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Expression of the Oncoprotein E5 from *Human papillomavirus* and miR-203 in Pre-Cancer Lesions and Cervical Cancer

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Abstract

High-risk *Human papillomavirus* (HPV) plays a key role in cervical cancer development due to its oncoprotein activities. The most frequent genotype in cervical lesions around the world is HPV-16, but other types are also founded, and the presence of multi-infection is associated with a higher risk of cervical cancer. E5 viral oncoprotein has a large range of tumorigenic attributes, including the modulation of microRNAs expression and previous *in vitro* studies have found an inverse relationship between E5 and microRNA-203, although no direct correlation was reported. Therefore, this study aimed to evaluate the profile of HPV infection and the possible correlation between E5 and microRNA-203 expression. Eighty-one fresh biopsies classified as normal tissue, cervical intraepithelial neoplasia grade (I, II, and III), and cancer were analyzed by qPCR. 83.95% of the samples were positive for HPV infection, and HPV-16 was the most prevalent, followed by HPV-31, HPV-58, HPV-18, and HPV-33. 29.41% of the samples were positive for more than one type (HPV-16 and HPV-31; HPV-16 and HPV58; HPV-31 and HPV-58; HPV-33 and HPV-58; HPV-18 and HPV-31; HPV-58 and HPV-18; HPV-16 and HPV-31 and HPV-18). We observed an increased expression of E5 in high-grade stages and cancer specimens, while microRNA-203 showed an opposite expression pattern from E5 mRNA, displaying reduced expression levels in cervical intraepithelial neoplasia III and cancer. These results help us to understand the HPV infection better, and even with no correlation, E5 may still alter miR-203 indirectly.

Keywords: *Human papillomavirus*; Cervical cancer; Gene expression; E5 oncogene; microRNA

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Introduction

HPV is closely related to the development of cervical cancer, which is one of the major cause of death by cancer, and 87% of cases occurs in developing countries [1,2]. More than 90% of them are associated with infection by high-risk HPVs [3,4]. In Brazil, as in most parts of the world, HPV-16 is the most prevalent [5], but other types were also found in cervical pre-cancer lesions and cancer, like HPV-31, HPV-33, HPV-35, HPV-45, HPV-52, HPV-18 and HPV-58 [6,7]. Besides the isolated type present in the lesion, the HPV multi-infection by two or more genotypes is an important data that must be taken to account for both epidemiologic studies and prevention programs. The risk of cervical carcinoma development is higher in a type of specific infection and type-specific multi-infection [8].

In order to promote cellular disorder and generation of malignant cells, the HPV genome encodes oncoproteins that can act separately or together when their effects become potentiated [9]. Such oncoproteins offer a wide range of interactions with regulatory proteins of the cell cycle, proliferation, differentiation, immune system, and cell metabolism. All these systems modified cooperate to cervical cancer generation at the last stage [10].

Among the oncoproteins, E5 was considered absent in cervical carcinoma due to its loss during viral integration into the host genome [10,11]. This integration is an established necessary step for persistent infection and because of that, E5 would only exert its potential in the progression of cervical lesions at early stages of infection [12-14]. Contradictorily, some studies showed that E5 was present in part of the studied samples, even after viral integration, in precancerous and cancerous

lesions [12,13].

As a consequence of viral oncoproteins activities, it was observed aberrant profile levels of some microRNAs (miR) in cervical neoplasia [1,15]. MicroRNAs are regulatory RNAs with a large scale of effects upon de expression of many genes at a post-transcriptional level. Some miR acts as tumor suppressors, like miR-203, and they act in a large number of human neoplasia through direct inhibition of gene products as ΔNp63, AKT2, Src, RUNX2 and ABL1 [16,17]. In normal cervical conditions, the expression levels of miR-203 increase from the basal to the upper layer, presenting higher expression in the most differentiated cells [18,19].

Greco et al. [14] evaluated the expression of several miR in HaCaT cells, and found altered expression of some miR in cells expressing E5. They observed that miR-203 was down-regulated when E5 oncoprotein was present. Few studies have included miR-203 and E5 expression at all stages of carcinogenesis (CIN I, CIN II, CIN III, and cancer), and none of them, until our knowledge, have evaluated the correlation between them in clinical samples with multi-infection complementary analysis.

