DELETION OF CYCLIN DEPENDENT KINASE INHIBITOR 2a GENE AS A MARKER OF OROPHARYNGEAL CARCINOMAS NON-ASSOCIATED WITH HUMAN PAPILLOMAVIRUS AND ITS PROGNOSTIC VALUE

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Abstract. Deletion of cyclin dependent kinase inhibitor 2a gene as a marker of oropharyngeal carcinomas non-associated with human papillomavirus and its prognostic value. Shponka I.S., Bondarenko O.O., Kovtunenko O.V., Rakhmanov V.V. Patients with human papilloma virus associated oropharyngeal squamous cell carcinoma generally have better treatment outcomes and prognosis compared to those with non-papillomavirus-associated oropharyngeal squamous cell carcinoma. However, prognostic evaluation for non-papillomavirus-associated oropharyngeal squamous cell carcinoma remains a problem that could be solved through the molecular mechanisms of squamous cell carcinoma for the purpose of further development of target therapies. Detection of cyclin dependent kinase inhibitor 2a gene deletion in oropharyngeal squamous cell carcinomas can have clinical significance as it may serve as a prognostic marker and potentially guide treatment decisions. To investigate and analyze cyclin dependent kinase inhibitor 2a gene alterations in oropharyngeal squamous cell carcinoma comparing with clinical data (age of the patient, TNM stage), their histological abnormalities respectively. Homozygous deletion of cyclin dependent kinase inhibitor 2a gene homozygous deletion had the highest risk of the nodal metastases development. Our findings suggest that not only detection of the loss of p16 expression, but also the evaluation of homozygous cyclin dependent kinase inhibitor 2a gene deletion might be predictive of worse outcome specifically in oropharyngeal squamous cell carcinomas.
Eventually the fact that the most common contributor in the development of oropharyngeal squamous cell carcinoma (OPSCC) is the human papillomavirus (HPV) offers the opportunities for preventive reduction of SCC incidence in this localization [1, 2, 3]. Furthermore, patients with HPV-associated OPSCC (OPSCC-HPV) generally have better treatment outcomes and prognosis compared to those with non-HPV-associated OPSCC [2]. Nevertheless, predicting the course and treatment effectiveness for the developed carcinomas, especially those that are not HPV-associated, remains an unresolved issue, where our hope relies on deciphering the molecular mechanisms of SCC for the purpose of further development of target therapies.

To date it is well known that non-HPV-associated OPSCCs harbor a spectrum of genetic mutations, including alterations in tumor suppressor genes, oncogenes, and genes involved in DNA repair and cell cycle regulation. Common mutations observed in non-HPV-associated OPSCC include mutations in TP53 (p53 gene), gene of cyclin-dependent kinase inhibitor 2A (CDKN2A, p16), notch receptor 1 gene (NOTCH1), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha gene (PIK3CA), and epidermal growth factor receptor gene (EGFR) [3]. These mutations disrupt key cellular pathways involved in controlling cell proliferation, apoptosis, and differentiation, leading to uncontrolled growth and tumor formation [4]. It is important to notice that although the mutated genes in non-HPV-associated OPSCC are TP53 and CDKN2A, the frequency of these genes alterations varies between 1 and 23% from case to case [5]. Deletion of CDKN2A has been implicated in the early stages of tumorigenesis in various cancers, including OPSCC’s, where it contributes to the initiation and development of malignant tumors.

Detection of CDKN2A deletion in OPSCCs can have clinical significance as it may serve as a prognostic marker and potentially guide treatment decisions [6, 7, 8, 9]. Additionally, therapies targeting the underlying molecular alterations associated with CDKN2A deletion, such as cyclin-dependent kinase (CDK) inhibitors or strategies to restore p53 function, are areas of active research and may hold promise for the treatment of OPSCC in the future [7].

Fluorescence in situ hybridization (FISH) analysis is a molecular technique used to detect and visualize specific DNA sequences within chromosomes. In the context of OPSCC, FISH analysis can be employed to assess certain genetic alterations in CDKN2A that may be observed in these tumors [8]. FISH probes specific to this gene can be used to assess copy number changes, such as deletions, which may have prognostic implications or therapeutic relevance [7, 8, 9, 10].

