Expression Profile of *MicroRNA-203* and its *ΔNp63* Target in Cervical Carcinogenesis: Prospects for Cervical Cancer Screening

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Abstract. *Background/Aim: Host molecules disturbed by human papillomavirus (HPV) oncoproteins have been shown to be potential biomarkers of cervical carcinogenesis and represent an alternative or supplementary aid to cytological testing and HPV detection. The miR-203 and one of its targets, ΔNp63, are known to be host molecules that interact with each other to control the proliferation and differentiation of keratinocytes; both have been found to be dysregulated in many cancers. As the role of p63 and miR-203 in cervical carcinogenesis is not yet well-understood, we have, thus, decided to evaluate the changes of expression of both in cervical carcinogenesis. Materials and Methods: This study was carried out by obtaining quantitative polymerase chain reaction (qPCR) data from cervical biopsies. Results: miR-203 and ΔNp63 displayed a similar expression pattern across cervical tissues and both targets showed statistically significant differences between low-grade squamous intraepithelial lesion (LSIL) x high-grade squamous intraepithelial lesion (HSIL); HSIL x Cancer. Additionally, we did not observe an inverse correlation between ΔNp63 mRNA and miR-203 levels as expected but, rather, a positive correlation between cervical tissues. Conclusion: Although preliminary, the expression levels of ΔNp63 mRNA and miR-203 seem to be promising for cervical cancer screening. In addition, positive correlation between miR-203 and ΔNp63 expression suggests the possible existence of some indirect pathways. However, further studies are needed to clarify the role of ΔNp63 and miR-203 in cervical carcinogenesis and, thus, determine how they can be applied in new strategies for diagnosis.*

Abbreviations: HPV, Human papillomavirus; CIN, cervical intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; *ΔNp63*, delta-Np63 isoform; mRNA, messenger ribonucleic acid; miRNA, micro ribonucleic acid; *miR-203*, microRNA 203; 3'-UTR, the three prime untranslated region; PCR, polymerase chain reaction; rRNA, ribosomal ribonucleic acid; RT, reverse transcriptase; *GAPDH*, Glyceraldehyde-3-Phosphate Dehydrogenase; *ACTB*, beta-actin; qPCR, quantitative polymerase chain reaction; *DGCR8*, DiGeorge syndrome critical region 8.

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Human papillomavirus (HPV) is a major risk factor for cervical cancer (1, 2) and responsible for about 275,000 deaths among approximately 529,000 women who are diagnosed throughout the world every year (3, 4). The entire HPV life-cycle depends on epithelial cell differentiation (5) where basal and parabasal cells are induced to proliferate by viral oncoproteins (6, 7). This results in neoplastic transformation that extends progressively from the lower to the upper layers of the epithelium (7). Depending on the extent of the affected area, the lesions can be classified as follows: cervical intraepithelial neoplasia (CIN) Grades 1, 2 and 3 (8) where CIN 1 is also classified as low-grade squamous intraepithelial lesions (LSIL) and CIN 2-3 as high-grade squamous intraepithelial lesions (HSIL) (9).

As CIN lesions usually develop slowly, cervical cancer is preventable and generally curable if detected early (10).

However, conventional screening tests, such as cervical cytology (11, 12) and HPV testing (13), still have a limited ability to provide an accurate diagnosis of each type of CIN or to predict the progression or regression of CINs. This can lead to serious problems in the management of CIN, *i.e.* the under- or overtreatment of affected women. Hence, there is a strong demand for more specific biomarkers to improve the screening of women with cervical neoplasia.

In view of this, studies have attempted to elucidate the complexity of the cellular pathways affected by the HPV cycle, both to obtain a better understanding of viral oncogenesis and identify new molecular markers of carcinogenic progression (14). For instance, Melar-New and Laimins, 2010 (15) evaluated the effects of HPV infection on one of the pathways involved in keratinocyte differentiation and proliferation. This study showed the capacity of HPV E7 oncoprotein to modulate *miR-203* expression levels. *miR-203* is a keratinocyte-specific miRNA (16) that targets *ΔNp63* mRNA at 3'-UTR, a predominant isoform of p63 protein in keratinocytes responsible for controlling the proliferation and differentiation program of these cells (17-19). The role of these two potential biomarkers in cervical carcinogenesis remains unclear.

Separate evaluations of the expression level of *ΔNp63* and *miR-203* have demonstrated dysregulation of both in cervical cancer (20-23). However, some discrepant results have suggested that these two targets might play both an oncogenic (20, 21) and a tumor suppressor role (22, 23). It should be mentioned that few studies have included all premalignant lesions (CIN 1, 2 and 3) in their analysis and, until now, none of them have evaluated the correlation between *ΔNp63* and *miR-203* in clinical samples. For this reason, little is known about the way these molecules alter in cervical carcinogenesis or the importance of these molecules at every stage of the process (*i.e.* from normal to CIN and from CIN to cancer).

In light of this, the aim of this study was to investigate the changes of expression in *ΔNp63* mRNA and *miR-203*, as well as their correlation, in clinical samples that include all premalignant conditions (CIN1, CIN2 and CIN3) and cancer.

