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MicroRNA-551b expression profile in low and high-grade cervical intraepithelial neoplasia

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Abstract. – OBJECTIVE: To evaluate the expression of microRNA (miR)-551b in patients with low and high grade cervical intraepithelial neoplasia (CIN) and to find an association with high-risk Human Papillomavirus (HR-HPV) infection-related prognostic biomarkers.

PATIENTS AND METHODS: The expression level of miR-551b was determined in 50 paraffin-embedded cervical specimens (10 normal squamous epithelium, 18 condylomas, 8 CIN1, and 14 CIN2-3) using quantitative Real-time polymerase chain reaction (qRT-PCR). χ^2-test compared miR-551b expression in different diagnosis groups. An Ordered Logistic Regression and a Probit correlation were made to correlate miR-551b expression levels with the cervical tissue histological findings. The immunohistochemical distribution of p16 and Ki-67 according to histopathological findings was also assessed.

RESULTS: The distribution of the miR-551b expression profile was significantly lower in CIN1-3 samples compared to other histological diagnosis groups (condyloma and negative). The expression levels were inversely correlated to the cervical pathological grade, from negative to CIN2-3. A 1% increase in miR-551b expression level produced an increase of 19% to the probability of a minor histological grade diagnosis in a range from negative to CIN2-3 and an increase of 13% to the probability of a negative histological grade diagnosis. Among the cases with miR-551b expression < 0.02 (considered as cut-off value) a significant statistical correlation was found between p16 and Ki-67 expression and the diagnosis of CIN2-3.

CONCLUSIONS: Our data showed a significant inverse correlation between miR-551b expression and the histological grading of the lesions, suggesting a tumor suppressive function in the different stages of cervical dysplasia.

Key Words
MicroRNA, miR-551b, Cervical intraepithelial neoplasia, p16, Ki-67.

Introduction

Cervical cancer is one of the very few cancers where a precursor stage lasts many years before becoming invasive cancer, providing a large opportunity for primary and secondary prevention, early detection and treatment. Unfortunately, although preventable, there is still a large number of women who die of cervical cancer in many countries (over 265,000 estimated deaths worldwide), with a large majority occurring in the less developed regions[1].

Persistent infection with high-risk Human Papillomavirus is the necessary but not the sufficient cause of cervical cancer. While high grade cervical intraepithelial neoplasia (CIN2-3) can be considered the true precursor of cervical cancer, low-grade cervical intraepithelial neoplasia (CIN1) and some CIN2 represent the cytopathological effect of a productive viral infection.

Women can develop CIN at any age; however, the age range associated to the higher prevalence is between 25 and 35 years[2]. Most cases of CIN remain stable, or undergo a presumably immunologically mediated spontaneous complete regression without the need of treatment. However, a small percentage of cases progresses to invasive cervical cancer. Estimates of regression and progression rates and the proportion of women who require treatment are needed. Because there is no validated method to predict the likelihood of histopathological regression or progression, the standard of treatment for CIN2-3 is the lesion excision[3]. An increasing number of biomarkers, such as p16, HPV genotype persistence, Ki-67, and mRNA are found to be important in the management of cervical pathology. The transforming HR-HPV infection of the cervical epithelium is characterized

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by the increased expression of E6 and E7 genes responsible for an uncontrolled cell proliferation, immortalization and genomic instability. While E6 acts through the inhibition of p53 and the raise of telomerase activity, E7 inactivates Rb (tumor suppressor protein retinoblastoma) and increases the transcription factor E2F inducing cell proliferation. Therefore, the enhanced expression of proliferation markers, such as Ki-67 and p16, a protein that promotes cell cycle arrest through Rb hyperphosphorylation prevention, is the consequence of Rb inactivation mediated by E7 gene. Among the epigenetic changes HR-HPV modifies the host DNA methylation status and aberrant miRNA expression silencing of tumor suppressor genes and overexpression of oncogenes[4].

