Original paper

# Nanog expression in patients with squamous cell carcinoma of oropharynx in relation to immunohistochemical score

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This study aimed to compare prognostic potential of Nanog expression analysed by three immunohistochemical scores in the group of 63 squamous cell carcinomas of oropharynx. Immunoreactivity of Nanog expression was analyzed by semiquantitative score, immunoreactive score and H-score. For all three scores, the cut-off points for Nanog overexpression and its lack, allowing for optimal separation of overall and disease free survival curves, were search by minimal p-value method. In semiquantitative score, the best separation of overall and disease free survival curves was obtain by cut-off point lack of staining vs. week/moderate/strong staining, although statistical significance was not reach (OS: HR = 1.016, p = 0.081, DFS: HR = 6.876, p = 0.061). The cut-off points for immunoreactive score and H-score were, respectively: 1 (OS: HR = 6.977, p = 0.014, DFS: HR = 6.002, p = 0.019) and 50 (OS: HR = 6.977, p = 0.014, DFS: HR = 6.002, p = 0.019). The cut-off points found for these two scores allow to identify the same subgroups of patients with lack of Nanog expression (11.1%) and its overexpression (88.9%). All patients with tumors characterized by lack of Nanog overexpression identifying by immunoreactive score and H-score survived 5 years without evidence of cancer progression. In multivariate analysis Nanog immunoreactivity analysed by QRS and IRS was independent prognostic factor for OS (HR = 10.195, p = 0.024). Immunohistochemical score using to distinguish Nanog overexpression or its lack has influence on prognostic potential of this biomarker.

**Key words:** oropharynx cancers, Nanog expression, immunohistochemical score, HPV infection, prognosis.

## Introduction

According to the GLOBOCAN, in 2020 there were 98 412 newly diagnoses cases of oropharynx cancers (0.5% of all sites) and 48 143 deaths from this disease (0.5% of all sites) worldwide [1]. The most common risk factors for this type of cancer include heavy smoking and alcohol use, chewing betel quid and infection with human papillomaviruses (HPV), especially HPV type 16

Table I. Studies on prognostic potential of Nanog expression among patients with head and neck cancers

| Reference | Patient' group | Antibody | Immunohistochemical score Cut-off point | Prognostic potential |
|---|---|---|---|---|
| Pedregal-Mallo et al. [6] | 348 patients with SCC of oropharynx, hypopharynx and larynx | Nanog, rabbit monoclonal antibody, Cell Signalling Technology | Semiquantitative score: 0 – absence of staining 1 – week/moderate staining 2- strong staining Cut-off point: 0 vs 1+2 | Nanog negativity – significant correlation with worse OS, lack of significant relation with DFS |
| Rizzo et al. [5] | 69 patients with SCC of oral cavity and oropharynx | Nanog, rabbit monoclonal antibody, Cell Signalling Technology | Semiquantitative score: 0 – no staining 1 – week/moderate staining 2 – strong staining Cut-off point: 0 vs 1+2 | Nanog negativity – significantly better DFS |
| De Vicente et al. [7] | 125 patients with SCC of oropharynx | Nanog, rabbit monoclonal antibody, Cell Signalling Technology | Semiquantitative score: 0 – no staining 1 – week/moderate staining 2 – strong staining Cut off point: 0 vs 1+2 | Nonog negativity –lack of significant relation with DFS |
| Rodrigo et al. [8] | 82 patients with SCC of larynx | No data | Semiquantitative score: 1 – week/moderate staining 2 – strong staining Cut-off point: 0+1 vs 2 | Lack of Nanog overexpression – significantly better CFS |
| Habu et al. [9] | 50 patients with SCC of tongue | Mouse anti-Nanog, Abnova | Score: no data Cut-off point: nuclear staining observed in 3% of tumor cells | Nanog negativity – significantly lower percentage of neck metastases |
| Lee et al. [10] | 57 patients with SCC of oral cavity | Abcam™ (ab109250), Cambridge, UK | Immunoreactive score: the sum of the staining intensity and percentage of positive tumor cell: –(negative, 0–1) + (weak, 2–3) ++ (moderate, 4–5) +++ (strong, 6–7); Cut-off point: IRS = 3 | Lack of Nanog overexpression – significantly better OS |

SCC – squamous cell carcinoma; OS – overall survival; DFS – disease free survival; CFS – cancer free survival