Materials and Methods

Patients and samples. A total of 95 cervical biopsies were collected from the Clinical Hospital of UFPE (HC-UFPE) and Institute of Integral Medicine - Prof. Fernando Figueira (IMIP) after signed consent forms had been obtained. The study was approved by the Research Ethics Committee of the Federal University of Pernambuco, Brazil (No.: 03606212.7.0000.5208). Fresh cervical biopsies included low-grade SIL or CIN1 (18); high-grade SIL or CIN2 (20) and CIN3 (21); cancer (15); and normal cervical tissues (21). After being collected, all the clinical samples were immediately preserved in RNAlater (Qiagen, Hilden, Germany) and stored at –80˚C. Women affected by human immunodeficiency virus (HIV) and/or pregnant, were excluded from this study. HPV detection was performed on samples by polymerase chain reaction (PCR) (24) after extraction and purification of total DNA with Trizol (Ambion, Life Technologies, Carlsbad, CA, USA) and a DNeasy Blood & Tissue Kit (Qiagen), respectively. All the samples from CIN and cancer were confirmed to be HPV-positive, whereas the normal cervical biopsies were confirmed to be HPV-negative.

Total RNA extraction and cDNA synthesis. All the preserved biopsies (25-100 mg) were macerated in liquid nitrogen and homogenized with 1 ml of Trizol (Invitrogen) for total RNA extraction. Purification of isolated RNA was performed with the aid of the miRNA Absolutely RNA Kit (Agilent Technologies, San Diego, CA, USA), in accordance with the manufacturer's instructions. This kit allows recovery of both miRNA and mRNA. The RNA quality was assessed by a NanoDrop 2000 Spectrophotometer (ThermoScientific, Wilmington, DE, USA) and 1% agarose gel electrophoresis (25-27). After this, 1 μg of RNA samples of a suitable quality (an OD260/280 from 1.8 to 2.1 and intact rRNA subunits - 28S and 18S) was used to generate the cDNA by means of a miScript II RT kit (Qiagen). A negative control RT reaction (no Reverse Transcriptase enzyme) was prepared for each sample to assess genomic DNA contamination.

Real-time quantitative PCR for ΔNp63 mRNA and miR-203. A QuantiTect SYBR Green PCR kit (Qiagen) was used to quantitate *ΔNp63* mRNA and *miR-203* expression in all cervical tissue conditions (normal, CIN 1, CIN 2, CIN 3 and cancer) using a Rotor Gene 6000 thermocycler (Qiagen). The qPCR efficiency for each primer pair was determined in a previous study by Leitão *et al*. (26), except for the *ΔNp63* primer pair, which was designed and validated by Marchini *et al*. (28). All the microRNA primers were purchased from a miScript primer assay (Qiagen). Reference genes that had been previously validated in cervical tissues were used to obtain miRNA and mRNA expression levels (26). In this way, the geometric mean of *GAPDH* and *ACTB* reference genes was used to calculate the relative expression of *ΔNp63* mRNA and the geometric mean of *miR-191* and *miR-23a* reference genes to calculate the miR-203 levels. Every qPCR reaction was run in duplicate for each sample to control pipetting error (29). Additionally, no template controls and no RT controls were added to detect contamination. For detailed information about the qPCR assay, see Leitão *et al*. (26).

Statistical analysis. All the analyses were carried out using GraphPad Prism software (GraphPad, San Diego, CA, USA) version 6.0. The D'Agostino-Pearson normality test was conducted to determine a Gaussian distribution of data. Welch's *t*-test was performed to compare the relative amounts of *ΔNp63* and *miR-203* in all cervical tissue conditions. The Pearson correlation coefficient was calculated to determine the relationship between *miR-203* expression and *ΔNp63* mRNA levels in cervical carcinogenesis. *p*-Values of <0.05 were considered to be statistically significant.

Results

Determination of the ΔNp63 mRNA expression pattern in cervical carcinogenesis. The molecular signature of *ΔNp63* mRNA in cervical cancer progression was investigated by obtaining the expression levels of this molecule through a qPCR in a series of biopsies including normal tissue, CIN

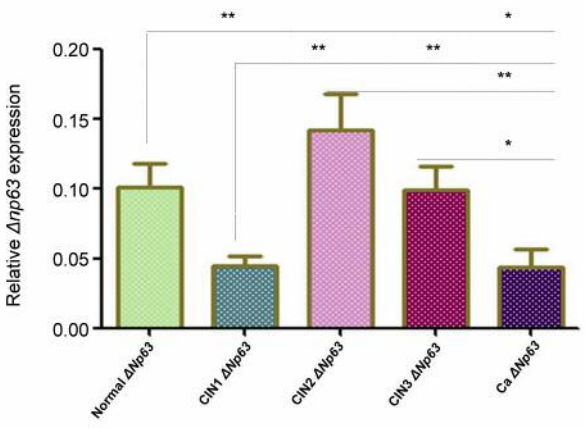

Figure 1. *Expression levels of ΔNp63 mRNA in cervical carcinogenesis. ΔNp63 showed a significant difference in expression between normal x LSIL (CIN1); LSIL x HSIL (CIN2-3); and HSIL x Cancer (Ca). The asterisks indicate a statistical significance in the differences between the cervical tissues (*p<0.05; **p<0.01).*

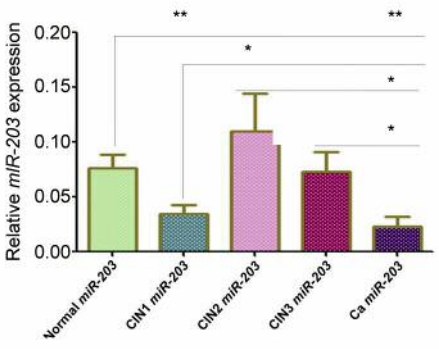

Figure 2. *Expression levels of miR-203 in cervical carcinogenesis. Statistically significant differences can be observed in the miR-203 levels from normal to LSIL (CIN1); from LSIL to HSIL (CIN2-3); and from HSIL to Cancer (Ca). The asterisks indicate a statistical significance in the differences between cervical tissues (*p<0.05; **p<0.01).*

