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THE STUDY OF PROGNOSTIC VALUE OF microRNAs (miR-10b AND -155) AND CDKN2A/P16INK4A IN ORAL SQUAMOUS CELL CARCINOMA

Background. Oral squamous cell carcinoma (OSCC) is one of the most common types of cancer worldwide. Discovering novel prognostic markers for OSCC can improve treatment outcomes by allowing for more effective therapy strategies. **Aim.** To identify the prognostic value of CDKN2A (p16INK4a) and miRNAs involved in its regulation as markers of OSCC. **Materials and Methods.** The work is based on the results of the examination and treatment of 70 patients with stage II—IV OSCC. miR-10b, -155, and *CDKN2A* mRNA expression in tumor samples was analyzed by real-time reverse transcription polymerase chain reaction. The expression of p16INK4a and Ki-67 proteins was determined immunohistochemically. **Results.** No association of *CDKN2A* mRNA and p16INK4a protein with Ki-67 expression in tumor tissue and clinical pathological parameters of OSCC patients was found. Most of the p16INK4a-positive cases were characterized by a high Ki-67 expression. We found a strong correlation of the studied miRNAs expression levels with lymph node metastasis ($r = 0.56$ for miR-10b and $r = 0.59$ for miR-155). Also, there was no difference in miR-10b and -155 expression between p16INK4a+ and p16INK4a– samples. The association of both miRNAs with lymph node metastases was not affected by p16INK4a status. **Conclusions.** The results indicate the relationship between miR-10b and -155 and the presence of lymph node metastases in OSCC patients, so these miRNAs can be considered as prognostic markers of the disease.

Keywords: oral squamous cell carcinoma miRNA, CDKN2A, p16INK4a prognosis.

The oral squamous cell carcinoma (OSCC) is one of the most common malignant tumors in the world. In most countries, the overall 5-year survival rate of patients with OSCC does not exceed 50%. Almost 60% of patients at the first visit to an oncologist are diagnosed with OSCC

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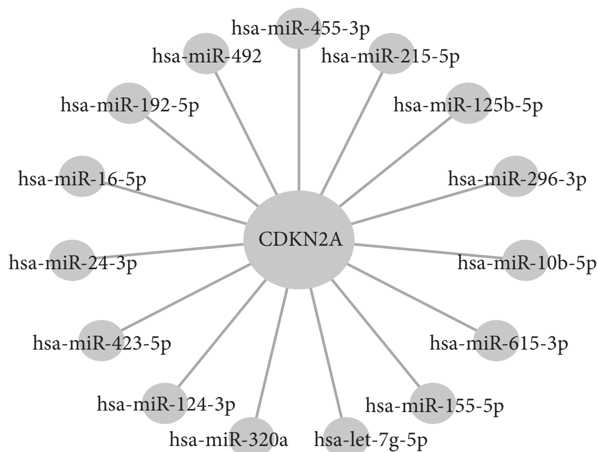

Fig. 1. miRNAs involved in post-transcriptional regulation of *CDKN2A* gene

at stages III—IV, which significantly reduces the likelihood of radical treatment or even makes it impossible. The median overall and recurrence-free survival of such patients is 27 months [1]. In Ukraine, in 2020—2021, the OSCC incidence was 5.3 per 100,000 population, and the mortality before 1 year after diagnosis reached 37.3%. At the same time, the incidence in men is much higher (9 per 100,000) than in women (2.1 per 100,000). In the structure of cancer incidence (except for non-melanoma malignant skin tumors) of the male population of Ukraine, OSCC ranks 10th place among all the nosological forms [2]. The presence of regional metastases in patients with head-and-neck tumors significantly worsens the treatment prognosis and survival rates. According to some authors, the 2-year survival rate in the group of patients without metastases is 56.8%, and with metastases corresponding to N1—N3 is only 18.8%, so already at the stage of diagnosis, it is important to assess the aggressiveness of the tumor for prevention of relapses and distant metastasis [3].