A class of promising non-invasive biomarkers to identify cancer at an early stage is represented by microRNAs (miRNAs). They are a group of short, non-coding, single-stranded RNAs involved in the regulation of gene expression at the post-transcriptional level by binding to complementary sites in the 3' untranslated region (3'-UTR) of specific mRNA transcripts, causing their degradation or translational repression. They may act as oncogenes or tumor suppressor genes depending on the targeted mRNAs, and themselves can be regulated by oncogenes or tumor suppressors[5,6]. Experimental evidence indicates that they play a crucial role on cell proliferation, differentiation, apoptosis, organ development and immune response in multicellular organism[7].

A growing number of dysregulated miRNAs has been documented in a variety of human cancer including different stages of CIN and cervical cancer[8-23]. Numerous miRNAs are in sites of genomic instability closed to the HPV integration hotspots and may be influenced by the integrated HPV DNA. Thus, characterization of altered miRNAs resulting from DNA copy number aberration could improve our understanding of tumor initiation and progression as well as provide molecular markers for early detection, prognosis, and therapy.

The microRNA-551b expression in different cancers[8-20] is shown in the Table I. This miRNA is located in the long arm of chromosome 3 (3q26), a region amplified in 77-90% of carcinomas of the cervix[21-23]. While the coding genes in the 3q26.2 amplicon are well studied, the potential role of miRNAs located at the 3q26 locus remains poorly characterized.

To the best of our knowledge, no studies exist about miR-551b and cervical preneoplastic lesions or cervical cancer. Thus, we investigated the miR-551b expression profile in the histological samples of patient with normal squamous epithelium, condyloma, CIN1, and CIN2-3 to evaluate its clinical hypothetical role in the management of these lesions.

Patients and Methods

From March to September 2013, 40 patients with abnormal cervical cytology and 10 negatives for cervical squamous intraepithelial neoplasia or abnormal cytology, were enrolled at the Colposcopy and the Low Genital Tract Pathology Unit at Sant'Andrea Hospital, Sapienza University of Rome.

We excluded women previously treated for cervical or vaginal intraepithelial lesions, those that had previously undergone total hysterectomy, chemotherapy and/or radiotherapy for gynecological or other cancers, and patients with acute genital infections, immunological disorders or HIV positivity.

The cytological exam was reported according to the Bethesda System 2011. All patients were submitted to colposcopy that was always performed by the same operator (L.A.). Cases with abnormal cervical-vaginal cytology had all adequate colposcopy and, in each case, a biopsy was obtained from the most significant colposcopy pattern. Instead, in cases with negative cytology and colposcopy, the colposcopist obtained the cervical biopsy sample directly from the uterus after a hysterectomy that was performed because of benign conditions such as fibromatosis, typical endometrial hyperplasia, adenomyosis or uterine prolapse.

Fifty cervical biopsy samples were processed and histological diagnoses were classified as follows: 10 negatives for cervical squamous intraepithelial lesions (normal squamous epithelium), 18 condylomas, 8 CIN1 and 14 CIN2-3. Microscopic evaluation was performed according to the CIN classification (CIN1, CIN2, and CIN3). This study was ethically approved by the local Institutional Review Board and all patients gave their informed consent.

RNA Extraction and qRT-PCR

Total RNA obtained by formalin-fixed paraffin-embedded tissue from 50 patients was isolated with High Pure miRNA Isolation Kit, in accordance with manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA). RNA concentrations were determined with a NanoDrop (Thermo Scientific, Waltham, MA, USA).

Table I. MicroRNA-551b expression in different human cancers.