(1, 2 and 3) and carcinoma. The expression of *ΔNp63* in the CIN1 lesion showed a significant decrease ($p<0.01$) when compared to matched normal tissues. Following this, a significantly increased expression of *ΔNp63* was observed in CIN2 when compared to CIN1 ($p<0.01$). After this, we observed a gradual decline in *ΔNp63* mRNA levels from CIN2 to CIN3 and from CIN3 to cancer ($p<0.05$). Hence, changes in expression levels were significant between the CIN1 and CIN2 lesions and it was possible to distinguish between LSIL and HSIL. Furthermore, this significance was also detected between CIN3 lesions and cancers ($p<0.05$), as can be seen in Figure 1.

Determination of the miR-203 expression pattern in cervical carcinogenesis. Interestingly, *miR-203* showed an expression profile similar to that of *ΔNp63* across all the cervical tissues despite the higher expression levels of *ΔNp63* mRNA (Figure 2). Thus, a significant decrease in *miR-203* levels was observed in CIN1 ($p<0.01$) when compared with normal tissues, as well as a significant increase in CIN2 when compared with the CIN1 samples ($p<0.01$). Subsequently, it was possible to observe a decrease in the levels of *miR-203* from CIN2 to CIN3 and from this last to cancer, where the significance was identified ($p<0.05$). This means that the changes in the *miR-203* expression levels also made it possible to distinguish between normal tissue and LSIL; LSIL and HSIL; and HSIL and cancer.

Correlation of miR-203 with ΔNp63 mRNA levels in cervical tissues. Once *ΔNp63* mRNA had been established as a direct

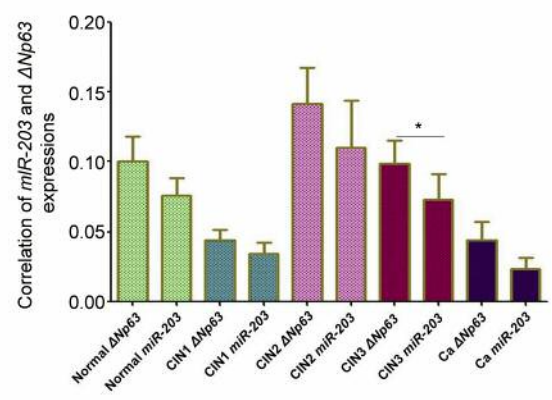

Figure 3. *Correlation of ΔNp63 mRNA and miR-203 levels. The inverse correlation of miR-203 and its ΔNp63 target was not observed in cervical carcinogenesis. The asterisk indicates a correlation with statistical significance (*p<0.05).*

target of *miR-203* (17-19), we were able to evaluate the correlation of both in cervical carcinogenesis to determine whether the expression levels of *ΔNp63* were affected by this miRNA in cervical tissues.

We observed a positive correlation pattern between the expression of *miR-203* and *ΔNp63* in all the cervical tissue conditions, which was as follows: normal tissue (r=0.310), CIN1 (r=0.346), CIN2 (r=0.345), CIN3 (r=0.518) and carcinomas (r=0.218). However, the correlation was only significant in CIN3 lesions ($p<0.05$), as can be seen in Figure 3.

Discussion

Molecular pathways have been described as participants in the progression of cervical cancer. However, little is known about the interaction between HPV and the host molecules in the progression of this type of cancer since there has been very little evaluation of mRNA and *miRNA* signatures in cervical dysplasia and carcinoma (30, 31). The evaluation of changes in mRNA and microRNA expression is important in cervical carcinogenesis since it allows important strategic targets to be set for diagnosis and molecularly targeted therapy.

ΔNp63 mRNA and *miR-203* are known to be two host molecules interacting with each other to control the proliferation and differentiation of keratinocytes (17-19) and have been cited as playing an important role in HPV-related cervical dysplasia and carcinoma (15). They have been evaluated separately in certain types of epithelial tumorigenesis and metastasis, while studies have shown dysregulated expression of these two targets in various types of cancer. Overexpression of *ΔNp63* has been found in ovarian, nasopharynx, head and neck, lung, bladder and breast cancers (28, 32-36). However, reduced expression of *ΔNp63* has been detected in bladder, esophagus and prostate cancers, as a result of poor prognosis and metastasis (37-40). Inconsistent data have also been related to *miR-203* since this has been shown to be overexpressed in endometrial carcinoma and pancreatic adenocarcinoma (41, 42), while, at the same time, being underexpressed in cancers of the esophagus, larynx, prostate, liver, among other organs (43-46).

The same contradiction can be observed in the roles played by *miR-203* and *ΔNp63* in cervical carcinogenesis. In the case of p63 expression in cervical tumorigenesis, it should be noted that studies have shown increased levels (20, 47-49), as well as reduced levels of the ΔNp63 isoform, in both cervical tissues and cervical cancer cell lines (22, 50). With regard to *miR-203*, some studies have found that it has a tumor suppressor role in cervical cancer (23, 51-56), while others have reported up-regulation of this miRNA in cervical cancer (21, 57, 58). Hence, *ΔNp63* and *miR-203* seem to play both an oncogenic role and a tumor suppressor role in the development of cervical cancer.

