

SIGNIFICANCE OF EXPRESSION OF TUMOR-ASSOCIATED MICRORNA-21 AND -375 FOR PREDICTING THE COURSE OF CANCER OF ORAL CAVITY

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Aim: To investigate the features of expression of miRNA-21 and miRNA-375 in tumor cells of patients with cancer of oral cavity (COC) and to determine the possibility of their use to predict the aggressiveness of COC course. **Materials and Methods:** The work is based on the results of examination and treatment of 50 patients with stage II–IV COC. miRNA expression in tumor cells was analyzed by real time reverse transcription polymerase chain reaction. **Results:** High levels of miRNA-21 expression (> 0.26 a.u.) and miRNA-375 (> 0.36 a.u.) were determined in 72.0% and 63.0% of cases. We revealed a tendency to decreased miRNA-21 expression and increased miRNA-375 expression in tumors of patients with recurrence-free survival less than 12 months and the presence of metastatic lesions in regional lymph nodes. In patients with COC of low differentiation grade, the level of miRNA-21 was 2.0 times lower compared with tumors of moderate differentiation grade ($p < 0.05$), while the expression of miRNA-375, on the contrary, was higher in tumors of low differentiation grade. A decreased expression of miRNA-21 (< 0.26 a.u.) against the background of decreased levels of miRNA-375 (< 0.36 a.u.) in tumor cells was associated with worse recurrence-free survival. **Conclusions:** The obtained results indicate the relation between the main clinical and pathological characteristics of patients with COC and the levels of miRNA-21 and -375 expression in tumor tissue, which indicates the involvement of these miRNAs in the formation of COC malignancy evidencing on their potential usefulness as additional prognostic markers.

Key Words: oral cancer, microRNA-21, microRNA-375, survival, aggressiveness.

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Cancer of oral cavity (COC) is the most common form of head and neck cancer, which includes cancer of the mucous membranes of the lips, cheeks, alveolar processes of the jaws, tongue and floor of the mouth. The tumors of this location share a common histological structure, which is almost homogeneous: 90–95% of COC is represented by squamous cell carcinoma. In most countries, the incidence of COC varies from 1 to 10 cases per 100,000 population. The prevalence of COC is relatively higher among men, especially the elderly [1].

Despite the improvement of the treatment approaches, long-term results of COC therapy vary widely and often remain unsatisfactory. The cause of such clinical polymorphism may be intertumor and intratumoral heterogeneity of COC, which depends on a complex of morphological, genetic and epigenetic changes that determine the phenotype of malignant cells. Therefore, the search for new markers that alone or in combination with other known indices could reveal additional mechanisms of COC development is relevant [2].

It is known that the occurrence and progression of malignant neoplasms are characterized by the changes in patterns of epigenetic biomarkers. Particular attention is paid to miRNAs as the main regulators of genes involved in carcinogenesis [3, 4]. MicroRNAs are small non-coding RNAs approximately 22 nucleotides in length that regulate the expression level of mes-

senger RNAs by interacting with their specific regions. Since microRNAs control expression of more than 30% of human genes, they are considered as the key regulators of biological processes. That is why the impairment of microRNA regulation can affect all stages of carcinogenesis — from the origin of malignant neoplasms to their progression [5, 6]. In this regard, further study of the miRNAs role in oncogenesis, search for markers of COC and identification of a range of key molecules that may be potential predictors of disease and survival of patients with this pathology is of great interest. In recent years, there has been found some evidence that the COC development may be associated with impaired expression of oncogenic miRNA-21 and oncosuppressive miRNA-375 [7].

The aim of this work was to investigate the patterns of miRNA-21 and miRNA-375 expression in tumor cells of patients with COC and to determine the possibility of their use to predict the aggressiveness of the COC course.

MATERIALS AND METHODS

The work is based on the results of examination and treatment of 50 patients with squamous cell COC, 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 an informed consent on the use of clinical data for scientific purposes. The general clinical and pathological characteristics of the patients are presented in Table. The tumor stage was

Table. Clinical and pathological characteristics of the patients with COC (n = 50)

Index	Number of patients	
	N	%
Average age, years	53.13 ± 8.43	
Age, range	34–64	
Sex		
Men	43	86
Women	7	14
T category by TNM		
T2	7	14
T3	32	64
T4	11	22
N category by TNM		
N0	22	44
N1	11	22
N2	17	34
Differentiation grade, G		
G2	40	80
G3	10	20
Localization		
Mucosa of the lower jaw	7	14
Mucosa of the floor of the oral cavity	14	28
Mucosa of the cheeks	7	14
Tongue	22	44

determined according to the International TNM Classification (7th edition, 2009). Histological type of tumor was established according to the WHO classification (2006). Depending on the clinical indications, patients underwent organ-sparing operations according to the treatment standards adopted in Ukraine, as well as chemoradiation therapy.

