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AURKAPS1, *HERC2P2* and *SDHAP1* pseudogenes: molecular role in development and progression of head and neck squamous cell carcinomas and their diagnostic utility

Tomasz Kolenda[1, 2], Joanna Kozłowska-Masłoń[3, 4], Patrycja Mantaj[5], Nina Grzejda[3, 6], Kacper Kamiński[7, 8, 9], Maria Dziuba[7], Małgorzata Czarnecka[7], Aleksandra Leszczyńska[7], Paulina Poter[10, 11], Kacper Guglas[3], Klaudia Dudek[3, 12], Katarzyna Regulska[2, 13], Paulina Gieremek[13, 14], Beata Stanisz[14], Anna Florczak-Substyk[7], Marlena Janiczek-Polewska[15, 16], Urszula Kazimierczak[7], Katarzyna Lamperska[2, 3], Zefiryn Cybulski[1, 2], Anna Teresiak[2, 3]

[1]Microbiology Laboratorium, Greater Poland Cancer Centre, Poznan, Poland
[2]Research and Implementation Unit, Greater Poland Cancer Centre, Poznan, Poland
[3]Laboratory of Cancer Genetics Poznan, Greater Poland Cancer Centre, Poznan, Poland
[4]Institute of Human Biology and Evolution, Faculty of Biology, Adam Mickiewicz University, Poznan, Poland
[5]Department of Radiation Protection, Greater Poland Cancer Centre, Poznan, Poland
[6]Faculty of Biology, Adam Mickiewicz University, Poznan, Poland
[7]Chair of Medical Biotechnology, Department of Cancer Immunology, Poznan University of Medical Sciences, Poznan, Poland
[8]Department of Histology and Embryology, Poznan University of Medical Sciences, Poznan, Poland.
[9]Doctoral School, Poznan University of Medical Sciences, Poznan, Poland.
[10]Department of Clinical Pathology, Poznan University of Medical Sciences and Greater Poland Cancer Centre, Poznan, Poland
[11]Department of Cancer Pathology, Greater Poland Cancer Centre, Poznan, Poland
[12]Department of Food Quality and Safety Management, Faculty of Food Science and Nutrition, Poznan University of Life Sciences, Poznan, Poland
[13]Pharmacy, Greater Poland Cancer Centre, Poznan, Poland
[14]Departament of Pharmaceutical Chemistry, Poznan University of Medical Sciences, Poznan, Poland
[15]Department of Clinical Oncology, Greater Poland Cancer Centre, Poznan, Poland
[16]Department of Electroradiology, Poznan University of Medical Sciences, Poznan, Poland

ABSTRACT

Background: Pseudogenes are epigenetic elements whose function is mostly unknown in cancer including head and neck cancers (HNSCCs). In our study we analyzed selected three pseudogenes, aurora kinase A pseudogene 1 (*AURKAPS1*), hect domain and RLD 2 pseudogene 2 (*HERC2P2*) and succinate dehydrogenase complex flavoprotein subunit A pseudogene 1 (SDHAP1), in the context of molecular function, biological role and potential utility as a biomarker in HNSCCs.

Materials and methods: Based on The Cancer Genome Atlas (TCGA) data we checked potential association of pseudogenes with pathological and clinical features, survival, cellular phenotype and involvement in pathways and cellular processes, and association with patients' response to radiotherapy.

Results: Only *AURKAPS1* pseudogene has significant upregulation in cancer than in normal samples and could be used as a diagnostic biomarker. Expression levels of all pseudogenes are dependent on cancer localization. *SDHAP1* are the most differentiated and associated with tumor subtypes, expressions of *AURKAPS1* do not depend on this tumor classification. Higher expression levels of *AURKAPS1*, *HERC2P2* and *SDHAP1* were associated with more aggressive phenotypes and associated with important cellular pathways and biological processes. Moreover, we observed that the expression of all pseudogenes were higher in human papilloma virus (HPV)(+) than in HPV(–) patients. Only *AURKAPS1* was associated with higher genome instability and worse response to radiotherapy. Patients with higher expression levels of *AURKAPS1* and *HERC2P2* displayed better survival.

Address for correspondence: Tomasz Kolenda, Greater Poland Cancer Centre, Research and Implementation Unit, Poznan, Poland; e-mail: tomasz.kolenda@wco.pl

Conclusions: *AURKAPS1* is a potential biomarker for HNSCC patients. This pseudogene is associated with changes in DNA repair, which should be more deeply analyzed in the future.

Keywords: HNSCC; pseudogene; lncRNA; biomarker; HPV; oncogene; suppressor; non-coding RNA; TCGA

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Introduction

In recent years, an increasing incidence of head and neck cancer has been observed, both globally and locally in the Greater Poland region [1, 2]. Head and neck squamous cell carcinomas (HNSCC) are located in three main anatomical regions, namely the oral cavity, larynx and pharynx, and are almost practically squamous cell carcinomas. The main factors for the development of HNSCC include smoking, alcohol consumption and human papilloma virus (HPV) infection [1, 3]. Due to their anatomical location, HNSCC are extremely difficult to treat; the presence of a large number of blood vessels and lymph nodes favor their rapid metastasis, both local and distant. Moreover, the location of tumors in the head and neck area makes resection difficult due to the large number of nerves, speech apparatus, organs of taste and smell, as well as the proper functioning of the digestive and respiratory systems [4]. The main methods of treatment for HNSCC, if resection is possible, are surgery with radiotherapy, often combined with chemotherapy. In the case of palliative treatment, for unresectable tumors, the only therapeutic options are radiotherapy and chemotherapy [5–13]. Among modern drugs, two immune checkpoint inhibitors, pembrolizumab and nivolumab, which are antibodies that bind to the PD-1 receptor, are used as the choice for the treatment of recurrent or metastatic HNSCC (R/M-HNSCC). It should be noted that pembrolizumab is a first-line therapy for unresectable tumors [7, 14]. Due to the very high molecular heterogeneity of HNSCC and the use of various therapies, both classical and modern, the use of appropriate biomarkers is the only option to avoid ineffective therapies at the beginning of and during therapy. Methods to assess the effectiveness of therapy are being increasingly implemented, but those based on molecular biomarkers are rare [14].

