RELATIONSHIP BETWEEN THE RESULTS OF IMMUNOHISTOCHEMICAL EXAMINATION (PHH3, Ki-67) AND POSITRON EMISSION TOMOGRAPHY DATA (SUVmax) OF OROPHARYNGEAL SQUAMOUS CELL CARCINOMA

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RELATIONSHIP BETWEEN THE RESULTS OF IMMUNOHISTOCHEMICAL EXAMINATION (PHH3, KI-67) AND POSITRON EMISSION TOMOGRAPHY DATA (SUVMAX) OF OROPHARYNGEAL SQUAMOUS CELL CARCINOMA

Lysak Andrii, Graduate Student of the Department of Pathological and Topographic Anatomy, Shupyk National Medical Academy of Postgraduate Education, Pathoanatomical Center of the National Military Medical Clinical Centre of the Main Military Clinical Hospital, Kyiv, ORCID ID: https://orcid.org/0000-0003-0917-2688

Diadyk Olena, Doctor of Medicine, Professor, Head of the Department of Pathological and Topographic Anatomy, Shupyk National Academy of Postgraduate Education, Kyiv

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ABSTRACT
The authors established the relationship between the indicators of immunohistochemical examination — proliferation markers (Ki67) and mitotic count (pHH3) with the indicators of positron emission tomography — SUVmax. The identified patterns of cancer cells differentiation and their metabolic activity are promising for the diagnosing and screening of tumours of varying degrees of progression and origin, which will allow forecasting the course of the disease at all stages of diagnostics. The study intends to assess the level of oropharyngeal squamous cell histopathological differentiation by immunohistochemical diagnostic methods, and their metabolic activity using positron emission tomography.

KEYWORDS
oropharyngeal squamous cell carcinoma, tumour differentiation, metabolic activity, immunohistochemistry, positron emission tomography.

1. Study of the activity of metabolic processes of transformed oropharyngeal cancer cells using positron emission tomography;
2. Determination of tumour proliferative potential by the number of positive stains to Ki-67 (%) and pH3 by immunohistochemical diagnostic methods;
3. Identification of the relationship between morphological changes of oropharyngeal cancer cells and their metabolic activity.

Materials and methods. We studied 130 samples of squamous cell carcinoma of the oropharyngeal area. We determined the level of metabolic activity — SUVmax — by positron emission tomography (PET) at the preoperative patient treatment stage. In the postoperative period, the proliferation index (Ki67) and the mitotic count (pHH3) were determined by histological and immunohistochemical methods. Upon assessment of the level of differentiation of tumour cells, patients were divided into three groups: Group I — patients having tumour cells in the G1 phase of mitosis (28 patients); Group II — tumour cells in the G2 phase of mitosis (48 patients); Group III — tumour cells in the G3 phase of mitosis (54 patients). We evaluated PET results according to the Maximum Standard Unit Value (SUVmax).

We determined the relationship between morphological changes and metabolic activity of tumour cells by 2-fluoro-[18F]-2-deoxy-D-glucose accumulation and immunohistochemical examination.

Results. We established a statistically significant difference between the groups (p<0.001 according to the Kruskal-Wallis test for all indicators). Thus, with decreasing Me level, the differentiation of SUVmax of tumours significantly (p<0.05) increases, which indicates an increase in the degree of malignancy of tumours. We evaluated the results of immunohistochemical examination by Ki67 and pH3 markers in the study groups. Comparing Ki67 and pH3, a statistically significant difference was found between the groups (p<0.001 according to the Kruskal-Wallis test for all indicators). Thus, with a decrease in the Me level, the differentiation of Ki67 increases significantly (p<0.05), and an increase in pH3 indicates an increase in the degree of malignancy of tumours.