Study	Sample	Evidence
Song, et al[8]	Epithelial-mesenchymal transition (EMT) and metastasis of gastric cancer	miR-551b inhibits EMT and metastasis in gastric cancer by inhibiting ERBB4. miR-551b and ERBB4 are thus potential therapeutic targets for the treatment of gastric cancer.
de Leeuw DC, et al[9]	Acute myeloid leukemia cells	High expression of miR-551b was highly predictive for poor overall survival and correlated with decreased complete remission rate after the first cycle of standard-dose remission induction therapy compared to patients with low expression.
Wei, et al[10]	Ovarian serous cystadenocarcinoma	miR-551b was up-regulated and correlated with the pathological grade of the malignancy. The inhibition of miR-551b increased the susceptibility to cisplatin and prolonged the survival of the host mice.
Chaluvally-Raghavan, et al[11]	High grade serous ovarian cancer	miR-551b was up-regulated and raised STAT3 mRNA levels, increasing proliferation of ovarian cancer cells and engendering resistance to chemotherapy.
Lin, et al[12]	Lung adenocarcinoma	miR-551b expression positively correlated to patient survival as an independent prognostic marker.
Kim, et al[13]	Terminal respiratory unit (TRU) and non-TRU types adenocarcinoma	miR-551b was down-regulated in non-TRU as compared to TRU-type adenocarcinoma.
Chen, et al[14]	Gastric cancer	miR-551b-3p was significantly downregulated in the gastric cancer tissues, and this downregulation was closely correlated with the degree of differentiation, TNM stage, and lymph-node metastasis.
Chong, et al[15]	Primary and recurrent serous cystadenocarcinoma	miR-551b was over-expressed in recurrent epithelial ovarian cancer and may be used as biomarkers for prediction of the recurrence of epithelial ovarian cancer.
Haldrup, et al[16]	Serum samples from prostate cancer, benign prostatic hyperplasia and control patients	A panel of circulating markers including several miRs (miR-562/miR-210/miR-501-3p/miR-375/miR-551b) was able to identify 84% of patients with prostate cancer
Xu X, et al[17]	Lung cancer cells with acquired chemoresistance	Increased miR-551b expression in cells with acquired apoptosis resistance inhibited the expression of catalase, potentiating reactive oxygen species (ROS) accumulation and the over-expression of the oncoprotein Mucin-1.
Swierniak, et al[18]	Papillary thyroid carcinoma and healthy thyroid specimen	miR-551b-3p was down-regulated in papillary thyroid carcinoma tissue compared with unaffected tissue adjacent to but not infiltrated by tumor from the same patient and non-cancerous thyroid specimen.
Huang, et al[19]	Papillary thyroid carcinoma and paracancerous normal tissue	miR-551b was up-regulated compared to normal tissue.
Dettmer, et al[20]	Follicular variant of papillary thyroid carcinoma and normal tissue	miR-551b was up-regulated in follicular variant of papillary thyroid carcinoma compared to normal tissue.

All quantitative Real-time polymerase chain reaction (qRT-PCR) were performed with Brilliant II QRT-PCR, AffinityScript Two-Step (Stratagene Products Division, La Jolla, CA, USA). Mature hsa-miR-551b was assayed using the Taq-Man MicroRNA Assays, in accordance with manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Briefly, RNA was reverse transcribed using miRNA-specific stem-loop primers (Applied Biosystems, Foster City, CA, USA). The mix was incubated at 16°C for 15 min, 42°C for 60 min and 75°C for 5 min in Applied Biosystems 9600 system. Subsequently, Real-time PCR was carried out on a MX3500P QPCR System (Agilent Technologies, Santa Clara, CA, USA). Samples were normalized to U6 snRNA (Applied Biosystems). Comparative Real-time PCR was performed in triplicate, including no-template controls. Relative expression was calculated using the comparative Ct method[20].