Although specific biomarkers based on DNA mutations have been known for years, they have not brought any breakthrough changes in the personalization of oncology. It should be emphasized that even The Cancer Genome Atlas (TCGA) project, which was based on whole-genome scanning of a large number of HNSCC patients, did not result in a spectacular improvement in clinical management [15, 16]. Currently, it seems that regulatory systems in the cell genome are more important. While the biological and diagnostic function of miRNAs is very well understood, lncRNAs are still a mysterious part of epigenetics. One specific type of long non-coding lncRNA molecules are pseudogenes [17–21]. Pseudogenes are non-coding RNA molecules that can be defined as an altered copy of an existing gene that has lost its protein-coding function. So far, it has been shown that the known pseudogenes function at different RNA, DNA and protein levels, and seem to be important elements of the epigenetic regulation network [22].

In this study, we analyzed three selected pseudogene transcripts named aurora kinase A pseudogene 1 (*AURKAPS1*), hect domain and RLD 2 pseudogene 2 (*HERC2P2*) and succinate dehydrogenase complex flavoprotein subunit A pseudogene 1 (*SDHAP1*) in the context of their potential utility as biomarkers in head and neck squamous cell carcinomas (HNSCCs) and their biological role. All pseudogenes have unknown functions in HNSCC or little is known in the case of other types of cancers. However, their parental genes are well described [23–25].

Materials and methods

To determine the diagnostic and biological significance of the pseudogenes *AURKAPS1*, *HERC2P2* and *SDHAP1*, transcriptome data retrieved from the TCGA database were used. The entire analysis included two basic steps: anal-

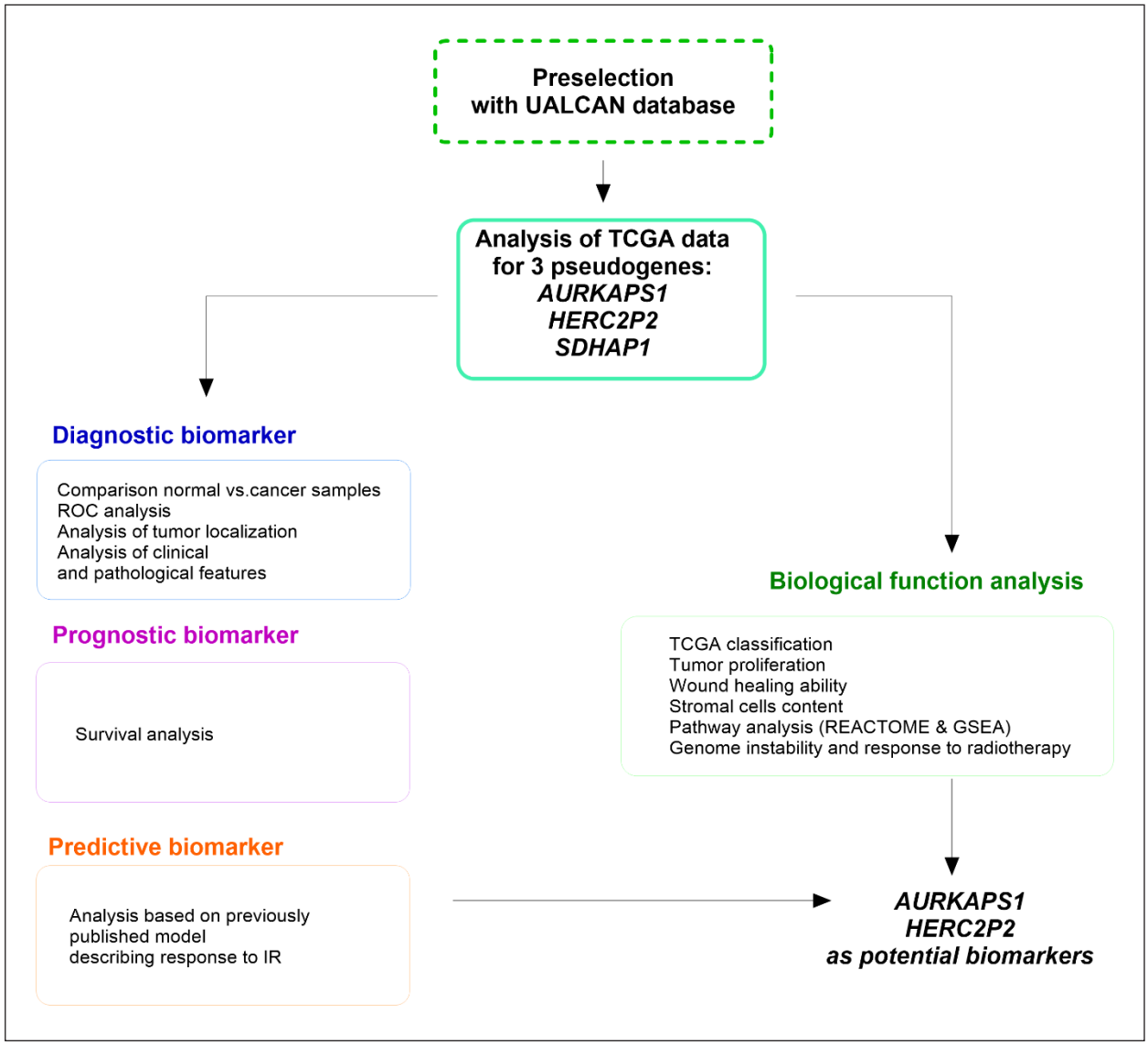

Figure 1. Analyses performed in this study to determine the diagnostic and biological significance of the *AURKAPS1*, *HERC2P2*, and *SDHAP1* pseudogenes. UALCAN — The University of ALabama at Birmingham CANcer data analysis Portal; TCGA — The Cancer Genome Atlas; ROC — receiver operating characteristic; GSEA — Gene Set Enrichment Analysis; IR — ionizing radiation

ysis for significance as a biomarker: diagnostic, prognostic and predictive, and a second step analysis for determination of biological significance. We used the individual steps of the methodology as presented in Figure 1.

Materials

Clinical and expression data of *AURKAPS1*, *HERC2P2* and *SDHAP1*, which were downloaded from the TCGA XenaBrowser (from the website of Santa Cruz University of California, https://xenabrowser.net/datapages/; cohort: TCGA Head and Neck Cancer (HNSC), accessed on January 2020), and as the graphs from the The University of ALabama at Birmingham CANcer data analysis Portal (UALCAN), Gene Expression Profiling Interactive Analysis version 2.0 (GEPIA2) tool databases and data from cBioPortal as well as from supplementary materials published by Thorsson et al. [26]. All of these datasets are connected and represent the same patients' samples collected during the TCGA project [27].

