denmark) and tertiary (ADVANCETM HRP Enzyme; Dako®, Glostrup, Denmark) antibodies for 20 min each, in a humid chamber at room temperature. 20 µl of the diaminobenzidine chromogen diluted in 1 ml of the buffer substrate (Dako®, Glostrup, Denmark) were used and were stained with Mayer's hematoxylin for 30 sec.

The slides were analyzed and photographed using the Zeiss camera microscope and the AxioVision program. Based on the article by Brummer et al. 2002, the cases were analyzed semiquantitatively through the intensity of reaction in more than 100 epithelial and mesenchymal cells in the control group and cervical lesions, being classified as: weak (reactivity between 0 and 10% of the cells epithelial) and strong (reactivity in more than 10% of epithelial cells).

**2.5 Statistical analysis**

Statistical analysis was performed using the analysis of variance test (ANOVA), and the Mann-Whitney non-parametric test to assess the correlation of expression between groups in the analysis of microRNA-29b. Williams' G-test was used to analyze MMP-2 expression between groups. And to evaluate the relationship of expression between microRNA-29b and MMP-2, a linear regression was performed. Graphpad Prism 5 and BioEstat 5.3 were used for this analysis. P values were considered significant when less than 0.05 ($p < 0.05$), in addition to $r^2$ between 0 and 1 in the regression analysis.
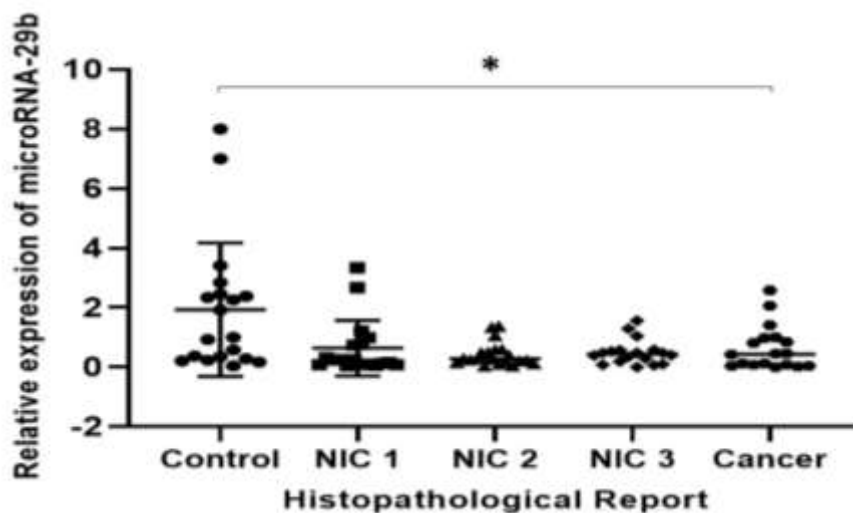
## 3. Results

### 3.1 Expression profile of microRNA-29b

The mean of the microRNA-29b expression profile, evaluated in qPCR, showed a decrease between the control samples and those with lesions and cancer. Graph 1 shows the gradual decrease in the mean expression of microRNA-29b in the control group for NIC 1 and NIC 2, followed by an increase for NIC 3 and cancer.

The evaluation of the difference in expression of microRNA-29b was made by analyzing all groups at the same time using the ANOVA statistical test and two by two using the Mann-Whitney test. The first test showed that there is a significant difference in expression of the microRNA-29b ($p = 0.0006$) (Figure 1) and the second showed statistical significance between the control x NIC 1 groups ($p = 0.0157$), control x NIC 2 groups ($p = 0.0097$), control x NIC 3 ($p = 0.0374$) and control x cancer ($p = 0.0255$).

**Figure 1 -** The relative expression of microRNA-29b in the different sample groups. * $p = 0.006$ ($> 0.05$).



NIC: Cervical Intraepithelial Neoplasm. Source: Authors.

No other statistical associations were found when comparing the remaining groups.

### 3.2 Expression profile of MMP-2

Of the 93 cases analyzed, immunohistochemistry for MMP-2 was performed on 42 samples. The samples in the NIC group showed lack of expression when compared to the control group, and a progressive increase in the expression of samples in the groups NIC 2, NIC 3 and cancer.

The immunohistochemical staining analysis was performed by evaluating the weak and strong expression groups. Of the total control samples, 80% were considered marked; for the NIC 1 group, no strong marking was found (0%), after a progressive increase in the marking of NIC 2 (8.3%), NIC 3 (30.8%) and cancer (37.5%) (Table I).