Immunohistochemistry[24]

The $p16^{INK4A}$ assessment was performed using a set of two different reagents (CINtec® *PLUS* Kit Roche, MTM Laboratories AG, Heidelberg, Germany): a primary monoclonal rabbit antibody clone 274-11AC3 directed against human Ki-67 protein, and a primary monoclonal mouse antibody clone E6H4 directed to human $p16^{INK4a}$ protein. A two-step immunohistochemical assay was performed: after microwave antigen retrieval and endogenous peroxidase block, a cocktail of the primary antibodies was added for 30 minutes at room temperature. Visualization reagents comprising a polymer reagent conjugated to horseradish peroxidase and goat anti-mouse antibody (15 min) and a polymer reagent conjugated to alkaline phosphatase and a goat anti-rabbit antibody (15 min) were used. The samples were then developed with two different chromogen reactions based on horseradish peroxidase-mediated conversion of a DAB chromogen (10 min), and alkaline phosphatase-mediated conversion of Fast Red chromogen (15 min) to visible reaction products at the respective antigen sites. A squamous cell carcinoma of the cervix with known positive staining for $p16^{INK4A}$ was used as the positive control; an immunoglobulin class-matched nonimmune antibody was substituted for the primary antibody in the negative control. After counterstaining, a two-step mounting protocol was applied: in a first step an aqueous mounting medium and, subsequently, a permanent mounting medium were used. The results were evaluated by light microscopy. The immunohistochemical procedure causes two different colored reaction products: a red staining of nuclei, which indicates Ki-67 expression and a brown precipitate (cytoplasm and/or nuclei) at the $p16^{INK4a}$ antigen sites.

Immunohistochemical Scoring

p16 was considered as a positive test result when a diffuse staining pattern (continuous staining of cells of the basal and parabasal cells layers, with or without staining of cells of superficial cells layers) was observed; a focal staining pattern (staining of isolated cells or small cells clusters) or a negative staining pattern was regarded as negative.

Scoring for Ki-67 included nuclear staining only, and was scored as 0 (no staining), + (focal basal/parabasal staining), ++ (diffuse staining confined to the bottom third), +++ (diffuse staining of the whole epithelium). Our study considered 0/+ and ++/+++ staining as negative or positive Ki-67 expression, respectively.

Statistical Analysis

To determine if there is any significant variation in miR-551b expression profile between histological diagnosis of negative, condyloma, CIN1 and CIN2-3 we applied the Chi-square test $(\chi)^2$ comparing different diagnosis groups in relation to the cut-off of miR expression profile. An Ordered Logistic Regression and a Probit correlation were made to identify if miR-551b expression profile was correlated to the cervical tissue histological grade while the age at diagnosis was used as control. The p-value < 0.05 was considered as statistically significant. Statistical data were obtained using the statistical program STATA 12 SE (StataCorp LLC, College Station, TX, USA).

Results

The mean age of the sample was 35.98 ± 9.5 years. The correlation between miR-551b expression and histological diagnosis in cervical samples showed a rapid increase of the expression at $\geq$ 0.02 level of the miR-551b distribution values that was considered the cut-off level (Figure 1).

The difference in the distribution of the miR-551b expression profile was significantly lower (p=0.05) in CIN1-3 samples compared to other histological diagnosis groups (condyloma and negative). Among the 11 cases with miR-551b expression $\geq$ 0.02, 9 (81.8%) had the histological diagnosis of condyloma or normal cervical tissue. Conversely, no significant statistical difference was found in the miR-551b expression profile between the following subgroups: CIN2-3 versus negative+condyloma+CIN1; CIN1 versus negative+condyloma+CIN2-3, condyloma versus negative+CIN1-3; negative versus condyloma+CIN1-3 (Figure 2). Moreover, the analysis of the distribution of the miR-551b expression profile within different histological groups through an "Ordered Logistic Regression" showed that the expression level of miR was inversely correlated to the cervical pathological grade, from negative to CIN2-3 (Figure 3). A 1% increase in miR-551b expression level produced an increase of 19% (p = 0.040) to the probability of a minor histological grade diagnosis in a range from negative to CIN2-3 (Table II). Probit regression analysis indirectly validated this result, showing that a 1% increase in miR-551b expression level produced an increase of 13% (p = 0.024) to the probability of a negative histological grade diagnosis (Table III). No significant correlation was found between the