Pathological and clinical analyses

We analyzed differences in the expression levels of *AURKAPS1*, *HERC2P2* and *SDHAP1* between

age, gender, alcohol consumption, smoking , pathological cancer stage, T-, N-cancer grade, perineural invasion, neck lymph node dissection, lymphovascular invasion and HPV status, similar as published previously [27, 28].

Survival analyses

Patients were divided into two groups depending on the expression levels of *AURKAPS1*, *HERC2P2* and *SDHAP1* (low- and high-expressing), using mean of expression as cutoff. For assessment of OS and DFS depending on all three pseudogenes together, overall survival (OS) and disease-free survival (DFS) gene survival signatures tool from the GEPIA2 portal were used [27, 28].

Phenotype analyses

First negatively and positively Spearman's correlation ($R < -0.3$ and $R > 0.3$, $p < 0.05$ and FDR adjusted p-value: q-value < 0.001) obtained from cBioportal and assessment of genes' list with cellular processes and pathways using REACTOME pathway browser with p-value ≤ 0.05 as a cut off value. In the second approach, HNSCC patients were divided into two subgroups based on the mean expression level of the specific pseudogene transcript and Gene Set Enrichment Analysis (GSEA) was performed as described previously [27, 28] and the results with $p \leq 0.05$ and FDR adjusted p-value: q-value ≤ 0.25 were considered as statistically significant.

Response to radiotherapy

For estimation if *AURKAPS1*, *HERC2P2* and *SDHAP1* pseudogenes levels differ depending on responses to radiotherapy we used a previously adapted model where HNSCC patients were divided into two groups with response and non-response to radiotherapy [28].

Statistical analyses

We used GraphPad Prism 8 (GraphPad, San Diego, CA, USA) for statistical analysis. Shapiro–Wilk normality test and next t-test or Mann–Whitney U test were used in all analyses. For analysis of three or more groups One Way ANOVA with proper posttest were calculated. In all analyses, $p < 0.05$ was used to determine statistical significance [27, 28].

Results

Expression of pseudogenes was upregulated in cancer tissue and depended on the tumor localization in HNSCC

Based on the data obtained from the The University of ALabama at Birmingham CANcer data analysis Portal (UALCAN) database, we determined that studied pseudogenes were significantly overexpressed in all primary HNSCC samples compared to normal tissue samples: *AURKAPS1* ($p < 0.0001$), *HERC2P2* ($p < 0.0001$), and *SDHAP1* ($p < 0.0001$) (Fig. 2A). However, the analysis of paired cancer and adjacent normal samples indicated a significant difference between expression levels only in the case of *AURKAPS1* ($p < 0.0001$) with a high ability to distinguish both types of samples [area under the curve (AUC) = 0.8986, 95% confidence interval (CI) = 0.8349 to 0.9623, $p < 0.0001$) (Fig. 2B).

Moreover, the pseudogenes expression showed a highly significant positive correlation between pairs of genes: *HERC2P2* and *SDHAP1* ($R = 0.626$, $p < 0.0001$), *HERC2P2* and *AURKAPS1* ($R = 0.233$, $p < 0.0001$) as well as *AURKAPS1* and *SDHAP1* ($R = 0.328$, $p < 0.0001$).

Interestingly, in the case of *AURKAPS1* and *SDHAP1*, we indicated that tumors localized in the oral cavity displayed significantly lower expression levels than those located in the pharynx and larynx (oral cavity *vs.* pharynx: $p = 0.001$ and $p = 0.0156$, respectively and for oral cavity *vs.* larynx: $p < 0.0001$ and $p = 0.0008$, respectively). For *HERC2P2*, we indicated differences only in expression levels between oral cavity and pharynx tumors ($p = 0.002$) (Fig. 2C).

Then, we examined the possible association of studied pseudogenes with atypical, basal, classical, or mesenchymal tumor subtypes based on the TCGA classification. In the case of *HERC2P2*, patients with an atypical type of tumor displayed higher expression levels than those with basal ($p = 0.0027$) or mesenchymal ($p = 0.0274$) subtypes. The most changed expression levels were indicated for *SDHAP1* in almost all variations of comparisons: an atypical *vs.* basal, atypical *vs.* classical, basal *vs.* classical (for all $p < 0.0001$), as well as between classical *vs.* mesenchymal ($p = 0.0012$) subtypes (Fig. 2D).