Conclusions. We statistically proved the relationship between Ki67, pH3 and SUVmax in oropharyngeal squamous cell carcinoma. We established the possibility of preoperative forecasting of the level of tumour differentiation and the use of pH3 immunohistochemical marker as a reliable criterion for assessing the level of tumour differentiation, including hardly diagnosable squamous cell carcinoma.
Introduction. Differentiation of tumour cells is a central aspect of histopathological classification of malignant neoplasms. The stages of differentiation are strongly related to the metabolic activity of the transformed cells and the tumour behaviour. It is known that undifferentiated tumour is more aggressive than its more differentiated analogue [1]. Tumour differentiation is directly related to the expression and function of genes that determine the metabolic activity and direction of development of transformed cells [2]. The mechanisms of differentiation of tumour cells, as well as the relationship of morphological changes in cells with their metabolic activity are poorly studied. Besides, the level of metabolic activity of transformed cells underlies the diagnosis and evaluation of the degree of tumour differentiation, which plays a huge role in the treatment of cancer patients [3].

Treatment of oropharyngeal tumours is a time-consuming and unresolved problem of modern oncology. Anatomical and functional features of this area are the tendency of malignant tumours of this localization to rapid infiltrative growth, while early metastasising to lymph nodes cause severe disease and create difficulties in diagnosing and treatment [4]. Diagnostics of oropharyngeal neoplasms includes two stages: preoperative and postoperative. Diagnostics in the preoperative period helps to establish the location and size of the tumour, without determining its degree of malignancy. Preoperative diagnostics of oropharyngeal tumours is performed using positron emission tomography (PET) and computed tomography (CT). These methods belong to non-invasive diagnostics — one of the priority areas of medical research [5, 6].

The immunohistochemical marker — Ki67 — is an important marker for the verification of malignant tumours, which is determined to establish the proliferation index of tumour cells. Ki67 belongs to the nuclear antigens that are involved in the regulation of the cell cycle and are found in cell nuclei in G1-, S- and G2 phases of the cell cycle, as well as during the mitotic phase [7]. Ki67 as a monoclonal proliferation marker gives a positive reaction in G1A, G1B, S, G2 and M-phases of the cell cycle, but is not detected in G0, G1T, G1a, G1b phases. Nowadays, antibodies to Ki67 have become the standard for assessing the proliferative activity of transformed cells [8]. It is proved that the increase in the expression of this marker is directly proportional to the formation of the aggressive phenotype of many malignant tumours [9]. In particular, the high level of Ki67 expression is a prognostically unfavourable factor for breast, ovarian, prostate cancer, melanocytic tumours, gastrointestinal stromal tumours [8, 10, 11].

Phosphohistone H3 (pHH3) may be another proliferation marker. pHH3 is a histone protein that is part of DNA chromatin, and plays an important role in chromosome condensation and cell cycle progression during mitosis and meiosis after phosphorylation of serine-10 and serine-28 residues. Phosphorylation occurs during the late G2 phase before the onset of prophase, while dephosphorylation occurs slowly from late anaphase to early telophase. Therefore, in the metaphase histone H3 is always strongly phosphorylated, which is manifested by a positive test for pHH3, while in the interphase there is minimal or no expression of pHH3 — a property that allows pHH3 to be detected only in mitotically active cells [11]. For this reason, we hypothesized that pHH3 may be a better prognostic marker than the Ki67 marker and better detect tumour cell metastasis.

The research objective is to assess the level of squamous cell histopathological differentiation of cells (Ki-67, pHH3) with oropharyngeal carcinoma by immunohistochemical diagnostic methods, and their metabolic activity using positron emission tomography (SUVmax).

Materials and research methods. The study involved 130 patients with oropharyngeal squamous cell carcinoma. Among patients, men accounted for 75% (98 men), women - 25% (32 women).
Diagnosis of the detected pathology was performed in the preoperative period using PET/CT examination using Philips Gemini PET/CT-System series C. The PET method determined the level of metabolic activity — \( \text{SUV}_{\text{max}} \) [12, 13].