The study of the role of miRNAs in oncogenesis and identification of a range of key molecules that may serve as potential prognostic markers of the disease course is of great interest [4]. According to Padhi et al. [5], the dysregulation of the *CDKN2A* gene and its protein product p16INK4a has been a frequent event in the development of OSCC. The *CDKN2A* gene is located on chromosome 9p21 and has three exons that encode for the tumor suppressor protein, commonly known as p16.

We used miRTargetLink 2.0 resource to define miRNAs involved in the regulation of the *CDKN2A* gene (Fig. 1). Among the spotted miRNAs, we chose miR-155 and -10b for further investigation, due to their involvement in OSCC progression [6, 7].

Thus, we aimed to identify the prognostic value of CDKN2A and miRNAs involved in its regulation as potential prognostic markers of OSCC.

Materials and Methods

The work is based on the results of the examination and treatment of 70 patients with OSCC, who were treated in the Research Department of Head and Neck Cancer of the National Cancer Institute of the Ministry of Health of Ukraine (Kyiv) from 2017 to 2020. All patients provided informed consent on the use of the clinical data for scientific purposes. The general clinical and pathological characteristics of the patients are presented in Table 1. The tumor stage was determined according to the International TNM Classification (8th edition, 2016). The histological type of tumor was established according to the WHO classification (2017). Depending on the clinical indications, patients underwent surgery according to the treatment standards adopted in Ukraine, as well as chemoradiation therapy.

The real-time reverse transcription polymerase chain reaction (RT-PCR) was used to study the expression of miR-10b and -155 and mRNA of CDKN2A in the tumor samples of patients with OSCC. The total RNA was isolated from paraffin blocks with tumor tissue using the commercial RNeasy FFPE Kit (QIAGEN, Germany). The quantity of isolated RNA was determined using a spectrophotometer “Nano-

Drop 1000 Spectrophotometer" (Thermo Fisher Scientific, USA). The purity of the isolated RNA was monitored by the ratio of the optical absorption values at the 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 quantitative detection system 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.

To determine mir-10b-5p, we used the stem-loop primer for synthesis of cDNA 5′- GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC CACAAA -3′, and for the real-time PCR, a forward primer was 5′- GGTACCCTGTAGAACCGAA -3′. For miR-155-3p detection, we used stem-loop primer 5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC TGTTAA -3′ primer and for the real-time PCR - forward primer 5′-GTTTGGCTCCTACATATTAGCA -3′.

According to the stem-loop miRNA RT-PCR technique, a universal reverse primer 5′-GTGCAGGGTCCGAGGT-3′ was used [4]. The primer sequences for the detection of miRNA-10b and -155 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. The primer sequences for RNU48 were synthesized by Metabion, Germany: forward 5′-AGTGATGATGACCCCAGGTAACTC-3′, reverse 5′-CTGCGGTGATGGCATCAG-3′. The relative expression of miRNAs-21 and -375 was determined by the comparative ΔCT method [4].

The primers for *CDKN2A* mRNA estimation were obtained from https://www.origene.com (HP226191).

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 $2^{-\Delta Ct}$ (a.u. hereinafter). The errors of the fold change calculations show a range of ΔCt values based on the inclusion of the standard deviation in these values.

The detection of p16INK4a and Ki-67 was performed by simultaneous immunohistochemical assessment in tumor cells as described earlier [8]. The monoclonal antibodies specific for p16INK4a (Thermo Fisher Scientific, USA) and Ki-67 (Dako Cytomation, Denmark) were used as primary antibodies in dilutions according to the manufacturer's instructions. p16INK4a expression was considered positive if the number of immunopositive tumor cells exceeded 10%. The number of cells with positive expression of Ki-67 was determined by immunohistochemical reaction intensity: "+++" — strong, "++" — moderate (these two categories were combined into "Ki-67 high" cohort), "+" — low, or no expression.