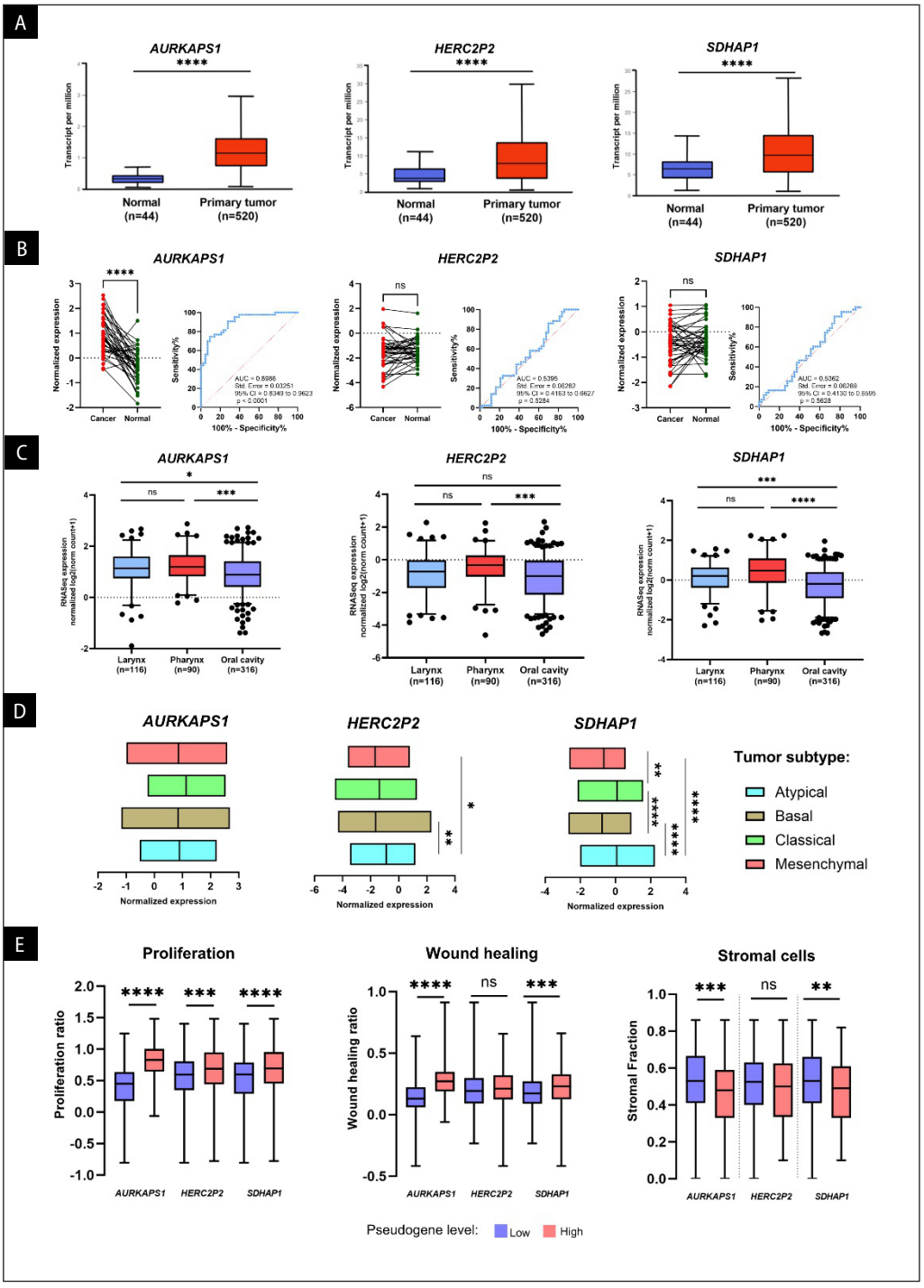

Figure 2. Expression levels of *AURKAPS1*, *HERC2P2* and *SDHAP1* in head and neck squamous cell carcinomas (HNSCC) patients. **A.** In primary tumor and normal tissue samples; unpaired samples; graphs from The University of ALabama at Birmingham CANcer (UALCAN) database, modified; **B.** Between matched adjacent normal and cancer samples with receiver operating characteristic (ROC) analysis; **C.** Depending on three main localization groups including larynx, pharynx and oral cavity, based on The Cancer Genome Atlas (TCGA) XenaBrowser dataset; box and whiskers with 5–95 percentile. Association of expression levels of *AURKAPS1*, *HERC2P2* and *SDHAP1* pseudogenes with (**D**) atypical, basal, classical or mesenchymal tumor subtype based on the TCGA classification and with tumor proliferation, wound healing ability and with stromal cells content within the patient's sample, based on the Thorsson et al. dataset. High and low expression levels divided based on mean expression level of specified pseudogene in an analyzed group of head and neck squamous cell carcinoma (HNSCC) patients; $p < 0.05$ set as statistical significance cutoff for one-way ANOVA with Tukey's multiple comparisons test or Mann Whitney test; ns — not significant ($p > 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$; AUC — area under the ROC curve; CI — confidence interval

Next, we observed that patients with higher levels of all studied pseudogenes had a higher proliferation ratio (*AURKAPS1*: $p < 0.0001$, *HERC2P2*: $p = 0.0002$, and *SDHAP1*: $p < 0.0001$) and wound healing ability ($p < 0.0001$, $p = 0.0516$, and $p = 0.0001$, respectively) than those with lower expression levels. Moreover, we determined that patients' samples with more abundant *AURKAPS1* and *SDHAP1* displayed lower levels of stromal cell content ($p = 0.0001$ and $p = 0.002$, respectively) (Fig. 2E).

Expression levels of pseudogenes differ depending on clinicopathological parameters

We compared pseudogenes' expression levels in groups distinguished based on clinicopathological parameters. Interestingly, *AURKAPS1* and *SDHAP1* expression significantly vary between the genders, resulting in lower values in the group of female patients ($p < 0.0001$ and $p = 0.0004$, respectively). Reduced levels of these pseudogenes were also more characteristic for the group of patients with I + II cancer stages than individuals with more advanced disease ($p < 0.0001$ and $p = 0.0252$, respectively).

Moreover, diminished amounts of both, *AURKAPS1* and *HERC2P2*, were associated with G1 + G2 subgroups ($p = 0.0038$ and $p = 0.0157$, respectively). Perineural invasion absence was linked with a higher expression level of *HERC2P2* ($p = 0.0264$), and lympho-vascular invasion presence was associated with elevated levels of *SDHAP1* ($p = 0.0037$). Surprisingly, we were able to distinguish between samples with negative and positive HPV status based on all of the analyzed pseudogenes expression values: *AURKAPS1* (AUC = 0.6521, $p = 0.0082$), *HERC2P2* (AUC = 0.7197, $p = 0.0001$), and *SDHAP1* (AUC = 0.7359, $p < 0.0001$) (Tab. 1).

Studied pseudogenes are associated with important molecular pathways and cellular processes

We determined that genes whose expression levels were negatively correlated with: 1) *AURKAPS1s*' were involved mostly in the formation of the cornified envelope, keratinization, and neutrophil degranulation; 2) *HERC2P2s*' were associated with intra-Golgi and retrograde Golgi-to-endoplasmic reticulum (ER) traffic, including the coat protein complex I (COPI)-independent pathway, as well as neutrophil degranulation; and 3) *SDHAP1s*' with response to elevated platelet cytosolic Ca2+, platelet degranulation, signaling by RAF1 mutants, and signaling by moderate kinase activity *BRAF* mutants.

In contrast, genes with expression positively correlated with levels of: 1) *AURKAPS1* were implicated in cell cycle and its control, including G2/M checkpoints, cellular division, regulation of chromatids and kinetochores as well as RHO GTPases activate formins pathway; and both 2) *HERC2P2* and 3) *SDHAP1* seem to be associated with RNA processing including RNA polymerase II transcription termination and mRNA 3'-end processing, Figure 3A.

