

Assessment of the Expression of Long Noncoding Mitochondrial RNAs (lncmtRNAs) During Cervical Cancer Progression and Cervical Carcinoma

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Abstract

Objective: Cervical cancer is the second most common cancer in women with high rates of mortality worldwide. Cervical cancer is slowly progressive and is preceded by pre-invasive intraepithelial lesions. Therefore, detection of premalignant lesions is key to preventing disease progression to advanced stages. The objective of the present study was examination and quantification of the differentially expressed non-coding mitochondrial RNAs during progression of disease.

Material and methods: The differential expression of S-ncmtRNA and AS-ncmtRNA was analyzed by in situ hybridization (ISH) using tissue macroarrays (TMA) from normal, CIN1, CIN2, CIN3 and invasive squamous carcinoma (SCC). PCNA and p16INK-4a were detected in consecutive biopsies by immunohistochemistry. Quantification of ISH signal was carried out with Image ProPlus 6.1 software and the results were expressed as percentage of Integrated Optical Density (IOD).

Results: We found a marked down-regulation of AS-ncmtRNA in 95% of tissues analyzed (CIN 1/2, CIN 3, and invasive squamous cancer). Moreover, differential expression of ASncmtRNA v/s S-ncmtRNA showed significant difference. Normal proliferating tissues did not display down-regulation of AS-ncmtRNA. Down regulation of AS-ncmtRNA correlated with the expression of the tumor suppressor protein p16INK-4a

Conclusions: We found down-regulation of AS-ncmtRNA in pre-malignant and tumor samples which could distinguish normal tissues from early lesions and tumor samples. These results suggest that the down-regulation of AS-ncmtRNA is a novel marker of early lesions and cervix neoplasia.

Keywords: Mitochondrial non coding RNAs; Cervical cancer; Differential expression; Human papilloma virus; Early detection

Introduction

Cervical cancer is the fourth most common cancer in women worldwide. In 2012, this disease accounted for 528,000 new cases and 266,000 deaths among females [1]. About 87% of cervical cancer deaths occur in developing countries, mainly in Melanesia, South America, East and Middle Africa [1]. The clinical evidence has unequivocally shown that Human Papilloma Virus (HPV) DNA can be detected in cervical cancer specimens in 90-100% of cases, with HPV 16 and 18 as the most frequent serotypes [2]. Cervical cancer is of slow progression and, according to histopathological studies there are at least three well-defined stages preceding cervical squamous carcinoma, known as cervical intraepithelial neoplasia (CIN). These stages (CIN1, CIN2 and CIN3) correspond to the progressive invasion of the cervical epithelium from the basal cell layer to the surface of the squamous epithelium [3].

The leading tools used in cervical screening programs are the Pap smear and liquid-based cervical cytology. However, both methods are not only labor-intensive but are also highly subjective and have low sensitivity and specificity for the detection of some high-grade significant lesions [4].

A large number of biomarkers have been identified that are overexpressed in cervical cancer cells with potential for cervical cancer screening, such as p16^{INK4A}, Ki-67, Cyclin E, p53, and survivin, among

others [5-7]. P16^{INK4A} is a tumor suppressor protein which is induced in cells upon expression of high-risk HPV E7 protein [8] and several reports indicate that overexpression of p16 is associated with the process of carcinogenesis in the cervical epithelium [9].

Long noncoding RNAs (lncRNA) are defined as non-coding transcripts of more than 200 mts in length. LncRNAs lack protein coding capacity, although a few of them have the capacity to code for small peptides that had not been identified previously [10]. LncRNAs are emerging as key players in different biological process, including cancer. In fact, lncRNAs have an advantage over protein-coding genes since their expression is more tissue specific, thus making them attractive as novel diagnostic tools [11].