Table 1. **Clinical and pathological characteristics of the patients with OSCC (n = 70)**

Index	Number of patients	
	N	%
Average age, years	53.13 ± 8.43	
Sex		
Male	59	84.29
Female	11	15.71
T category by TNM		
T2	10	14.29
T3	45	64.29
T4	15	21.42
N category by TNM		
N0	31	44.29
N1	15	21.42
N2	24	34.29
Differentiation grade, G		
G1	12	17.14
G2	42	60.00
G3	16	22.86

The statistical analysis of the obtained results was performed using the program Statistica 6.0 (Statistica Inc., USA) taking into account the nature of the distribution of the obtained data. The data are presented as M ± m where M is the arithmetic mean, m is the standard error of the mean or a percentage for relative values. The Mann—Whitney U-test was used to compare two independent groups on a quantitative basis. Pearson's correlation coefficient was used to establish the association between studied parameters. The critical level of statistical significance was taken to be 0.05.

Results and Discussion

Nowadays in standard clinical practice in Ukraine, only the presence/absence of p16INK4a expression in tumor tissue is considered as a prognostic marker due to the estimation of OSCC-associated papillomavirus (HPV) infection. However, novel studies prove that in p16INK4a-positive tumors, HPV DNA is detected in 70% of cases [9].

Among our samples, 30 patients were p16INK4a-positive. We performed estimation of CDKN2A mRNA expression in this cohort. The average expression levels were established as 44.5 ± 13.3 a.u. No association of both mRNA and protein expression with age, gender, and clinical parameters (TNM and G) was found (Table 2). Most of p16INK4a-positive patients were characterized by high Ki-67 expression, but no correlation with clinical parameters was found (Table 2). Omer et al. [10] also found no significant association of p16INK4a and Ki-67 protein expression in OSCC with clinical parameters.

Table 2. **Association of CDKN2A/p16INK4a expression with clinical and pathological characteristics of the patients with OSCC (n = 70), r**

Parameter	*CDKN2A* (mRNA)	p16INK4a (protein)
Age	0.36	0.39
T by TNM	0.47	0.46
N by TNM	0.35	0.32
Differentiation grade, G	0.29	0.22
Proliferative activity (Ki-67)	0.49	0.48

Note: * $p < 0.05$

It should be noted that Prigge et al. [11] observed a co-expression of p16INK4a/Ki-67 only in cancer cells of head-and-neck tumors but not in non-cancerous cells. In their study, the combined expression of p16INK4a/Ki-67 was consistently associated with a diffuse pattern of the p16INK4a expression. All head-and-neck squamous cell carcinomas expressing HPV oncogenes showed a co-expression of p16INK4a/Ki-67 [11].

Due to the bioinformatic prediction of the miR-10b and -155 involvement in *CDKN2A* regulation, we tried to find possible correlations of their levels in the OSCC samples.

We estimated the miRNA levels in tumor tissue samples of OSCC patients (Fig. 2). The average levels of miR-10b were 11.8 ± 3.9 a.u. and miR-155 — 6.78 ± 2.92 a.u. Both miRNAs were elevated in the tumors of patients with lymph node metastases. We found a strong correlation of studied miRNAs expression with lymph node metastasis (r = 0.56 for miR-10b and r = 0.59 for miR-155).

We then analyzed the studied miRNAs levels depending on the p16INK4a status of tumors (Fig 3. *a, b*). The average level differences between these groups were statistically insignificant, but the cohort with p16INK4a+ tumors was characterized by slightly lower levels of both studied miRNAs. In these patients, the levels of miR-10b were 9.5 ± 3.5 a.u. and levels of miR-155 — 5.76 ± 2.5 a.u. In p16INK4a-negative tumors, the levels of the studied miRNAs were 13.78 ± 4.9 and 8.47 ± 3.6 a.u., respectively. An interesting fact was that the association of both miRNAs with lymph node metastases remained the same, as well as the absence of any relation

to other clinical-pathological parameters. Also, miR-10b levels were significantly lower in G3 patients in comparison with G1 tumors ($p < 0.05$).

Further, we analyzed the correlation between miR-10b and -155 and the levels of *CDKN2A* and did not find any reliable associations.

The obtained data coincide with the results of other authors. For example, Golabek et al. [12] also found no association between the *CDKN2A*/p16INK4a gene expression levels, age, gender, smoking, and alcohol consumption and clinical-pathological parameters in the tumor and margin samples, even though a bigger number of patients were analyzed.