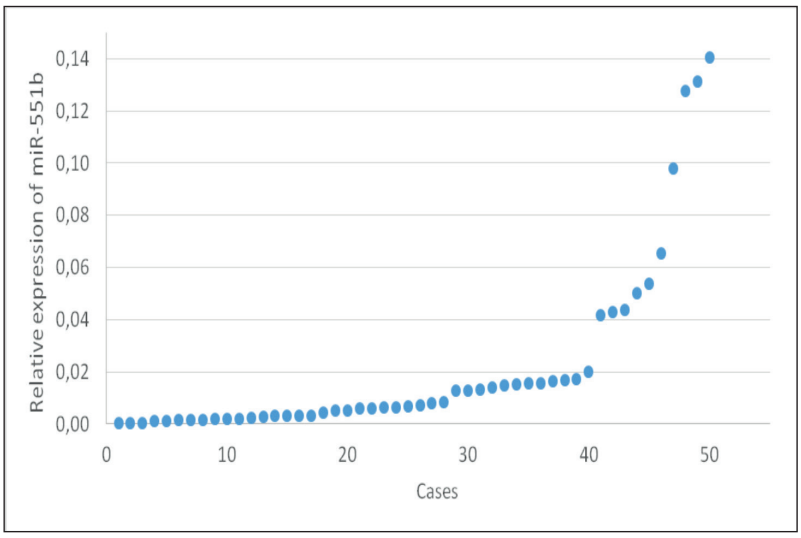

Figure 1. Distribution of the miR-551b expression levels.

miR-551b expression levels and the patient's age ($p = 0.064$ and $p = 0.282$; Tables II and III).

The immunohistochemical distribution of p16 and Ki-67 according to histopathological findings are summarized in Table IV. Overall, 25 of 50 cases (50%) were positive for p16 expression, assessed by p16/Ki-67 immunoreaction. Positive dual-staining immunoreactivity was mainly found in high-grade lesions (93%) while condyloma lesions and CIN1 were positive in 39% and 25%, respectively. Regarding the proliferation index, the diagnostic categories with the higher Ki-67 score were represented by CIN2-3 (71%) and CIN1 lesions (62%) while condylomas showed decreasing proliferating score (33%). Considering the 39 cases with miR-551b expression < 0.02 a significant statistical correlation was found between p16 expression and CIN2-3 ($p = 0.001$) and between Ki-67 expression and CIN2-3 ($p = 0.039$) (Table V). Among the 11 cases with miR-551b expression ≥ 0.02 no significant statistical correlation was found between histological diagnosis