Subsequently, based on GSEA analysis, we indicated that patients with higher expression levels of *AURKAPS1* have enriched genes implicated in: RB pathway, G2M checkpoint, CSR late response (genes upregulated in late serum response of CRL 2091 cells), E2F1 pathway, response to vascular endothelial growth factor (VEGF) treatment in HUVEC cells, as well as such hallmarks of cancer as E2F2 associated pathways, mitotic spindle, and DNA repair. For the second pseudogene, patients displayed differences in expression pattern similar to genes changed after the knockdown of the *JAK2* gene, after over-expressing of SRC, changed in an mTOR pathway, or similar to these caused by knockdown of EZH2, and connected with CSR early phenotype. Surprisingly, in the case of the *SDHAP1* pseudogene, we observed variations in the enrichment of gene expression similar to these after the knockdown of the *JAK2* gene and CSR early phenotype (Fig. 3B).

Involvement of AURKAPS1 pseudogene in response to ionizing radiation

We determined that patients with increased expression levels of *AURKAPS1* and *SDHAP1* had higher ratios of the silent and nonsilent mutations ($p = 0.0058$ and $p = 0.034$; $p = 0.0043$ and $p = 0.0058$, respectively).

We also calculated the association of analyzed pseudogenes with aneuploidy scores, and only

Table 1. Expression levels of *AURKAPS1*, *HERC2P2* and *SDHAP1* pseudogenes depending on clinicopathological parameters analyzed in all localizations of head and neck squamous cell carcinomas (HNSCC) based on The Cancer Genome Atlas (TCGA) XenaBrowser dataset; t-test or Mann-Whitney U test; $p < 0.05$ considered significant

Parameter	Group	*AURKAPS1*		*HERC2P2*		*SDHAP1*	
		Mean ± SEM	p-value	Mean ± SEM	p-value	Mean ± SEM	p-value
Age	> 60	0.9933 ± 0.0468	0.8989	−0.9433 ± 0.0788	0.7750	−0.1068± 0.0536	0.4887
	≤ 60	1.0020 ± 0.04505		−0.9507 ± 0.0807		−0.0539 ± 0.0603	
Sex	Female	0.7759 ± 0.0597	< 0.0001	−1.0000 ± 0.1054	0.5861	−0.3082 ± 0.0729	0.0004
	Male	1.0760 ± 0.03771		−0.9254 ± 0.0665		−0.0002 ± 0.0473	
Alcohol	Positive	1.0360 ± 0.0395	0.1752	−0.9628 ± 0.0657	0.3926	−0.0808 ± 0.0494	0.5977
	Negative	0.9045 ± 0.0588		−0.9352 ± 0.1100		−0.1130 ± 0.0729	
Smoking	No/Ex	0.9557 ± 0.0399	0.0749	−0.9037 ± 0.0705	0.4998	−0.1166 ± 0.0516	0.2000
	Yes	1.0790 ± 0.0562		−0.9816 ± 0.0953		−0.0114 ± 0.0654	
Cancer stage	I + II	0.5872 ± 0.0470	< 0.0001	−1.0780 ± 0.1251	0.3032	−0.2624 ± 0.0876	0.0252
	III + IV	1.0650 ± 0.0423		−0.9303 ± 0.0683		−0.0588 ± 0.0487	
T Stage	T1 + T2	1.0010 ± 0.0543	0.2882	−0.8666 ± 0.0961	0.2220	−0.0787± 0.0699	0.8675
	T3 + T4	0.9273 ± 0.0457		−1.0030 ± 0.0756		−0.1026 ± 0.0535	
N Stage	N0	1.0470 ± 0.0554	0.2977	−0.8782 ± 0.0971	0.5845	−0.1153 ± 0.0666	0.2941
	N1 + N2 + N3	0.9840 ± 0.0478		−0.9369 ± 0.0811		−0.0224 ± 0.0599	
Grade	G1 + G2	0.9348 ± 0.0383	0.0038	−1.0560 ± 0.0679	0.0157	−0.1388 ± 0.0471	0.0993
	G3 + G4	1.1440 ± 0.0670		−0.7528 ± 0.1066		0.0086 ± 0.0818	
Perineural invasion	Positive	0.9943 ± 0.0534	0.7826	−1.1700 ± 0.0937	0.0264	−0.1778 ± 0.0706	0.2043
	Negative	0.9726 ± 0.0564		−0.8709 ± 0.0929		−0.0334 ± 0.0655	
Lymph node neck dissection	Positive	0.9843 ± 0.0369	0.4771	−0.9546 ± 0.0633	0.7573	−0.0902 ± 0.0438	0.7023
	Negative	1.0720 ± 0.0658		−0.8724 ± 0.1232		−0.0279 ± 0.1008	
Lympho-vascular invasion	Positive	1.0630 ± 0.0631	0.0507	−0.9429 ± 0.1143	0.4409	0.0746 ± 0.0775	0.0037
	Negative	0.9010 ± 0.0506		−1.0410 ± 0.0826		−0.2079 ± 0.0597	
HPV status	Positive	1.2490 ± 0.1102	0.0103	−0.3343 ± 0.1739	0.0003	0.5613 ± 0.1604	< 0.0001
	Negative	0.8972 ± 0.0789		−1.1870 ± 0.1393		−0.2942 ± 0.1173	

SEM — standard error of the mean; HPV — human papilloma virus

patients with elevated levels of *AURKAPS1* had higher aneuploidy scores compared to those with low expression levels ($p < 0.0001$). Surprisingly, we indicated that patients with increased expression levels of all studied pseudogenes have higher homologous recombination defect ratios than those with decreased levels of *AURKAPS1*, *HERC2P2*, and *SDHAP1* ($p < 0.0001$, $p = 0.0341$ and $p < 0.0001$, respectively) (Fig. 3C).

Next, we checked the association between expression levels of analyzed pseudogenes and response to radiotherapy. We indicated that patients with diminished levels of *AURKAPS1* displayed better responses to applied treatment than those with increased expression levels ($p = 0.0364$) (Fig. 3D).

Estimation of expression level of SDHAP1 alone and pseudogene pairs: AURKAPS1 with HERC2P2, and SDHAP1 with HERC2P2 could help in the assessment of patients survival

First, overall and progression-free survivals were calculated between groups of patients with high and low expression levels of *AURKAPS1*, *HERC2P2*, and *SDHAP1*. Significantly longer overall survival was linked with low expression levels of *HERC2P2* ($p^A = 0.1184$ and $p^B = 0.041$) and *SDHAP1* ($p^A = 0.0211$ and $p^B = 0.0384$) (Fig. 4A).