Therefore the main objective of this study was to investigate and analyze CDKN2A alterations in oropharyngeal squamous cell carcinomas comparing with clinical data (age of the patient, TNM stage), their histological features and occurrence of HPV infection markers (p16 expression).

MATERIALS AND METHODS OF RESEARCH

The study examined biopsies and material after transoral radical surgery of oropharyngeal tumors from 26 patients (all males) admitted to Metchnikov Dnipro Regional Clinical Hospital. Age ranged from 41 to 77 years, with an average 57.35±10.33 years.

The study was conducted according to the legislated consent of the participants and in accordance with the principles of bioethics mentioned in the Helsinki Declaration for Ethical Principles for Medical Research Involving Human Subjects and the Universal Declaration of Bioethics and Human Rights (meeting minutes of the Biomedical Ethics Committee of Dnipro State Medical University No. 2 dated 26.10.2021).

All cases were grouped according to the occurrence of nodal metastases: OPSCC without metastases 9 (34.6%) and OPSCC with nodal metastases 17 (65.4%); no recurrent OPSCC or cases with distant metastases were observed within selected patients.

Histological method

Formalin-fixed and paraffin-embedded samples were taken from the biobank of the Dnipro Regional Pathological Bureau. Paraffin sections of 4 μm were obtained on a Microm HM-340 microtome and stained with hematoxylin and eosin according to the standard method [11]. Multilayered squamous epithelium without dysplastic changes was used as an

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internal control. Microscopy was performed using a ZEISS "Axio Imager" light microscope (×10, ×20, ×40 objectives). Digital images were processed with the licensed software ZEN 2 blue edition.

**Immunohistochemical method**

Paraffin sections were applied to SuperFrost Plus adhesive slides. After deparaffinisation, rehydration, heat-induced antigen retrieval and inhibition of endogenous peroxidase activity, sections were incubated with primary antibodies in humid chamber at 4°C overnight. The primary monoclonal antibody to p16\(^{\text{INK4}}\) (clone MX007, Master Diagnostica, Spain) and the UltraVision Quanto imaging system (LabVision) were used. To identify the reaction, a solution of chromogen 3-diaminobenzidine tetra-chloride (Quanto, LabVision) was applied under the control of a microscope for a 20 seconds to 3 minutes, with a brown colouration. The nuclei were additionally stained with Mayer's haematoxylin for 1-3 minutes. Following the recommendations of Ferreira et al. (2021), the expression of the p16 marker, which is approved as a surrogate marker of OPSCC-HPV, was considered positive only if it demonstrates strong diffuse nuclear-cytoplasmic staining in more than 75% of cells [12].

**Fluorescent in situ hybridization**

Fluorescence in situ hybridization (FISH) was performed for assessment of the CDKN2A gene (at 9p21) using ZytoLight SPEC CDKN2A/CEN 9 Dual Color Probe (ZytoVision, GmbH, Germany) consisting of polynucleotides (~10 ng/μl), which target sequences mapping in 9p21.3 (chr9:21,742,629-22,056,853) harboring the CDKN2A gene region labeled with ZyGreen (excitation 503 nm/emission 528 nm) and polynucleotides (~1.5 ng/μl), which target sequences mapping in 9q12 specific for the classical satellite III region D9Z3 of chromosome 9 labeled with ZyOrange (excitation 547 nm/emission 572 nm). A following counting strategy was used for assessment of the CDKN2A gene: a signal pattern of two red and two green signals indicated two intact CDKN2A loci on chromosome 9; one red and two green signals indicated heterozygous deletion of CDKN2A, and no red and two green signals indicated a homozygous deletion of CDKN2A. It was scored a total of 100 tumor cells for each specimen [13].