Materials and Methods

Patients and samples

Eighty-one cervical biopsies were collected from the Institute of Medicine - Professor Fernando Figueira (IMIP) and Clinical Hospital of UFPE (HC-UFPE), after patients signed consenting terms. All the women were between 18 and 70 years old and were from the north-east of Brazil.

All grades of cervical lesions specimens were obtained: Cervical intraepithelial neoplasia I - CIN I (n=19), cervical intraepithelial neoplasia II - CIN II (n=20), cervical intraepithelial neoplasia III - CIN III (n=19) and cancer (n=14). A control group without lesion or HPV infection (n=9) was also obtained. RNA later solution (Qiagen) was used for the preservation of fresh biopsies and stored at -80ºC until the extraction procedure. Women with Human Immunodeficiency Virus (HIV) and/or pregnant were excluded from this study.

All procedures performed were in accordance with the ethical standards of the institutional Research Ethics Committee of the Federal University of Pernambuco, Brazil, (Number: 03606212.7.0000.5208) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

DNA extraction and HPV detection

Extraction and purification of DNA were made by Trizol (Invitrogen) and DNeasy Blood & Tissue Kits (Qiagen), respectively. The integrity of the DNA was assured by the amplification of the β-globin human gene using the PC04 and GH20 primers [20].

Detection of HPV was made by PCR with MY09/11 primers [21], and the genotype was performed by PCR with specific primers for the region E7 from 5 specific types previously described as present in the region [6,20] (Table 1). After an initial hold at 95°C for 3 min, 30 amplification cycles were performed (95°C for 15 s and annealing for 60 s), followed by final elongation step at 72°C for 1 min. Amplicons were visualized by UV light after electrophoresis on a 2.5% agarose gel stained with ethidium bromide.

RNA extraction and cDNA synthesis

Total RNA extraction was performed. All biopsies (25 mg to 100 mg) were macerated and homogenized using liquid nitrogen and 1 ml de Trizol (Invitrogen). Purification of isolated total RNA was performed through miRNA Absolutely RNA Kit (Agilent Technologies) following manufacturer's instructions, which permits the recovery of both miRNA and mRNA. The RNA's quality was assured by a NanoDrop 2000 Spectrophotometer (Thermo Scientific Wilmington, USA) and electrophoresis on a 1% agarose gel [22-24]. Next, 1 µg of purified RNA of adequate quality (an OD260/280 from 1.8 to 2.1 and intact rRNA subunits - 28S and 18S) was used to synthesize cDNA by means of miScript II RT kit (Qiagen). For each sample, a negative control RT reaction (no Reverse Transcriptase enzyme) was prepared.

Primers: Design and efficiency estimation to qPCR

Primers for genotyping and E5 detection were designed through CLCbio Main Workbench software version 5.7.1 (QIAGEN). For E5 detection and quantification the primers sequences were: E5 HPV-16 (F: A C T G G C T G C T T T T T G C T T T G; R: G A C A C A G A C A A A A G C A G C G G); E5 HPV-18 (F: C G C T T T T G C C A T C T G T C T G T; R: A C A C A A A T A C C A A T A C C C A T G C) E5 HPV-31 (F: G C T G T C T G T G T C G G T A T A T; R: A A A A C A A C G T A A T G G A G A G G); E5 HPV-33 (F: C T A T G C T T G G T T G C T G G T G T; R: G A G A T C C C A C A A A C A C C C A A A); E5 HPV-58 (F: G G G T C G G C T C T A C G A A T T T T; R: C T T G T T G G G T T A A G T A T T G T G C). MicroRNAs primers were obtained from miScript primer assay (Qiagen). All reference genes used to acquire miR-203 expression levels (miR-191 and miR-23a) and E5 HPV 16 (GAPDH and ACTB) were previously validated in cervical tissues [23]. Primer pair's efficiency was evaluated by serial dilution of 10 potencies, and it was used an actual cDNA of an HPV positive cervical sample to exemplify the real assay condition.