The real-time reverse transcription polymerase chain reaction was used to study the expression of miRNAs-21 and -375 in tumor cells of patients with COC. 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 “NanoDrop 2000c Spectrophotometer” (ThermoScientific, USA). The purity of the isolated RNA was monitored by the ratio of 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 real-time reverse transcription polymerase chain reaction was performed on a quantitative detection system QuantStudio 5 Dx Real-Time PCR System (ThermoScientific, USA) using a commercial kit for RT-PCR TaqMan MicroRNA Assay (ThermoScientific, USA) according to the manufacturer’s protocol. To determine miR-21 we used the stem-loop primer for synthesis of cDNA 5′-GTTGG CTCTGGTGCAGGGTCCGAGGTATTCGACACAGAG CCAACTCAACA-3′ and for real-time PCR forward primer was 5′-GTTTGGTAGCTTATCAGACTGA-3′. For miR-375 detection we used stem-loop primer 5′-GT CGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGA TACGACTCACGCG-3′ primer and for real-time PCR forward primer 5′-CACAAAATTTGTTCTGTTGGCT-3′.

According to the stem-loop miRNA RT-PCR technique, a universal reverse primer 5′-GTGCAGGGTCCGAGGT-3′ was used [8]. Primer sequences for the detection of miRNA-21 and -375 were obtained using the resource <http://genomics.dote.hu:8080/mirnadesigntool/> and synthesized by Metabion, Germany.

RNU48 microRNA was used as an endogenous control to objectify expression parameters. Primer sequences are taken from <https://www.ncbi.nlm.nih.gov> and synthesized by Metabion, Germany: RT-primer: 5′-CTCTGACC-3′, forward 5′-AGTGATGATGACCCCAGGTAATC-3′, reverse 5′-CTGCGGTGATGGCATCAG-3′. The relative expression of miRNAs-21 and -375 was determined by comparative ΔCT method (a.u.) [9].

The threshold cycle was averaged in all technical and biological replicas in the middle of each line. The fold difference (fold change, fold difference) between the expression of the studied miRNAs was calculated by the formula $2^{-\Delta\text{Ct}}$ (hereinafter — a.u.). Errors for fold difference calculations show a range of ΔCt values based on the inclusion of standard deviation in these values. For the convenience of presenting the results, the miRNA-375 was calculated using the coefficient $10 \times 2^{-\Delta\text{Ct}}$. High expression was considered to be the expression above the median: for miRNA-21 and -375 > 0.26 and > 0.36 a.u., respectively, low level of expression was < 0.26 and < 0.36 a.u., respectively.

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 $M \pm m$, where M is the arithmetic mean, m is the standard error of the mean or as a percentage for relative values. Mann — Whitney U-test was used to compare two independent groups on a quantitative basis. The critical level of statistical significance was taken to be 0.05. Survival of patients was analyzed by the Kaplan — Meier method, reliability between curves — using a logarithmic rank test.

RESULTS AND DISCUSSION

Analysis of miRNA-21 and -375 expression levels in tumor tissue of patients with COC has revealed their significant variability. The average expression level of miRNA-21 was 0.36 ± 0.24 a.u. with individual fluctuations from 0.02 a.u. to 1.01 a.u., while the expression of miRNA-375 was 0.47 ± 0.61 a.u. with individual fluctuations from 0.01 to 0.89 a.u. It was found that a characteristic feature of the studied COC samples were high levels of expression of miRNA-21 (> 0.26 a.u.) and miRNA-375 (> 0.36 a.u.), which were determined in 72.0% ($p < 0.05$) and 63.0% ($p < 0.05$) of cases.

Depending on the survival rates, the patients with COC were divided into 2 groups. The first group consisted of 23 patients in whom disease progression was observed within 12 months, and the second group — 27 patients in whom no signs of recurrence were detected during the first 12 months after treatment. When analyzing the expression of miRNA-21 in COC cells, depending on the indices of recurrence-free survival of patients, it was found that in the 1st group of patients its average level was 0.30 ± 0.05 a.u. and was 1.56 ($p < 0.05$) times lower compared with patients of group 2 — 0.47 ± 0.08 a.u., respectively. The miRNA-375 expression levels in patients with recurrences during the first 12 months were 2.4 times ($p < 0.05$) lower than in patients of group 2 (Fig. 1 a, b).