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Previously, we reported that human cells express a unique family of mitochondrial long ncmtRNAs (ncmtRNAs) [12,13]. One of these transcripts, named sense ncmtRNA (SncmtRNA) is expressed in normal proliferating cells and tumor cells but not in resting cells, suggesting a functional role in cell cycle progression [12]. Normal proliferating cells express, in addition to the S-ncmtRNA, two antisense mitochondrial ncmtRNAs (AS-ncmtRNA-1 and AS-ncmtRNA-2) where both AS-ncmtRNAs are down-regulated in human cancer cells regardless of tissue of origin. In order to analyze the expression pattern of these ncmtRNAs in cervical cancer, we performed a qualitative pilot study in cervical biopsies encompassing the progression of the disease. We found that AS-ncmtRNAs are down-regulated in mild, moderate and severe neoplasia and in invasive cervical carcinoma [14]. In the present study a quantitative analysis of the differential expression of the S-ncmtRNA and the ASncmtRNAs was performed in biopsies of CIN1 to CIN3 and invasive cervical carcinoma, correlating it with the expression of the tumor suppressor p16^{INK 4A} and proliferating cell nuclear antigen (PCNA).

Methods

Tissue specimens

All the tissues were purchased from Pantomics Inc. (Richmond, CA, USA), corresponding to Tissue Macroarrays (TMA) containing normal cervix, CIN1, CIN2, CIN3 and invasive squamous carcinoma (SCC). For normal specimens, RAFTs were obtained from the laboratories of L.L-V. and E.B. All specimens were analyzed by experienced pathologists (F.G and J.R) and the entire group classified as normal in the original TMA was reclassified as “non tumoral”.

Chromogenic in situ hybridization (ISH)

The slides were deparaffinized in 2 consecutive 5 min xylene washes. Afterward, sections were rehydrated in two 3 min washes each in 98%, 90% and 70% ethanol, and once in DEPC-treated water for 5 min. Sections were then incubated in 2.5 µg/ml of proteinase K (Invitrogen) at RT for 20 min and then washed twice for 3 min in DEPC-treated water. Hybridization was carried out with digoxigenin-labeled probes P1 (5' TGATTATGCTACCTTTGCACGGT 3') to detect the S-ncmtRNA and digoxigenin-labeled probe P2 (5' ACCGTGCAAAGGTAGCATAATCA 3') complementary to the AS-ncmtRNAs. As positive control, digoxigenin-labeled P3 probe (5' TGTAATTGGAATGAGTCCA 3'), complementary to 18S rRNA, was used.

A negative control was performed without probe (Mock)

Hybridization mixtures contained 35 pmoles/mL of the corresponding probe in hybridization solution (50% formamide, 150 µg/ml herring sperm DNA, 4X SSC, 1X Denhardt's solution and 2 mM EDTA). Hybridization was carried out overnight at 37°C. Post hybridization washes and color development were carried out as described before [10,14]. Afterwards, the sections were counterstained with contrast red (KPL).

Immunohistochemistry (IHC) for p16^{INK 4A} and PCNA

The sections were deparaffinized and hydrated as described above and permeabilization was performed for 20 min at 95°C in Target Retrieval Solution (DAKO) and then washed twice for 3 min in PBS. Afterwards, the sections were blocked in 2% goat serum in PBS for 30 min at RT. Detection of p16 was carried out using a 1:200 dilution of the corresponding antibody (Santa Cruz, USA) in blocking buffer

for 1hr at RT. Immunodetection was performed using Ultravision LP Detection System (Thermo Scientific) according to manufacturer specifications. Finally, the sections were counterstained with contrast blue (KPL). PCNA detection was carried out using the PCNA staining Kit (Invitrogen), according to manufacturer's instructions.

Annotation of ISH and IHC tissues

The staining pattern obtained by chromogenic ISH corresponds to an intense blue-purple staining of the cells. In order to evaluate the expression of ncmtRNAs, we analyzed the percentage of positivity and the signal strength of the cells using the software ImageProPlus 6.1. We defined as positive the presence of a blue reaction product in the nucleus or cytoplasm, or both, which was expressed as a percentage of Integrated Optical Density (IOD).

The intensity of IHC was manually evaluated and scored using an annotation system similar to that described before [15], with a four grade scale; where 0 denotes absence of signal, 1+ presence of weak signal, 2+ presence of moderate signal and 3+ presence of a strong signal.