**Table 1 -** Evaluation of immunohistochemistry expression for MMP-2, according to histological reports.

| Histology | MPP-2 | | | | | | P value |
| | + | | ++ | | Total | | |
| | n | % | n | % | n | % | |
|---|---|---|---|---|---|---|---|
| Control | 1 | 20,0 | 4 | 80,0 | 5 | 100,0 | |
| NIC 1 | 4 | 100,0 | 0 | 0,0 | 4 | 100,0 | p$^{(1)}$ = 0,0438* |
| NIC 2 | 11 | 91,7 | 1 | 8,3 | 12 | 100,0 | |
| NIC 3 | 9 | 69,2 | 4 | 30,8 | 13 | 100,0 | |
| Cancer | 5 | 62,5 | 3 | 37,5 | 8 | 100,0 | |
| **Total group** | **30** | **71,4** | **12** | **28,6** | **42** | **100,0** | |

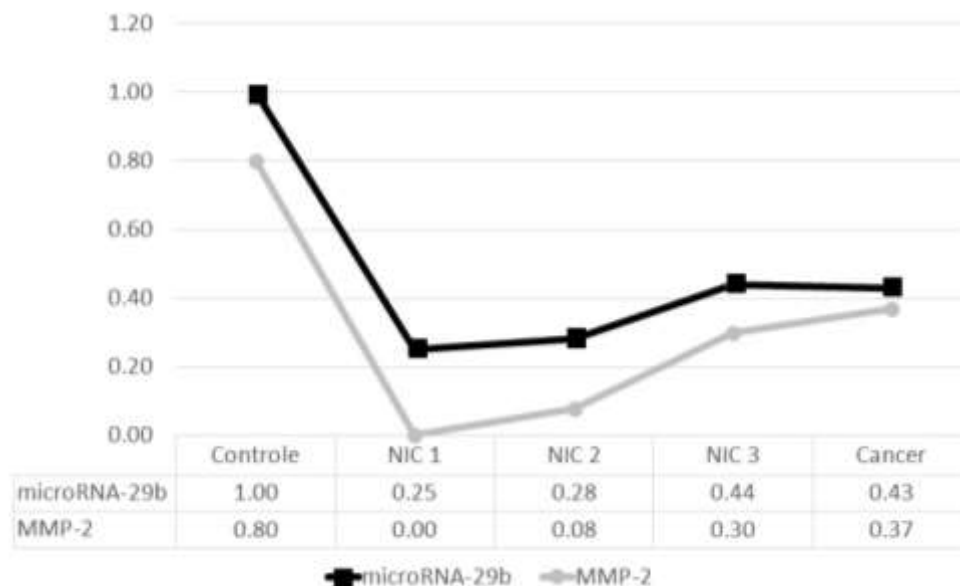 (+) low expression, (++) high expression. (*) Significant association at the level of 5.0%
(1) Williams G-Test.
Source: Authors.

### 3.3 Analysis of the relationship between microRNA-29b expression and MMP-2

The analysis of the relationship between the expression of microRNA-29b and MMP-2 was equivalent, showing the highest expression in the control group with a decrease in cases of CIN 1 and a progressive increase in expression in CIN 2, CIN 3 and cancer, however remaining less expressed than the control group (Figure 2).
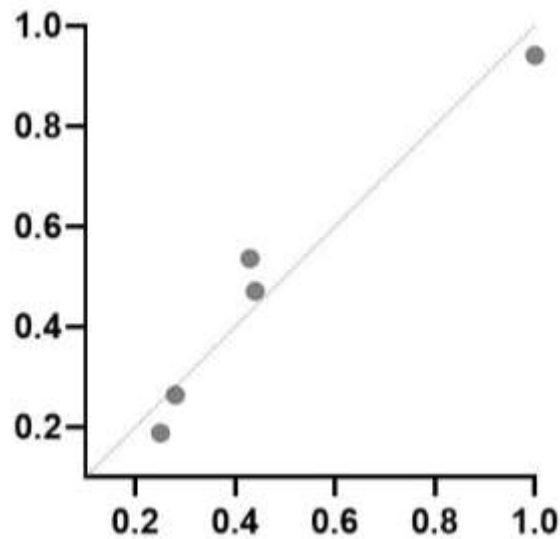
**Figure 2 -** Relationship of microRNA-29b x MMP-2 expression.



| | Controle | NIC 1 | NIC 2 | NIC 3 | Cancer |
|---|---|---|---|---|---|
| microRNA-29b | 1.00 | 0.25 | 0.28 | 0.44 | 0.43 |
| MMP-2 | 0.80 | 0.00 | 0.08 | 0.30 | 0.37 |

NIC: Cervical Intraepithelial Neoplasm. Source: Authors.

The statistical evaluation of this relationship was performed using the linear regression test, showing statistical significance with a p = 0.0054 and r^2 = 0.9461 (Figure 3).