In the postoperative period, pieces of transformed tissue were taken from the tumour for histological and immunohistochemical examinations. The fixation was performed in a 10% solution of neutral formalin. Proliferation index (Ki67) and mitotic count (pHH3) were determined by histological and immunohistochemical methods.

In determining the degree of differentiation of tumour cells we used the classification of Anneroth et al. and Bryne et al. [14], presented in Table 1.

| Criterion                                      | Number of points |
|------------------------------------------------|------------------|
| The level of keratinization (% of cells)       | 1                |
| >50                                            | 20 –50           |
| 5 –20                                          | 0 –5             |
| Polymorphism of nuclei (% of mature cells)     | 2                |
| > 75                                           | 50 –75           |
| 25 –50                                         | 0 –25            |
| Mitotic count                                  | 3                |
| 0 –1                                           | 2 –3             |
| 4 –5                                           | >5               |
| Type of invasion                               | 4                |
| clearly demarcated edges                       | infiltrating areas|
| groups of infiltrating cells (n<15 cells)       | widespread areas of dissociated cells in groups or as single cells (n>15 cells) |
| Inflammation as a result of lymphoplasmacytic infiltration | 5                |
| severe                                         | moderate         |
| insignificant                                   | no inflammation  |

Taking into account the criteria shown in Table 1, we analysed the phases of the cell cycle of the studied tumours, with: 4-8 points — tumours with cells in G1 phase of the cell cycle; 9-12 points — G2 tumours; 13-16 points — G3 tumours.

Assessing the level of differentiation of tumour cells, we divided patients into three groups: Group I — patients with tumour cells in G1 phase of mitosis (28 patients); Group II — tumour cells in G2 phase of mitosis (48 patients); Group III — tumour cells in the G3 phase of mitosis (54 patients).

The mitotic activity of tumour cells was assessed by the proliferation index Ki67 and pH3, which we determined by immunohistochemistry.

During the immunohistochemical study, we used paraffin blocks to prepare histological sections with a thickness of 4 μm, which were obtained using a rotary microtome HM 355S, Section-Transfer-System, Walldorf.

Upon removal of paraffin and rehydration of sections, we performed temperature unmasking of antigens. We performed incubation of sections with primary antibodies in humid chambers for 30 minutes at 23-25°C, followed by staining of the test sections with hematoxylin-eosin in Ventana BenchMark machine (Ventana Medical Systems, Ventana, USA), and application to SuperFrost Plus slides. Ki67 monoclonal antibodies (monoclonal, murine anti-Ki67 antigen) were used as primary antibodies. We studied stained preparations on a digital light microscope Leica DM 6000 B, Leica Microsystems CMS GmbH using x5, x10, x20, x40, x100 lenses.

Reaction with the pHH3 marker was performed using rabbit polyclonal antigen and was determined in the number of positive nuclei counted during pH3 staining.

We performed statistical processing of the results using the Kruskal-Wallis test to compare indicators in the three groups. We used Dunn’s multiple comparison test during a posteriori comparisons [15]. The critical level of significance was \( \alpha_{\text{crit}} = 0.05 \).

To determine the values of \( \text{SUV}_{\text{max}} \), we analysed the constructed Receiver Operating Characteristic curves (ROC curves). We assessed the reliability of the test by the area under the ROC curve [15].

We determined the threshold for making a decision on the test by Youden index Inex=Max. We evaluated the prognostic characteristics of the test at the appropriate threshold by the specifics (95%) and sensitivity (95%) of the test.
We used the ROC curve method to assess the prognostic characteristics of the Ki67 and pHH3 markers [16].

**Results.** Analysing the morphological characteristics of tumours with cells at different stages of the life cycle, we found significant changes between the study groups.

The results of studies showed that at least 50% of mature cells we found in tumours with cells in G1 phase of the cell cycle (Group I) (Figure 1).