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(HPV16). For patients with HPV16+ squamous cell carcinoma of oropharynx (OPSCC), the significantly better prognosis has been well documented [2]. In relation to this finding, different strategies of treatment de-intensification (less toxic) are being tested in clinical trials. However, de-escalated strategies should be focused mainly on the low-risk HPV(+) category of patients. Therefore, basic researches are needed to indicate prognostic factors, which will be helpful in identification of those patients. Some studies suggest that cancer stem cells (CSC) biomarkers, such as transcrptional factors: Oct, SOX and Nanog may play a role as such prognostic factors [3]. Among them, Nonog play a key role in self-renewal of embryonic stem cells and in maintenance of their pluripotency. In normal cells, its activity is supress by binding of P53, what promotes cell differentiation and apoptosis.[4] Because in cancer cells with active HPV infection (with expression of viral oncoproteins E6 and E7) P53 is degraded, viral presence can influence Nanog expression. Meanwhile, according to our best knowledge, correlation of Nanog expression and HPV presence in OPSCC was assessed in one study. Rizzo *et al.* [5], in the small group of 10 OPSCC, have found that HPV positivity was significantly related with lack of Nanog expression. However, prognostic potential of Nanog expression in the light of results obtained so far is unclear (Table I). Several reasons for these conflicting results can be identified. One of the reason of conflicting results presented in Table I is simultaneous analysis subgroups with HPV positivity and HPV negativity. Second reason may be related to differences in immunohistochemical staining procedure, particularly in scoring systems and cut-off points used to distinguish tumors with Nanog overexpression or its lack. Some authors have used three points semiquantitative scale (SQS), which takes into account only intensity of staining [5, 6, 7, 8]. Other authors have applied scores, which based on intensity of staining and the percentage of positive staining cells, such as immunoreactive score (IRS) [9, 10] and H-score (HS) [11]. Moreover, in case of all these scores there is also a lack of consensus to the value of the cut-off point using to distinguish tumours with Nanog overexpression or its lack. Therefore, in the light of all above-mentioned facts, the aim of the present study was to assess prognostic potential of Nanog expression in the group of 63 patients with SCC of oropharynx in relation to three immunostaining scores: SQS, IRS and HS. We decided also to search cut-off point for these three scores using minimal p-value method. Additionally, we investigated the correlations between Nanog expression and other previously assessed clinicopathological variables including patient's age, gender, clinical stage, grade, degree of keratinization, HPV16 status identified by quantitative polymerase chain reaction, P16 expression and expression of CD44, CD98 and ALDH1/2 assessed in our earlier paper [12].

## Material and methods

### Patients

A series of 63 patients with SCC of oropharynx (T1-2, N1-2, M0 between 2001–2005 at Centre of Oncology, Krakow Branch, Poland were included into the study. Details concerning study population, inclusion and exclusion criteria, treatment type have been presented previously [12]. Additionally, Table IV in the present paper summarizes all details concerning clinical and histopathological characteristics of patients involved in this study.

### Preparation of tissue

Immunohistochemical assessment of Nanog expression was performed on formalin fixed and paraffin-embedded sections (FFPE). As we described earlier [12], before staining each sample undergone histopathological verification based on typical eosin/hemotaoxylin stained slides. Histopatologists indicated also for further analysis FFPE section, in which tumor component covered > 50% of the slide. For application of the immunohistochemistry (IHC) serial 4-$\mu$m sections were processed.

### Immunohistochemistry

Sections were deparaffinized in xylenes and rehydrated trough graded alcohol steps. To quench the endogenous peroxidase activity, the slides were treated with 0.3% hydrogen peroxide in methanol for 30 min. Nanog expression status were evaluated IHC. For antigen unmasking, 50 min incubation in Target Retrieval Solution, (pH = 6.1, DAKOCytomation, Glostrup, Denmark), preheated to 96°C was applied. Next, the incubation with the primary rabbit monoclonal antibody (Cat. No 3579, Cell Signalling Technology) was applied for 60 min at 37°C. The antigen-primary antibody complex was detected by BrightVision system (Immunologic, Duiven, Netherlands) and visualised using 0.01% 3.3-diaminobenzidine tetrahydrochloride (Vector Laboratories, Inc., Burlingame, CA, USA) and 0.015% hydrogen peroxide. The slides were counterstained with Mayer's hematoxylin. For negative control, Tris buffered saline (TBS) was substituted for primary antibody. Positive control includes cervical cancer exhibiting high expression of Nanog.
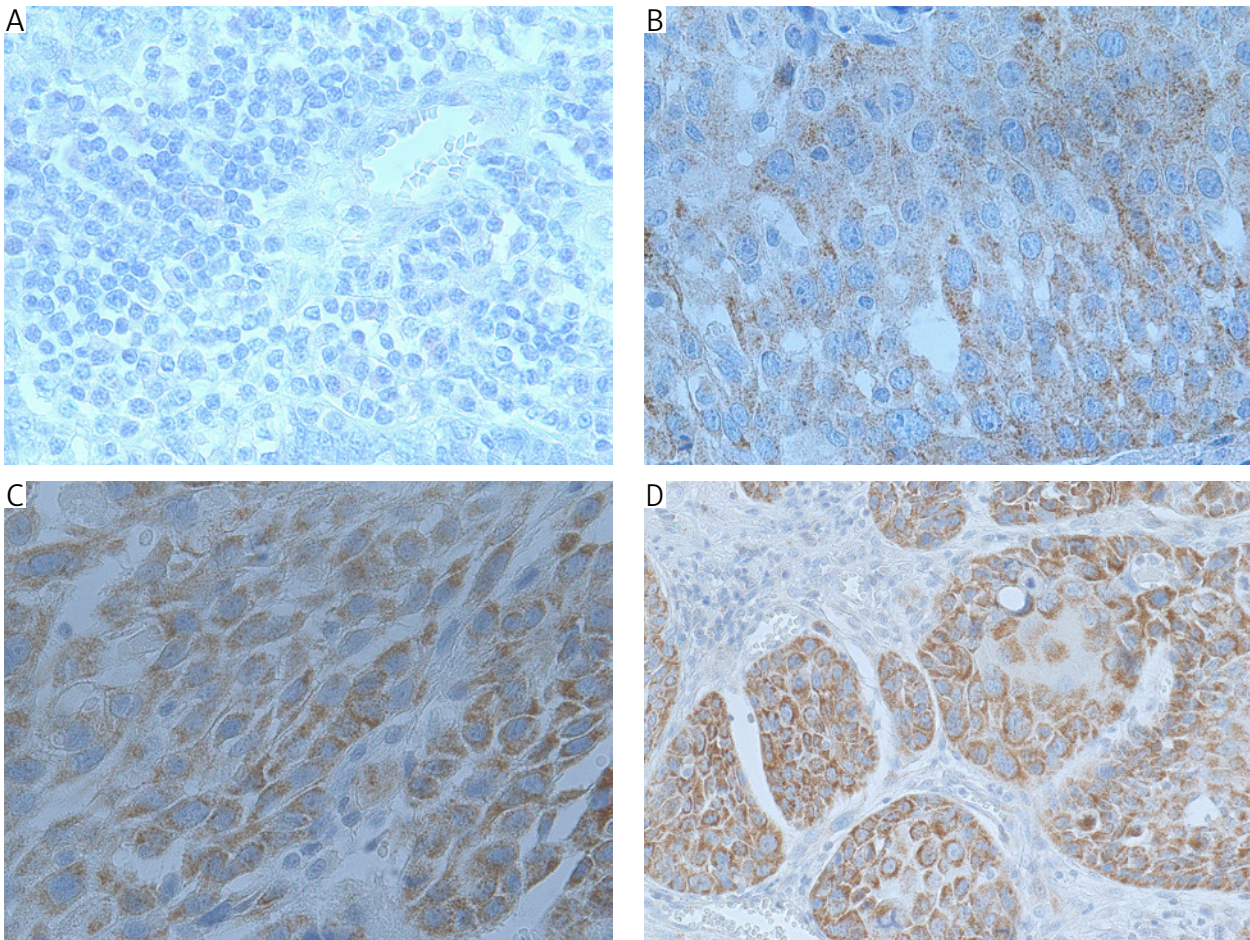
**Fig. 1.** Tumour slides stained immunohistochemically against Nanog transcriptional factor (primary rabbit monoclonal antibody (Cat. No 3579, Cell Signalling Technology). A) Nanog immunoreactivity assessed in semiquantitative score (SQS) as week staining (categories 1, according to cut-off point 0 vs. 1 + 2 classified as cancer with Nanog overexpression), in immunereactive score (IRS) as 1 (according to cut-off point IRS ≤ 1 classified as lack of Nanog overexpression) and in H-score (HS) assessed as 10 (according to cut-off point HS ≤ 50 classified as lack of Nanog overexpression); B) tumor classified in SQS score as 2, on IRS score as 4 and in HS as 60; C) tumor classified in SQS score as 2 on IRS score as 6 and in HS as 120; D. tumor classified in SQS score as 3 on IRS score as 12 and in HS as 210. Microphotographs were taken at 10 × 40 (objective) magnification