Analytical methods show variations in studies where p63 expression in cervical cancer is evaluated. For instance, several expression studies fail to distinguish between p63 isoforms in cervical carcinogenesis (30, 59, 61), which makes it difficult to conclude if ΔNp63 is the predominant isoform in a certain obtained profile. Additionally, only two of the studies evaluating *ΔNp63* expression included premalignant lesions (20, 47). The inclusion of CIN1, CIN2 and CIN3 in expression studies can provide a more detailed understanding of the role of potential biomarkers in cervical carcinogenesis, as well as their usefulness, in giving support to screening tests. In our results, we observed that the ΔNp63 isoform showed the expression profile declined from normal to CIN1, as well as from CIN2 to cancer, unlike the increasing levels that have been found in other studies, which have evaluated the same p63 isoform in cervical cancer cell lines and cervical tissues (20, 47, 48, 50). On the other hand, reduced levels of *ΔNp63* were detected in cervical carcinoma, as well as in cervical cancer cell lines (HeLa, SiHa and C33A), and it has been suggested that this decline can be combined with tumor progression (22). Likewise, a previous study by Yugawa *et al.* (50) reported that HPV-positive cell lines, such as HeLa, CaSki and SiHa, showed very low or undetectable *ΔNp63* levels, while other cervical cancer cell lines (QG-H, SKGIIIb and ME180) overexpressed the *ΔNp63* isoform. In our study we observed an overexpression of *ΔNp63* from LSIL (CIN1) to HSIL (CIN2 and CIN3). According to Higashikawa *et al.* (62) and Yugawa *et al.* (50) the overexpression of *ΔNp63* may be linked to early stages of tumorigenesis, while reduction or absence of expression can be found in late stages of cancer development, such as tumor invasion. Similarly, Herfs *et al.* (63) found that *ΔNp63* silencing leads to a loss of cell-cell adhesion and an increase in the migratory properties of cancer cells. Some studies, that have attempted to understand the effects of changes in p63 expression in progressive cancer, have put forward some controversial explanations. Recently, King *et al.* demonstrated that the increase in *ΔNp63* causes up-regulation of the expression of target genes while maintaining the proliferation of keratinocytes, as well as causing the up-regulation of anti-apoptotic genes (64). Conversely, other studies have shown that *ΔNp63* adopts an anti-proliferative strategy by inducing apoptosis both in human keratinocyte cells and in human squamous carcinoma cells (65, 66). According to Zhu *et al.* (66), increased expression of *ΔNp63* in various cancers may be a compensatory cell mechanism to prevent a high rate of tumor cell proliferation, although it is unsuccessful. Hence, further studies are needed to determine the underlying mechanisms by which *ΔNp63* affects cervical carcinogenesis, *i.e.* to determine to what extent this p63 isoform predominates at each stage of the development of the malignant cervical cells.

With regard to the role played by *miR-203* in cervical carcinogenesis, most studies have been restricted to evaluating this miRNA in different cervical cancer cell lines or between normal and cancer tissues (21, 23, 51, 54-57). As a result, the *miR-203* role in cervical cancer remains unclear because of discrepancies in the results between different studies; this is perhaps owing to differences in the high-throughput platforms and methods employed or ethnic variations in the evaluated population (26, 52, 67). To date, few studies have included samples from premalignant lesions

that can allow the expression of this miRNA in cervical carcinogenesis to be evaluated (52-54, 68). Overall, all these studies have formed an expression profile of *miR-203* that declines from normal to CIN and from CIN to cancer. These studies were corroborated by our results, where there was a reduction in the levels of *miR-203* from normal to CIN1 and from CIN2 to cancer. Greco *et al*. (69) found that in vitro keratinocytes expressing HPV E5 oncoprotein had lower levels of *miR-203* than cells that were not expressing the oncoprotein. It is known that the E5 oncoprotein is of particular importance in the early stages of cervical neoplasia, since the E5 gene is deleted after HPV DNA has been integrated into the host genome (51). Additionally, in our findings there was an up-regulation of *miR-203* from CIN1 to CIN2. Some studies have reported overexpression of this miRNA in cervical cancer samples (21, 57, 58). Pereira *et al*. provided the only study that assessed expression of miRNAs in all the pre-malignant lesions (CIN 1, 2 and 3), demonstrating that there was a progressive reduction in levels of *miR-203* depending on the degree of the lesion (52). The same profile was observed in other studies involving premalignant neoplasias (53, 54, 68). However, in the studies of Cheung *et al*. 2012 (53) and Wilting *et al*. 2013 (54), the CIN2 and CIN3 lesions were lumped together as a single class, while in the study of Mo et al., CIN2 lesions were not included (68). Hence, further studies that include all premalignant lesions are needed to clarify the *miR-203* signature in cervical carcinogenesis, as well as to understand how *miR-203* dysregulation leads to cervical carcinogenesis.

