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EXPRESSION OF miRNAs IN THE PRESENCE OF HPV INFECTION IN CERVICAL DYSPLASIA SAMPLES: A PILOT STUDY

Background. Cervical cancer is a major health concern, with human papillomaviruses (HPV) infection being a key risk factor. However, not all HPV-infected individuals develop cancer, suggesting the additional factors may be involved. This study **aims** to evaluate the differences in the miR-155 and -205 expression in cervical tissue with dysplasia depending on the presence of HPV and confirmed cancer diagnosis. **Materials and Methods**. The expression of miR-155 and -205 in 30 formalin-fixed paraffin-embedded primary cervical tissue biopsy samples was evaluated using RT-PCR. **Results**. The expression levels of miRNA-155 and -205 in cervical dysplasia samples without malignant transformation was lower than these in carcinoma in situ tissues (0.74 ± 0.21 and 1.65 ± 0.42 vs. 1.37 ± 0.18 and 2.35 ± 0.32, respectively). In carcinoma in situ cases, we found higher levels of miRNA-155 and -205 (1.6 and 1.38 times, respectively) in CIN-3/HSIL samples compared to CIN-2/HSIL samples. The expression of both miRNAs tended to increase in HPV-positive cases and in the presence of malignant transformation compared to HPV-negative dysplasia and dysplasia without signs of malignant transformation, respectively. **Conclusions**. The obtained data indicate a potential relationship between the presence of HPV infection and the expression profile of miRNA-155 and -205.

Keywords: cervical cancer, cervical dysplasia, HPV, miRNA.

Cervical cancer (CCa) is the fifth most prevalent malignancy among women in Ukraine [1] and is primarily linked to persistent infection with high-risk human papillomaviruses (HR-HPV). The viral oncoproteins play a pivotal role in cervical carcinogenesis by interfering with the key cellular regulatory pathways, targeting tumor suppressors such as pRb and p53, and thus leading to a decrease in the expression and the subsequent loss of the cell cycle control [2].

While HR-HPV infection is the primary driver of CCa, recent studies have highlighted a significant proportion of cases that are HPV-negative despite improved detection methods. This suggests that other factors may contribute to CCa development in those cases [3].

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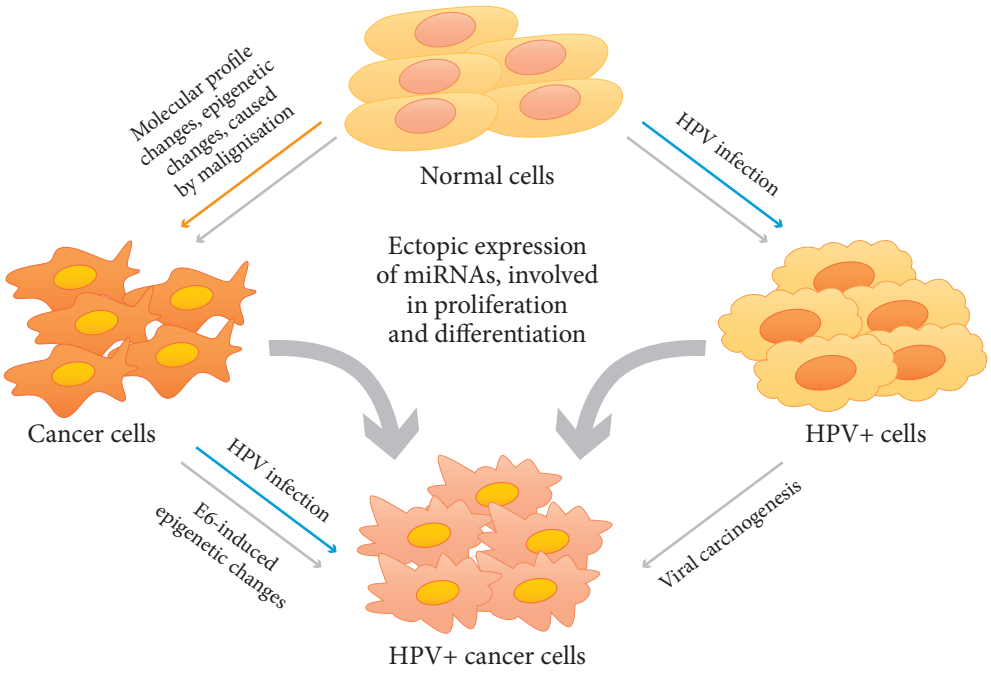

