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PROGNOSTIC BIOMARKERS AND THEIR IMPACT ON TREATMENT STRATEGY SELECTION FOR PATIENTS WITH ADVANCED STAGES OF OROPHARYNGEAL CANCER

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Ключові слова: *онкологія, рак ротоглотки, плоскоклітинний рак, статус вірусу папіломи людини, імуногістохімічне дослідження, біомаркери, рецептор епідермального фактора росту, Cyclin D1, Ki-67, ген інгібітор циклін-залежної кінази 2а, прогноз, лікування*

Abstract. Prognostic biomarkers and their impact on treatment strategy selection for patients with advanced stages of oropharyngeal cancer. Rakhmanov V.V., Shponka I.S. *The study of prognostic biomarkers is crucial for personalising and enhancing the effectiveness of oropharyngeal cancer treatment, as they help identify patients with aggressive disease progression and determine the optimal patient management strategy. Purpose: Analysis of the effectiveness of the use of biomarkers in the context of human papillomavirus status for predicting oncological events, functional outcomes to optimise treatment approaches for patients with advanced oropharyngeal cancer. The study examined the results of examination and treatment of 120 patients aged 37 to 80 years, of both sexes diagnosed with stage III-IV oropharyngeal cancer ($T_{3-4}N_{0-3}M_{0-1}$). The clinical and morphological characteristics and the outcomes of histological and immunohistochemical analyses of biopsy and surgical samples were assessed. Human papillomavirus (HPV) status was determined using immunohistochemistry for the p16 oncoprotein. Statistical analysis was performed using R Commander (https://www.r-project.org/) and MedCalc Statistical Software trial version 22.030 (https://www.medcalc.org/), applying descriptive and analytical statistics, rank correlation, logistic regression, and receiver operating characteristic (ROC) analysis. In late stages of oropharyngeal cancer, increased expression of Cyclin D1 and epidermal growth factor receptor (EGFR) was significantly more common in HPV-negative cases compared to HPV-positive ones ($p<0.05$). HPV-negative tumours were associated with homozygous deletion cyclin dependent kinase inhibitor 2a gene (CDKN2A). The identification of biomarkers as prognostic criteria for different HPV statuses confirms their role in choosing a treatment strategy. Biomarkers like Ki-67 and homozygous deletion of CDKN2A were prognostic for metastasis, while Ki-67 and Cyclin D1 were linked to recurrence. EGFR showed the highest prognostic accuracy for predicting masticatory muscle trismus. Increased expression of Cyclin D1 and Ki-67 in late stages of oropharyngeal neoplasms correlated with combined treatment, increasing the odds by 4.42 times and 25%, respectively ($p<0.05$). Evaluation of biomarkers such as p16, EGFR, Cyclin D1, and Ki-67 provides essential information into disease prognosis and response to treatment, enabling risk stratification, tailored treatment planning, and the development of personalized strategies for managing advanced oropharyngeal cancer.*

Реферат. Прогностичні біомаркери та їх вплив на вибір стратегій лікування пацієнтів з пізніми стадіями раку ротоглотки. Рахманов В.В., Шпонька І.С. *Вивчення прогностичних біомаркерів є актуальним для персоналізації та підвищення ефективності лікування раку ротоглотки, оскільки вони допомагають ідентифікувати пацієнтів з агресивним перебігом захворювання та визначити оптимальну стратегію ведення пацієнтів. Мета: аналіз ефективності використання біомаркерів з урахуванням статусу вірусу папіломи людини для прогнозування онкологічних подій, функціональних результатів та оптимізації підходів до лікування хворих з пізніми стадіями раку ротоглотки. Проведено аналіз результатів обстеження та лікування 120 пацієнтів від 37 до 80 років, обох статей, хворих на рак ротоглотки III-IV стадії ($T_{3-4}N_{0-3}M_{0-1}$). Оцінювалися клініко-морфологічні характеристики пацієнтів та результати гістологічних й імуногістохімічних вибіркових досліджень біопсійного та операційного матеріалу. Імуногістохімія для онкопротеїну p16 використовувалася*