ΔNp63 is known to be a target of *miR-203* which acts by controlling keratinocyte proliferation and epidermal differentiation (17-19). Some studies have demonstrated that the correlation of the expression of these two targets in the epithelium appears to be inverse, *i.e*. while the *miR-203* levels increase by proliferating undifferentiated basal keratinocytes to non-proliferative differentiated suprabasal keratinocytes, the *ΔNp63* levels decrease in the same way (17, 18, 70). According to a study by Melar-New and Laimins (15), *miR-203* levels are down-regulated by the HPV E7 oncoprotein in infected keratinocytes and this results in p63 protein overexpression. However, so far no studies have evaluated the correlation between *miR-203* and *ΔNp63* in cervical clinical samples. Our results showed that changes in *miR-203* expression did not have an inverse correlation with the target. Melar-New and Laimins also found that *miR-203* had a minimal effect on *ΔNp63* mRNA levels in HPV-positive keratinocytes. They suggest that this may be due to the mechanism of post-transcriptional regulation by microRNAs (71), which does not always lead to the degradation of the mRNA target (72). Furthermore, a positive correlation in all types of evaluated samples was observed in our results, although this correlation was only significant in the CIN3 lesions. Interestingly, a study by Miles and co-workers (73) also identified positively correlated microRNA/mRNA pairs in both ovarian tumors and normal samples, thus suggesting the possible existence of some indirect pathways in tumorigenesis. On the other hand, Chakravarti *et al*. (74) reported that *ΔNp63*-deficient epidermal cell lines resulted in a global down-regulation of miRNAs (including the *miR-203*); this is an event that may be linked to the transcriptional activation of *DGCR8* (a component of the microRNA processing machinery) by *ΔNp63* (74).

To date, the search for biomarkers that assist in distinguishing and carrying out a prognosis of premalignant lesions remains a priority. There are still limitations to the screening tests for cervical cancer that often make it difficult to conduct a reliable diagnosis of the CIN of non-neoplastic lesions or differentiate each type of CIN. This can lead to both under- or overtreatment of affected women (75, 76). In view of this, when we focused on diagnosis, we observed that the expression changes of *ΔNp63* mRNA and *miR-203*, in our study, showed significant differences between the samples: normal x CIN1 (LSIL); CIN1 (LSIL) x CIN2 (HSIL); CIN1 (LSIL) x CIN3 (HSIL); and CIN3 (HSIL) x Cancer. Although preliminary, the expression levels of *ΔNp63* mRNA and *miR-203* seem to be promising in terms of diagnosis of cervical carcinogenesis stages. However, further validation in larger sample sizes or in multiple cohorts are needed to determine the true clinical value of these potential biomarkers and, perhaps, whether they can be incorporated into a clinical diagnosis.

Conflicts of Interest

The Authors declare that they have no competing interests.

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References

1 zur Hausen H: Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer *2*: 342-350, 2002.
2 Bosh FX and de Sanjosé S: Chapter 1: human papillomavirus and cervical cancerburden and assessment of causality. J Natl Cancer Inst Monogr *31*: 3-13, 2003.

3 Parkin M: The global health burden of infection-associated cancers in the year. Int J Cancer *118*: 3030-3044, 2006.

4 Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin *61*: 69-90, 2011.

5 Stanley MA: Human papillomavirus and cervical carcinogenesis. Best Pract Res Clin Obstet Gynaecol *15*: 663-676, 2001.

6 Thomas JT, Hubert WG, Ruesch MN and Laimins LA: Human papillomavirus type 31 oncoproteins E6 and E7 are required for the maintenance of episomes during the viral life cycle in normal human keratinocytes. Proc Natl Acad Sci USA *96*: 8449-8454, 1999.

7 Thomison J, Thomas LK and Shroyer KR: Human papillomavirus: molecular and cytologic/histologic aspects related to cervical intraepithelial neoplasia and carcinoma. Hum Pathol *39*: 154-166, 2008.

8 Waxman AG, Chelmow D, Darragh TM, Lawson H and Moscicki AB: Revised terminology for cervical histopathology and its implications for management of high-grade squamous intraepithelial lesions of the cervix. Obstet Gynecol *120*: 1465-1471, 2012.

9 Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, Prey M, Raab S, Sherman M, Wilbur D, Wright T Jr, Young N, Forum Group Members and Bethesda 2001 Workshop: The 2001 Bethesda System: terminology for reporting results of cervical cytology. JAMA *287*: 2114-2119, 2002.

10 Holowaty P, Miller AB, Rohan T and To T: Natural dysplasia of the uterine cervix. J Natl Cancer Inst *91*: 252-258, 1999.

11 Derchain SFM, Longatto Filho A and Syrjanen KJ: Neoplasia intra-epitelial cervical: diagnóstico e tratamento. Rev Bras Ginecol Obstet *27*: 425-433, 2005.

12 Pinto AP, Degen M, Villa LL and Cibas ES: Immunomarkers in gynecologic cytology: the search for the ideal biomolecular Papanicolaou test. Acta Cytol *56*: 109-121, 2012.

13 Ronco G, Segnan N, Giorgi-Rossi P, Zappa M, Casadei GP, Carozzi F, Dalla Palma P, Del Mistro A, Folicaldi S, Gillio-Tos A, Nardo G, Naldoni C, Schincaglia P, Zorzi M, Confortini M, Cuzick J and New Technologies for Cervical Cancer Working Group: Human papillomavirus testing and liquid-based cytology: results at recruitment from the new technologies for cervical câncer randomized controlled trial. J Natl Cancer Inst *98*: 765-774, 2006.

14 Freitas AC, Coimbra EC and Leitão MD: Molecular targets of HPVoncoproteins: Potential biomarkers for cervical carcinogenesis. Biochim Biophys Acta *1845*: 91-103, 2014.

15 Melar-New M and Laimins A: Human papillomaviruses modulate expression of microRNA 203 upon epithelial differentiation to control levels of p63 proteins. J Virol *84*: 5212-5221, 2010.

16 Sonkoly E, Wei T, Janson PC, Sääf A, Lundeberg L, Tengvall-Linder M, Norstedt G, Alenius H, Homey B, Scheynius A, Ståhle M and Pivarcsi A: MicroRNAs: novel regulators involved in the pathogenesis of psoriasis? PLoS One *2*: e610, 2007.