Statistical analysis

Results of ISH quantitation were analyzed by the Student's t-test and represent average ± SEM. Significance (p-value) was set at the nominal level of p < 0.05 or less.

Results

Expression of p16 and PCNA during cervical cancer progression

Table 1 shows the pathological evaluation of specimens through H&E diagnosis. The immunohistochemical staining results are summarized in Table 2 and Figure 1. Both CIN 3 and invasive SCC tissues displayed a strong signal for both markers, while a varying degree of labeling intensity was observed in CIN1/2 (Table 2). For non-tumoral tissues, PCNA showed a positive signal in over 70% of samples (Figure 1 and Table 2). We observed that in the most severe lesions (CIN 3 and SCC) there is a strong staining for both PCNA and p16, corresponding to a nuclear pattern plus a diffuse cytoplasmic signal (Figure 1). For CIN 1/2 specimens, PCNA shows intense nuclear staining with low or no cytoplasmic staining (Figure 1), throughout practically all the epithelia. Most interestingly, the signal obtained for p16 in some tissues was very strong, with nuclear and diffuse cytoplasmic localization (Figure 1). In mild lesions such as CIN 1/2 or non-tumoral tissues, p16 is practically absent, 80% of tissues are negative for this marker and, PCNA shows a varying degree of staining with a strictly nuclear localization (Figure 1 and Table 2).

Evaluation of differential expression of ncmtRNAs

We evaluated the expression of the S-ncmtRNA and AS-ncmtRNA by colorimetric ISH in serial biopsy sections, throughout the progression of cervical cancer. As normal tissue we used organotypic (raft) cultures of keratinocytes. This system allows cells to proliferate and differentiate at an air-liquid interface and normal keratinocytes stratify and fully differentiate in a similar way to the normal squamous epithelial tissue [16]. The ISH shows that AS-ncmtRNA is expressed 1, 47-fold over S-ncmtRNA (Figures 2 and 3), similarly to previous results [12,13]. S-ncmtRNA is expressed at all stages of cervical cancer progression, from non-tumoral up to SCC (Figure 2), with a switch in cellular localization of the staining from nuclear (raft and non-tumoral) to mainly cytoplasmic from CIN 1/2 up to SCC.

Diagnosis by H&E						
Specimen type	Normal	“Non-tumoral”	CIN 1 – 2	CIN 3	Invasive SCC	Total
Punch biopsy	0 (0)	20 (20,8)	18 (18,7)	17 (17,7)	21(21,8)	93 (100)
RAFT	3 (3,1)	0 (0)	0 (0)	0 (0)	0 (0)	3 (100)
All cases	3 (3,1)	20 (20,8)	18 (18,7)	17 (17,7)	21 (21,8)	96 (100)

Values are presented as number (%). CIN: Cervical intraepithelial neoplasia; SCC: Squamous cell carcinoma; RAFT; Organotypic keratinocytes cultures

Table 1: Frequency of diagnostic criteria according to diagnosis established on H&E stained slides.

Diagnosis by H&E	p16				PCNA			
	0	1+	2+	3+	0	1+	2+	3+
Normal	0 (0)						3 (100)	
Non tumoral	16 (80)	4 (20)	0 (0)	0 (0)	5 (25)	4(20)	7 (35)	4 (20)
CIN 1 – 2	8 (44)	3 (17)	0 (0)	7 (39)	4 (22)	2 (11)	7 (39)	5 (28)
CIN 3	1 (6)	0 (0)	0 (0)	16 (94)	0 (0)	0 (0)	4 (24)	13 (76)
Invasive SCC	1 (4,7)	0 (0)	0 (0)	20 (95,3)	0 (0)	0 (0)	2 (9,5)	19 (90,5)

Values are presented as number (% row). PCNA: Proliferating cell nuclear antigen; CIN: Cervical intraepithelial neoplasia; SCC: Squamous cell carcinoma

Table 2: Intensity of p16 and PCNA immunohistochemical staining of H&E-diagnosed tissues.