для визначення вірусу папіломи людини (ВПЛ) статусу. Статистичний аналіз проводився за допомогою R Commander (https://www.r-project.org/) та MedCalc Statistical Software trial version version 22.030 (https://www.medcalc.org/download/) та включав описову й аналітичну статистику, ранговий кореляційний аналіз, простий і множинний логістичний регресійний аналіз та receiver operating characteristic (ROC) аналіз. Було визначено, що на пізніх стадіях раку ротоглотки підвищена експресія Cyclin D1 та рецептора епідермального фактора росту (EGFR) є суттєво вищою у ВПЛ-негативних випадках порівняно з ВПЛ-позитивними (p<0,05). ВПЛ-негативний статус асоціюється з гомозиготною делецією гена інгібітор циклін-залежної кінази 2a (CDKN2A). Визначення біомаркерів у якості прогностичних критеріїв для різних ВПЛ-статусів підтверджує їх роль у виборі стратегії лікування. Прогностичними критеріями метастазування визначено значення Ki-67 та гомозиготну делецію CDKN2A, для передбачення рецидиву – маркери Ki-67 і Cyclin D1. Найкращі прогностичні характеристики для прогнозу тризму жувальної мускулатури показав EGFR. Підвищена експресія Cyclin D1 та Ki-67 на пізніх стадіях новоутворень ротоглотки асоціюється з комбінованим лікуванням, збільшуючи шанси в 4,42 раза та на 25% відповідно (p<0,05). Оцінка біомаркерів, таких як p16, EGFR, Cyclin D1 і Ki-67, надає цінну інформацію про прогноз захворювання і відповідь на лікування, допомагаючи в стратифікації ризиків, плануванні лікування та розробленні персоніфікованої стратегії лікування раку ротоглотки.

Oropharyngeal cancer constitutes a significant portion of malignant neoplasms of the head and neck, averaging 10% to 25% of all cases, and reaching up to 30% in certain populations [1, 2].

According to global cancer statistics (GLOBOCAN) in 2022, the number of new cases of oropharyngeal cancer in 185 countries worldwide amounted to 106,316, ranking 24th (0.5% of all cancer types), with 52,268 deaths, ranking 23rd (0.5% of all causes). The age-standardized incidence risk for men was 1.9%, while the mortality risk was 0.9%, exceeding the rates for women, which were 0.4% and 0.2%, respectively [3].

As with other head and neck neoplasms, squamous cell carcinoma accounts for 80-90% of all oropharyngeal cancers. Risk factors include human papillomavirus (HPV) infection, tobacco use, alcohol consumption, ultraviolet radiation, lifestyle, dietary deficiencies, and others [3, 4, 5]. Oropharyngeal cancer is more commonly diagnosed in older individuals, typically after the age of 50. However, in recent years, there has been an increase in incidence among younger age groups, primarily associated with HPV infection, particularly HPV type 16 (HPV 16) [4, 5].

Approximately 70% of oropharyngeal cancer cases are diagnosed at late stages, reducing the 5-year survival rate from 83.7% for early-stage localized cancer to 38.5% when metastases are present at diagnosis [6]. In Ukraine in 2022, advanced stages of malignant neoplasms of the tonsils (76.1%) and oropharynx (82.0%) were most frequently detected [7].

Despite the adoption of the 2023 Evidence-Based Clinical Guideline «Cancer of the Oral Cavity, Oropharynx, Hypopharynx, Larynx, and Advanced Head and Neck Cancer» and the Unified Clinical Protocol for Primary and Specialized Medical Care «Oropharyngeal Cancer» (Order of the Ministry of Health of Ukraine dated October 20, 2023, No. 1831), the treatment of patients with advanced stages of oropharyngeal cancer remains a critical issue [8].

The study of prognostic biomarkers aids in personalising oropharyngeal cancer treatment and improving its effectiveness. Biomarkers associated with clinical outcomes are used to identify patients with more aggressive disease progression. To date, more than 75 biomarkers have been investigated as indicators for predicting head and neck cancer outcomes [2, 5, 6, 8], with particular emphasis on the p16 oncoprotein as an indicator of HPV infection. According to the new World Health Organization classification, HPV-positive oropharyngeal squamous cell carcinoma is a distinct oncological disease with its staging criteria and a better prognosis compared to HPV-negative squamous cell carcinoma [2].

Biomarkers that regulate the cell cycle and play key roles in tumor development and progression are being actively studied. These include the cell proliferation marker Ki-67, which reflects tumor growth rate; matrix metalloproteinases (MMPs) as enzymes that promote invasion and metastasis of tumor cells; vascular endothelial growth factor (VEGF), associated with tumor neoangiogenesis; Cyclin D1 as a key regulator of the cell cycle; epidermal growth factor receptor (EGFR), a critical membrane protein involved in regulating cell growth, survival, proliferation, and differentiation; and oncogenic proteins such as p53, p27, and Bcl-2, as well as the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene and TP53 gene, among others [9, 10, 11].

Despite the growing number of studies on biomarkers as diagnostic and prognostic parameters, no indicators have yet surpassed the classical prognostic factors used in clinical practice, such as TNM staging and HPV infection status [9]. This underscores the need for further detailed research on biomarkers with the potential to enhance the effectiveness of oropharyngeal cancer treatment, considering the HPV status of the tumor, which determined the relevance of this study.

Analysis of the effectiveness of the use of biomarkers in the context of human papillomavirus status for predicting oncological events, functional outcomes to optimise treatment approaches for patients with advanced oropharyngeal cancer.