17 Lena AM, Shalom-Feuerstein R, Rivetti di Val Cervo P, Aberdam D, Knight RA, Melino G and Candi E: miR-203 represses 'stemness' by repressing DeltaNp63. Cell Death Differ 15: 1187-1195, 2008.

18 Yi R, Poy MN, Stoffel M and Fuchs E: A skin microRNA promotes differentiation by repressing 'stemness'. Nature *452*: 225-229, 2008.

19 Jackson SJ, Zhang Z, Feng D, Flagg M, O'Loughlin E, Wang D, Stokes N, Fuchs E and Yi R: Rapid and widespread suppression of self-renewal by microRNA-203 during epidermal differentiation. Development *140*: 1882-1891, 2013.

20 Lin Z, Liu M, Li Z, Kim C, Lee E and Kim I: DeltaNp63 protein expression in uterine cervical and endometrial cancers. J Cancer Res Clin Oncol *132*: 811-816, 2006.

21 Muralidhar B, Goldstein LD, Ng G, Winder DM, Palmer RD, Gooding EL, Barbosa-Morais NL, Mukherjee G, Thorne NP, Roberts I, Pett MR and Coleman N: Global microRNA profiles in cervical squamous cell carcinoma depend on Drosha expression levels. J Pathol *212*: 368-377, 2007.

22 Zhou Y, Xu Q, Ling B, Xiao W and Liu P: Reduced expression of ΔNp63α in cervical squamous cell carcinoma. Clin Invest Med *34*: E184-191, 2011.

23 Lee JW, Choi CH, Choi JJ, Park YA, Kim SJ, Hwang SY, Kim WY, Kim TJ, Lee JH, Kim BG and Bae DS: Altered MicroRNA expression in cervical carcinomas. Clin Cancer Res *14*: 2535-2542, 2008.

24 Chagas BS, Batista MV, Guimarães V, Balbino VQ, Crovella S and Freitas AC: New variants of E6 and E7 oncogenes of human papillomavirus type 31 identified in Northeastern Brazil. Gynecol Oncol *123*: 284-288, 2011.

25 Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J and Wittwer CT: The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem *55*: 611-622, 2009.

26 Leitão Mda C, Coimbra EC, de Lima Rde C, Guimarães Mde L, Heráclio Sde A, Silva Neto Jda C and de Freitas AC: Quantifying mRNA and microRNA with qPCR in cervical carcinogenesis: a validation of reference genes to ensure accurate data. PLoS One *9*: e111021, 2014.

27 Rueda-Martínez C, Lamas O, Mataró MJ, Robledo-Carmona J, Sánchez-Espín G, Jiménez-Navarro M, Such-Martínez M and Fernández B: Selection of reference genes for quantitative real time PCR (qPCR) assays in tissue from human ascending aorta. PLoS One *9*: e97449, 2014.

28 Marchini S, Marabese M, Marrazzo E, Mariani P, Cattaneo D, Fossati R, Compagnoni A, Fruscio R, Lissoni AA and Broggini M: DeltaNp63 expression is associated with poor survival in ovarian cancer. Ann Oncol *19*: 501-507, 2008.

29 Nolan T, Hands RE and Bustin SA: Quantification of mRNA using real-time RT-PCR. Nat Protoc *1*: 1559-1582, 2006.

30 Royse KE, Zhi D, Conner MG, Clodfelder-Miller B, Srinivasasainagendra V, Vaughan LK, Skibola CF, Crossman DK, Levy S and Shrestha S: Differential Gene Expression Landscape of Co-Existing Cervical Pre-Cancer Lesions Using RNA-seq. Front Oncol *4*: 339, 2014.

31 Del Pino M, Svanholm-Barrie C, Torné A, Marimon L, Gaber J, Sagasta A, Persing DH and Ordi J: mRNA biomarker detection in liquid-based cytology: a new approach in the prevention of cervical cancer. Mod Pathol *28*: 312-320, 2015.

32 Crook T, Nicholls JM, Brooks L, O'Nions J and Allday MJ: High level expression of ΔN-p63: a mechanism for the inactivation of p53 in undifferentiated nasopharyngeal carcinoma (NPC)? Oncogene *19*: 3439-3444, 2000.

33 Yamaguchi K, Wu L, Caballero OL, Hibi K, Trink B, Resto V, Cairns P, Okami K, Koch WM, Sidransky D and Jen J: Frequent gain of the p40/p51/p63 gene locus in primary head and neck squamous cell carcinoma. Int J Cancer *86*: 684-689, 2000.