AS-ncmtRNA was down-regulated in 90% of non-tumoral tissues, and for CIN 1/2, CIN 3 and invasive SCC, down-regulation of AS-ncmtRNA was observed in 94%, 95% and 95% of tissues, respectively (Figures 2 and 3). The color staining pattern of each tissue was quantified using the Image Proplus 6.1 software and expressed as percentage of Integrated Optical Density (IOD). Signal quantification in all samples of different groups (non-tumoral, CIN 1/2, CIN 3 and invasive SCC) show a strong and robust down-regulation of the AS-ncmtRNA as early as CIN 1 (Figure 3). However, in the tissues classified as non-tumoral (see Discussion) a 5-fold reduction of AS-ncmtRNA was observed, which was more marked in tissues corresponding to CIN 1/2, where a 12-fold reduction was observed. At CIN 3 this reduction was only 6-fold, corresponding to half of CIN 1/2. Finally, for invasive SCC, a 4-fold reduction in the AS-ncmtRNA, compared to S-ncmtRNA is observed (Figure 3 and Table 3). In all analyzed tissues, an internal control was included; corresponding to 18S rRNA, in order to make sure that the quality of target RNA in the biopsy sections was well preserved (Figure 2).

Discussion

In a previous work we reported a qualitative study of the differential expression of the S-ncmtRNA and AS-ncmtRNA in tissue sections of CIN and cervical cancer [14]. We showed down-regulation of the AS-ncmtRNA as early as mild dysplasia (CIN 1). However, this previous study lacked the quantitative assessment of this differential expression in order to obtain evidence based on a more accurate study.

In the present analysis, we used commercially available Tissue Macroarrays (TMA), in order to perform a more accurate analysis of the expression of these ncmtRNAs. The reason for selecting this approach was because TMA offers a flexible way to analyze tissue expression of large gene sets and all the steps required for tissue permeabilization, antibody or probe incubation, washing steps and detection are identical in time, temperature, probe and antibody dilution, therefore maximizing the homogeneity of the procedure for all the samples under analysis [15].

After pathological evaluation, the entire group classified initially as “normal” in the original TMA were reclassified as “non-tumoral”, due to 1) presence of cervicitis, 2) difficulty to classify some tissues as normal or CIN 1 and vice-versa or 3) the clear observation of koilocytes in some tissues. These last types of cells correspond to typical HPV-infected [17]. Similarly, CIN 1 and CIN 2 were classified in one group

(CIN1/2) due to the low number of samples and some difficulties in the final stage annotation. Therefore, as normal tissues we used organotypic raft cultures of human foreskin keratinocytes (HFk) in order to obtain a normal proliferating epithelium and avoiding the issues related to miss-classification and/or early HPV infection in samples classified as normal.

Different biomarkers have been evaluated to determine their usefulness in cervical cancer diagnosis [16]. p16^{INK4a} (p16) protein indicates over-expression of the viral oncoproteins E6 and E7 and hence cellular transformation induced by HPV infection [8]. Recently, the College of American Pathologists included p16 immunohistochemistry to categorize lower anogenital tract squamous lesions [18]. Proliferating cell nuclear antigen (PCNA) is essential for nucleic acid replication and repair machinery and used as a surrogate marker for cell proliferation [19]. Its expression has been associated with severity and progression of cervical neoplasia [20].

In the present work we found that both p16 and PCNA protein expression correlated well with the progression of cervical neoplasia (Figure 1). Some exceptions are provided for p16 expression in the CIN 1/2 group, where only 39% of tissues showed strong labeling, in contrast to other published studies where over 75% even 100% of tissues displayed high expression [20-22]. A plausible explanation for this discrepancy may be because a certain percentage of infections are thought to be caused by low-risk HPV types. Because the affinity of the E7 protein from low-risk HPV for Rb is much lower than that of high-risk HPV types, in this case there would not be an overexpression of p16^{INK4a} [23]. In CIN 3 as well as invasive SCC a strong staining of over 90% was obtained, in accordance with previous reports [24,25].

For PCNA, an increase in the degree of staining was observed in correlation to the increase of the cervical lesions CIN 1/2 (29% 3+), CIN 3 (76% 3+) and SCC (90.5% 3+), in agreement with previous reports [23,26]. However, it is important to mention that the expression of PCNA in normal and non-tumoral tissues in this work and others [27,28] suggest that the role of PCNA is more suitable as a marker for proliferation activity than as a marker for cancer progression.