Interestingly, we found a correlation in miR-10b expression with Ki-67 levels in both p16INK4a+ and p16INK4a– samples (Fig 3 *a, b*), as well as in the total group (Fig. 2) (r = 0.53, 0.54, and 0.55, respectively).

The association between miR-10b and Ki-67 levels in tumor tissue is well studied, for example, in breast cancer. Zhang et al. [13] have proved that the expression of miR-10b is inversely correlated with the Ki-67 score.

On the other hand, there are reports that miR-10b is down-regulated in OSCC, but still is responsible for the activation of cell proliferation [14].

Other authors reported the involvement of miR-10b in OSCC metastasis. According to Lu et al. [6], the silencing of miR-10b significantly decreased cellular migration and the invasive ability of OSCC cells *in vitro* [6]. According to a recent study [15], the miR-10b overexpression significantly accelerates OSCC tumor growth. So, miR-10b plays a critical role in regulating the tumor cell invasion and migration, which might partially explain how miR-10b influences OSCC development and progression *in vivo*.

Manikandan et al. [16] claim that high levels of miR-155 are associated with the habit of chewing tobacco/betel quid, but not with any clinical-pathological parameters of OSCC in the Indian population.

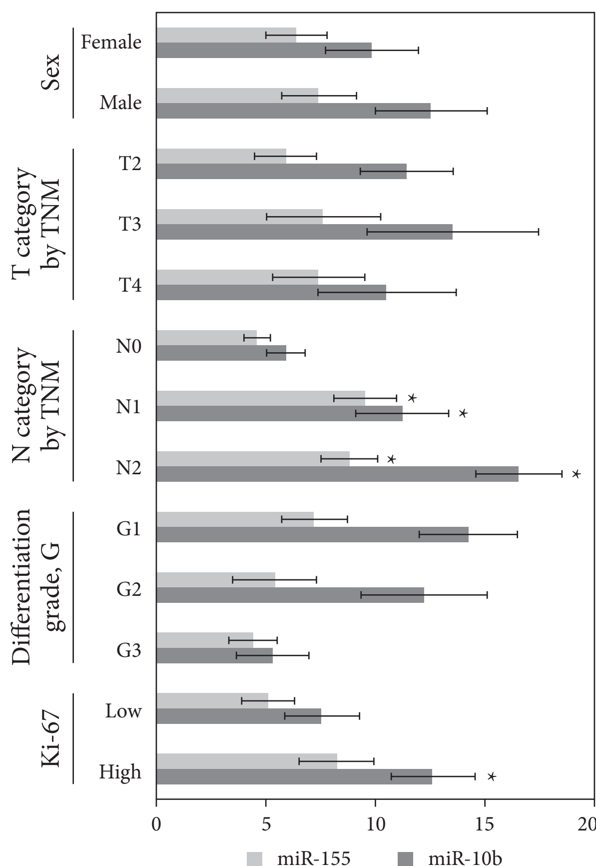

Fig. 2. Relationship between expression levels of microRNA-10b and -155 and clinical-pathological features of OSCC: * $p < 0.05$ compared to N0 category

On the other hand, Ni et al. [17] found that the miR-155 expression level is significantly higher in the OSCC tissues than in the adjacent non-neoplastic oral tissues. In addition, the expression of miR-155 was associated with tumor histological grade, and correlated with the proliferative activity of tumor cells.

Dioguardi et al. [18] performed a meta-analysis of the available data from recent studies that investigated the tissue expression of miR-155 as a prognostic biomarker of OSCC. They showed that this miRNA can be a highly valuable prognostic marker in terms of the survival rates of OSCC patients.