## Evaluation of staining

Each section was assessed blind without any knowledge of the patient's previous investigations or treatment outcome trough two independent observers (M.K-R and B.B.). (Olympus Optic Co., Ltd, Tokyo, Japan). During microscopic analysis (Olympus Optic Co., Ltd, Tokyo, Japan) attention has been paid on intensity of staining and the percentage of positive stained cells. To distinguish lack of overexpression or overexpression three scoring system were used: (1) semiquantitative score (SQS), (2) immunoreactive score (IRS) and (3) histological score (HS) (Fig. 1A-D). SQS include three categories of staining intensity: (1) lack of positive cytoplasamatic staining, (2) week or moderate cytoplasamatic staining in tumours areas and (3) strong cytoplasmatic staining in tumours areas (Fig. 1) [5, 6, 7, 8]. IRS is a product of multiplication of percentage of positive stained cells (five categories: 0 – 0%, 1 – < 25%, 2 – 25-50%, 3 – 50-75%, 4 – > 75%) and staining intensity (three categories, similar like in SQS), giving a range from 0 to 12 [10]. HS was calculated according to the formula: H-score = (1 × percentage of weakly positive cells) + (2 × percentage of moderately positive cells) + (3 × percentage of strongly positive cells), giving a range from 0 to 300 [11].

## Statistical analysis

Descriptive statistics was used to determine mean and median values of continuous variables (IRS and HS) and standard errors of means (SE). The differences between means were analyzed by T-student test. The correlation between continues variables were assessed by R Spearman coefficient. The cut-off points for three scores applying to assess immunostaining have been searched by minimal $P$-value

**Table II.** Correlation between Nanog expression analysed by semiquantitative score, immunoreactive score and H-score

| SEMIQUANTITATIVE SCORE | | IMMUNOREACTIVE SCORE | H SCORE |
|---|---|---|---|
| CATEGORIES | N (%) | MEAN ± SE | MEAN ± SE |
| 0 | 7 (11.1) | 0.14 ± 0.11 | 2.86 ± 1.86 |
| 1 | 39 (61.9) | 3.54 ± 2.81* | 87.49 ± 7.40* |
| 2 | 17 (27.0) | 7.65 ± 0.54* | 191.71 ± 15.64* |

*significant differences between mean values in categories of semiquantitative score: 0 vs. 1, 0 vs. 2
and 1 vs. 2 in T-student test, p ≤ 0.000*

*SE – standard error*

method (Table III). At the beginning of this strategy, the mean, median, and percentiles: $90^{th}$ $75^{th}$, $25^{th}$ and $10^{th}$ were analysed. Associations between categorical variables were analyzed using Pearson $\chi^2$ test. The primary endpoints for the study were overall survival (OS), defined as the percentage of patients who are alive five years after their diagnosis and disease free survival (DFS), defined as the percentage of patients alive five years after their diagnosis without cancer progression (locoregional recurrence, distant recurrence or second malignancy). The median duration of OS and DFS was calculated using the Kaplan-Meier method. Comparisons between groups were evaluated using log-rank test. Multivariate analysis was carried out using the Cox proportional hazards model. Two-sided p-values of < 0.05 were considered significant. All statistical analyses were carried out using Statistica v.13.3 software.