![Fig. 1. Squamous cell carcinoma of the oral cavity in G1 phase (hematoxylin-eosin staining, x50 magnification).](image)

The level of keratinization of tumour cells of squamous cell carcinoma was not less than 20%. Moderate-expressed (weak) polymorphism was visualized on histological sections. There was a clear growth of solid fields. We found a small number of cells that are in the stage of mitosis in the tumour tissue — from 0 to 3 cells in the field of vision. Lympho-plasmacytic infiltration is expressed by moderate inflammation (Figure 1).

In the tissues of squamous cell carcinoma, the cells of which are in G2 phase of the cell cycle (Group II), the level of keratinization was not less than 5% (Figure 2).

![Fig. 2. Oral squamous cell carcinoma cells in the G2 phase (hematoxylin-eosin staining, x100 magnification).](image)

The number of mature cells was not less than 25%. Histological sections showed moderate polymorphism of nuclei. We established that infiltrative growth is characteristic of G2 tumours. This growth was expressed in the localization of small groups of cells, but there were at least 15 cells in the group. The sections revealed a moderate number of mitotic cells, and a slight inflammatory reaction or its complete absence (Figure 2).
The results of the study of squamous cell carcinoma in Group III patients, having the transformed cells in G3 phase, showed at least 25% of mature cells (Figure 3). This group includes tumours with a low level of keratinization and pronounced polymorphism of the nuclei. The number of mitotic cells was 5 or more in the field of vision. As for the G2 phase, invasive processes in the form of small groups of cells were observed in G3 phase. However, there were less than 15 cells in the group or single cells were detected in G3 tumours. Inflammation was not observed (Figure 3).

Fig. 3. Oral squamous cell carcinoma cells in G2 phase (hematoxylin-eosin staining, x50 magnification)

The metabolic activity of tumour cells was determined by the standardized uptake value (SUV) in PET [17].

With the help of PET diagnostics we determined both the minimum and maximum SUV in patients of all study groups [18]. The mean SUV_{\text{min}} in all patients was 5.8; the mean SUV_{\text{max}} was 48.7.

Analysing the standardized uptake values — SUV_{\text{min}} and SUV_{\text{max}} depending on the degree of tumour differentiation revealed changes in these values in different groups of patients. Thus, in the group of G1 tumours the minimum value of SUV_{\text{min}} is 5.6, while the maximum value of SUV_{\text{max}} is 27. In Group II patients — with G2 tumours — the minimum value of SUV_{\text{min}} is 7, the maximum value of SUV_{\text{max}} is 28.2. In the group of G3 tumours, the minimum value of SUV_{\text{min}} is 5.7, the maximum value of SUV_{\text{max}} — 48.5 (Table 2).

As the results of the study presented in Table 2 show, SUV_{\text{max}} increases as the cell cycle of the tumour cells progresses.

Table 2. The value of standardized accumulation (SUV) of squamous cell carcinoma of the oral cavity in the Me format (Q_{I}\pm Q_{III})

| Index | Me (Q_{I}\pm Q_{III}) | G1 (n=28) | G2 (n=48) | G3 (n=54) | Significant difference between groups |
|-------|------------------------|-----------|-----------|-----------|--------------------------------------|
| SUV_{\text{max}} | 25.7^{*} (18.6\pm33.3) | 28.2^{*} (22.5\pm30.1) | 48.5^{*} (43.8\pm55.0) | <0.001 |
| SUV_{\text{min}} | 5.6^{*} (3.2\pm8.3) | 7.0^{*} (4.5\pm13.1) | 5.7^{*} (3.8\pm11.0) | <0.001 |

Note: * – statistically significant difference compared with G1 tumours, p<0.05; # - statistically significant difference compared with G2 tumours, p<0.05; ^ – statistically significant difference compared with G3 tumours, p <0.05.