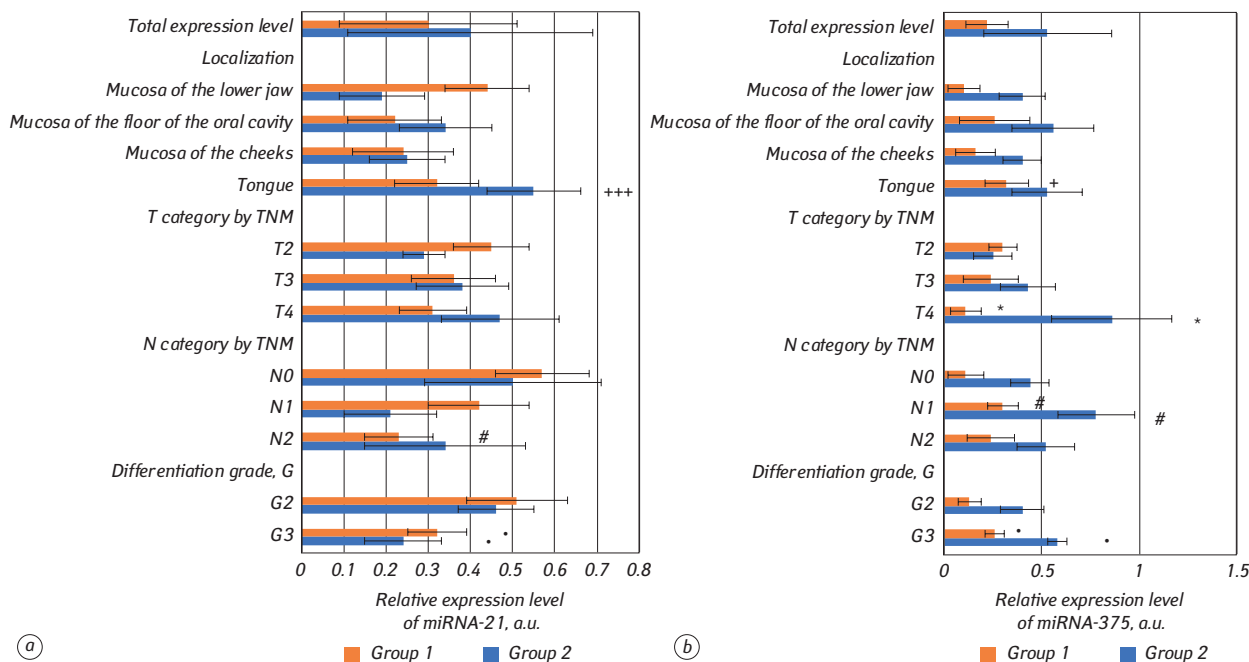


Fig. 1. Relation between expression levels of microRNA-21 (a) and -375 (b) and clinical-pathological features of COC cases: $p < 0.05$; *in comparison with indices of COC of the mucosa of the lower jaw; **compared with COC of the cheek mucosa; *in comparison with the indices of patients from group 1, respectively; #in comparison with indices of COC of N0 category; • $p < 0.05$ compared with COC of moderate differentiation grade

At the next stage, we have analyzed the expression of miRNA-21 and -375 depending on the clinical and pathological parameters of patients from both study groups. As can be seen from Fig. 1, the lowest expression of miRNA-21 was observed in patients of group 2 with tumors localized on the mucosa of the lower jaw (0.19 ± 0.11 a.u.), the highest — in the same group of patients with tumors localized on the tongue (0.55 ± 0.22 a.u.), which is 4.0 times lower ($p < 0.05$) and 1.7 times higher ($p < 0.05$) compared with similar indices of patients from group 1, respectively. No differences in the expression of miRNA-21 in patients with tumors of the cheek mucosa and oral cavity floor between groups 1 and 2 were found.

We found that in group 1 the level of miRNA-375 expression was significantly higher in tumors localized on the tongue compared to that in neoplasms of the lower jaw mucosa. In group 2 we revealed significantly higher levels of miRNA-21 in tumors localized on the tongue compared with those in the neoplasms of the lower jaw mucosa ($p < 0.05$) and the cheek mucosa ($p < 0.05$).