## Results

### Patient's characteristics

Patient characteristic has been previously presented in details [13]. Briefly, there were 63 patients in age from 32 to 78 years with SCC of oropharynx in clinical stage T1N1 (54.7 %), T1N2 (23.3%), T2N1 (18.0%) and T2N2 (4.0%). In this group, most patients (n = 28, 44.4%) received concurrent chemoradiotherapy with cisplatin (CisPt-CRT), which was used as definitively (*n* = 22, 78.6%) or after surgery (n = 6, 21.4%). Cisplatin was administered during radiotherapy according to two regimens: 100 mg CisPt/m² every $3^{rd}$ week of RT in 2 – 3 courses or 40 mg CisPt/m² every week of RT in 3-6 courses, depending on patient's condition and early normal tissue response. In 19 patients (30.2%) radiotherapy was used definitively (n = 6, 31.6%) or after surgery (n = 13, 23.3%). Total dose of RT was 20.0-74.0 Gy, with mean value of 59.5Gy, fraction dose of 1.8-4.0 Gy, and number of fractions of 5-40. Altogether, 19 patients (30.2%) underwent surgery. Among 63 patients, 16 (25.4%) were treated with induction

chemotherapy (cisplatin + 5-fluorouracil + taxanes) followed by radiotherapy (total dose: 20-70 Gy, with mean value of 59.5 Gy, fraction dose: 1.8-4 Gy, number of fractions: 5-40).

The mean follow-up time was 42.0 months ± 4.4 and ranged from 0 to 113 months. Among 63 patients, 45 s (71.4%) had cancer regression. Cancer progression (2 treatment failure, 12 local recurrence and 4 distant metastases) was noticed in 18 (28.6%) patients from 0 to 39 months after completing treatment (mean: 12.0 months ± 2.5).

### Nanog expression in the group of 63 tumors with SCC of oropharynx

In the group of 63 OPSCC, according to SQS, there were 7 tumors (11.1%) with lack of Nanog staining (category 0), 39 (61.9%) with week/moderate staining (category 1) and 17 (27.0%) with strong staining (category 2) (Table II). The mean and median values of IRS were 4.3 ± 0.4 (SE) and 4.0, with range 0.0-12.0. Regarding HS, the mean and median values were 106.2 ± 9.6 and 100, ranging from 0.0 to 300.0. There were statistically significant differences between mean values of IRS and HS in particular categories of SQS. The correlation between IRS and HS, considered as continuous variables, was also significant (R = 0.854, p = 0.000).

### The search of optimal cut-off points for Nanog expression in semiquantitative score, immunoreactive score and H score

In order to stratify patients into subgroups with tumors characterized by lack of Nanog overexpression and Nanog overexpression as well as to obtain optimal categorization of OS and DFS curves, we decided to search for cut-off points in SQC, IRS and HS by minimal p-value method. In SQS, two cut-off points were tested: 0 vs. 1 + 2 and 0 + 1 vs. 2 (Table III). For both cut-off points, we did not found any significant differences in OS and DFS. However, the best separation of OS and DFS curves [(the lowest p value, the highest hazard ratio (HR) and the reasonable number of patients)] was noticed when tumours were dichotomized as follows: category 0 (lack of positive

**Table III.** Searching for optimal cut-off points for Nanog immunoreactivity in three scores: semiquantitative score, immunoreactive score and H-score.

| | Overall survival | | | | Disease free survival | | | |
|---|---|---|---|---|---|---|---|---|
| | Response N (%) | HR | 95% CI | Log-rank p | Response N (%) | HR | 95% CI | Log-rank p |
| **SEMIQUANTITATIVE SCORE** | | | | | | | | |
| 0 vs. 1 + 2 | | | | | | | | |
| 0 | 6/7 (85.7) | 1.000 | | | 7/7 (100.0) | 1.000 | | |
| 1 + 2 | 26/56 (50.0) | 4.016 | 1.007-1.025 | 0.081 | 38/56 (74.3) | 6.876 | 3.248-14.555 | 0.061 |
| 0 + 1 vs. 2 | | | | | | | | |
| 0 + 1 | 6/17 (35.3) | 1.612 | 0.993-2.032 | 0.104 | 10/17 (58.8) | 7.254 | 3.655- 2.255 | 1.118 |
| 2 | 28/46 (60.9) | 1.000 | | | 35/46 (76.1) | 1.000 | | |
| **IMMUNOREACTIVE SCORE** | | | | | | | | |
| Mean value | | | | | | | | |
| ≥ 4.3 | 10/23 (43.5) | 1.609 | 0.772-3.352 | 0.205 | 15/23 (65.2) | 1.738 | 0.669-4.515 | 0.256 |
| < 4.3 | 24/40 (60.0) | 1.000 | | | 31/40 (77.5) | 1.000 | | |
| Median value | | | | | | | | |
| ≥ 4.0 | 10/23 (43.5) | 1.609 | 0.772-3.352 | 0.205 | 15/23 (65.2) | 1.738 | 0.669-4.515 | 0.256 |
| < 4.0 | 24/40 (60.0) | 1.000 | | | 31/40 (77.5) | 1.000 | | |
| 75th percentile | | | | | | | | |
| ≥ 6.0 | 2/11 (18.2) | 2.614 | 1.181-5.790 | 0.021* | 6/11 (54.6) | 2.329 | 0.818-6.628 | 0.121 |
| < 6.0 | 32/52 (61.5) | 1.000 | | | 40/52 (76.9) | 1.000 | | |
| 25th percentile | | | | | | | | |
| ≥ 2.0 | 19/42 (45.2) | 2.473 | 1.005-6.084 | 0.033* | 29/42 (69.1) | 2.081 | 0.676-6.403 | 0.175 |
| < 2.0 | 15/21 (71.4) | 1.000 | | | 17/21 (81.0) | 1.000 | | |
| 90th percentile | | | | | | | | |
| ≥ 8.0 | 0/6 (0.0) | 3.194 | 1.276-7.996 | 0.017* | 3/6 (50.0) | 2.597 | 0.737-9.151 | 0.160 |
| < 8.0 | 34/57 (59.7) | 1.000 | | | 43/57 (75.4) | 1.000 | | |
| 10th percentile | | | | | | | | |
| ≥ 1.0 | 25/53 (47.2) | 6.997 | 0.951-11.512 | 0.014* | 36/53 (67.9) | 6.002 | 1.484-9.112 | 0.019* |
| < 1.0 | 9/10 (90.0) | 1.000 | | | 10/10 (100.0) | 1.000 | | |