Fig. 1. Schematic representation of the potential influence of HIV infection on the epigenetic profile during cervical carcinogenesis

MicroRNAs (miRNAs) are small, non-coding RNA molecules, which play a critical role in regulating gene expression. By binding to the 3' untranslated region of target mRNAs, miRNAs can inhibit translation or induce mRNA degradation. Dysregulation of miRNA expression has been linked to a variety of human cancers, including CCa [4].

Several miRNAs have been identified as potential biomarkers and therapeutic targets of CCa. Such miRNAs can function as either oncogenes or tumor suppressors, depending on their target genes and the cellular context. For example, oncogenic miRNAs such as miR-21, miR-221, and miR-222 promote cell proliferation, invasion, and metastasis by targeting tumor suppressor genes. Tumor suppressor miRNAs such as miR-34a, miR-145, and let-7 inhibit cell proliferation and promote apoptosis by targeting oncogenes.

HPV infection can disrupt the expression of various miRNAs, leading to dysregulated gene expression and cellular transformation. Some studies have shown that HPV E6 and E7 oncoproteins can directly or indirectly affect miRNA expression, thereby contributing to cervical carcinogenesis (Fig. 1).

Persistent HPV infection, particularly with HR-HPV types, can lead to significant alterations in the miRNA expression profiles. This dysregulation plays a crucial role in the multi-step process of cervical carcinogenesis. Several studies have investigated the impact of HPV infection on miRNA expression in CCa [5].

The microarray and next-generation sequencing analyses have revealed widespread changes in miRNA expression in HPV-positive CCa cells and tissues. Several miRNAs, such as miR-21, miR-34a, miR-145, and miR-155, have been consistently implicated in HPV-induced cervical carcinogenesis [6] and the functional studies have demonstrated that they can regulate the expression of the key oncogenes and tumor suppressor genes, thereby promoting cell proliferation, invasion, and metastasis [7].

The dysregulation of specific miRNAs is closely linked to the expression of HPV E5, E6, and E7 oncoproteins. For instance, silencing these oncoproteins in HPV-positive CCa cell lines leads to the upregulation of miRNAs such as miR-148a-3p, miR-199b-5p, miR-190a-5p, and miR-18a [8]. These findings suggest that these miRNAs may serve as potential biomarkers for HPV-related CCa. Additionally, the studies have shown that the HPV oncoproteins can directly or indirectly downregulate specific miRNAs such as miR-3156-3p and miR-377, contributing to tumorigenesis [5]. In particular, Park et al. [2] have demonstrated that the

overexpression of miR-155 is linked to the HPV presence in CCa patients and may be used as a potential biomarker. Yu et al. [9] evaluated the diagnostic accuracy of miR-145 and miR-205 in CCa and showed their significant association with HPV infection and tumor stage.

Thus, identifying miRNA biomarkers holds promise for the early detection, risk stratification, and prognosis of CCa. Moreover, miRNAs can be exploited as therapeutic targets for developing novel anticancer therapies.

We aimed to evaluate the differences in miR-155 and -205 expression in the cervical tissue with dysplasia depending on the HPV presence and confirmed cancer diagnosis.