34 Massion PP, Taflan PM, Rahman SMJ, Yildiz P, Shyr Y, Edgerton ME, Westfall MD, Roberts JR, Pietenpol JA, Carbone DP and Gonzalez AL: Significance of p63 amplification and overexpression in lung cancer development and prognosis. Cancer Res *63*: 7113-7121, 2003.
35 Compérat E, Bièche I, Dargère D, Ferlicot S, Laurendeau I, Benoît G, Vieillefond A, Verret C, Vidaud M, Capron F, Bedossa P and Paradis V: p63 gene expression study and early bladder carcinogenesis. Urology *70*: 459-462, 2007.
36 Hanker L, Karn T, Ruckhaeberle E, Gaetje R, Solbach C, Schmidt M, Engels K, Holtrich U, Kaufmann M and Rody A: Clinical relevance of the putative stem cell marker p63 in breast cancer. Breast Cancer Res Treat *122*: 765-775, 2010.
37 Koga F, Kawakami S, Fujii Y, Saito K, Ohtsuka Y, Iwai A, Ando N, Takizawa T, Kageyama Y and Kihara K: Impaired p63 expression associates with poor prognosis and uroplakin III expression in invasive urothelial carcinoma of the bladder. Clin Cancer Res *9*: 5501-5507, 2003.
38 Morita M, Uramoto H, Nakata S, Ono K, Sugaya M, Yoshimatsu T, Oyama T, Hanagiri T, Sugio K and Yasumoto K: Expression of DeltaNp63 in squamous cell carcinoma of the esophagus. Anticancer Res *25*: 3533-3539, 2005.
39 Parsons JK, Saria EA, Nakayama M, Vessella RL, Sawyers CL, Isaacs WB, Faith DA, Bova GS, Samathanam CA, Mitchell R and De Marzo AM: Comprehensive mutational analysis and mRNA isoform quantification of TP63 in normal and neoplastic human prostate cells. Prostate *69*: 559-569, 2009.
40 Karni-Schmidt O, Castillo-Martin M, Shen TH, Gladoun N, Domingo-Domenech J, Sanchez-Carbayo M, Li Y, Lowe S, Prives C and Cordon-Cardo C: Distinct expression profiles of p63 variants during urothelial development and bladder cancer progression. Am J Pathol *178*: 1350-1360, 2011.
41 Chung TK, Cheung TH and Huen NY: Dysregulated microRNAs and their redicted targets associated with endometrioid endometrial adenocarcinoma in Hong Kong women. Int J Cancer *124*: 1358-1365, 2009.
42 Ikenaga N, Ohuchida K, Mizumoto K, Yu J, Kayashima T, Sakai H, Fujita H, Nakata K and Tanaka M: MicroRNA-203 expression as a new prognostic marker of pancreatic adenocarcinoma. Ann Surg Oncol *17*: 3120-3128, 2010.
43 Feber A, Xi L, Luketich JD, Pennathur A, Landreneau RJ, Wu M, Swanson SJ, Godfrey TE and Litle VR: MicroRNA expression profiles of esophageal cancer. J Thorac Cardiovasc Surg *135*: 255-260, 2008.
44 Tian L, Li M, Ge J, Guo Y, Sun Y, Liu M and Xiao H: MiR-203 is downregulated in laryngeal squamous cell carcinoma and can suppress proliferation and induce apoptosis of tumours. Tumour Biol *35*: 5953-5963, 2014.
45 Boll K, Reiche K, Kasack K, Mörbt N, Kretzschmar AK, Tomm JM, Verhaegh G, Schalken J, von Bergen M, Horn F and Hackermüller J: MiR-130a, miR-203 and miR-205 jointly repress key oncogenic pathways and are downregulated in prostate carcinoma. Oncogene *32*: 277-285, 2013.
46 Liu Y, Ren F, Rong M, Luo Y, Dang Y and Chen G: Association between underexpression of microrna-203 and clinicopathological significance in hepatocellular carcinoma tissues. Cancer Cell Int *15*: 62, 2015.
47 Quade BJ, Yang A, Wang Y, Sun D, Park J, Sheets EE, Cviko A, Federschneider JM, Peters R, McKeon FD and Crum CP: Expression of the p53 homologue p63 in early cervical neoplasia. Gynecol Oncol *80*: 24-29, 2001.
48 Wang TY, Chen BF, Yang YC, Chen H, Wang Y, Cviko A, Quade BJ, Sun D, Yang A, McKeon FD and Crum CP: Histologic and immunophenotypic classification of cervical carcinomas by expression of the p53 homologue p63: a study of 250 cases. Hum Pathol *32*: 479-486, 2001.
49 Lin Z, Nan Y, Zhang X, Zhao Y, Kim C and Kim I: Reverse transcription-polymerase chain reaction and western blotting analysis for detection of p63 isoforms in uterine cervical cancers. Int J Gynecol Cancer *16*: 1643-1647, 2006.
50 Yugawa T, Narisawa-Saito M, Yoshimatsu Y, Haga K, Ohno S, Egawa N, Fujita M and Kiyono T: DeltaNp63alpha repression of the Notch1 gene supports the proliferative capacity of normal human keratinocytes and cervical cancer cells. Cancer Res *70*: 4034-4044, 2010.
51 Wang X, Tang S, Le SY, Lu R, Rader JS, Meyers C and Zheng ZM: Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. PLoS One *3*: e2557, 2008.
52 Pereira PM, Marques JP, Soares AR, Carreto L and Santos MA: MicroRNA expression variability in human cervical tissues. PLoS One *5*: e11780, 2010.
53 Cheung TH, Man KN, Yu MY, Yim SF, Siu NS, Lo KW, Doran G, Wong RR, Wang VW, Smith DI, Worley MJ Jr, Berkowitz RS, Chung TK and Wong YF: Dysregulated microRNAs in the pathogenesis and progression of cervical neoplasm. Cell Cycle *11*: 2876-2884, 2012.
54 Wilting SM, Snijders PJ, Verlaat W, Jaspers A, van de Wiel MA, van Wieringen WN, Meijer GA, Kenter GG, Yi Y, le Sage C, Agami R and Meijer CJ: Altered microRNA expression associated with chromosomal changes contributes to cervical carcinogenesis. Oncogene *32*: 106-116, 2013.
55 Zhao S, Yao DS, Chen JY and Ding N: Aberrant expression of miR-20a and miR-203 in cervical cancer. Asian Pac J Cancer Prev *14*: 2289-2293, 2013.
56 Zhu X, Er K, Mao C, Yan Q, Xu H, Zhang Y, Zhu J, Cui F, Zhao W and Shi H: miR-203 suppresses tumor growth and angiogenesis by targeting VEGFA in cervical cancer. Cell Phys Biochem *32*: 64-73, 2013.
57 Witten D, Tibshirani R, Gu SG, Fire A and Lui WO: Ultra-high throughput sequencing-based small RNA discovery and discrete statistical biomarker analysis in a collection of cervical tumours and matched controls. BMC Biol *8*: 58, 2010.
58 Gocze, K, Gombos K, Juhasz K, Kovacs K, Kajtar B, Benczik M, Gocze P, Patczai B, Arany I and Ember I: Unique microRNA expression profiles in cervical cancer. Anticancer Res *33*: 2561-2567, 2013.
59 Martens JE, Arends J, Van der Linden PJ, De Boer BA and Helmerhorst TJ: Cytokeratin 17 and p63 are markers of the HPV target cell, the cervical stem cell. Anticancer Res *24*: 771-775, 2004.
60 Vasilescu F, Ceauşu M, Tănase C, Stănculescu R, Vlădescu T and Ceauşu Z: P53, p63 and Ki-67 assessment in HPV-induced cervical neoplasia. Morphol Embryol *50*: 357-361, 2009.
61 Cheung AN, Tsun KL, Ng KM, Szeto E, Siu MK, Wong ES and Ngan HY: P634A4 and TAp73 immunocytochemistry in liquid-based cervical cytology – potential biomarkers for diagnosis and progress prediction of cervical neoplasia. Mod Pathol *23*: 559-566, 2010.