**Table III.** Cont.

| | | OVERALL SURVIVAL | | | | | DISEASE FREE SURVIVAL | | |
| | RESPONSE N (%) | HR | 95% CI | LOG-RANK P | RESPONSE N (%) | RESPONSE N (%) | HR | 95% CI | LOG-RANK P |
|---|---|---|---|---|---|---|---|---|---|
| | | | | H - SCORE | | | | | |
| **Mean value** | | | | | | | | | |
| ≥ 106.2 | 11/27 (40.7) | 4.283 | | | 18/27 (66.7) | 18/27 (66.7) | 2.810 | | |
| < 106.2 | 23/36 (63.9) | 1.000 | 1.636-9.302 | 0.115 | 28/36 (77.8) | 28/36 (77.8) | 1.000 | 1.688-2.686 | 0.293 |
| **Median value** | | | | | | | | | |
| ≥ 100.0 | 12/29 (41.4) | 5.157 | | | 20/29 (69.0) | 20/29 (69.0) | 6.866 | | |
| < 100.0 | 22/34 (64.7) | 1.000 | 1.970-11.466 | 0.122 | 26/34 (76.5) | 26/34 (76.5) | 1.000 | 1.477-9.034 | 0.459 |
| **75th percentile** | | | | | | | | | |
| ≥ 141.0 | 5/15 (33.3) | 2.428 | | | 10/15 (66.7) | 10/15 (66.7) | 1.756 | | |
| < 141.0 | 29/48 (60.4) | 1.000 | 1.122-5.255 | 0.033* | 36/38 (75.0) | 36/38 (75.0) | 1.000 | 0.616-5.003 | 0.322 |
| **25th percentile** | | | | | | | | | |
| ≥ 50.0 | 25/53 (47.2) | 6.997 | | | 36/53 (67.9) | 36/53 (67.9) | 6.002 | | |
| < 50.0 | 9/10 (90.0) | 1.000 | 0.951-11.512 | 0.014* | 10/10 (100.0) | 10/10 (100.0) | 1.000 | 1.484-9.112 | 0.019* |
| **90th percentile** | | | | | | | | | |
| ≥ 210.0 | 3/6 (50.0) | 1.381 | | | 4/6 (66.7) | 4/6 (66.7) | 1.552 | | |
| < 210.0 | 31/57 (54.4) | 1.000 | 0.417-4.574 | 0.626 | 42/57 (73.7) | 42/57 (73.7) | 1.000 | 0.354-6.803 | 0.587 |
| **10th percentile** | | | | | | | | | |
| ≥ 20.0 | 25/53 (47.2) | 6.997 | | | 36/53 (67.9) | 36/53 (67.9) | 6.002 | | |
| < 20.0 | 9/10 (90.0) | 1.000 | 0.951-11.512 | 0.014* | 10/10 (100.0) | 10/10 (100.0) | 1.000 | 1.484-9.112 | 0.019* |

*The statistical significance limit for p-value was accepted as p < 0.05, significant results are written in bold font

HR – hazard ratio; CI – confidence interval

**Table IV.** Relation between Nanog expression in semiquantitative score, immunoreactive score and H-score and epidemiological and clinical features of 63 patients with squamous cell carcinoma of oropharynx