Materials and Methods

The study was conducted on the biopsy material of 30 patients with cervical intraepithelial neoplasia (CIN) 2—3/high-grade squamous intraepithelial lesion of the cervix (HSIL) and suspicion of CCa, who were treated at the NPO "National Cancer Institute" and the Medical Center of Colposcopy "LyNa", Kyiv, within 2020—2024. All subjects provided an informed consent on the use of their clinical data for the scientific purpose, and the research approved by the Medical Ethical Committee of the NPO "National Cancer Institute" was carried out in conformity with the guidelines of the Declaration of Helsinki. Before the study, all patients did not receive treatment and were examined using conventional clinical and laboratory methods according to the standards of diagnosis and treatment of cancer patients approved by Order No. 554 of 17.09.2007 of the Ministry of Health of Ukraine. The final diagnosis and HPV status were established using an immunohistochemical analysis according to the Standard of Medical Care "Cervical Cancer Screening. Management of patients with abnormal screening results and precancerous conditions of the cervix" (Order of the Ministry of Health of Ukraine dated June 18, 2024 No. 1057 approved). Tissue samples were encoded and depersonalized. The general clinical characteristics of the patients are presented in Table 1.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA from CCa tissue was isolated using the commercial kit RNeasy FFPE Kit (QIAGEN, Germany) according to the manufacturer's recommendations. The amount of isolated RNA was determined using a NanoDrop 2000c Spectrophotometer (Thermo

Table 1. **Clinical characteristics of BC cases**

Characteristics	Number of patients	
	n	%
Total number of patients	30	100
Average age, years	41.5 ± 13.02	
Age fluctuation, years	22—79	
Menstrual function is preserved	18	60.00
Menopause	12	40.00
Cervical intraepithelial neoplasia grade		
CIN 2/HSIL	10	33.33
CIN 2—3/HSIL	10	33.33
CIN 3/HSIL	10	33.33
HPV status		
HPV-positive, non-cancerous	4	13.34
HPV-positive, carcinoma in situ	6	20.00
HPV-negative, non-cancerous	10	33.33
HPV-negative, carcinoma in situ	10	33.33

Table 2. **Primers used in the study**

Primer	Sequence 5'—3'
miRNA-155-3p-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTC-GCACCAGAGCCAAC TGTTAA
miRNA-155-3p-F	GTTTGGCTCCTACATATTAGCA
miRNA-205-5p-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCG CACCAGAGCCAACCAGACT
miRNA-205-5p-F	GTTTGGTGAGAACTGAATTCCA
Universal Reverse primer	GTGCAGGGTCCGAGGT
RNU48-R	CTGCGGTGATGGCATCAG
RNU48-F	AGTGATGATGACCCCAGGTAACTC

Scientific, USA). The purity of the isolated RNA was monitored by the ratio of the optical absorption values at wavelengths of 260 and 280 nm. RNA was dissolved in Tris-EDTA buffer and stored at –20 °C until use. The RT-PCR was performed on a QuantStudio 5 Dx Real-Time PCR System (Thermo Fisher Scientific, USA) using a commercial kit for RT-PCR TaqMan MicroRNA Assay (Thermo Fisher Scientific, USA) according to the manufacturer's protocol.

The primer sequences (Table 2) for the detection of miRNA-155 and -205 were obtained using the resource genomics.dote.hu:8080/mirnadesigntool/ and synthesized by Metabion, Germany. RNU48 microRNA was used as an endogenous control to objectify expression parameters [9]. The relative expression of miR-155 and -205 was determined by the comparative ΔCT method. The threshold cycle was averaged in all technical and biological replicas in the middle of each line. The fold change in the expression of the studied miRNAs was calculated by the formula $2^{-\Delta Ct}$. The errors of the fold change calculations show a range of ΔCt values based on the inclusion of the standard deviation in these values.

Statistical methods. Statistical processing of the obtained results was carried out using STATISTICA 6.0 program (Statistica Inc., USA). Standard descriptive, parametric, and non-parametric statistical methods were used. A comparison of the reliability of differences between the mean values was carried out using Student's *t*-test (for a parametric distribution) or the Mann — Whitney U-test (for a non-parametric distribution). The data are presented as M ± m, where M is the arithmetic mean and m is the standard error of the mean, or as a percentage for relative values. Differences at $p \leq 0.05$ were considered significant.