Real-time qPCR for E5 mRNA and miR-203

E5 mRNA and miR-203 from normal and all stages of carcinogenesis (CIN I, CIN II, CIN III, and cancer) were quantified using Quanti Tect SYBR Green PCR kit (Qiagen)and the amplification performed by Rotor-Gene 6000 thermocycler (Qiagen, Hilden, Germany). This way, the geometric mean of GAPDH and ACTB reference genes was used to calculate the relative expression of E5 from HPVs mRNA, and the same was done regarding miR-203, using miR-191 and miR-23a as reference genes [25]. Every qPCR reaction was performed in duplicate for each sample [26]. Additionally, no template controls were added to detect contamination. For more details about qPCR assay, see Leitao et al. [23].

Statistical analysis

Statistical analysis was performed using Graph Pad Prism (version 5.0) and Stata/SE (Version 12.0) software. Shapiro-Wilk test was made to determine if the data has or has not a Gaussian distribution. Kruskal-Wallis test and Dunn's comparison test were conducted to compare the expression levels in all tissue conditions at the same time. Correlation between E5 mRNA and miR-203 expression was evaluated by the Spearman correlation test. P-values lower than 0.05 were considered statistically significant.

Results

Detection, genotype and multi-infection analysis

Sixty-eight samples from patients with cervical lesions were positive for one or more than one type of HPV, and thirteen samples with lesion were negative for HPV infection (2 CIN I, 6 CIN II, 2 CIN III and 3 Cancer). The HPV 16 was the most prevalent, followed by HPV-31, HPV-58, HPV-18, and HPV-33 (Table 2).

Table 1: Primers and annealing temperature for HPV genotype.

Primers	Sequence	Amplicon	Annealing temperature
HPV 16	F:AGCTCAGAGGAGGAGGATGA R:GAGAACAGATGGGGCACAAC	199 pb	60°C
HPV 18	F:CAACACGGCGACCCTACAA R:AGCATGGGGTATACTGTCTCT	170 pb	52.5°C
HPV 31	F:CGTTTTCGGTTACAGTTTTACAAGC R:AGCTGGACTGTCTATGACAT	728 pb	55°C
HPV 33	F:ACTGAGGAAAAACCACGAAC R:GATAAGAACCGCAAACACAGT	200 pb	61°C
HPV 58	F:GAAATAGGCTTGGACGGGC R:GTTCGTACGTCGGTTGTTGT	131 pb	60°C

Table 2: Positive samples for each HPV type most frequent in north east of Brazil.

HPV	Lesion stage	16	31	58	18	33
Number of samples	CIN I	9	9	3		1
	CIN II	7	7	3	2	1
	CIN III	9	7	7		1
	Cancer	10	4		1	

A total of 20 samples were positive for more than one HPV type, and the most common multi-infection Types was HPV-16 and HPV-31 followed by HPV-16 and HPV-58; HPV-31 and HPV-58; HPV-33 and HPV-58; HPV-18 and HPV-31; HPV-58 and HPV-18; HPV-16 and HPV-31 and HPV-18 (Table 3).

mRNA expression profile of E5 oncogene

E5 HPV mRNA expression profile was evaluated comparing all lesion groups simultaneously and also comparing two groups by turn. From the most incident to the least, E5 HPV-16 was detected in 29 samples from the 35 that were positive for HPV-16 (9 CIN I, 3 CIN II, 10 CIN III, and 7 cancer). There was a significant statistical difference between the E5 HPV-16 expression among the groups studied (p=0.0041). The oncoprotein showed a progressive rising from CIN I to cancer and significant differential expression between CIN I and CIN III (p<0.001); CIN I and cancer (p<0.01); CIN II and CIN III (p<0.05) (Figure 1).

E5 HPV 31 mRNA was detected in 10 samples of the 27 positives for HPV 31(1 CIN I, 3 CIN II, 3 CIN III and 4 cancer). The expression increased from CIN I to CIN II, followed by a decrease in CIN III and then was up again in cancer. No statistical difference was found between the lesions groups.