| | All (%)[a] | Overexpression of Nanog | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | Semiquantitative score | | | Immunoreactive score and H-score | | |
| | | Yes N (%)[b] | No N (%) | Test $\chi^2$ P | Yes N (%)[b] | No N (%) | Test $\chi^2$ P |
| All | 63 (100.0) | 56 (88.9) | 7 (11.1) | | 53 (84.1) | 10 (15.9) | |
| Age | | | | | | | |
| Female | 28 (23.8) | 27 (96.4) | 1 (3.6) | | 27 (96.4) | 1 (3.6) | |
| Male | 35 (76.2) | 29 (82.9) | 6 (17.1) | 0.089 | 26 (74.3.7) | 9 (25.7) | 0.017* |
| Gender | | | | | | | |
| Female | 15 (23.8) | 11 (73.3) | 4 (26.7) | | 9 (60.0) | 5 (40.0) | |
| Male | 48 (76.2) | 45 (93.7) | 3 (6.3) | 0.028* | 44 (91.7) | 4 (8.3) | 0.003* |
| Status in the Karnofsky scale | | | | | | | |
| < 80% | 26 (41.3) | 23 (88.5) | 3 (11.5) | | 19 (73.1) | 6 (17.6) | |
| ≥ 80% | 37 (58.7) | 33 (89.2) | 4 (10.8) | 0.928 | 25 (86.2) | 4 (13.8) | 0.676 |
| The level of smoking-Brinkman index[d] | | | | | | | |
| ≤ 520[c] | 34 (54.0) | 14 (41.2) | 20 (58.8) | | 28 (82.3) | 13 (38.2) | |
| > 520 | 29 (46.0) | 11 (37.9) | 18 (62.1) | 0.858 | 22 (75.9) | 7 (24.1) | 0.231 |
| The level of drinking[e] | | | | | | | |
| Low | 28 (44.4) | 23 (82.1) | 5 (17.9) | | 20 (71.4) | 8 (28.6) | |
| High | 35 (55.6) | 33 (94.3) | 2 (5.7) | 0.127 | 33 (94.3) | 2 (5.7) | 0.014* |
| T stage | | | | | | | |
| 2 | 15 (23.8) | 13 (86.7) | 2 (13.3) | | 12 (80.0) | 3 (20.0) | |
| 3 | 32 (50.8) | 28 (87.5) | 4 (12.5) | | 27 (84.4) | 5 (15.6) | |
| 4 | 16 (25.4) | 15 (93.7) | 1 (6.3) | 0.771 | 14 (87.5) | 2 (12.5) | 0.848 |
| N stage | | | | | | | |
| 0 | 10 (15.9) | 10 (100.0) | 0 (0.0) | | 10 (100.0) | 0 (0.0) | |
| 1 | 13 (20.6) | 11 (84.6) | 2 (15.4) | | 10 (76.9) | 3 (23.1) | |
| 2 | 35 (55.6) | 31 (88.6) | 4 (11.4) | | 29 (82.9) | 6 (17.1) | |
| 3 | 5 (7.9) | 4 (80.0) | 1 (20.0) | 0.595 | 4 (80.0) | 7 (20.0) | 0.476 |
| Grade | | | | | | | |
| 1 | 25 (39.7) | 22 (88.0) | 3 (12.0) | | 21 (84.0) | 4 (16.0) | |
| 2 | 33 (52.4) | 29 (87.9) | 4 (12.1) | | 27 (81.8) | 6 (18.2) | |
| 3 | 5 (7.9) | 5 (100.0) | 0 (0.0) | 0.712 | 5 (100.0) | 0 (0.0) | 0.584 |
| Keratinization | | | | | | | |
| Yes | 35 (44.4) | 32 (91.4) | 3 (8.6) | | 29 (82.9) | 6 (17.1) | |
| No | 28 (55.6) | 24 (85.7) | 4 (14.3) | 0.473 | 24 (85.7) | 4 (14.3) | 0.758 |
| HPV16 infection (qPCR) | | | | | | | |
| Yes | 25 (39.7) | 21 (84.0) | 4 (16.0) | | 18 (72.0) | 7 (28.0) | |
| Not | 38 (60.3) | 35 (92.1) | 3 (7.9) | 0.316 | 35 (92.1) | 3 (7.9) | 0.033* |
| P16 immunopositivity | | | | | | | |
| Yes | 27 (42.9) | 22 (77.8) | 5 (18.5) | | 19 (79.4) | 8 (29.6) | |
| Not | 36 (57.1) | 34 (94.4) | 2 (5.6) | 0.105 | 34 (94.4) | 2 (5.6) | 0.010* |