We analysed the SUV_{\text{max}} index in the primary tumour and determined the dependence of the SUV_{\text{max}} index on the main characteristics of the oncological process, such as the depth of invasion of the tumour process, histological structure, and tumour differentiation [19].
The comparison of $\text{SUV}_{\text{max}}$ revealed an increase in the value of this indicator with a decrease in the level of differentiation of tumour tissue ($p<0.001$ according to the Kruskal-Wallis test). The lowest value of $\text{SUV}_{\text{max}}$ was observed in the group of patients with G1 tumours, and the highest — in patients of G3 group. An intermediate value of $\text{SUV}_{\text{max}}$ was observed in patients with tumours with cells in G2 phase of the life cycle. The values of this group were statistically lower than the indicators characteristic of G3 group, and statistically higher than the indicators characteristic of G1 group (Figure 4).

**Fig. 4.** The value of $\text{SUV}_{\text{max}}$ depending on the degree of differentiation of squamous cell carcinoma of the oral cavity

Analysis of the degree of differentiation of squamous cell carcinoma of the oral cavity revealed a low or medium degree of differentiation of transformed cells in patients with G2 and G3 tumours. For patients with G1 tumours, we observed a high degree of tumour cell differentiation, which indicates a favourable prognosis in the treatment of squamous cell carcinoma of the oral cavity.

During the analysis of samples according to $\text{SUV}_{\text{max}}$, we revealed the adequacy of the forecasting model (however, AUC=0.78, which is 95%, BI is 0.70–0.85, statistically significant value, $p<0.001$ exceeds 0.5). Figure 5 shows the ROC curve of the test, which indicates an important prognostic value of the indicator. The ROC curve of the test shows the degree of differentiation of tumour tissue (G3, G2 vs G1) by $\text{SUV}_{\text{max}}$, and indicates the forecasting of the tumour process [20]. Figure 5 indicates the sensitivity and specificity of the test, as well as the optimal decision threshold (according to Youden Index).

**Fig. 5.** ROC curve of the test for forecasting a high degree of tissue differentiation (G3, G2 vs G1) by $\text{SUV}_{\text{max}}$

*Note: the optimal decision threshold, sensitivity and specificity of the test are indicated.*
When choosing the optimal threshold, we set a critical value of the indicator — SUV\textsubscript{crit}, which equals 14. In case of exceeding this value, G2 or G3 phases of the life cycle of tumour cells are predicted. At this threshold, the sensitivity of the test is 71.7% (95% BI 61.8-80.3%). At the same time, the specificity of the test is 86.4% (95% BI 65.1-97.1%).

In the second stage of experimental studies, we observed the degree of differentiation of tumour cells in patients with G3 tumours. The forecast was favourable (not the case) for patients with G1 or G2 tumours.

We also revealed the adequacy of the forecasting model during the analysis of the studied samples according to SUV\textsubscript{max}. However, AUC=0.70 95% CI 0.61–0.78. Figue 6 shows the ROC curve of the test. Its result allows asserting the importance of the quality of this indicator in the forecast of oncogenesis.

When selecting the optimal threshold (according to the Youden Index), we established the critical value of the indicator, sensitivity and specificity. SUV\textsubscript{crit} is 17.2. It should be noted that exceeding this value forecasts G3 phase of the life cycle of tumour cells. At this threshold, the sensitivity of the test is 68.5% (95% BI 54.4–80.5%), while the specificity of the test is 70.2% (95% BI 57.7–80.7%).

The next step was to establish a relationship between Ki67, pHH3 markers, which indicate the differential state of oropharyngeal carcinoma cells, and the metabolic activity of these cells, which was established by PET-CT in oropharyngeal squamous cell tumours.