There was observed a tendency to decreased expression of miRNA-21 depending on the size of the tumor in patients from group 1. In particular, the level of this miRNA in patients with T2 category tumors was 0.42 ± 0.11 a.u. and did not differ significantly from that in patients with T3 and T4 neoplasms (0.36 ± 0.10 and 0.31 ± 0.08 a.u., respectively). In tumors of patients from group 2, its levels, on the contrary, increased — in T4 cases this index was significantly higher than in T2 cases, and was 0.47 ± 0.14 a.u.

The expression of miRNA-375 in the tumor tissue of patients from group 1 significantly decreased with increasing size of tumors. At the same time, in group 2, there was observed an increase in the levels of miR-

NA-375 expression with increasing tumor size. In particular, the lowest indices of its expression (0.25 ± 0.10 a.u.) were found in T2 tumors, while in T3 tumors its average values reach 0.43 ± 0.14 a.u., and in T4 — 0.86 ± 0.31 a.u.

In the group of patients with recurrence-free survival less than 12 months, a tendency to decreased levels of miRNA-21 expression and increased levels of miRNA-375 expression in the presence of metastatic lesions of regional lymph nodes was revealed (see Fig. 1). In the group of patients without recurrence, the presence of metastases in regional lymph nodes, in contrast, was associated with low levels of miRNA-21.

We found a significant increase in miRNA-375 expression levels by 1.77 times in the COC cases with N1 status ($p < 0.05$) compared with the cases without metastases. However, no significant difference between the expression of this miRNA in malignant neoplasms of the oral cavity of patients with N2 and N0 categories was found.

In patients from both study groups with tumors of low differentiation grade, the level of miRNA-21 was 2.0 times lower compared with tumors of moderate differentiation grade ($p < 0.05$), while the expression levels of microRNA-375 were higher in tumors with a low differentiation grade.

Given the data on the relation between the expression of miRNA-21 and -375 with such indices of tumor aggressiveness as the presence of regional lymph node metastasis and differentiation grade, we have analyzed the recurrence-free survival of patients with COC taking into account the expression of these microRNAs.

The expression profiles of miRNA-21 and 375 allow us to determine the risk of recurrence within 1 year after diagnosis. Recurrence-free survival in the presence of miRNA-21 expression < 0.26 a.u. and miRNA-375 $<$

0.36 a.u. in tumor cells was lower than in the case of the expression of miRNAs-21 > 0.26 a.u. and miRNA-375 > 0.36 a.u. (Fig. 2). Recurrences in the group of patients with the expression profile of miRNA-21 < 0.26 and miRNA-375 < 0.36 a.u. in tumor cells were detected 1.7 times more often than in patients with higher expression of these microRNAs.

COC belongs to the highly heterogeneous malignant neoplasms, in the molecular pathogenesis of which a large number of signaling pathways is involved, providing high plasticity of tumor cells and, as a consequence, resistance to cancer therapy. MiRNAs play an important role in the development of COC. According to recent studies, the histological type of COC, resistance to therapy, tumor progression, metastasis or recurrence of the disease are associated with impaired expression of many microRNAs such as miRNA-100, -130a, -197, -181b, -181d, -101, -195 [10], -136, 147, -220a, -323-5p, -503, -632, -646, -668, -877, -1250 [11], etc.

We have shown that the development of COC is associated with increased levels of miRNA-21 and miRNA-375, which coincides with the literature data. In particular, significantly higher expression of miRNA-21 in tumor tissue of patients with COC compared with normal tissue was reported by Lamperska *et al.* [12]. Higher levels of miRNA-375 expression in the tissue of COC compared with untransformed tissue reported by Schneider *et al.* [13], however, other publications reported on the decrease of its expression [14, 15].