**Table IV.** Cont.

| | All (%)[a] | Overexpression of Nanog | | | | | |
|---|---|---|---|---|---|---|---|
| | | Semiquantitative score | | | Immunoreactive score and H-score | | |
| | | Yes N (%)[b] | No N (%) | Test $\chi^2$ P | Yes N (%)[b] | No N (%) | Test $\chi^2$ P |
| **Nanog expression (intensity of staining)** | | | | | | | |
| Overexpression | 56 (88.9) | | | | 53 (94.6) | 3 (5.4) | |
| Lack of overexpression | 7 (11.1) | | | | 0 (0.0) | 7 (100.0) | 0.000* |
| **Nanog expression – immunoreactive score or H score** | | | | | | | |
| Overexpression | 53 (84.1) | 53 (100.0) | 0 0.0) | | | | |
| Lack of overexpression | 10 (15.9) | 3 (30.0) | 7 (70.0) | 0.503 | | | |
| **CD44 expression** | | | | | | | |
| Overexpression | 43 (68.3) | 39 (90.7) | 4 (9.3) | | 38 (88.4) | 5 (11.6) | |
| Lack of overexpression | 20 (31.7) | 17 (85.0) | 3 (15.0) | 0.090 | 15 (75.0) | 5 (25.0) | 0.176 |
| **CD98 expression** | | | | | | | |
| Overexpression | 30 (47.6) | 29 (96.7) | 1 (3.3) | | 29 (66.7) | 1 (3.3) | |
| Lack of overexpression | 33 (52.4) | 27 (81.8) | 6 (18.2) | 0.061 | 24 (72.7) | 9 (27.3) | 0.009* |
| **ALDH1/2 expression** | | | | | | | |
| Overexpression | 33 (47.6) | 30 (90.9) | 3 (9.1) | | 29 (87.9) | 4 12.1) | |
| Lack of overexpression | 30 (52.4) | 26 (86.7) | 4 (13.3) | 0.593 | 24 (80.0) | 6 (20.0) | 0.393 |
| **Treatment** | | | | | | | |
| Definitive CisPt-CRT or surgery + CisPt-CRT | 28 (44.4) | 22 (46.4) | 6 (21.4) | | 20 (71.4) | 8 (28.6) | |
| Definitive RT or surgery + RT | 19 (30.2) | 18 (94.7) | 1 (5.3) | | 18 (94.7) | 1 (5.3) | |
| Induction CT + definitive RT | 16 (25.4) | 16 (100.0) | 0 (0.0) | 0.059 | 15 (93.7) | 1 (6.3) | 0.057 |
| **Treatment outcome** | | | | | | | |
| Regression of cancer disease | 45 (71.4) | 38 (84.4) | 7 (15.6) | | 35 (77.8) | 10 (22.2) | |
| Treatment failure | 2 (3.2) | 2 (100.0) | 0 (0.0) | | 2 (100.0) | 0 (0.0) | |
| Local recurrence | 12 (19.1) | 12 (100.0) | 0 (0.0) | | 12 (100.0) | 0 (0.0) | |
| Distant metastases | 4 (6.3) | 4 (100.0) | 0 (0.0) | 0.785 | 4 (100.0) | 0 (0.0) | 0.529 |
| **Survival** | | | | | | | |
| Alive at the last follow-up | 34 (54.0) | 27 (50.0) | 7 (100.0) | | 24 (70.6) | 10 (29.4) | |
| Death from cancer disease | 15 (20.0) | 15 (100.0) | 0 (0.0) | | 15 (100.0) | 0 (0.0) | |
| Death from others reasons | 14 (35.7) | 14 (100.0) | 0 (0.0) | 0.168 | 14 (100.0) | 0 (0.0) | 0.039* |

*The statistical significance limit for p value was accepted as $p < 0.05$, significant results are written in bold font

CisPt-CRT concurrent chemoradiotherapy with cisplatin, CT chemotherapy

[a] Colunm percentage

[b] Row percentage

[b] Median value

[d] Number of cigarettes per day $\times$ years of smoking

[e] Low level of drinking – no alcohol and occasional drinkers (at most two drinks a day, especially with a meal) high level of drinking – more than 15 drinks high percentage alcohol in a week and alcoholics

**Table V.** Multivariate Cox proportional hazard model carried out in whole group of 63 patients with squamous cell carcinoma of oropharynx

| | OVERALL SURVIVAL | | | DISEASE FREE SURVIVAL | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | P-VALUE | HR | 95% CI | P-VALUE |
| **T stage** | | | | | | |
| 2+3 | 1.000 | | | 1.000 | | |
| 4 | 4.070 | 1.859-8.909 | 0.000* | 5.649 | 2.085-15.306 | 0.001* |
| **Nanog overexpression – immunoreactive score or H-score** | | | | | | |
| Yes | 10.195 | | | | | |
| No | 1.000 | 1.362-16.306 | 0.024* | | | |
| **P16 immunoreactivity** | | | | | | |
| Yes | | | | 1.000 | | |
| No | | | | 3.963 | 1.011-7.253 | 0.027* |

*\* The statistical significance limit for p value examined by the Cox proportional hazard model for multivariate survival analysis and accepted as p < 0.05, significant results are written in bold font*

*HR – hazard ratio; CI – confidence interval*

staining) vs. categories 1 + 2 (week/moderate and strong staining) and this stratification was applied in further analysis.

In IRS and HS, at the beginning of searching, we analyzed as cut-off points the mean, median values as well as $90^{th}$ $75^{th}$, $25^{th}$, and $10^{th}$ percentiles. Next, other values were tested (Table III). In case or IRS, the best separation of OS and DFS curves was observed at the value IRS = 1 ($10^{th}$ percentile) and this cut-off point was applied in further analysis. Regarding HS, significantly higher OS and DFS was found for patients with tumors characterized by lack of Nanog overexpression defined as HS = 50 ($25^{th}$ percentile) and we decided to assume this value as a cut-off point. It should be noticed that the cut-off points found for IRS and HS allow to identify the same subgroups of patients with lack of Nanog expression and its overexpression, therefore in the further analysis the results concerning these two scores will be presented together. All patients (n = 10) with tumors characterized by lack of Nanog overexpression identifying by IRS and HS survived 5 years without evidence of cancer progression.

## Nanog expression and clinical and histopathological data

According to the SQS, in the group of 63 SCC of oropharynx, there were 56 (88.9%) tomurs with Nanog overexpression (week/moderate and strong intensity of staining) and 7 (11.1%) with lack of Nanog staining (lack of staining). The proportion of tumors with Nanog overexpression significantly increased in male patients (p = 0.028) (Table IV). We did not observe any other significant association between Nanog expression assessed by SQS and remaining clinicopathological variables. In IRS and HS, there were 53 (84.1%) cancers overexpressing Nanog and 10 (15.9%) without overexpression. In these two scores, the distribution of tumors with different Nanog expression was significantly related with patient's age (p = 0.017), gender (p = 0.003), the level of drinking (p = 0.014), HPV16 infection (p = 0.033), P16 expression (p = 0.010) and CD98 expression (p = 0.009) (Table IV). In these two scores, other clinicopathological features did not correlated with distribution of Nanog overexpressing or not overexpression tumors.