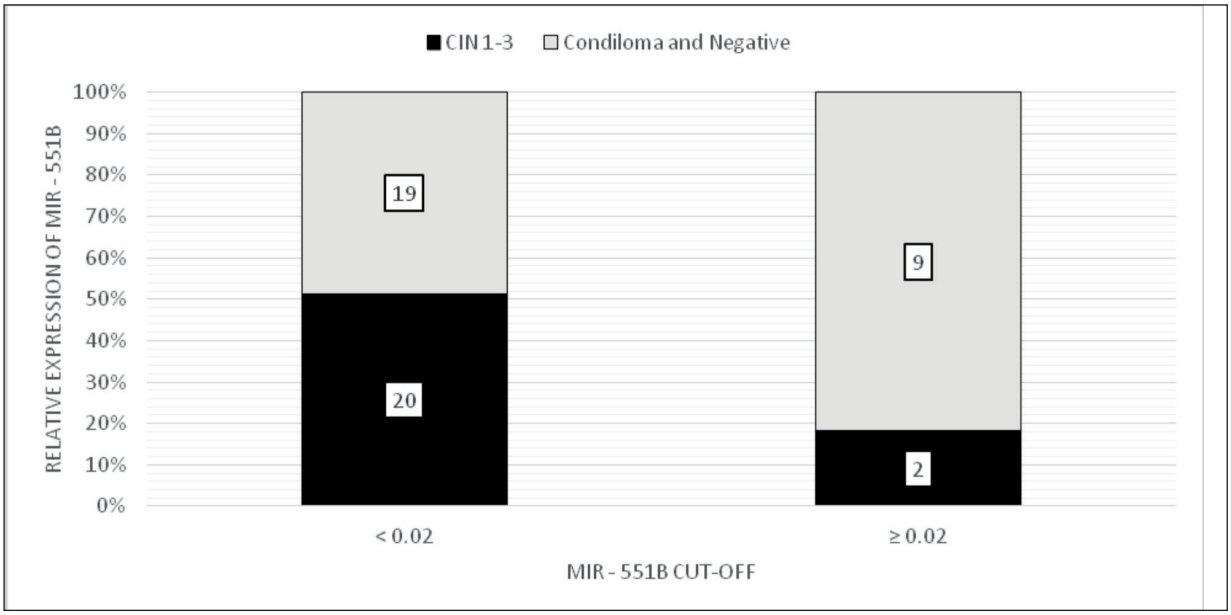

Figure 2. Correlation between miR-551b expression profile and histological diagnosis of cervical samples. Y-axis: the expression profile of miR-551b is represented in percentage. X-axis: the cut-off <0.02 and ≥0.02 of miR-551b (p=0.05; Pearson χ^2=3.8151).