**Figure 3 -** Linear regression for microRNA-29b X MMP-2. p = 0.0054.



Source: Authors.

## 4. Discussion

The cervical carcinogenic process begins after HPV infection and replication within the cervical cells, characterizing the lowest level of the carcinogenic process, NIC 1. The persistence of this virus will lead to the integration of its DNA into the host cell genome and consequent expression of its oncoproteins, which act by modifying metabolic routes that control the cell cycle, establishing a major degree lesion, NIC 2, which progresses temporarily to an NIC 3 lesion and later to cancer (Zur Hausen 2002, Basu et al. 2018).

In our study, is was seen that the lesion that had the lowest level of microRNA-29b expression was the one with the lowest degree (NIC 1), we suggest that this fact may be related to the presence of HPV in these cells, since with progression of lesion for NIC 2, where there is a decrease in the number of viral particles, there is a small increase in the expression of this microRNA, which continues to increase more mildly until cancer. However, the HPV-infected cell has its cell cycle control routes modified, which can contribute to the microRNA-29b expression remaining on average three times lower than the expression in the control tissues. This decrease in miRNA-29b expression was statistically significant and differentiated in the histopathological subtypes of cervical carcinogen.

The decrease in microRNA-29b expression has also been reported in other types of tumors (Sur et al. 2019, He et al. 2020, Fu et al. 2020), however, only the study by Li et al. 2017, had the expression of this microRNA in cervical samples was evaluated. Despite not considering all histopathological levels of cervical carcinogenic progression, they observed that the expression of microRNA-29b was suppressed when compared to normal tissues, corroborating our results.

We also evaluated the functional significance of microRNA-29b, for this, we consulted the microRNA databases (TargetScan, miRanda and miRTarBase) that indicated MMP-2 as a potential target for the action of this microRNA. MMP-2 is a molecule involved in the carcinogenic progress of the uterine cervix, where it acts by degrading extracellular matrix and basal lamina, contributing to the establishment of cancer and even metastasis (Libra et al. 2009, da Silva Cardeal et al. 2012).

Our results show that the level of expression of MMP-2 accompanied the level of expression of microRNA-29b, since in relation to the control sample there was a decrease in the expression of the protein in the initial lesion, NIC 1, and progressively increased in the lesions of greater degree until cancer, but not being expressed in the same quantity of the control samples, which was also found in the studies by Branca et al. 2006 and Vasconcelos 2015. The relationship between these

expressions was statistically significant, and showed that 94% of the MMP-2 level is explained by the action of microRNA-29b, therefore there is a strong relationship between them.

The increased expression of MMP-2 justifies the progression of the lesions to the onset of cancer, since the greater the activity of the MMPs, the greater the degradation of the basal lamina (Chang & Werb 2001, Björklund & Koivunen 2005). However, it is worth remembering that MMP-2 activity is controlled by its inhibitor, TIMP-2. Therefore, for the lesions to progress, a proportional imbalance in the production of these proteins would be necessary (Chakraborti et al. 2003).

In our sample, the control group samples showed the highest levels of expression for MMP-2, which, due to their action, should be found in lesions with a worse prognosis. However, this fact can be explained, since in tissues free of injury or cancer, there is a balance between the expression of MMPs and their inhibitors, thus there is no degradation of the extracellular matrix and maintaining the preservation of tissue structure. However, as a microRNA can act by regulating several target genes (Chen et al. 2015), we speculate that microRNA-29b may play a role in the proportional imbalance between these two proteins, so that it can suppress even more the expression of TIMP-2, without the increase occurring if its expression in higher levels of injury as seen for MMP-2.

In our research, a direct relationship between the expression of microRNA-29b and MMP-2 was observed. No studies were found to assess this relationship in the same tissue type. However, differently from what we found, studies carried out on liver samples (Fang et al. 2011), colon (Poudyal et al. 2013), lung (Wang et al. 2015) and prostate (Ivanovic et al. 2018), showed a relationship of inverse expression between these molecules. However, studies were carried out on tissues that do not have the presence of an oncogenic virus, which in themselves interfere with cell metabolism. We therefore suggest that HPV, in addition to modifying cell cycle control, inhibiting proteins such as pRb and p53 (Zur Hausen 2002), can act on other metabolic pathways and modulate the expression of microRNA-29b, from MMP- 2 and TIMP-2.

## 5. Conclusion

In conclusion, our data show that microRNA-29b is a promising molecule for understanding the cervical carcinogenic process, in addition to what has been described so far. In addition, it has an important potential to establish itself as a biomarker for the diagnosis of cervical lesions and cancer. However, we suggest that further studies be carried out in order to understand a possible relationship between microRNA-29b and matrix metalloproteinases and their inhibitors in this carcinogenic process.

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