## Multivariate analysis

In multivariate Cox multivariate analysis, we applied two classes of Nonog expression according to the IRS and HS. In this analysis, we additionally included other parameters, which were tested in our earlier paper and which significantly affected OS and DFS in univariate analysis [12]. For OS, there were: gender, level of smoking, alcohol abuse, T and N stages, keratinization status, P16 immunoreactivity and treatment type. In the case of DFS, level of smoking, T stage, keratinization status, HPV16 infection, P16 immunoreactivity, CD98 overexpression and treatment type were included. For OS, T stage as well as Nanog expression (analysed by QRS and IRS) were independent prognostic factors (Table V). For DFS such factors were T stage and P16 immunoreactivity.

## Discussion

The present study aims to determine the significance of immunohistochemical expression of Nanog as a prognostic factor in the group pf 63 patients with SCC of oropharynx. To address this objective we decided to apply three immunohistochemical scores us-

ing in the literature: semiquantitative score [5, 6, 7, 8], immunoreactive score [9, 10], and H-score [11]. We have shown, according to our best knowledge for the first time, 100% of DFS for patients having tumours with lack of Nanog overexpression identifying by IRS and HS (Table III). Using these two scores we have also found significantly better OS for patients with lack of Nanog overexpression then for those with its overexpression. When immunohistochemical expression of Nanog was analysed by SQS, we were not able to show significant differences in OS and DFS. Based on IRS and HS we identified the same subgroup of patients with Nanog overexpression or its lack (Table III). Subgroup of patients with Nanog overexpression in SQS was differ by 3 cases in which week intensity of staining was found, however percentage of positive cells was very small. Similar to us, Lee *et al*. [10] who analysed Nanog expression by IRS in the group of 57 SCC of oral cavity treated by surgery only, surgery combined with adjuvant radiotherapy or concurrent chemoradiotherapy and radiotherapy only, have shown that patients with lack of Nanog expression (IRS ≤ 3) had better survival rates than those with NANOG. In turn, de Vicente *et al*. [7] in the group of 125 patients with SCC of oral cavity who underwent surgical treatment, when analysing Nanog expression in SQS score (cut-off point 0 *vs* 1 + 2) did not obtain significant difference in DFS. Habu *et al*. [9] in the group of 50 patients with SCC of tongue, have also shown that Nanog negativity, defined as the percentage of staining cells below 3%, was significantly related to lower percentage of neck metastases. All these findings suggest that scoring system affects results concerning prognostic potential of different biomarkers, including Nanog expression. The scoring system that should be considered has to include both staining intensity and the number of positive staining cells. It seems that expression of Nanog in a few cells has no meaningful impact on the tumour to therapy. It should be also noticed that using SQS, contrary results concerning prognostic value of Nanog expression was obtain. Some authors have found significantly higher survival for patients with tumours having lack of Nanog overexpression identifying by SQS with cut-off point: 0 *vs* 1 + 2 (Table I). Pedregal-Mallo *et al*. [6] have noticed that Nanog overexpression (categories 1 + 2 in SQS) was significantly correlated with OS in the subgroup of patients with pharyngeal cancers, but not laryngeal tumors. However, they included in the study patients with various tumor localization, such as: oropharynx, hypopharynx and larynx, which differ in percentage of HPV positivity. Among HNSCC, the highest HPV positivity is noticed in OPSCC, meanwhile hypopharynx and laryngeal cancers are overwhelmingly HPV negative [13]. Meanwhile, Pedregal-Mallo *et al*. [6] did not analyze HPV status in their patient's cohort

and did not report any details concerning treatment regime in the analyzed group of patients. In the present study, we have found the significant correlation between HPV negativity and Nanog overexpression (Table IV). According to our best knowledge, the correlation between HPV infection and Nanog expression was analysed in one study of Rizzo *et al*. [5]. Similar to us, they have found that HPV positive OPSCC are characterized by lower expression of Nanog in the cytoplasm of cancer cells than HPV negative OPSCC. The significance of these findings for the biology of HPV positive HNSCC cancers and treatment response is unknown. However, same authors suggest that Nanog overexpression in HNSCC correlates with cisplatin resistance. Tsai *et al*. [14], in the group of ten cisplatin chemosensitive cell lines of SCC of oral cavity and ten cisplatin resistance lesions, have namely found that cisplatin resistant cells were characterized by Nanog overexpression. Moreover, in the present study, among 10 patients with lack of Nanog overexpression, who survived 5 years without cancer progression, 8 ones (80.0%) were treated with CisPt-CRT (Table IV). These results suggest the relation between cisplatin sensitivity and lack of Nonog overexpression. Therefore, contrary results concerning prognostic potential of Nanog expression can be partly explain by heterogeneity in analysed patient's group according to treatment type. However, this hypothesis should be confirm by further *in vitro* studies.

Summarized, we have shown that immunohistochemical score using to distinguish Nanog overexpression/ist lack has influence on prognostic potential of this CSC biomarker. In the group of 63 patients with SCC of oropharynx we have found, according to our best knowledge for the first time, 100% of DFS for OPSCC patients with that lack of Nanog expression identifying by immunoreactive score and H score, but not by semiquantitative score. We have also shown that lack of Nanog expression identifying by immunoreactive score and H score is significantly correlated with HPV16 positivity.

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The authors declare no conflict of interest.

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