Early down-regulation of AS-ncmtRNA is observed in CIN 1/2, and maintained throughout the progression of the disease (Table 3). However, due to the low number of samples analyzed in this study it is not possible to conclude with accuracy if the variations on the fold of down-regulation of AS-ncmtRNA in the different stages reflect

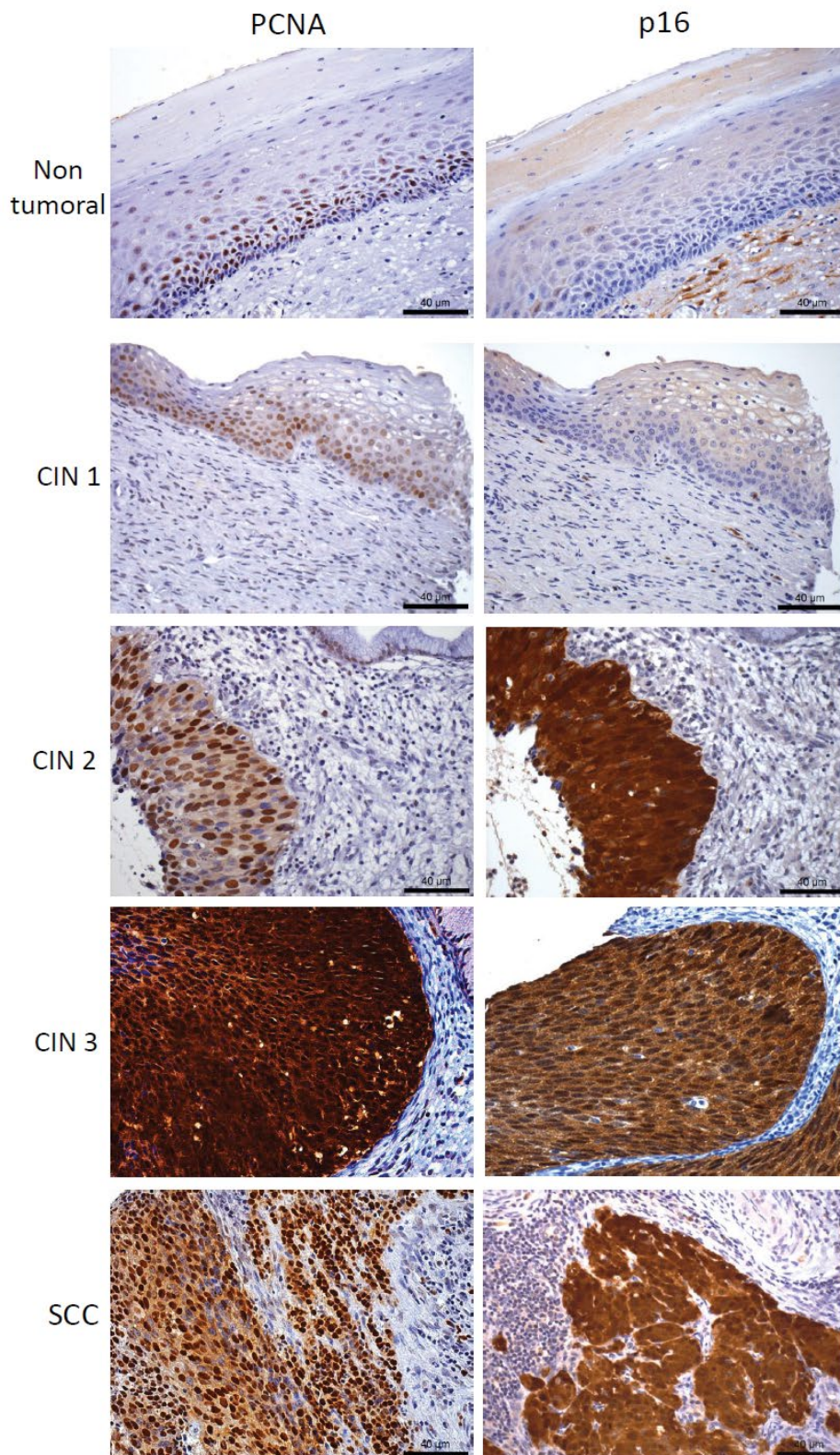


Figure 1: Representative image of immunohistochemical analysis of p16INK4a (p16) and proliferating cell nuclear antigen (PCNA) expression in cervical tissue (x200). Non-tumoral and CIN 1 show nuclear PCNA staining with absence of p16 signal. Varying degree of staining intensity was observed in cervical intraepithelial neoplasia (CIN 1/2). Both CIN 3 and invasive squamous cell carcinoma tissues displayed diffuse and strong staining for all markers.