From the 13 samples positive for HPV 58, the E5 oncogene was detected in 6 (1 sample CIN II and 5 CIN III). Because of the small number of E5 positive samples, no statistical analysis could be performed.

For both HPV 18 and 33, E5 mRNA was detected only in 2 samples, each one. Due to that, no statistical analysis was applied.

Expression profile of miR-203

The statistic evaluation methods applied were the same used for E5 mRNA. At first, Kruskal-Wallis test showed no significant difference between all groups. Evaluating two groups by two through Dunn's test, we observed a decreased expression level of miR-203

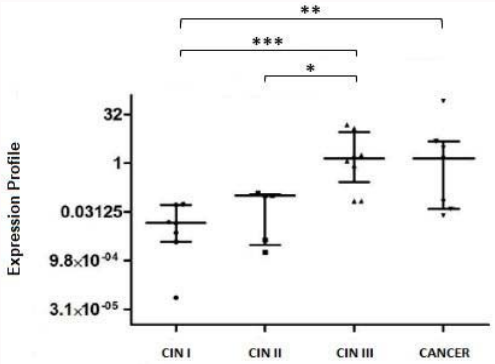

Figure 1: Quantitative relative expression of E5 HPV-16 in precancerous and cancerous lesions. The applied statistical tests were Kruskal-Wallis, which compares all groups simultaneously (p=0.0041) and Dunn's comparison test to see if there is differences in the expression profile between two groups. Significant p values were obtained when comparing: CIN I *vs.* CIN III (p<0.001); CIN I *vs.* cancer (p<0.01) and CIN II *vs.* CIN III (p<0.05).
*p<0.05; **p<0.01; ***p<0.001

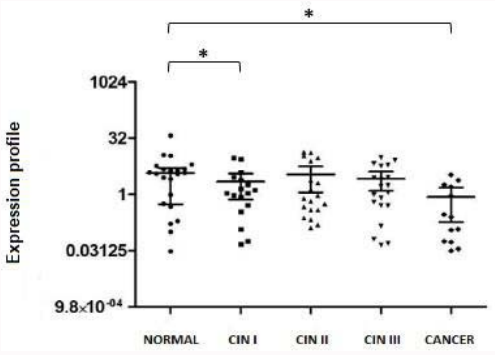

Figure 2: Quantitative relative expression of microRNA-203 in normal, precancerous, and cancerous lesions. The applied statistical test was Kruskal-Wallis and Dunn's comparison test. Kruskal-Wallis test showed no significant difference between all groups however, Dunn's test showed different expression profiles between normal *vs.* (p<0,05) CIN I and normal *vs.* cancer showed statistical significance (p<0,05).
*p<0.05; **p<0.01; ***p<0.001

Table 3: Multi-infection profile.

HPV	Lesion stage	16/31	16/58	31/58	33/58	18/31	58/18	16/31/18
Number of samples	CIN 1	2	1	2				
	CIN 2	2	1				1	1
	CIN 3	2	2	1	1			
	Cancer	3				1		
Total	**20**	**9**	**4**	**3**	**1**	**1**	**1**	**1**

Table 4: Rho coefficients from the comparison between E5 from HPV-16 and miR-203. No p value was lower than p<0.05.

	CIN I E5 HPV-16	CIN II E5 HPV-16	CIN III E5 HPV-16	Cancer E5 HPV-16
CIN I miR-203	-0.4285714			
CIN II miR-203		0.7		
CIN III miR-203			-0.0958101	
Cancer				0.086

Table 5: Rho coefficients of lesions rates comparison between E5 from HPV 31 and miR-203. No p-value was lower than p<0.05.

	CIN II E5 HPV-31	CIN III E5 HPV-31	Cancer E5 HPV-31
CIN II miR-203	0.500		
CIN III miR-203		0.500	
Cancer			0.800

with statistical significance between normal group *vs.* CIN I ($p<0.05$) and normal group *vs.* cancer ($p<0.05$) (Figure 2).