Subsequently, we calculated the possible link between overall survival and studied molecule abundance using expression signatures of combinations of two and all three pseudogenes using the GEPIA2

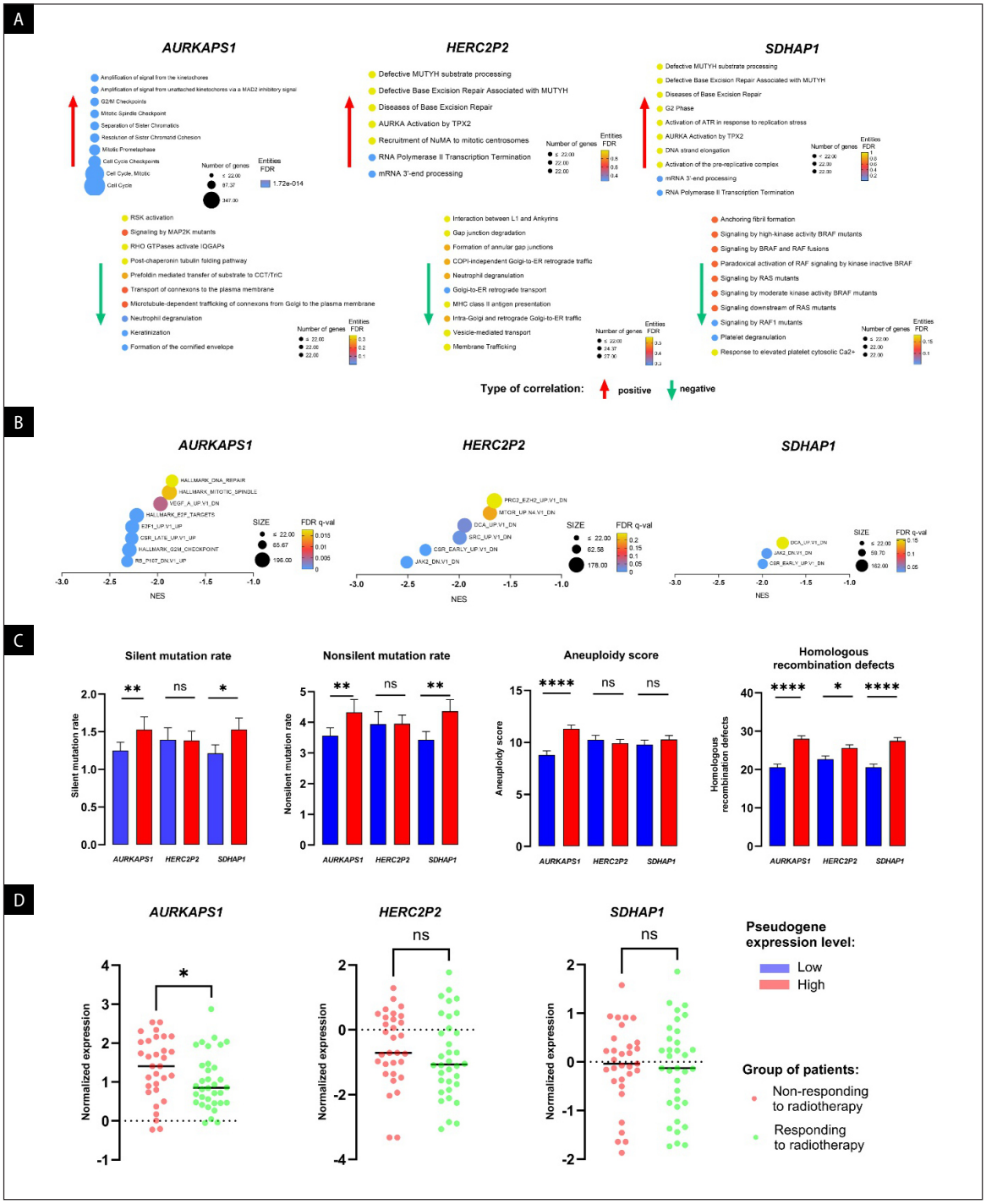

Figure 3. Association of *AURKAPS1*, *HERC2P2* and *SDHAP1* pseudogenes with molecular pathways and cellular processes. **A.** REACTOME pathway analysis based on the lists of negatively (red color) and positively (blue color) correlated genes [Spearman's correlation R < –0.3 and R > 0.3, p < 0.05 and false discovery rate (FDR) adjusted p-value: q-value < 0.001, based on the cBioPortal, The Cancer Genome Atlas (TCGA) data]; only results with p-value < 0.05 were shown; **B.** Gene set enrichment analysis (GSEA) between the groups of patients with low and high expression of *AURKAPS1*, *HERC2P2* and *SDHAP1* pseudogenes, based on the TCGA XenaBrowser dataset (mean expression set as cutoff); only results set with p ≤ 0.05 and FDR adjusted p-value (q-value) ≤ 0.25 were shown; **C.** With genome instability and response to radiotherapy of head and neck squamous cell carcinoma (HNSCC) patients: silent and nonsilent mutation rates, aneuploidy score and homologous recombination defects in the group of patients with low and high expression levels of specified pseudogenes; **D.** differences in the response to radiotherapy depending on the expression levels of *AURKAPS1*, *HERC2P2* and *SDHAP1* pseudogenes assessed based on the model described by Paszkowska et al. [36], based on the Thorsson et al. dataset; t-test or Mann-Whitney U test; ****p < 0.0001, **p < 0.01 and *p < 0.05 considered as significant; ns — not significant; NES — normalized enrichment score; SIZE — number of genes that are enriched in a specified deregulated process