62 Higashikawa K, Yoneda S, Tobiume K, Taki M, Shigeishi H and Kamata N: Snail-induced down-regulation of DeltaNp63alpha acquires invasive phenotype of human squamous cell carcinoma. Cancer Res *67*: 9207-9213, 2007.
63 Herfs M, Hubert P, Suarez-Carmona M, Reschner A, Saussez S, Berx G, Savagner P, Boniver J and Delvenne P: Regulation of p63 isoforms by snail and slug transcription factors in human squamous cell carcinoma. Am J Pathol *76*: 1941-1949, 2010.
64 King KE, Reddi DM, Ponnamperuma RM, Gerdes M and Weinberg WC: Dysregulated ΔNp63α negatively regulates the maspin promoter in keratinocytes via blocking endogenous p73 binding. Mol Carcinog *53*: 698-710, 2014.
65 Dohn M, Zhang S and Chen X: p63alpha and DeltaNp63alpha can induce cell cycle arrest and apoptosis and differentially regulate p53 target genes. Oncogene *20*: 3193-3205, 2001.
66 Zhu L, Rorke EA and Eckert RL: DeltaNp63alpha promotes apoptosis of human epidermal keratinocytes. J Invest Dermatol *127*: 1980-1991, 2007.
67 Rao Q, Shen Q, Zhou H, Peng Y, Li J and Lin Z: Aberrant microRNA expression in human cervical carcinomas. Med Oncol *29*: 1242-1248, 2012.
68 Mo W, Tong C, Zhang Y and Lu H: microRNAs' differential regulations mediate the progress of Human Papillomavirus (HPV)-induced Cervical Intraepithelial Neoplasia (CIN). BMC Syst Biol *9*: 4, 2015.
69 Greco D, Kivi N, Qian K, Leivonen SK, Auvinen P and Auvinen E: Human papillomavirus 16 E5 modulates the expression of host microRNAs. PLoS One *6*: e21646, 2011.
70 Truong AB, Kretz M, Ridky TW, Kimmel R and Khavari PA: p63 regulates proliferation and differentiation of developmentally mature keratinocytes. Genes Dev *20*: 3185-3197, 2006.
71 Soifer HS, Rossi JJ and Saetrom P: MicroRNAs in disease and potential therapeutic applications. Mol Ther *15*: 2070-2079, 2007.
72 Guo H, Ingolia NT, Weissman JS and Bartel DP: Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature *466*: 835-840, 2010.
73 Miles GD, Seiler M, Rodriguez L, Rajagopal G and Bhanot G: Identifying microRNA/mRNA dysregulations in ovarian cancer. BMC Res Notes *5*: 164, 2012.
74 Chakravarti D, Su X, Cho MS, Bui NH, Coarfa C, Venkatanarayan A, Benham AL, Flores González RE, Alana J, Xiao W, Leung ML, Vin H, Chan IL, Aquino A, Müller N, Wang H, Cooney AJ, Parker-Thornburg J, Tsai KY, Gunaratne PH and Flores ER: Induced multipotency in adult keratinocytes through down-regulation of ΔNp63 or DGCR8. Proc Natl Acad Sci USA *111*: E572-81, 2014.
75 Al-Nafussi AI and Colquhoun MK: Mild cervical intraepithelial neoplasia (CIN 1): a histological overdiagnosis. Histopathology *17*: 5575-61, 1990.
76 Creagh T, Bridger JE, Kupek E, Fish DE, Martin-Bates E and Wilkins MJ: Pathologist variation in reporting cervical borderline epithelial abnormalities and cervical intraepithelial neoplasia. J Clin Pathol *48*: 59-60, 1995.
77 Kim SM, Lee JU, Lee DW, Kim MJ and Lee HN: The prognostic significance of p16, Ki-67, p63, and CK17 expression determined by immunohistochemical staining in cervical intraepithelial neoplasia 1. Korean J Obstet Gynecol *54*: 184-191, 2011.

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