Correlation between the relative expression of E5 and miR-203

We applied the Spearman's correlation test to evaluate the supposed association between the expression of E5 mRNA and miR-203. Due to the number of samples positives for E5 from HPV 58, 18, and 33, no correlation analysis could be performed.

No correlation was observed between E5 HPV 16 and miR-203, for $p<0.05$ (Table 4).

The analysis of the correlation between E5 HPV 31 and miR-203 was also performed, but without de CIN I group due to the lower number of E5 samples. Also, no correlation was found for $p<0.05$ (Table 5).

Discussion

HPV multi-infection in cervical biopsies

The most prevalent HPV types in the northeast population of Brazil were found in the studied samples, and all of them are high-risk HPV types linked to a higher probability of cancer formation [6]. Besides that, the presence of two or more HPV types is often founded in cervical cancer patients, and women with multiple types of HPV have a higher prevalence of abnormal cytology [27]. In our data, 29.4% of infected patients presented infection by two HPV types at the same time - HPV-16 and HPV-31; HPV-16 and HPV-58; HPV-31 and HPV-58; HPV-33 and HPV-58; HPV-18 and HPV-31; HPV-58 and HPV-18 or by three HPVs - HPV-16 and HPV-31 and HPV-18. A study in India found that HPV multi-infection is associated with a higher risk of cervical cancer development, and infection by two or more types excluding HPV-16 and HPV-18 had an Oddis Ratio (OR) of 5.87 and 2.5, respectively. Also, multi-infection by HPV α9 (such as HPV-16, -31, -33, -58) have an OR=5.3 and by α7 (like HPV-18) an OR=2.5 [8].

The multi-infection by those HPVs not covered by the vaccines has 2.94 folds higher chance to lead to cervical carcinoma; luckily the most founded types in the population are targeted by both Gardasil and Cervarix vaccines. Interestingly, HPVs from different phylogenetic branches in the multi-infection situation is negatively associated with cervical cancer, which in our results are the least prevalent types combined -HPV-18 and HPV-31; HPV-58 and HPV-18 [8].

Even with vaccine coverage, a significant number of women are not protected since the vaccination in Brazil is only for girls between the ages of 9 and 14 years old and because there is no effect against installed infection. Other combat methods have been developed; however, most of them are based on specific HPV type's oncoproteins or capside [28-30]. Therefore, profile studies In the populations of each country must be taken into account as part of a prognostic measure and therapeutic new approaches that target specific HPVs oncoproteins.

Expression profile of E5 in clinical biopsies

All oncoproteins play critical roles in cervical lesion development and carcinoma, yet E5 requires considerable attention since its mechanisms in cervical carcinogenesis are not entirely understood. Some reports have observed E5 contribution to carcinogenesis by promoting virus replication, cell cycle continuation [31-33], inhibition of apoptosis [34], cell adhesion and motility disruption [35,36], EGFR surface expression [37,38] and host immune system depression [12,39]. Liao et al. [40], for example, found evidence that E5 supports proliferation, migration, and invasion of cancer cells *in vitro* and cell growth *in vivo*. Still, little is known regarding E5 relative expression in cancer or precancerous lesions.

In this paper, E5 oncogene mRNA expression was measured, and it was observed an expression increase as the lesions became more severe. There was a statistically significant difference in expression between CIN I × CIN III, CIN I × cancer, and CIN II × CIN III. The difference founded between CIN II and CIN III as mentioned above, suggests that CIN II and CIN III lesions have different molecular patterns of infections and maybe HPV oncogene's analysis provides a form of differentiation in cases of histopathological doubts.

Our results are contrary to those in the literature which points that E5 expression is greater in low-grade lesions than in high grade and cancer, due to viral genome integration, a known cause of E5 loss, and thus this oncogene would mostly act in the early stage of carcinogenesis [41-44]. This contrast in E5 expression may be due to differences in the integration pattern, which is more frequently observed in CIN III, in addition to cancer, than in the CIN II stage [45]. However, E5 expression was found before in cancer and high-grade stages in accordance with our findings [46-50]. Hafner et al. [48], for example, concluded that E5 expression was not correlated to specimen histological grade, but only to viral physical status (whether episomal or integrated).