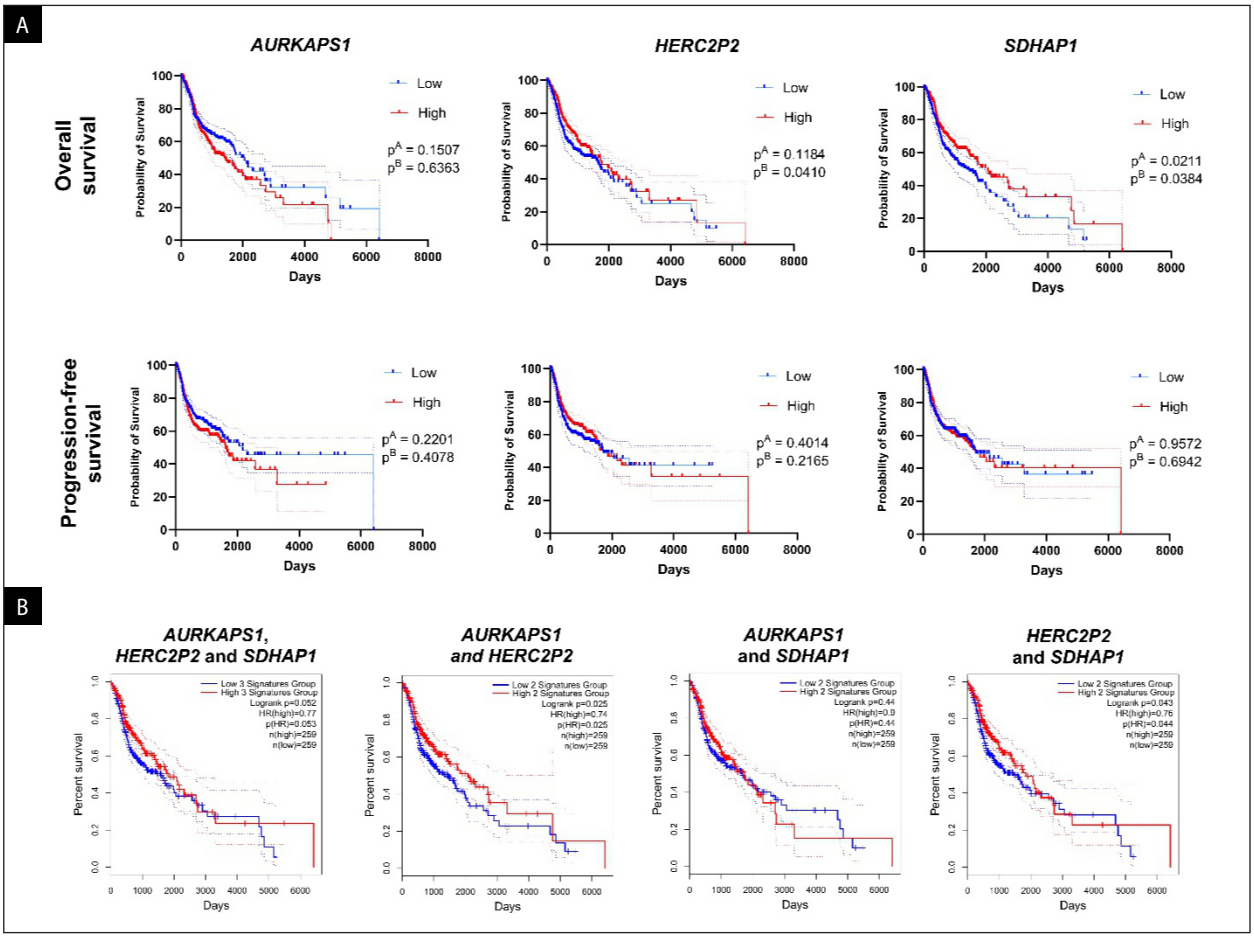

Figure 4. Association of pseudogenes with survival rate of head and neck squamous cell carcinoma (HNSCC) patients with all localizations of tumors depending (**A**) single expression levels of *AURKAPS1*, *HERC2P2* and *SDHAP1* and overall and progression free survivals; [A]Log-rank (Mantel-Cox) test; [B]Gehan-Breslow-Wilcoxon test; and (**B**) calculated for signatures expression of all three and two pseudogenes using GEPIA2 tool. Low and high subgroups of patients divided based on mean expression; darker blue and red lines represents patients' survival and lighter blue and red lines represent 95% confidence interval (CI); $p < 0.05$ considered significant

tool. In the case of the *AURKAPS1* and *HERC2P2* pair and *HERC2P2* and *SDHAP1* pair, we determined the association of higher expression levels with longer patients' overall survival ($p = 0.025$ and $p = 0.043$, respectively) (Fig. 4B).

Discussion

Radiotherapy is one of the main treatment strategies in HNSCC [29] and other types of cancers [30–34]. During the last decades different types of radiotherapy personalization was made including development of imaging systems as well as changes in therapy planning [35–43]. However, those approaches are not strictly based on the specific patients and in our opinion searching for biological biomarkers for radiotherapy personalization is an urgent need and a challenge. Among biological biomarkers we can distinguish cellular based biomarkers such as red cell distribution width (RDW), neutrophil-to-lymphocyte ratio (NLR), and platelet-to-lymphocyte ratio (PLR) [44, 45] and molecular based like change of telomerase activity [46]. The first analyzed pseudogene was *AURKAPS1*, also known as *AURKAP*1, which is a pseudogene of *AURKA*, and is located on the long arm of chromosome 1 (1q41). Our analysis revealed that *AURKAPS1* is upregulated in HNSCC patients and this is the first report related to this pseudogene in HNSCC. However, the parental genes, *AURKA*, as well as *AURKB*, are well described and were found upregulated in HNSCC. Elevated Aurora-A activity causes increased cell migration, mesenchymal-to-epithelial transition (MET) process, inva-

sion and cell survival. Moreover, *AURKA* could be used as a potential target for therapies [47]. In hepatocellular carcinoma, *AURKAPS1* expression was significantly higher compared to normal hepatocytes. Moreover, the level of *AURKAPS1* expression was positively correlated with tumor size and TNM stage, which suggests that *AURKAPS1* may be involved in tumor invasion. In addition, Li et al. identified that *AURKAPS1* by sponging the *miR-182*, *miR-155* and *miR-142*, and by increasing the protein level of RAC1, may promote the activation of ERK [48]. However, we observed no association *AURKAPS1* with tumor subtype based on the TCGA classification, but patients with higher levels of *AURKAPS1* have high proliferation and wound healing ability and high stromal cells content within analyzed samples. The mechanism of *AURKAPS1* action in HNSCC cells is feasible, but requires experimental validation.