We suggest several reasons to explain the absence of correlation between miR-10b and -155

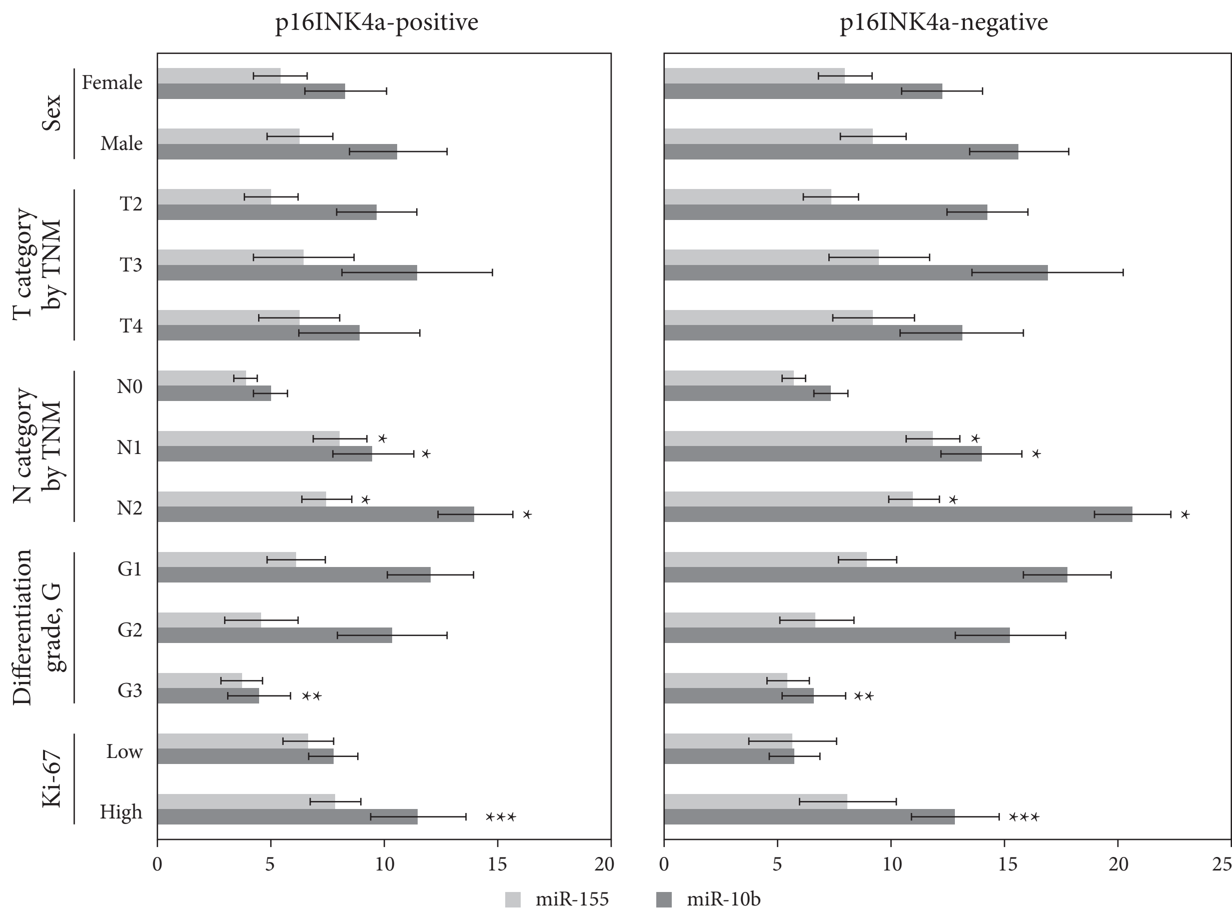

Fig. 3. Relationship between expression levels of microRNA-10b (a) and -155 (b) and clinical-pathological features of OSCC in dependence of p16INK4a status: * $p < 0.05$ compared to N0 category; ** $p < 0.05$ compared to G1 category; *** $p < 0.05$ compared to low Ki-67 category

levels and their experimentally validated target *CDKN2A* in the studied cohort of OSCC patients:

The levels of these miRNA are somehow affected by the presence of HPV, and their expression is regulated by their downstream target. Lajer et al. [19] found several miRNAs that were perturbed in the HPV-positive pharyngeal OSCC samples compared with the HPV-negative pharyngeal OSCC. The observed fold changes were described as modest, and the false discovery rates were relatively high. This is at least partly due to a prominent infiltration of the normal tissue in the HPV-negative OSCC and a low number of HPV-positive samples.