Several hypotheses have been created trying to explain these opposite results. The ones who found low expression levels in high grade and cancer stages rely on the hypothesis of physical loss of E5 after viral integration into the host genome. Other researchers who defend the activity of E5 in late stages of carcinogenesis explain that viral episomal form also exists in high grade and cancer tissue, and such cells are responsible for E5 expression.

E5 expression here was not only found in cancer and high-grade specimens but was higher at these stages than in low-grade lesions,

occurring a gradual increase as lesions become closer to carcinoma or total transformation process. Possibly, E5 oncogene also supports carcinogenesis at later stages, and the episome coexists with the integrated form in the same cell, helping each other to induce cell transformation. Another possibility is that there are cells with different HPV status, and the E5 expression increased substantially, causing a final relative amount higher than the earlier precancerous stages. Cancer and CIN III relative expression data showed a larger distribution pattern than CIN II and CIN I groups, with a higher inter quartile range and variation coefficient. This attribute is consistent with tissues in pre-cancer or cancer state that have many alterations and heterogeneity and supports the idea of possible different viral forms. Still, another hypothesis may be mentioned. Sahab et al. [49] reported that E5 could be expressed even in cells exclusively containing integrated HPV genome, what could also explain high expression levels of this oncogene in several samples found in studies such as Hafner et al. [48], Chen et al. [50] and ours. Previous studies that motivate Sahab et al. [49] had already observed E5 transcript in cells with an integrated HPV genome [46,51]. It seems that other hot spots in the viral genome may represent important fragile sites of gene breaking besides E5 and E2.

Variations in results between studies may also be due to other reasons derived from different experimental models used, number of samples, *in vivo* models and the differences in models themselves, such as *in vitro vs. in vivo*, the relative quantitative expression method or the chosen standard control used. Furthermore, variations between and into populations and between individuals (clinical and biological differences) can also alter data insight.

Expression profile of microRNA-203 in clinical specimens

MicroRNAs are essential small molecules for the regulation of gene expression and have achieved great importance in cancer study, whether in prognostic, diagnostic or in therapeutic approaches. In cervical carcinogenesis, there are several key microRNAs that regulate the cell cycle, proliferation, differentiation, apoptosis, and are distinctly expressed, including miR-203 [52,53]. It has been reported that miR-203 expression inhibits cell proliferation, both *in vitro* [54-56] or *in vivo* [57], and it is known to be critical in controlling proliferation and differentiation rate of keratinocytes [53,55,58]. This microRNA is specific of epithelial tissue [18] and plays an essential role in the development of stratified epithelium [57].

In this work, it was demonstrated that miR-203 relative expression levels were decreased in cancers specimens compared to normal. The normal group also showed different expression patterns with statistical significance when compared to CIN I group. MiR-203 levels have been reported to be decreased in cancer, in previous *in vitro* and clinical studies. Wilting et al. [56] revealed augmented methylation levels in CIN III and in squamous cervical carcinoma clinical samples. They also showed increased methylation in cervical cancer and HPV immortalized cell lineages, and the methylation status was indirectly associated with miR-203 expression levels *in vitro*.

miR-203 is situated on a CpG island and can frequently undergo local DNA hypermethylation. Aberrant miRNA methylation causing altered expression profile is frequent in cervical cancer [59-61], mostly due to hrHPV presence [1,62-64]. It had been demonstrated that HPV-16 oncogenes could regulate fundamental epigenetic mechanisms and enzymes, such as DNA methyltransferases - Jimenez-Wences et al. [65] describes it well - which changes the expression profile of central host genes, such as tumor suppressor genes like p53 and microRNAs [66-69]. These variations cause cell transformation and the subsequent cancer event. On the other hand, Wilting et al. [56] concluded that miR-203 expression was not correlated to hrHPV infection, however hrHPV oncogenes expression were not measured, and a detailed evaluation with appropriate data about hrHPV influence upon miR-203 expression was absent.