MATERIALS AND METHODS OF RESEARCH

Analysis of the examination and treatment results of 120 patients aged 37 to 80 years, with a mean age of 59.3 years (95% CI 57.6 – 61.1; SD=9.9), of both sexes (67.5% male and 32.5% female), diagnosed with stage III-IV oropharyngeal cancer ($T_{3-4}N_{0-3}M_{0-1}$) treated at the ENT-2 (Oncology) Department of the «Dnipropetrovsk Regional Clinical Hospital named after I.I. Mechnikov» of the Dnipropetrovsk Regional Council during 2018-2023.

All patients were included in the study after providing their informed written consent. The study was conducted in compliance with the Helsinki Declaration and other international and national guidelines on bioethical principles of scientific research (excerpt from the protocol of the Biomedical Ethics Committee of Dnipro State Medical University, No. 9 dated October 26, 2021).

Clinical and morphological characteristics of the patients were evaluated, including age, sex, disease stage, tumor differentiation and keratinization degree, morphological variants of the tumor, oncological events (recurrence and metastasis), and functional outcomes such as the presence or absence of masticatory muscle trismus.

The study analysed the morphological examination results for all patients, histological and immunohistochemical evaluations of biopsy and surgical materials from 70 patients, and fluorescence in situ hybridization (FISH) results from 26 histological samples. Morphological, histological, immunohistochemical (IHC), and FISH methods were employed to assess CDKN2A gene anomalies. Detailed methodologies for marker expression evaluation have been described in previous publications [10, 11].

Morphological and histological examinations were performed using the ZEISS «Primo Star» light microscope (objectives ×10, ×20, ×40). Digital images were obtained using the Zeiss Primo Star-Axiocam ERC 5s camera with ZEN 2 blue edition software.

For IHC studies, antibodies against p16 (RTU), p53 (RTU), EGFR (RTU), Cyclin D1 (EP12, RTU), Ki-67 (sp6, RTU), and p27 (sp1, RTU), as well as the UltraVision Quanto visualization system (LabVision), were used. The reaction was identified using a chromogen solution of 3-diaminobenzidine tetrachloride (Quanto, LabVision), visualized with a microscope, and marked by brown staining. Nuclei were counterstained with Mayer's hematoxylin for 1-3 minutes.

The results of IHC reactions were assessed visually using a semi-quantitative method by counting positively stained cells of varying intensity. IHC for the p16 oncoprotein was used to determine HPV status – p16(+) indicated HPV (+) status. Biomarker expression was evaluated on a scale ranging from a negative reaction to high (strong) levels of expression, following expert recommendations.

The expression of biomarkers was assessed in a range from negative reaction to high (strong) levels of expression according to the recommendations of leading experts. Marker expression thresholds were as follows: p53 was considered elevated (positive) if ≥25% of tumor cells demonstrated nuclear expression, Cyclin D1 at ≥10% of stained cells, Ki-67 at ≥50% of cells, and high expression levels of EGFR and p27 were scored as (+2, +3). Homozygous deletion of CDKN2A was studied as an anomaly of the cyclin-dependent kinase inhibitor 2A gene [10, 11].

Statistical analysis was performed using tools implemented in R Commander (https://www.r-project.org/), including calculations of means (M) and relative values (%), 95% confidence intervals (CI), standard deviation (SD), evaluation of statistical differences using Pearson's Chi-square test (χ^2), including Yates' correction for continuity for small frequencies and proportions (near 0 or 100), Spearman's rank correlation coefficients (r_s), simple and multiple logistic regression analyses with odds ratios (OR) and 95% CIs, and receiver operating characteristic (ROC) analysis.

The ROC analysis included calculating sensitivity (Se), specificity (Sp), and area under the ROC curve (AUC). AUC values were interpreted as follows: 0.9–1.0: excellent accuracy; 0.8–0.9: very good; 0.7–0.8: good; 0.6–0.7: average; 0.5–0.6: poor; 0.5: unsuitable method [12]. ROC analysis and ROC curve plotting were performed using MedCalc Statistical Software trial version 22.030 (MedCalc Software, Ostend, Belgium; https://www.medcalc.org/download/).

The critical level of statistical significance (p) was set at <5% ($p<0.05$) for all analyses.

RESULTS AND DISCUSSION

In all 120 examined cases, oropharyngeal cancer was represented by squamous cell carcinoma. Among these, 78 cases (65.5%) were classified as moderately differentiated carcinoma, while 80 cases (66.7%) were non-keratinizing carcinoma.

All patients received treatment according to the current clinical guidelines and national regulatory documents. Predominantly, surgical treatment was applied in 17 cases (14.2%), radiation therapy and chemoradiotherapy (CRT) in 67 cases (55.8%), and comprehensive treatment involving a combination of

surgery, radiation therapy, chemotherapy, and other methods in 36 cases (30.0%).