The expression of *CDKN2A*, as well as miRNAs, involved in its regulation can be affected by other mechanisms, both on transcriptional and translational levels. For example, Lee et al. [20] established that HPV16 suppresses miR-181 expression by inhibiting its promoter activity, indicating for the first time that miR-181a and miR-181d are novel transcription targets of HPV16. Several biomarkers have been identified and used for diagnosing HPV-associated cancers, such as p16INK4a and TOP2A, and surviving but this phenomenon requires further investigation.

Several recent studies highlight the importance of another epigenetic mechanism of gene expression regulation, namely DNA methyla-

tion. Avram et al. [21] evaluated the impact of the differentially methylated microRNAs in the OSCC transcriptome. They concluded that in contrast to the general decrease in DNA methylation across the entire genome in OSCC, microRNA loci may exhibit an increase in their methylation status. This can lead to variations in the expression of their mature forms. The authors performed the bioinformatic analysis indicating that the methylation-dependent regulation of the microRNA expression can significantly impact the response pathways to the viral infections, the ECM-receptor interaction pathway, and the AGE-RAGE signaling pathway. These pathways could be relevant for the development, progression, and pathogenesis of OSCC.

In conclusion, miR-10b and -155 can be suggested as prognostic markers of OSCC without any relation to the *CDKN2A*/p16INK4a status, but their value requires further validation.

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ПРОГНОСТИЧНЕ ЗНАЧЕННЯ мікроРНК (miR-10B ТА -155) І CDKN2A/P16INK4A ЯК МАРКЕРІВ ПЕРЕБІГУ ПЛОСКОКЛІТИННОГО РАКУ ПОРОЖНИНИ РОТА

Плоскоклітинний рак ротової порожнини (ПКРР) є одним із найпоширеніших видів злоякісних новоутворень в усьому світі. Ідентифікація нових прогностичних маркерів може допомогти клініцистам зрозуміти біологічні характеристики ПКРР і створити більш персоналізовані плани лікування для пацієнтів на основі їхніх факторів ризику. Сучасні прогностичні маркери ПКРР обмежені у своїй здатності точно передбачати результати пацієнтів, що може призвести до надмірного або недостатнього лікування. Таким чином, відкриття нових прогностичних маркерів ПКРР може допомогти покращити результати лікування, дозволяючи застосовувати більш точні та ефективні стратегії терапії, що зрештою приведе до покращення показників виживаності та якості життя пацієнтів з ПКРР. **Мета.** Виявити прогностичне значення CDKN2A та мікроРНК, які беруть участь у його регуляції, як маркерів ПКРР. **Матеріали та методи.** Робота базується на результатах обстеження та лікування 70 хворих на ПКРР II—IV стадії. Експресію мікроРНК-10b, -155 і мРНК CDKN2A у зразках пухлини аналізували за допомогою полімеразної ланцюгової реакції в реальному часі. Експресію білків p16INK4a та Ki-67 визначали за допомогою імуногістохімічного аналізу. **Результати.** Не виявлено жодного зв'язку між рівнями мРНК CDKN2A та білка p16INK4a з експресією Ki-67 в пухлинній тканині, а також клініко-патологічними характеристиками пацієнтів з ПКРР. Більшість p16INK4a-позитивних пацієнтів характеризуються високою експресією Ki-67. Ми виявили кореляцію експресії мікроРНК з метастазами в лімфатичні вузлах ($r = 0{,}56$ для мікроРНК-10b і 0,59 для мікроРНК-155). Крім того, не встановлено відмінностей рівнів мікроРНК-10b і -155 у хворих із p16INK4a+ і p16INK4a- пухлинами. **Висновки.** Отримані результати вказують на зв'язок експресії мікроРНК-10b та -155 з наявністю метастазів у лімфатичних вузлах у хворих на ПКРР, що дозволяє розглядати ці мікроРНК як прогностичні маркери захворювання.

Ключові слова: плоскоклітинний рак ротової порожнини, мікроРНК, CDKN2A, прогноз.