Results and Discussion

We examined the expression of miR-155 and -205 in biopsy samples from patients with CIN 2—3/HSIL depending on the HPV status and their final diagnosis. Non-cancerous samples were characterized by lower levels of the mentioned miRNAs (0.74 ± 0.14 and 1.67 ± 0.33, respectively) compared to cancer in situ samples (1.38 ± 0.21 and 2.36 ± 0.28, respectively). The expression of studied miRNAs was not related to menstrual status of patients.

We detected higher levels of miR-155 and -205 (1.6 and 1.38 times, respectively) in CIN 3/HSIL compared to CIN 2/HSIL tissue samples from patients with carcinoma *in situ* (Table 3).

As seen from Fig. 2, miR-155 and -205 levels were higher in HPV-positive cases compared to the HPV-negative cases. At the same time, both miRNAs were elevated in cancerous tissue compared to non-malignant dysplasia cases, with no association with HPV status. Nevertheless, no significant differences between studied cohorts were established, probably, because of a small number of HPV-positive patients in our study.

Park et al. [2] assumed the diagnostic role of miR-9, -21, and -155 as markers of CCa, comparing normal and cancerous tissues without mentioning dysplasia and pre-cancerous cases. Thus, this matter requires more investigation. Authors claimed that a combination of miR-155 expression with HPV E6/E7 mRNA testing could improve the diagnostic accuracy for HPV E6/E7-negative CCa. Fur-

Table 3. **miR-155 and -205 levels in CIN 2—3/HSIL biopsy samples depending on the final diagnosis, fold change**

	Non-cancerous		Carcinoma *in situ*	
	miR-155	miR-205	miR-155	miR-205
Menstrual status				
Menstrual function is preserved	0.69 ± 0.24	1.34 ± 0.45	1.37 ± 0.51	2.19 ± 0.33
Menopause	0.78 ± 0.35	1.97 ± 0.38	1.39 ± 0.49	2.56 ± 0.19
Cervical intraepithelial neoplasia grade				
CIN 2/HSIL	0.57 ± 0.22	1.23 ± 0.34	1.04 ± 0.23	1.89 ± 0.21
CIN 2—3/HSIL	0.71 ± 0.25	1.79 ± 0.43	1.39 ± 0.56	2.56 ± 0.18
CIN 3/HSIL	0.98 ± 0.26	2.04 ± 0.39	1.69 ± 0.23*	2.61 ± 0.11*

Note: *$p < 0.05$ compared to CIN 2/HSIL samples.

thermore, miR-155 was identified as a prognostic marker, with its higher levels associated with an increased risk of CCa, especially in HPV E6/E7-positive patients [2].

In *in vivo* studies, Paiva et al. [10] showed that miR-155 expression might modulate tissue susceptibility to cancer development.

Yu et al. [9] stated that compared to normal cervical tissue, cervicitis, and cervical intraepithelial neoplasia, CCa tissue samples exhibited significantly lower miR-145 expression and significantly higher miR-205 expression. Both miRNAs were significantly associated with HPV infection: HPV+ patients had significantly lower miR-145 expression and significantly higher miR-205 expression compared to HPV-negative patients [9].

McKenna et al. [11] investigated the impact of HPV presence on miRNA expression and examined the effect of E6 and E7 oncoproteins on miR-24 and miR-205 in keratinocytes. They found that E6 and E7 expression led to the increased miR-24 levels and decreased miR-205 levels, suggesting that HPV infection can disrupt the normal regulation of these miRNAs. Moreover, HPV infection interfered with the upregulation of miR-24 and miR-205 during differentiation of keratinocytes. However, the authors stated the oncosupressive role of miR-205 in their study, and, as known, miRNAs can change their functions depending on the tissues and are shown to be oncogenic in cervical tissues [12]. Expression of mir-155 and -205 is shown to be induced by E6-mediated p53 inactivation, therefore promoting inflammation and cell proliferation and-inhibiting apoptosis [13].