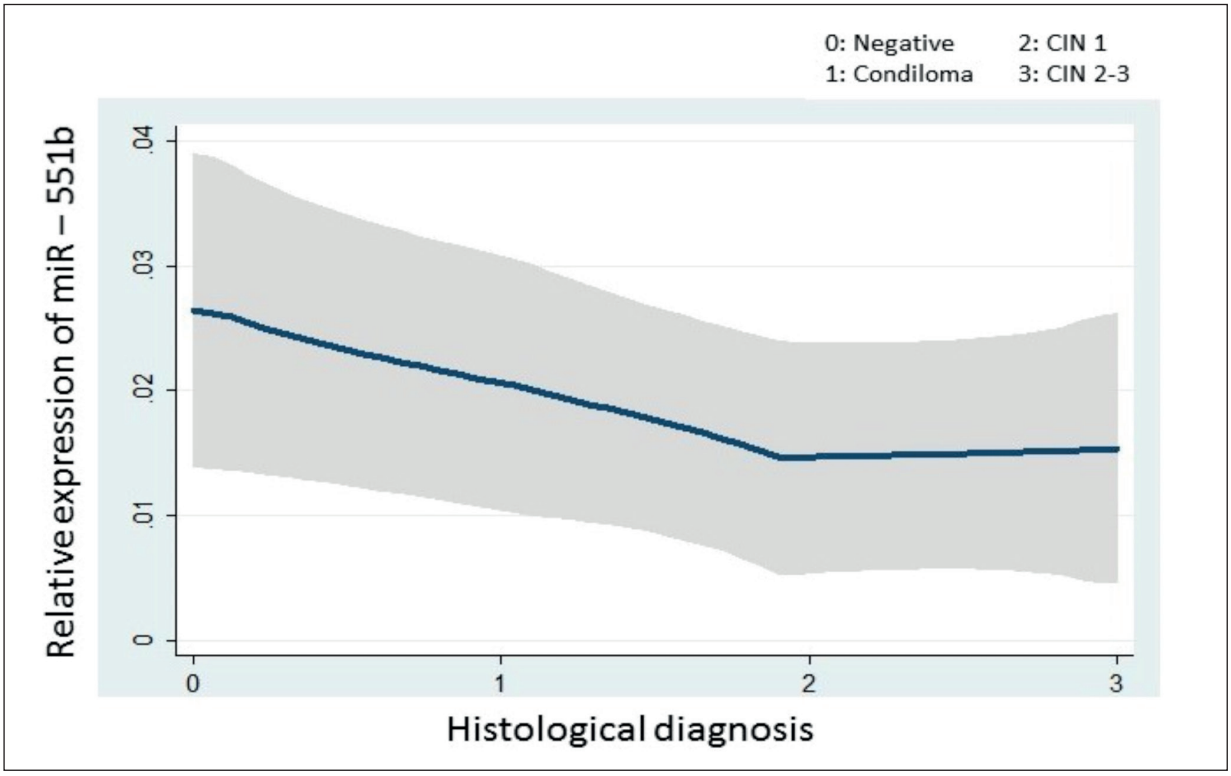

Figure 3. Order Logistic Regression: correlation between miR-551b expression profile and histological diagnosis of cervical samples. Y-axis: the relative expression of miR-551b, X-axis: histologic diagnosis of negative (0), condyloma (1), CIN1 (2), CIN2-3 (3).

and p16 expression. The 2 cases of CIN2-3 with miR-551b expression ≥ 0.02 showed no expression of Ki-67 and only one was p16 positive.

Discussion

To date, the prognostic value of miRNAs in cervical cancer genesis has not been fully investigated. Recent studies[21,25,26] indicate that multiple miRNAs have altered expression in Human Papillomavirus (HPV) integrated cervical cancer cells compared with HPV-negative cervical cancer cells or normal cervical tissues, revealing potential interactions between dysregulated miRNAs and HPV oncoproteins. In addition, altered miRNA expression profiles have also been reported in CIN and carcinomas as compared with normal cervix[21,27-36].

We focused on miR-551b because its gene is located on the long arm of the chromosome 3 which is amplified in several cases of cervical cancer. We found that an expression of miR-551b higher than 0.02 increased the probability of "negative" histological finding. Moreover, our data showed a significant inverse correlation between miR-551b expression and the histological grading of the lesions (condyloma, CIN1, CIN2-3), suggesting a tumor suppressive function.

These findings agree with a recent systemat-

Table II. Ordered Logistic Regression between miR-551b expression levels and histological diagnosis subgroups (negative, condyloma, CIN1, CIN2-3).

Variable	Coefficient	Z	*p*-value
miR-551b	-0.1988	-2.05	0.040
Age (patient's age at diagnosis date)	-0.0503	-1.85	0.064
Total cases 50			

Table III. Probit correlation between negative and other histological diagnoses (condyloma, CIN1, CIN2-3) with miR-551b expression levels.

Variable	Coefficient	Z	*p*-value
miR-551b	0.1327	2.27	0.024
Age	0.0246	1.07	0.282
Constant factor	-2.0854	-2.35	0.019
Total cases 50			

Table IV. Correlation between p16 and Ki-67 and miR-551b expression according to histological diagnoses.

Histology	p16+/tot	Ki-67+/tot	miR-551b	
			<0.02/tot	>0.02/tot
Negative	3/10 (30%)	4/10 (40%)	6/10 (60%)	4/10 (40%)
Condyloma	7/18 (38.9%)	6/18 (33.3%)	13/18 (72.2%)	5/18 (27.8%)
CIN1	2/8 (25%)	5/8 (62.5%)	8/8 (100%)	0/8 (0%)
CIN2-3	13/14 (92.8%)	10/14 (71.4%)	12/14 (85.7%)	2/14 (14.3%)
Total	25/50 (50%)	25/50 (50%)	39/50 (78%)	11/50 (22%)

ic study of He et al[21] showing differentially expressed miRNAs in consecutive stages of cervical cancer development. The authors reported very few changes in miRNA expression in CIN1 compared to normal tissue, whereas in CIN2 and CIN3 more significant changes in miRNA expression were observed, indicating a "miRNA signature" associated with different levels of cellular and nuclear abnormalities.