Among the 120 patients, metastases were detected in 61 individuals, with the metastatic rate of oropharyngeal cancer at late stages amounting to 50.8% (95% CI: 41.5 – 60.0%). Recurrence was observed in 8 patients, with a recurrence rate of 6.7% (95% CI: 2.9 – 12.8%). Trismus of the masticatory muscles was identified in 15 patients, corresponding to a trismus prevalence of 12.5% (95% CI: 7.2–19.8%), which aligns with international studies reporting a trismus prevalence ranging from 5% to 38% following treatment of head and neck malignancies [13].

A similar event frequency was observed in the subset of cases subjected to histological and immunohistochemical (IHC) studies (Table 1).

In the comparison groups based on HPV status, no significant differences were found in the frequency of trismus of the masticatory muscles, metastases, or recurrences ($p>0.05$). However, it is noteworthy that the recurrence rate was 5.2% higher in patients with p16 (-) cancer (95% CI -10.8% to 17.7%), although it did not reach statistical significance due to the limited number of adverse events, aligning with findings reported by other researchers [14].

It was determined that advanced-stage oropharyngeal cancer is predominantly characterized by elevated expression of Cyclin D1 (67.1% of cases) and EGFR (75.7%), with fewer cases showing increased expression of p53, p27, and Ki-67. Regarding the elevated expression of biomarkers about HPV status, no significant differences were observed for p27 and Ki-67. In contrast, a statistically significant ($p<0.05$) higher level of expression of p53, Cyclin D1, and EGFR was identified in the HPV-negative group compared to the HPV-positive group.

Homozygous deletion of CDKN2A was observed exclusively in HPV-negative cases with p16 (-) status, while it was absent in p16 (+) cases ($p<0.001$).

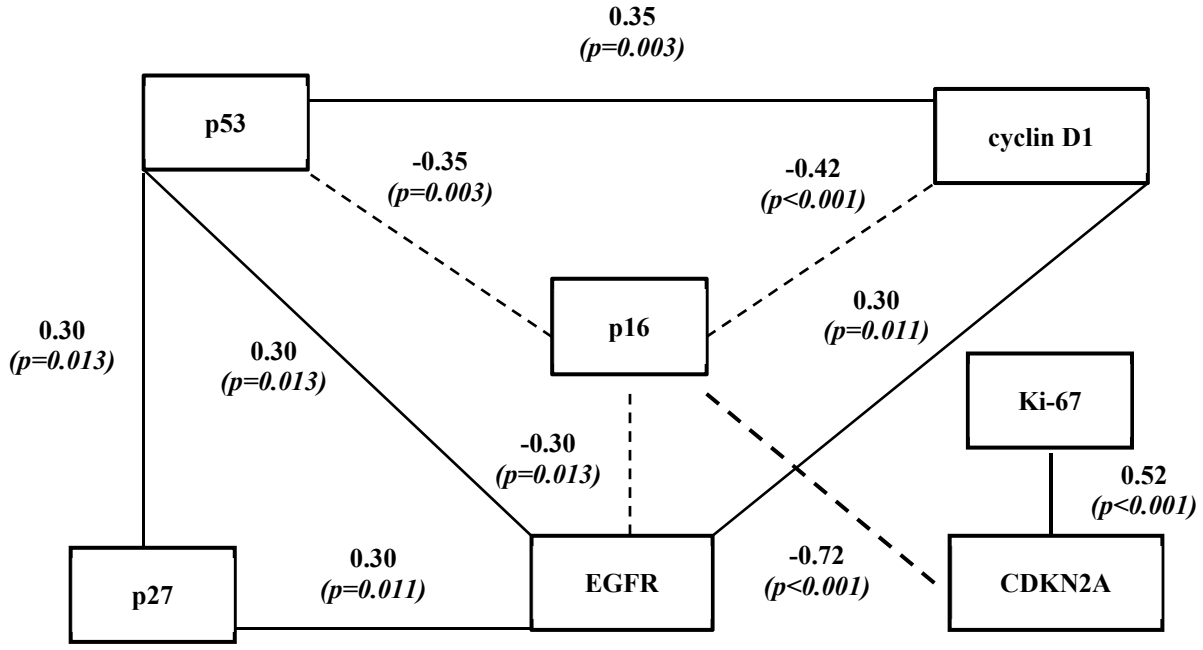
Table 1

Clinical outcomes and biomarker expression levels in patients with advanced oropharyngeal cancer by HPV status