Correlation analysis was used to determine the relationship between Ki67 and pHH3 and SUV\textsubscript{max} [21]. In this regard, the results of Spearman’s Rank correlation coefficient and the level of significance of its difference from 0 were expressed in the form of a correlation matrix presented in Table 3.

| Indicator | Ki-67       | pHH3               | SUV\textsubscript{max} |
|-----------|-------------|--------------------|------------------------|
| Ki-67     | 0.814 (p<0.001) | 0.214 (p=0.019)    |                        |
| pHH3      | 0.814 (p<0.001) | 0.301 (p<0.001)    |                        |
| SUV\textsubscript{max} | 0.214 (p=0.019) | 0.301 (p<0.001)    |                        |

Analysis of the results showed a strong positive correlation between the proliferation index (Ki67) and the mitotic count (pHH3) (r=0.814 at p<0.001). At the same time, we found a weak positive correlation between SUV\textsubscript{max} and Ki-67 (r=0.214 at p=0.019) and pHH3 (r=0.301 at p<0.001).
As Figure 7 shows, a strong positive correlation is observed between Ki67 and pHH3. It follows that changes in the proliferation index of tumour cells directly depend on their mitotic activity.

Therefore, we found the following during the method of tracing ROC curves for the analysis of the prognostic characteristics of tumour cells. In the presence of 50.0% of positive cells in the test sample, which are determined by the level of Ki67 marker, we detected a medium or low level of differentiation of oropharyngeal squamous cell carcinoma.

The pHH3 also indicates the differential state of tumour cells. Thus, 4 mitoses in the test sample, determined using pHH3 marker, indicates a medium and low level of differentiation of these tumours.

An increase in the SUV\textsubscript{max} value greater than 14 indicates an average level of differentiation of the studied tumours, and when the value is over 17.2 — a low level [22]. During the assessment of the relationship between the studied immunohistochemical markers, we established a positive correlation between Ki67 and pHH3 (r=0.814 at p<0.001) and a weak positive correlation between SUV\textsubscript{max} and Ki67 (r=0.214) at p=0.019) and pHH3 (r=0.301 at p <0.001).

**Discussion.** The obtained results indicate not only the complexity of assessing the functional status of cells involved in the tumour process in the oropharyngeal area, but also the importance of identifying key diagnostic criteria, in particular, such as metabolic activity of tumour cells. Taking into account these results will allow forecasting the level of differentiation of transformed squamous cell carcinoma in the preoperative stage, followed by the choice of effective treatment tactics.

The dependence of SUV\textsubscript{max} on the level of differentiation of tumour cells has also been found for lung cancer [23], tumours of the stomach and esophagus, lymphoma, endometrial cancer [24]. The results of these studies, including ours, may be the basis for creating the latest diagnostic classification of tumours based on the results of PET research.

In addition, immunohistochemical markers, in particular pHH3 and Ki67 levels, make it possible to accurately determine the aggressiveness of tumours, regardless of individual features of the tumour or laboratory features of material staining.

**Conclusions.** 1. In our work, we statistically proved the possibility of predicting the degree of differentiation of oropharyngeal carcinoma cells by the level of intake of 2-fluoro-[18F]-2-deoxy-D-glucose by tumour tissues. The results indicate the dependence (p<0.001) of SUV\textsubscript{max} and the level of differentiation of transformed cells. 2. The proliferation index Ki67 and pHH3 depend (p<0.001) on the number of mitotic cells in the tumour tissue and the level of histological differentiation of the tumour. 3. We proved that pHH3 marker can be used as a reliable criterion for assessing the level of tumour differentiation, which may form the basis for the diagnosis of squamous cell carcinoma. 4. We found a direct relationship between Ki67, pHH3 markers and the results of PET-CT in oropharyngeal squamous cell tumours.

**Prospects for further research.** In our opinion, further study of the patterns of differentiation of cancer cells and their metabolic activity is a promising area for the diagnosing and screening of tumours of varying degrees of progression and origin, including oropharyngeal tumours. This gives
the prospects of their implementation in practice. An important area is also the creation of a new system for studying the degree of tumour differentiation depending on SUV_{max}, which indirectly indicates the level of tumour differentiation. For morphological diagnosis and detection of tumours of different origin, it is important to determine the proliferation potential of tumours by the number of positively stained cells to Ki67 (%) and pHH3 of oropharyngeal carcinoma.

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