In some reports[21,35,37,38], HPV E7 was reported to modulate the expression of miR-15a, miR-15b, miR-16, miR-20a-5p, miR-155, miR-203 levels via E2F transcription factors.

Like other investigated miRNAs[36-44], we can postulate that high-risk HPV infection may affect the miR-551b expression through epigenetic changes mediated by oncoproteins E6 and E7. These two proteins inactivate p53 and retinoblastoma (RB1) protein respectively, leading to uncontrolled cell growth. E7 mediates degradation of RB1 protein releasing E2F transcription factor which induces transcription of many genes that control cellular proliferation.

The role of common single nucleotide polymorphisms (SNPs) of E2F transcription factors 1 and 2 (E2F1 and E2F2) in association with risk of squamous cell carcinoma of the head and neck (SCCHN) was evaluated. The authors concluded that E2F1 and E2F2 genetic variants might jointly play important roles in head and neck carcinogenesis[44].

Table V. Distribution of p16 and Ki-67 expression between different histological subgroups (negative/condyloma/CIN1 and CIN2-3) with miR-551b expression < 0.02.

	< CIN2-3 (n=27) No. (%)	CIN2-3 (n=12) No. (%)
p16		
–	16 (59.3)	0 (0)
+	11 (40.7)	12 (100)
	Chi² = 12.06, p = 0.001	
Ki-67		
–	14 (51.9)	2 (16.7)
+	13 (48.1)	10 (83.3)
	Chi² = 4.25, p = 0.039	

In relation to the p16 expression as a biomarker of high-risk HPV infection, p16 positivity in condyloma and CIN1 cases was 34.6%. This percentage was slightly smaller than both what stated in literature from metanalysis of Tsoumpou et al[45] (resulting in 45%) and what included in one of our previous study[46] which showed a positivity level for low-grade lesions of 40.2%. In our work, the p16 expression was useful to discriminate low-grade lesions from CIN2-3, showing a strong sensitivity (93%), but it resulted rather weak in differentiating negative cases (30%) from low-grade lesions (34.6%). Such conclusion is shared with data from the literature, in particular Galgano et al[47] reported that p16 was useful to distinguish high-grade lesions (CIN2-3) *vs.* ≤ CIN1 but it was not useful to discriminate CIN1 from the healthy cervix.

The identification of one isolated p16 negative CIN2-3 case could be explained considering the methylation of the p16 promoter resulting in silencing the p16 gene[48]. Promoter hypermethylation of p16 might be an alternative pathway in cervical dysplasia.

The Ki-67 proliferation index expression was higher in CIN lesions compared to condylomas and negative samples. In literature, this data was also confirmed by reports from Kruse et al[49], Galgano et al[47], and Lim et al[50] where Ki-67 expression at epithelium's second and third superior was significantly higher in CIN2-3 compared to CIN1, in which it was isolated to the basal layer (epithelium's third inferior).

Finally, cases with miR-551b expression with cut-off <0.02 showed a significant correlation between moderate and severe dysplasia and proliferation index Ki-67. Correlation appeared even more consistent taking in consideration the p16 expression. In the literature, there are no studies on the involvement of miR-551b in the cervical dysplasia process, however, from our results ap-

pear that a cut-off of 0.02 might discriminate different levels of cervical dysplasia.

Conclusions

We showed that the miR-551b could play an important role as oncosuppressor in the different stages of cervical dysplasia through the control on cell cycle. Despite there have been several findings about the connection between miRs and carcinogenesis, it is still necessary to understand all target regulated by miRs, in order to increase our understanding of cervical dysplasia and carcinogenesis. Some of the analyzed miRNA could be used as biomarkers not only for early diagnosis and monitoring of cervical cancer development but also for choosing the best therapeutic strategy for cervical dysplasia.

Conflict of Interest

The authors do not have any conflicts of interest to declare

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