**Statistical analysis**

For statistical analysis OPSCCs were categorized by patients’ age divided on groups of 10-year periods: 40-49, 50-59, 60-69, and 70-77 year old patients. Data were compared between groups (p16-positive versus p16-negative expression and homozygous deletion of CDKN2A versus other variants) by using. Age was compared with Mann-Whitney test. Patients with nodular metastases versus metastasis-free cases were also estimated with Fisher’s exact tests and compared with p16 expression and FISH by using relative risk test (RR) with calculation of 95% confidence intervals (CI) using Koopman asymptotic score. P-values less than 0.05 were considered statistically significant [14]. All data analyses were performed in GraphPad Prism version 8.0.2 (263) for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

**RESULTS AND DISCUSSION**

Histological examination demonstrated the presence of moderately differentiated SCCs with keratinization in sixteen cases, the remaining cases appeared as moderately differentiated SCC without keratinization (Fig., A-B). Immunohistochemically in eleven cases p16 expression was revealed as the positive diffuse nuclear-cytoplasmic staining in more than 75% of cells. The other fifteen cases were evaluated as negative (Fig., C-D). Six out of eleven p16-positive SCCs (54.5%) were non-keratinized and predominately had nested arrangements of tumor cells. Respectively, in five cases of p16-positive SCCs the presence of keratinization was found. In contrast, a keratinized/non-keratinized tumor ratio comprised 11/4 in p16-negative cohort. The patients with p16-positive SCCs were overall younger than the patients with p16-negative tumors (mean age 55.3 and 58.9 respectively), though it was not statistically meaningful (p=0.3916). Furthermore, five patients with p16-positive OPSCC clearly revealed the presence of nodal metastases.

CDKN2A analysis was conducted successfully by FISH in all selected patients. Ten out of 26 tumors (38.5%) exhibited homozygous deletion of CDKN2A (Fig., E-F). Eleven out of 26 cases (42.3%) harbored normal (two) copies of CDKN2A. Five cases (19.2%) revealed a single copy deletion of CDKN2A.

Loss of p16 protein expression correlated with homozygous CDKN2A deletion (p<0.00012). In all ten cases with homozygous CDKN2A deletion, p16 expression was absent (Table). The remaining five of the fifteen p16-negative OSCCs (33.3%) had two copies in three cases or a single copy deletion of CDKN2A in two remaining cases. No homozygous deletion of CDKN2A was detected in a tumor which expressed p16, however two cases revealed single CDKN2A copy deletion.

Further we evaluated p16 expression and CDKN2A gene status of OPSCC separately regarding the age of the patient and occurrence of nodal metastases. Patients with OPSCC lacking p16 expression were older at time of surgery than patients with tumors expressing p16 though it was not statistically significant (median age 60 versus 52 years, p=0.3491). Similarly, patients with non-
HPV OPSCC showing homozygous CDKN2A deletion (n=10) were overall older at time of surgery than patients with five non-HPV-associated tumors not harboring CDKN2A loss (median age 65.5 versus 49 years, p=0.0263). In entire cohort of patients with OPSCC, loss of p16 expression was associated with higher risk of nodal metastases when compared with patients with p16 expression though it was not statistically significant (RR for p16-positive versus p16-negative patients = 0.682, 95% CI: 0.34–1.17, p=0.218). Within p16-negative OPSCC group patients with homozygous deletion of CDKN2A showed highest risk of nodal metastasizing in comparison with patients without CDKN2A homozygous deletion (RR for CDKN2A loss versus patients with at least one copy of CDKN2A=2.5, 95% CI: 2.18–2.56, p=0.022).

Comparison of histological and histogenetic features of HPV and non-HPV-associated OPSCC.
A, C, E – HPV-associated SCC with non-keratinizing nested appearance, p16-positivity and normal number of CDKN2A gene copies; B, D, F – keratinizing non-HPV-associated SCC demonstrates a lack of p16 expression and homozygous deletion of CDKN2A gene. A,B – H&E staining (∗200); C,D – p16 expression (∗200); E, F – CDKN2A/CEN9 FISH, orange – chromosome 9 labelling, green – CDKN2A labeling: homozygous deletion is notable due to lack of green labels (∗1000)
Distribution of $CDKN2A$ deletion in patients with OPSCC, n

<table>
<thead>
<tr>
<th>Group/Feature</th>
<th>n</th>
<th>p16+</th>
<th>p16–</th>
<th>$CDKN2A$ loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) 40-49</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>50-59</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>60-69</td>
<td>8</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>70-80</td>
<td>4</td>
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<td>3</td>
<td>3</td>
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<tr>
<td>With nodal metastases</td>
<td>17</td>
<td>5</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Without metastases</td>
<td>9</td>
<td>6</td>
<td>3</td>
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<tr>
<td>TOTAL</td>
<td>26</td>
<td>11</td>
<td>15</td>
<td>10</td>
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</tbody>
</table>

Notes: n – number of patients; “p16+” – cases with positive p16-immunostaining; “p16–” – cases with negative p16-immunostaining; “$CDKN2A$ loss” – homozygous $CDKN2A$ deletion.