Clinical and IHC results	All patients	HPV - negative cancer/ p16 (-)	HPV - positive cancer/ p16 (+)	p
Total number, n (%)	70 (100.0%)	44 (62.9%)	26 (37.1%)	-
Trismus of masticatory muscles, n (%)	11 (15.7%)	5 (11.4%)	6 (23.1%)	0.193
Metastases, n (%)	36 (51.4%)	19 (43.3%)	17 (65.4%)	0.072
Recurrence, n (%)	5 (7.1%)	5 (9.1%)	1 (3.9%)	0.410
Biomarker Expression Levels, n (%)				
Elevated expression of p53>25%, n (%)	23 (32.9%)	20 (45.5%)	3 (11.5%)	0.004
Elevated expression of Cyclin D1>10%, n (%)	47 (67.1%)	38 (86.4%)	9 (34.6%)	<0.001
High EGFR expression (+2, +3), n (%)	53 (75.7%)	37 (84.1%)	16 (61.5%)	0.033
High expression of p27 (+2, +3), n (%)	22 (31.4%)	13 (29.6%)	9 (34.6%)	0.659
High expression of Ki-67>50%, n (%)	17 (24.3%)	10 (22.7%)	7 (26.9%)	0.692
Homozygous deletion of CDKN2A, N, (n, %)	n=26 10 (38.5%)	n=14 10 (71.4%)	n=12 0 (0.0%)	0.001

Note. p values represent differences between comparison groups according to Pearson's χ^2 test, including Yates' correction where applicable.

Correlation analysis of the expression of the investigated biomarkers (Fig. 1) confirmed that the carcinogenesis of oropharyngeal neoplasms is a multifaceted process. A negative correlation was observed between p16 and p53, EGFR, and Cyclin D1, while a positive correlation was noted among EGFR, p53, p27, and Cyclin D1. This indicates a coordinated interaction among these biomarkers in the regulation of the cell cycle and proliferation processes.



The numbers represent Spearman's correlation coefficients (r_s): $0.25 \leq |r_s| < 0.70$ – moderate correlations; solid lines indicate direct correlations; dashed lines indicate inverse correlations.

Fig. 1. Correlation of biomarker expression in patients with late-stage oropharyngeal cancer

The CDKN2A gene, encoding the p16 protein, exhibits reduced or absent expression, which is illustrated by a strong inverse Spearman's rank correlation between p16 expression and homozygous CDKN2A deletion (r_s=-0.72; p<0.001). This deletion directly correlates with Ki-67 levels (r_s=0.75; p<0.001), indicating that the loss of the CDKN2A gene is associated with increased cell proliferation.

Studies by Pakistani researchers on the association of human papillomavirus (HPV) with other biomarkers in head and neck cancer carcinogenesis revealed correlations between p16 and Cyclin D1, p53, and EGFR [15].

To identify biomarker expression that may assist in risk assessment for poor prognosis and in selecting an optimal treatment strategy, an ROC analysis was conducted, including the consideration of HPV status (Table 2).

Regarding masticatory muscle trismus, the epidermal growth factor receptor demonstrated the best prognostic performance among the studied markers, both in the overall cohort and in the HPV-positive subgroup. The area under the ROC curve showed good predictive characteristics: AUC=0.710 (p=0.036) and AUC=0.721 (p=0.048), respectively. Elevated EGFR expression is associated with aggressive tumor growth and invasion into surrounding tissues, potentially leading to trismus.

It is noteworthy that for several markers, the sensitivity of the tests approaches or reaches the maximum of 100% in predicting masticatory muscle trismus: p53, EGFR, and homozygous CDKN2A deletion for the entire cohort; p53, EGFR, p27, and Ki-67 for the HPV-negative subgroup. This indicates that these tests almost always accurately identify individuals with this functional impairment, emphasizing their importance in the broader context regardless of other factors.

Previous studies have established links between excessive Cyclin D1 expression and the development of regional lymph node metastases in HPV-positive cases, as well as increased EGFR expression with worse prognosis and more aggressive tumor behaviour [16].

Statistically significant areas under the ROC curves and corresponding predictive capabilities for metastasis were identified for Ki-67 (moderate predictive performance: AUC=0.603; p=0.048) and homozygous CDKN2A deletion (good predictive performance: AUC=0.735, p=0.013). In p16 (-) cases, the predictive capability of the latter marker improves to a good level, while for Ki-67, it does not reach clinically or statistically significant levels, although the protein's sensitivity in this context reaches 100%. The cell proliferation marker maintains its predictive ability for metastasis in p16 (+) cases.