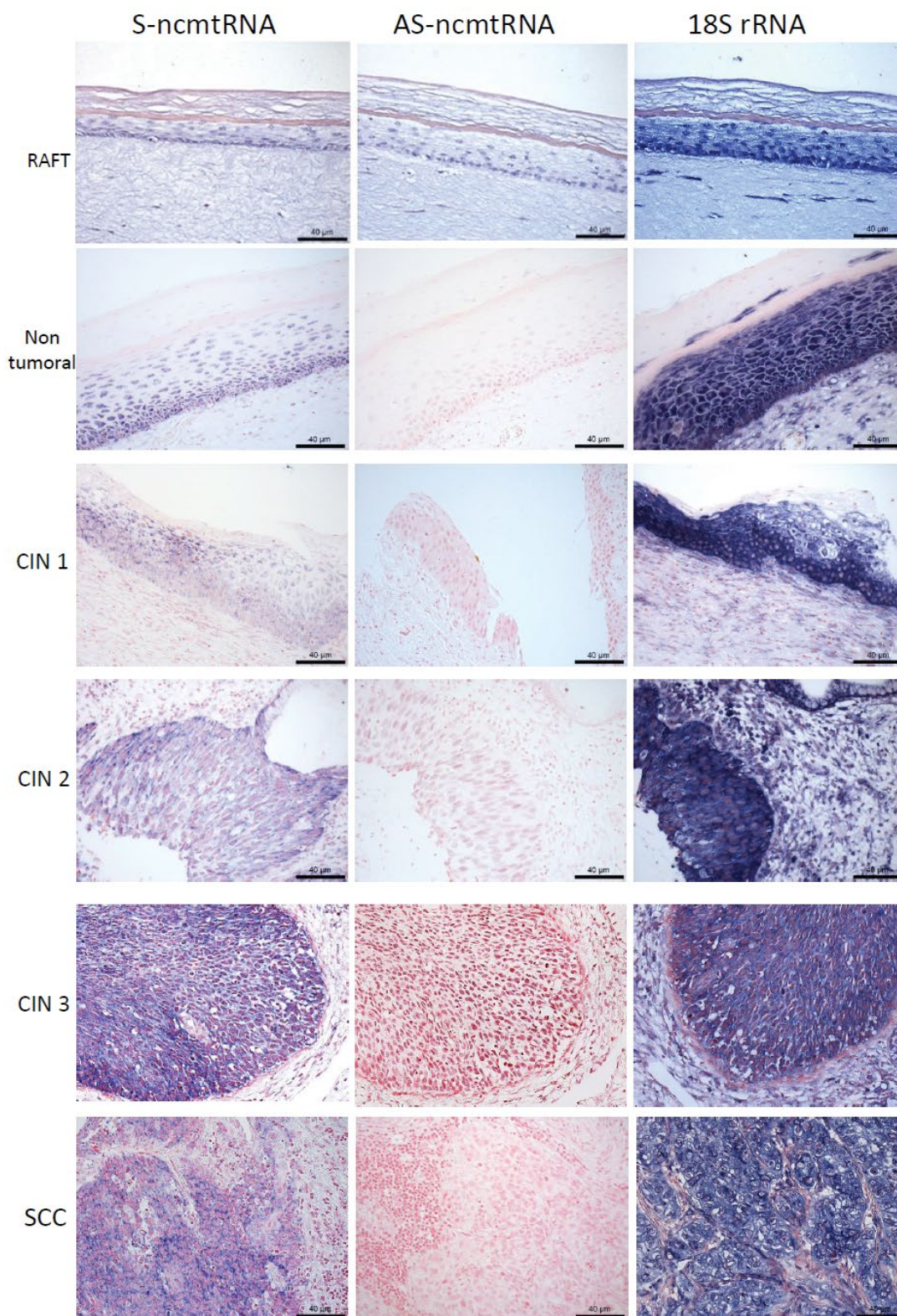


Figure 2: Representative image of in situ hybridization analysis for S-ncmtRNA and AS-ncmtRNA during cervical cancer progression (x200). Normal RAFT organotypic cultures showed expression of both transcripts. Non-tumoral samples showed down-regulation of AS-ncmtRNA. In stages CIN 1, CIN 2, CIN 3 and invasive squamous cancer (SCC), a clear cytoplasmic staining is observed for S-ncmtRNA with down-regulation of AS-ncmtRNA in all these stages. As positive control; parallel sections of each tissue were hybridized with a probe complementary to 18S rRNA.