Increased methylation pattern in the miR-203 gene was also encountered by Botezatu et al. [1] in biopsy tissues collected from patients with cervical precursor lesions and tumors, but they did not measure the expression levels of this microRNA. Other studies showed significant miR-203 down regulation in high-grade lesions compared to the normal cervix [70], in invasive squamous cell carcinoma biopsy specimens [71], and in atypical dysplasia and cancer samples [72].

Previous studies evaluated the relative expression of HPV oncogenes and miR-203. McKenna et al. [55] observed in cell culture models that E6 expression was associated with miR-203 decrease through p53 degradation, creating an imbalance on proliferation and differentiation rates. They also revealed miR-203 regulation by E7 following DNA damage and affecting differentiation. E7 activity upon miR-203 was also reported before through MAPK/PKC pathway and/or by a mechanism involving PMA - phorbol 12-myristate 13-acetate [53]. In this same study, the authors suggested miR-203 could be involved in HPV genome amplification and had its expression reduced in cervical cancer, which may justify findings in the cancer group, where the reduction in miR-203 expression levels was evident.

MiR-203 overexpression has also been demonstrated in cervical cancer, including overexpression in the serum of patients with cervical adenocarcinoma and squamous cervical cell carcinoma [72,73,15]. The conflicting results regarding miR-203 expression in cancer may be due to the chosen normalization method, among other factors, such as differences in the population (e.g. genetic, aging, healthy, latent viral infections) or in sample processing, subjective lesion degree classification and chosen experimental methods (e.g. *in vitro* × *in vivo*; Taqman × Syber Green). Data from biopsy specimens, for example, have a higher standard deviation than a cell model controlled experiment, which has a significant influence upon statistical analyses. Leitão et al. [23] showed that the expression profile might change depending on which and how many reference genes are used. They conclude that, at least, two most stable miRNAs or mRNA must be used for proper normalization method and expression quantification.

Correlation between E5 and miR-203 expression profiles

Greco et al. [32] observed miR-203 expression in cancer cells stably transfected with E5 and concluded that in E5 positive cancer cell lines, this microRNA presented lower levels than cancer cells negative for E5 from HPV-16. These lower levels of miR-203 raised the hypothesis that E5 may act down regulating this microRNA, and based on their study, we evaluated the existence of a correlation between E5 and miR-203 expression. We evaluated each sample expressing E5 of HPV 16 and miR-203 at all stages of carcinogenesis, observing any association between different groups of lesions.

CIN I showed a negative Rho coefficient between E5 and miR-203, indicating opposite expression patterns. The same was observed when compared both targets in CIN III GROUP, but in this case, E5 mRNA expression levels were elevated while miR-203 was decreased.

Rho coefficient in CIN II and in cancer was positive: In the first, both targets increased its levels together, and in the second group even with a weakly positive value, its visual tendency is for a negative relation, with miR-203 decreasing and E5 increasing its expression.

None of the comparisons showed statistical significance, which can be explained either by the fact that several other molecules are interacting with the studied targets or by the real absent correlation. Many factors are involved in gene expression in a complex net of protein relations. Besides, E5 may have indirectly effect on miR-203 through several others pathways, like methylation [1,65], disruption of miRNA-203 biogenesis (by DROSHA/DICER modulation) at the post-transcriptional level [75] or activation of AKT/PI3K pathway, that also is targeted by miR-203 [17,76], among others.

Conclusion

Our results showed the down regulation of miR-203 in cervical cancer. Its expression was also able to differentiate low grade, CIN I lesion, from normal cervix reassuring its possible role as a biomarker. In turn, E5 HPV-16 demonstrated increased expression in high-grade lesions and cancer compared to low-grade ones, which can be useful in differentiating the cervical lesions, a challenge for our current method applied, and shows that E5 may have an essential role at late stages of infection. No correlation between the targets was found, although the indirect effect of E5 on miR-203 expression cannot be discarded. In this view, miR-203 and E5 are potential candidates for studies aiming for its use in the diagnosis, prognosis, and as a target for cervical lesions and cancer treatment.

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