Table 2

Prognostic potential of biomarkers in late-stage oropharyngeal cancer: an ROC analysis

Markers	Masticatory muscle trismus		Metastases		Recurrence	
	AUC (95% CI), *p*	Se/Sp, %	AUC (95% CI), *p*	Se/Sp, %	AUC (95% CI), *p*	Se/Sp, %
All patients						
p16	0.601 (0.478-0.714) p=0.193	54.6/65.6	0.597 (0.475-0.711) p=0.086	47.2/72.2	0.566 (0.444-0.683) p=0.607	75.0/38.2
p53	0.547 (0.423-0.666) p=0.600	100.0/17.0	0.567 (0.443-0.685) p=0.338	74.3/51.4	0.578 (0.454-0.695) p=0.685	25.0/100.0
Cyclin D1	0.558 (0.434-0.676) p=0.553	54.6/71.2	0.595 (0.471-0.711) p=0.173	40.0/85.7	0.805 (0.694-0.890) p<0.001	100.0/64.6
EGFR	0.710 (0.500-0.869) p=0.036	90.9/66.7	0.570 (0.446-0.688) p=0.281	31.4/82.9	0.640 (0.517-0.751) p=0.036	75.0/60.6
p27	0.520 (0.397-0.641) p=0.836	36.4/76.3	0.505 (0.383-0.627) p=0.945	77.1/31.4	0.509 (0.387-0.631) p=0.937	100.0/25.8
Ki-67	0.508 (0.386-0.630) p=0.932	18.2/91.5	0.603 (0.479-0.718) p=0.048	71.4/51.4	0.650 (0.526-0.760) p=0.042	50.0/90.9
Deletion of CDKN2A	0.615 (0.491-0.729) p=0.149	80.0/27.1	0.735 (0.527-0.887) p=0.013	52.9/88.9	n/d	n/d
HPV – negative cancer/p16 (-)						
p53	0.621 (0.462-0.762) p=0.304	100.0/28.2	0.566 (0.408-0.715) p=0.452	61.1/61.5	0.593 (0.435-0.739) p=0.653	33.3/100.0
Cyclin D1	0.574 (0.416-0.722) p=0.653	60.0/ 4.4	0.561 (0.403-0.710) p=0.513	61.1/61.5	0.792 (0.532-0.823) p=0.030	100.0/57.5
EGFR	0.579 (0.421-0.727) p=0.509	100.0/18.0	0.544 (0.387-0.695) p=0.629	22.2/88.5	0.756 (0.603-0.873) p=0.009	100.0/51.2
p27	0.574 (0.416-0.722) p=0.512	100.0/25.6	0.549 (0.392-0.699) p=0.589	83.3/34.6	0.585 (0.427-0.732) p=0.581	66.7/68.3
Ki-67	0.554 (0.397-0.704) p=0.667	100.0/25.6	0.590 (0.431-0.736) p=0.297	100.0/26.9	0.650 (0.492 0.788) p=0.049	66.7/87.80
Deletion of CDKN2A	0.625 (0.335-0.861) p=0.588	50.0/75.0	0.825 (0.535-0.971) p=0.023	90.0/75.0	n/d	n/d
HPV – positive cancer/p16 (+)						
p53	0.646 (0.435-0.822) p=0.337	66.7/75.0	0.520 (0.317-0.718) p=0.872	41.2/77.8	n/d	n/d
Cyclin D1	0.621 (0.411-0.802) p=0.298	100.0/35.0	0.575 (0.368-0.765) p=0.533	58.8/77.8	n/d	n/d
EGFR	0.721 (0.512-0.877) p=0.048	50.0/85.0	0.503 (0.302-0.704) p=0.979	0/88.9	n/d	n/d
p27	0.500 (0.299-0.701) p=0.986	66.7/15.0	0.520 (0.317-0.718) p=0.889	70.6/55.6	n/d	n/d
Ki-67	0.517 (0.314-0.715) p=0.916	33.3/90.0	0.709 (0.509-0.869) p=0.050	70.6/77.8	n/d	n/d
Deletion of CDKN2A	0.621 (0.411-0.802) p=0.298	100.0/35.0	0.575 (0.368-0.765) p=0.533	58.8/77.8	n/d	n/d

Notes:. n/d – not determined; results were not provided due to insufficient data.

The adjusted odds ratio, determined through simple logistic regression analysis, showed that the presence of homozygous deletion of the CDKN2A gene in HPV-negative cancer increases the chances of metastasis by 27 times: OR=27.0 (95% CI 1.26-578.39; p=0.035).

In the HPV-positive status group, the prognostic ability of biomarkers to predict recurrence could not be determined due to the insufficient number of such unfavourable events in this group. The statistically prognostic ability for recurrence in HPV-negative cancer was found for Cyclin D1 (AUC=0.792; p=0.030), EGFR (AUC=0.756; p=0.009), and Ki-67 (AUC=0.650; p=0.049). Both in the p16 (-) status and across all studied cases, the best prognostic properties were observed for Cyclin D1: AUC=0.805 (p<0.001). For EGFR and Ki-67, although the operational characteristics reached statistical significance, they did not have clinical relevance – the areas under the ROC curves were AUC<0.700 (Fig. 2).