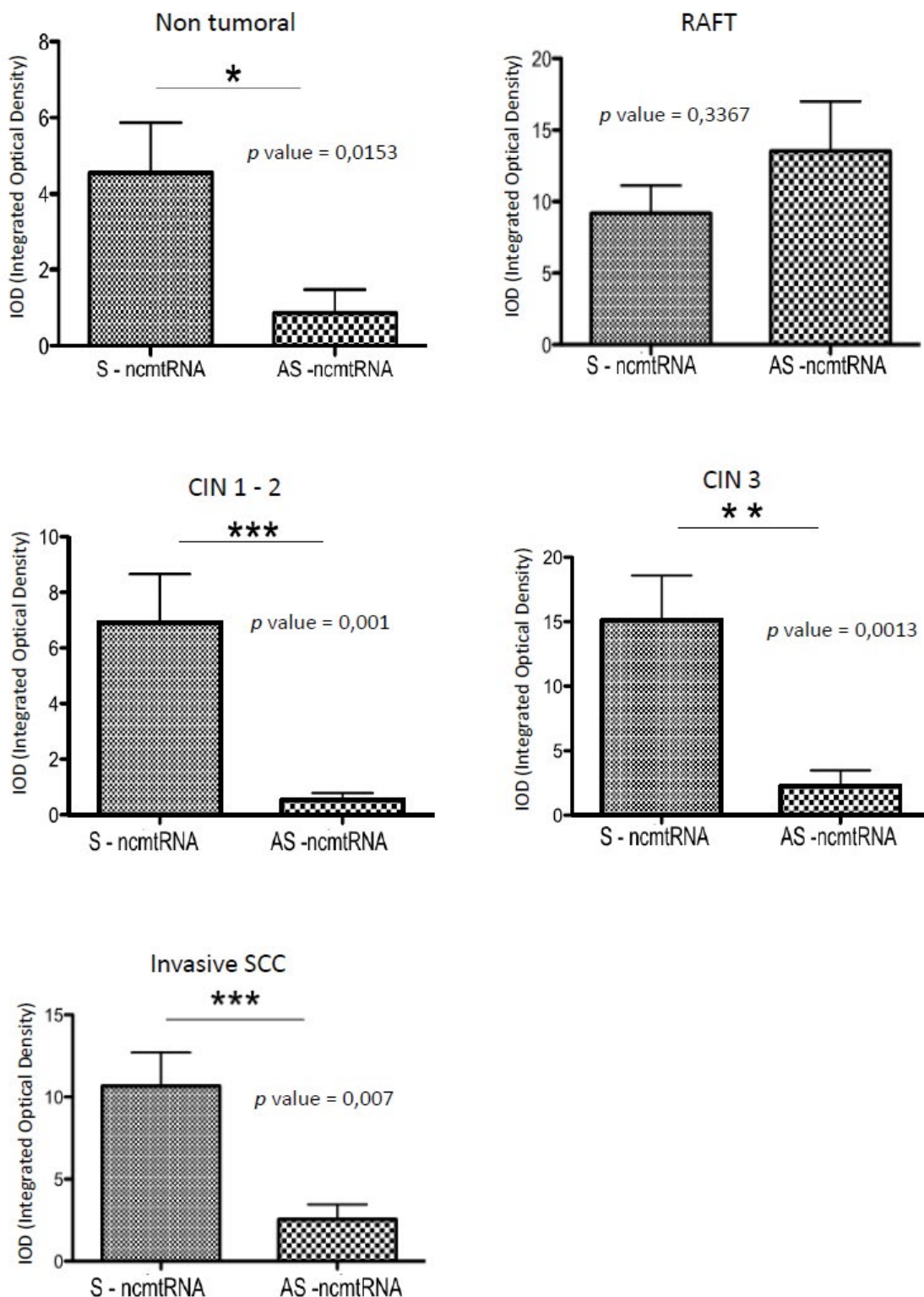


Figure 3: Quantitative Analysis of the expression S-ncmtRNA and AS-ncmtRNAs in samples representing cervical cancer progression. Non-tumoral samples showed a lower expression of AS-ncmtRNA with a significant difference (p-value = 0.0153), whereas normal raft organotypic culture showed high expression of both transcripts with no significant difference (p-value = 0.3367). A significant lower expression of AS-ncmtRNA was observed in CIN 1 and CIN 2 (p-value = 0.001), CIN3 (p-value = 0.0013) and invasive squamous cancer samples (p-value = 0.007). Down-regulation of AS-ncmtRNA was observed in all samples from the three later stages.

Diagnosis	Number cases	Mean ± SEM of S-ncmtRNA	Mean ± SEM of AS-ncmtRNA	Fold of down-regulation AS-ncmtRNA	p value
Normal (RAFT)	3	9.178 ± 1.947	13.53 ± 3.482	0.678 ± 0.559	0.3367
Non tumoral	20	4.551 ± 1.320	0.8635 ± 0.603	5.27 ± 2.18	0.0153
CIN 1 – 2	18	6.906 ± 1.749	0.5477 ± 0.234	12.6 ± 7.47	0.001
CIN 3	17	15.13 ± 3.442	2.275 ± 1.205	6.65 ± 2.85	0.0013
Invasive SCC	21	10.67 ± 2.029	2.554 ± 0.897	4.17 ± 2.26	0.0007

Noncoding mitochondrial RNAs and cervical cancer progression

Table 3: Summary of the quantification of AS-ncmtRNA down-regulation throughout cervical cancer progression.

the reality of AS-ncmtRNA negative regulation. The exception of the characteristic stages of cervical cancer progression is represented by the non-tumoral group, where a down-regulation of AS-mtncRNA is also evidenced. However, in the case of a true normal proliferative condition, represented by the raft model, expression of both ncmRNAs was observed, according to previous results in different models, including cervix [13,29,30].

Finally, the role of lncmtRNAs in cellular physiology is still limited but a growing body of evidence suggests that these noncoding RNAs may play different roles in normal and pathological conditions and could be used as novel biomarkers in several diseases. Recently, in a very exciting review, Dietrich [31] pointed out the emerging different roles of organellar noncoding RNAs. The author indicates that several groups have reported the presence of miRNAs in the mitochondria and their relationship with cancer [32,33]. This review also describes the finding of mitochondrial lncRNAs in different cell types such as HeLa cells, breast cells and ventricular tissue [34,35,36], fulfilling different roles as regulators of translation or as novel biomarkers of disease progression. Among these, LIPCAR (Long Intergenic noncoding RNA Predicting CARDiac remodeling) is worthy of mention, as it is up-regulated at late stages of left ventricular remodeling and is elevated in patients with chronic heart failure, suggesting this mtlncRNA as a prognostic indicator for cardiovascular mortality [37].

Therefore, despite the limitations of this study, our results point out that the differential expression of the ncmRNAs correlated with the expression of p16 protein, could constitute the basis for the development of a novel diagnostic procedure for cervix cancer in a future hypothesis that warrants further investigation.

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