Deletion or inactivation of the $CDKN2A$ gene is a common genetic alteration observed in various types of cancers, including OPSCC [5-9]. The $CDKN2A$ gene, located on chromosome 9p21, encodes two important tumor suppressor proteins, $p16^{INK4a}$ and $p14^{ARF}$ [5]. In the context of OPSCCs, deletion of the $CDKN2A$ gene can lead to loss of function of both $p16^{INK4a}$ and $p14^{ARF}$ proteins, contributing to further tumorigenesis [7]. $p16^{INK4a}$ functions as a negative regulator of the cell cycle by inhibiting the activity of CDKs, specifically CDK4 and CDK6. Inactivation of $p16^{INK4a}$ due to $CDKN2A$ deletion results in dysregulated CDK activity, leading to unchecked cell cycle progression and increased cell proliferation [5, 7]. $p14^{ARF}$ is another product of the $CDKN2A$ gene and plays a crucial role in stabilizing the tumor suppressor protein $p53$ [6]. Activation of $p53$ by $p14^{ARF}$ leads to cell cycle arrest, apoptosis, and senescence in response to various cellular stresses [5, 6]. Deletion of $CDKN2A$ can disrupt the $p14^{ARF}$-$p53$ pathway, impairing the cell's ability to respond to DNA damage and leading to genomic instability [5, 6, 7, 8, 9].

This study confirmed the involvement of $CDKN2A$ homozygous deletion as a common pathway of carcinogenesis in non-HPV-associated OPSCC. Our study shows that loss of $p16$ expression is not significantly associated with higher risk of nodal metastases development in oropharyngeal squamous cell carcinomas, at least within the represented cohort of the patients; however, larger cohorts of investigated patients reveal that this parameter does not worsen the general outcome of these patients [10]. Furthermore, the patients with non-HPV-OPSCCs that harbored $CDKN2A$ homozygous deletion had the highest risk of the nodal metastases development. In addition, loss of $p16$ expression was associated with older age of patients with OPSCC; homozygous $CDKN2A$ deletion in OPSCCs also tended to occur in older patients. Our findings suggest that not only detection of the loss of $p16$ expression, but also the evaluation of homozygous $CDKN2A$ deletion might be predictive of worse outcome specifically in oropharyngeal SCC that was confirmed in other studies [9]. Moreover $CDKN2A$ evaluation could be used as an independent of known prognostic parameters such as staging and complete resection, and is generally associated with older age of the patients with OPSCC [9, 15]. Certain amount of non-HPV-associated $p16$-negative OPSCC does not reveal $CDKN2A$ deletion therefore it should be suggested and investigated another mechanism (e.g. frameshift mutation, methylation etc) for $p16$ inactivation that was described in previous studies [7, 8, 9].

**CONCLUSION**

In conclusion, homozygous deletion of cyclin dependent kinase inhibitor 2a gene could be suggested as a promising prognostic biomarker for non-papillomavirus-associated oropharyngeal squamous cell carcinomas in terms of increased risk of nodal metastasis development. Besides that, loss of cyclin dependent kinase inhibitor 2a gene is associated with older age of patients. Furthermore, on the basis of our findings, certain amount of non-papillomavirus-associated $p16$-negative oropharyngeal squamous cell carcinomas does not reveal $CDKN2A$ deletion therefore it should be suggested and investigated another mechanism (e.g. frameshift mutation, methylation etc) for $p16$ inactivation that was described in previous studies [7, 8, 9].

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Contributors:

Shponka I.S. – project administration, supervision, resources, data curation, validation, writing – review & editing;
Bondarenko O.O. – methodology, investigation, formal analysis, visualization, validation;
Kovtunenko O.V. – project administration, supervision, resources, data curation, validation, writing – review & editing;

Rakhmanov V.V. – conceptualization, methodology, funding acquisition, resources, writing – original draft.

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REFERENCES


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