In HPV-negative status, an increase in Ki-67 expression above 15% (the cutoff point determined by ROC analysis) increases the chances of recurrence by 12.0 times (95% CI 1.13-153.89; p=0.046). Without considering the HPV status, in all studied cases, an increase in Ki-67 expression above 40.4% raises the chances of recurrence by 8.4 times (95% CI 1.27-55.39; p=0.027).

The identification of informative biomarkers allows for the selection of patients with an increased risk of unfavourable outcomes in oropharyngeal cancer and helps to determine the optimal treatment strategy, including complex treatment for the high-risk group [16].

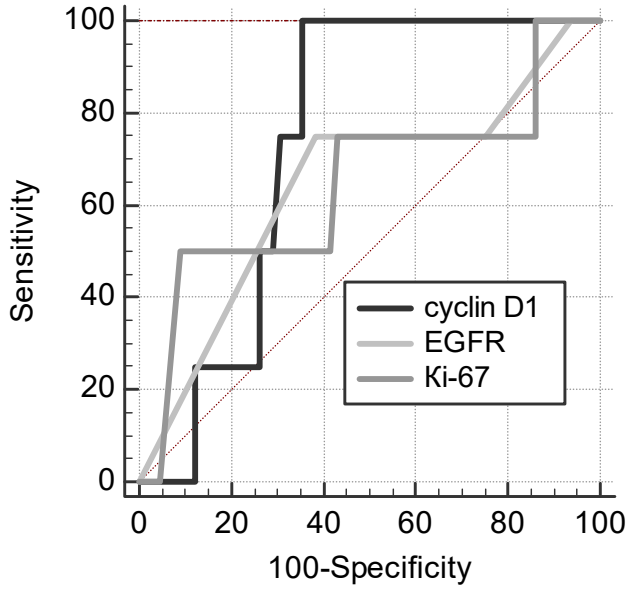


Fig. 2. Assessment of prognostic capabilities for predicting metastasis based on Cyclin D1, EGFR, and Ki-67 in oropharyngeal cancer at advanced stages

To confirm this approach, a multiple logistic regression analysis was performed with stepwise inclusion of independent variables. Biomarkers were studied to determine the treatment strategy, considering the following options: monotherapy (standard chemoradiotherapy or surgical treatment) or combined therapy (a combination of surgical treatment, radiotherapy, chemotherapy, and other methods).

As a result of the stepwise inclusion of independent variables, the parameters of the logistic regression equation, presented in Table 3, showed that Cyclin D1 and Ki-67 had statistically significant prognostic values for selecting the treatment strategy in the studied patient group.

Considering the obtained data, the equation for selecting the type of treatment for patients with advanced-stage oropharyngeal cancer is as follows:

$$y=\exp(-2{,}67-0{,}08*x_1+0{,}225*x_2)/[1+\exp(-2{,}67-0{,}08*x_1+0{,}225*x_2)],$$

where – y is the outcome, ranging from 0 (standard treatment: chemoradiotherapy or surgery) to 1 (combined treatment); β_0=-2,67 is the intercept of the logistic regression equation; x_1 – Cyclin D1 (%) expression; x_2 – Ki-67 (%) expression.

Table 3

Assessment of approaches to selecting treatment options for patients with advanced-stage oropharyngeal cancer based on biomarker determination (according to stepwise multiple logistic regression analysis)

Prognostic variables	β	m (β)	Wald χ^2	p Wald χ^2	OR	95% CI
Intercept	-2.670					
Cyclin D1 (%) – x_1	-0.080	0.040	3.99	0.042	4.42	1.15-11.91
Ki-67 (%) – x_2	0.225	0.103	4.763	0.029	1.25	1.02-1.53

Notes: β – regression coefficients; m (β) – standard errors of the regression coefficients β; Wald χ^2 (p) – Wald χ^2 statistic; OR (95% CI) – adjusted odds ratios; table presents statistically significant predictors.

Regardless of the regression coefficients or predictor values, the predicted value (y) will always range from 0 to 1. If the calculated result exceeds 0.5, combined treatment can be recommended.

According to Nagelkerke's coefficient of determination, 60.4% of the variation in the choice of treatment strategy for oropharyngeal cancer is explained by the expression of biomarkers included in the model (Cyclin D1 and Ki-67). The remaining 39.6% is attributed to other factors not included in the model.

According to the adjusted odds ratios, accounting for the effects of other factors, the influencing factors on the choice of treatment among the studied biomarkers are Cyclin D1 expression (OR=4.42; 95% CI 1.15-11.91) and Ki-67 expression (OR=1.25; 95% CI 1.02-1.53). This shows that for each unit increase in Cyclin D1 expression, the likelihood of combined treatment increases 4.42 times, while for Ki-67, the likelihood increases by 25% (1.25 times).