The obtained data indicate an important role of miRNA-21 and -375 in the formation of malignant phenotype of tumor cells. Thus, according to the literature and the results of bioinformatics analysis, we found that there is a relationship between the expression level of miRNA-21 or miRNA-375, clinical and pathological characteristics of patients and their relapse-free survival may be due to the indirect effect of miRNA-21 on the cell cycle [16], and the effect of miRNA-375 on the expression of a number of proteins associated with the tumor growth, invasion and

migration. Using the online tool TargetScan, we found that the target of miRNA-21 in COC is a gene encoding the oncogenic protein PTEN, which, in turn, is involved in the regulation of PI3K/AKT signaling pathway and migration of tumor cells. Therefore, in COC, miRNA-21 may act as an oncosuppressor. These data could explain the decrease in miRNA-21 levels along with increasing tumor size in patients with disease recurrence within 12 months. In addition, the targets of miRNA-375 are mRNA for p53, p21, Rb, c-MYC, v-MYC, the expression of which is associated with carcinogenesis not only of tumors of the head and neck, but also tumors of other localizations. The inverse relationship between the indices of the studied markers and the survival of patients with COC is confirmed by the available literature data on oral cancer [17, 18].

Therefore, unsatisfactory results of treatment of patients with COC indicate the need to search for prognostic markers, which at the early stages, during therapy, and after treatment may help to identify patients at high risk of recurrence. The results indicate the possibility of using the expression of miRNA-21 and -375 in tumor cells as markers of COC aggressiveness.

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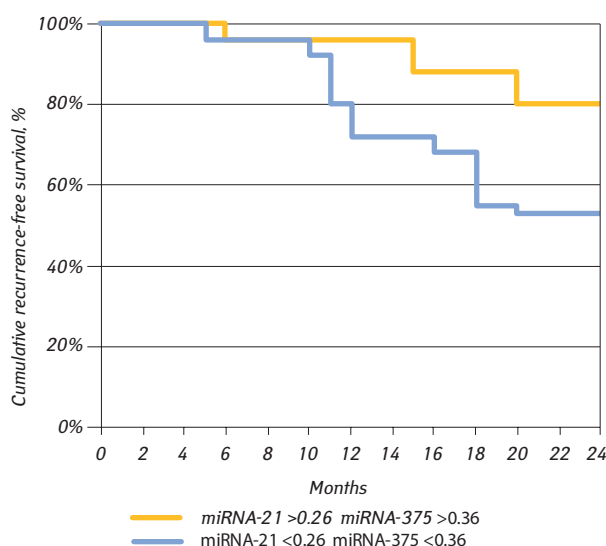


Fig. 2. Recurrence-free survival of patients with COC depending on the expression of miRNAs in tumor tissue, a.u.

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ЗНАЧЕННЯ ЕКСПРЕСІЇ ПУХЛИНОАСОЦІЙОВАНИХ МІКРОРНК-21 ТА -375 ДЛЯ ПРОГНОЗУВАННЯ ПЕРЕБІГУ РАКУ ПОРОЖНИНИ РОТА

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Мета: Дослідити особливості експресії мікроРНК-21 та

мікроРНК-375 у пухлинних клітинах хворих на рак порожнини рота (РПР) та визначити можливість їх використання для прогнозування агресивності перебігу РПР.

Матеріали та методи: Робота заснована на результатах обстеження та лікування 50 хворих з II–IV стадією РПР. Експресію мікроРНК у клітинах пухлини аналізували за допомогою кількісної полімеразної ланцюгової реакції. **Результати:** Високий рівень експресії мікроРНК-21 (> 0.26 у.о.) та мікроРНК-375 (> 0.36 у.о.) було визначено у 72.0% та 63.0% випадків відповідно. Ми виявили тенденцію до зниження експресії мікроРНК-21 та підвищення експресії мікроРНК-375 у пухлинах пацієнтів з безрецидивною виживаністю менше 12 міс та наявністю метастатичних уражень у регіонарних лімфатичних вузлах. У пацієнтів з РПР низького ступеня диференціювання рівень мікроРНК-21 був у 2.0 рази нижчим порівняно з пухлинами помірного ступеня диференціювання ($p < 0.05$), тоді як експресія мікроРНК-375, навпаки, була вищою у пухлинах низького ступеня диференціювання. Зниження експресії мікроРНК-21 (< 0.26 у.о.) на тлі зниженого рівня мікроРНК-375 (< 0.36 у.о.) в пухлинних клітинах асоціювалося з гіршою безрецидивною виживаністю. **Висновки:** Отримані результати вказують на зв'язок між основними клініко-патологічними характеристиками пацієнтів з РПР та рівнем експресії мікроРНК-21 та -375 у тканині пухлини, що свідчить про участь цих мікроРНК у формуванні злоякісності РПР та про їх потенційну практичну значимість як додаткових прогностичних маркерів.

Ключові слова: рак ротової порожнини, мікроРНК-21, мікроРНК-375, виживаність, агресивність.