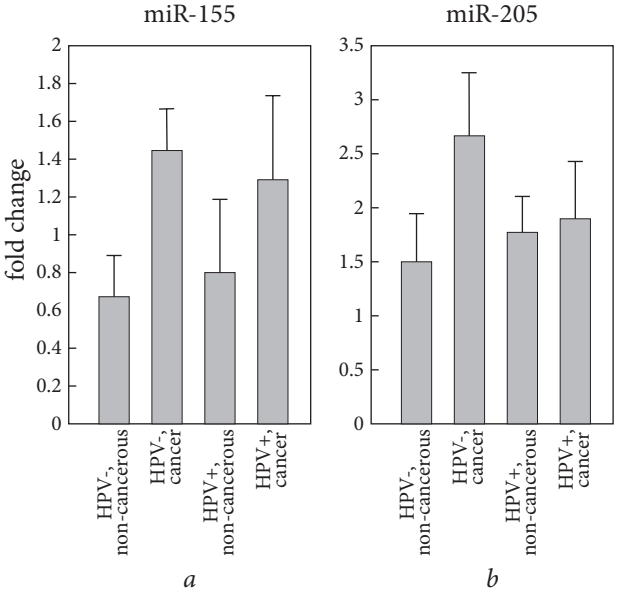

Fig. 2. Expression of miR-155 (*a*) and miR-205 (*b*) in biopsy samples depending on HPV status and cancer diagnosis in CIN 2-3/HSIL patients

To sum up, the interplay between miR-155, -205 and HPV infection is a complex and dynamic process that may contribute to the development and progression of CCa. By understanding this relationship, researchers and clinicians can develop more effective strategies for prevention, early detection, and treatment of this disease.

Taking into account the limitations of our study regarding a small number of patients with the HPV+ status, the obtained data should be validated on a larger sample.

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АСОЦІАЦІЯ ЕКСПРЕСІЇ МІКРОРНК ІЗ ВПЛ СТАТУСОМ У ЗРАЗКАХ ДИСПЛАЗІЇ ШИЙКИ МАТКИ: ПІЛОТНЕ ДОСЛІДЖЕННЯ

Вступ. Рак шийки матки є актуальною проблемою онкології, причому інфекція вірусу папіломи людини (ВПЛ) є ключовим фактором ризику. Однак не у всіх людей, інфікованих ВПЛ, розвивається рак, що свідчить про наявність додаткових факторів. Це дослідження спрямоване на оцінку відмінностей у експресії мікроРНК-155 і -205 у тканині шийки матки з дисплазією залежно від присутності ВПЛ та підтвердженого діагнозу «*carcinoma in situ*». **Матеріали та методи**. Експресію мікроРНК-155 і -205 у 30 фіксованих у формаліні залитих парафіном первинних зразках біопсії тканини шийки матки оцінювали за допомогою ЗТ-ПЛР. **Результати**. При аналізі експресії мікроРНК-155 і -205 у зразках дисплазії шийки матки без злоякісної трансформації зафіксовано низький рівень зазначених мікроРНК (відповідно 0,74 ± 0,21 і 1,65 ± 0,42) порівняно з тканиною карциноми *in situ* (відповідно 1,37 ± 0,18 і 2,35 ± 0,32). У пацієнтів з карциномою in situ ми встановили вищі рівні мікроРНК-155 і -205 (відповідно в 1,6 і 1,38 рази) при CIN-3/HSIL порівняно зі CIN-2/HSIL. Обидві мікроРНК були підвищені у ВПЛ-позитивних новоутвореннях, а також за наявності злоякісної трансформації порівняно з ВПЛ-негативною дисплазією та дисплазією без ознак злоякісної трансформації відповідно. **Висновки**. Отримані дані свідчать про потенційний зв'язок між наявністю інфекції ВПЛ та профілем експресії мікроРНК-155 і -205 та вказують на необхідність проведення подальших досліджень на більшій вибірці хворих.

Ключові слова: рак шийки матки, дисплазія шийки матки, ВПЛ, мікроРНК.