The evaluation of the logistic regression model using the Chi-square value (χ^2=13.68; p<0.033), percentage of concordance (83.3%), Hosmer-Lemeshow goodness-of-fit test (p=0.946), and ROC analysis (sensitivity=83.3%, specificity=88.9%, area under the ROC 0.889 (95% CI 0.790-0.952)) showed very good predictive ability of the obtained regression equation.

Regarding the choice of treatment depending on oncological events and functional outcomes, a pattern of more frequent use of combined treatment in the presence of recurrences is observed. Patients with recurrence almost always received combined treatment (87.5%), which statistically significantly differs from patients without recurrence, of whom 59.8% received chemoradiotherapy (p=0.008).

The obtained results of the study confirm that late-stage oropharyngeal cancer has specific molecular markers that can serve as indicators of disease aggressiveness and prognostic factors. Increased expression of Cyclin D1 and EGFR in patients with late-stage disease aligns with data from other studies, confirming their importance in regulating the cell cycle and their association with tumor cell proliferation [9, 16]. These results highlight their role in the aggressive course of oropharyngeal cancer, particularly in metastasis and recurrences. A decrease in the expression levels of p53, p27, and Ki-67, in turn, may indicate disruptions in cell cycle control mechanisms, which also have prognostic significance, especially for assessing the risk of disease progression [10, 13, 17]. According to correlation analysis, similar to the work of other researchers (Tariq N. et al., 2023), there is coordination between the molecular markers p16, EGFR, p53, and Cyclin D1 in the processes of cell proliferation and tumor growth [15].

Analysis of the relationship between biomarker expression levels and HPV status revealed significant differences between the HPV-positive and HPV-negative patient groups. In particular, the expression of p53, Cyclin D1, and EGFR was higher in HPV-negative cases, supporting the existing concept of two molecular subtypes of oropharyngeal cancer with different carcinogenic mechanisms [5, 6, 8]. This has important clinical implications, as HPV-negative patients generally have a worse prognosis compared to HPV-positive patients [2, 14].

According to our study, prognostic factors such as the expression level of Ki-67 and homozygous deletion of CDKN2A were significant in predicting metastasis, recurrences, and trismus of the masticatory muscles. These results, which align with the views of various researchers (Pekarek L. et al., 2023), highlight the importance of molecular stratification for determining the risk of metastasis and selecting optimal treatment [16].

According to our data, the presence of trismus, metastasis, or recurrence influences the choice of treatment strategy, with combination therapy being more commonly applied in cases with negative prognostic factors.

When determining treatment strategies for patients with late-stage oropharyngeal cancer, it is also necessary to consider other factors such as tumor spread, patient age, comorbidities, and others [4, 5].

Further research should focus on studying the interaction of molecular markers, genetic changes, and improving personalized treatment methods for oropharyngeal cancer, taking into account HPV status and individual patient characteristics.

CONCLUSION

1. It was determined that at the advanced stages of oropharyngeal cancer, there is increased expression of Cyclin D1 and EGFR, which is significantly higher in HPV-negative cases compared to HPV-positive cases ($p<0.05$).
2. The assessment of the correlation between biomarkers revealed that HPV-negative status p16(-) is associated with homogeneous deletion of the CDKN2A gene, which in turn correlates with increased cell proliferation activity (elevated Ki-67 levels), potentially contributing to a more aggressive course of oropharyngeal cancer.
3. Prognostic criteria for oncological events were identified as follows: for metastasis prediction – Ki-67 and homogeneous deletion of the CDKN2A gene (in HPV-negative cancer, increasing the likelihood of metastasis by 27 times; $p=0.035$); for recurrence prediction – Ki-67 (expression levels exceeding 40.4% increase the likelihood of recurrence by 8.4 times; $p=0.027$); for HPV-negative status – Cyclin D1 (AUC=0.805; $p<0.001$), EGFR, and Ki-67, with Cyclin D1 demonstrating the best prognostic characteristics. For functional outcomes, specifically trismus of the masticatory muscles, the epidermal growth factor was a significant predictor in both the overall cohort and HPV-positive cases.
4. In the multiple logistic regression analysis, it was found that the choice of treatment strategy for oropharyngeal tumors at advanced stages is associated with Cyclin D1 and Ki-67, with elevated expression of these markers increasing the likelihood of combined the treatment by 4.42 times and 25%, respectively ($p<0.05$).
5. The current strategy for treating oropharyngeal cancer requires a comprehensive multimodal approach, with a deeper understanding of carcinogenesis and tumor progression mechanisms. The evaluation of biomarkers, including p16, EGFR, Cyclin D1, and Ki-67, provides valuable information on disease prognosis and treatment response. These biomarkers can aid in risk stratification, treatment planning, and the development of targeted therapies for patients with oropharyngeal cancer.

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Rakhmanov V.V. – formal analysis, investigation, resources, writing – original draft, visualization;

Shponka I.S. – conceptualization, methodology, validation, project administration, writing – review & editing

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