Next, we checked the biological role of *HERC2P*. It is a pseudogene of *HERC2* (HECT and RLD domain containing E3 ubiquitin protein ligase 2). *HERC2* functions as a ubiquitin in the DNA damage pathway, regulates ubiquitin-dependent retention of repair proteins on damaged chromosomes and is recruited to sites of DNA damage in response to ionizing radiation. RNA splicing process is involved in cancer proliferation and it was suggested that lncRNA *HERC2P2* may have influence on tumor progression by upregulating genes involved in RNA splicing by the ceRNA (competing endogenous RNA) mechanism [49]. We observed that pseudogene *HERC2P2* is overexpressed in HNSCC patients and seems to be a tumor suppressor, associated with longer OS of patients. The knowledge about *HERC2P2* in HNSCC is limited. Only Peng et al. indicated that *HERC2P2* is downregulated in vocal cord leukoplakia as compared with adjacent non-tumorous tissue [50]. In different tumors, *HERC2P2* is downregulated in high grade gliomas and acts as tumor suppressor. Overexpression of *HERC2P2* inhibits proliferation and metastasis of glioma cells [49]. We indicated that patients with atypical type of tumor displayed a higher expression level of *HERC2P2* than those with tumors that were classified to basal or mesenchymal subtypes. Moreover, strong association between *HERC2P2* expression and higher proliferation ratio were noticed. Hou et al. indicated that *HERC2P2* is downregulated and could serve as a potential blood-based biomarker in breast cancer but its biological role has not been shown so far [51]. The last one analyzed by us was succinate dehydrogenase complex flavoprotein subunit A pseudogene 1. *SDHAP1* is a 2591 nucleotide long gene located on chromosome 3, which encodes lncRNA *SDHAP1*. Moreover, it should be noted that the parental gene *SDHA* (succinate dehydrogenase complex subunit A) is a large transcript and has two more pseudogenes *SDHAP2* and *SDHAP3* [52]. Our results indicated that the above lncRNA was upregulated in cancer tissue. Surprisingly, the most changed expression levels were indicated in an atypical *vs*. basal, atypical *vs*. classical and basal *vs*. classical as well as between classical *vs*. mesenchymal tumor subtypes when the *SDHAP1* pseudogene was analyzed. Moreover, patients with higher levels of this pseudogene displayed higher tumor proliferation and wound healing ability and stromal cells content within the patient's sample in comparison to patients with lower levels of *SDHAP1*. Unfortunately, there are no reports on *SDHAP1* in HNSCC. Moreover, there are not many studies on *SDHAP1* in other types of cancers. However, it was shown that lncRNA *SDHAP1* is upregulated in ovarian cancer and correlates with chemotherapeutic resistance. Overexpression of *SDHAP1* in PTX-resistant ovarian cancer cells led to downregulation of *miR-4465*, which caused upregulation of *EIF4G2* (eukaryotic translation initiation factor 4 gamma 2) and resulted in PTX-resistance of ovarian cancer [53].

According to the previous report, Li et al. indicated that HPV integration in endocervical adenocarcinomas tissues caused 52 genetic fusions including *SDHAP2-SDHAP1* pseudogenes [54]. However, the biological consequences of this alteration has not been described yet.

Next, we analyzed genes positively and negatively correlated with *AURKAPS1, HERC2P2* and *SDHAP1,* and genes enrichment between groups of patients with low and high expression level of specific pseudogene. We found that *HERC2P2* and *SDHAP1* pseudogenes positively correlated with genes involved in AURKA activation by TPX2. It binds to aurora kinase A (AURKA) at centrosomes and promotes its activation by changing AURKA to its active conformation and allows autophosphorylation of the AURKA threonine residue T288 [55]. AURKA is a paren-

tal gene of *AURKAPS1* pseudogene, which we also analyzed, and we hypothesize that positive correlation between *HERC2P2* and *SDHAP1* pseudogenes and AURKA activation by TPX2 pathway also affects *AURKAPS1* pseudogene activation. Our results support the above since *AURKAPS1* expression was strongly and positively correlated with both *HERC2P2* and *SDHAP1* pseudogenes. Furthermore, *AURKAPS1* has a strong positive correlation with almost 300 genes involved in various pathways connected to cell cycle regulation and cell cycle checkpoints. Deregulation of any of those genes can lead to genomic instability [55]. Even though *AURKAPS1* correlates with increased expression of genes involved in cell cycle checkpoints, which are thought to prevent genomic instability, it can still lead to cell cycle deregulation and, eventually, result in cancer development [56]. Above results suggest that *AURKAPS1* is an oncogenic pseudogene and *HERC2P2* and *SDHAP1* can play an oncogenic role through activation of *AURKAPS1*. Next, the GSEA analysis of gene set enrichment indicated that patients with high expression of *AURKAPS1, HERC2P2* and *SDHAP1,* have dysfunctions in carcinogenesis pathways, which suggests they should be referred to as oncogenic pseudogenes. In the group of GSEA "hallmark gene sets" are genes classified as MYC targets, E2F targets, phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway and connected with processes, such as DNA repair, angiogenesis, oxidative phosphorylation or unfolded protein response. However, the enrichment of genes connected with suppressive pathways are also associated with the p53 pathway or G2M checkpoint. Accordingly, we need to consider that in our studies only cancer patients were compared in terms of differences between low- and high- pseudogene expression levels. Moreover, we observed that patients with lower expression of analyzed pseudogenes displayed lower enrichment of genes involved in "oncogenic signature gene sets" in comparison to high expressing groups. The cellular pathways, which are often dysregulated in cancer may lead to aggressive cellular phenotype. Our patients with lower expression of specific pseudogenes may display profiles like cells with BMI1 knockdown [57] or downregulated genes associated with epithelial-to-mesenchymal transition process or longer survival, indicating oncogenic function of studied pseudogenes in HNSCC.

The next issue we analyzed was the potential association of *AURKAPS1*, *HERC2P2* and *SDHAP1* pseudogenes with genome instability and response to radiotherapy of HNSCC patients. Little is known about association of pseudogenes and response to ionizing radiation and genome instability [22]. Our research is the first to describe the *AURKAPS1*, *HERC2P2* and *SDHAP1* pseudogenes in this context. We observed that patients with higher levels of *AURKAPS1* responded worse to the radiotherapy than those with lower level of this pseudogene. It could be connected with the fact that patients with positively correlated genes, which, we observed, were involved in the control cell cycle such as checkpoints, separation of chromatid and mitotic spindle checkpoint, amplification of signal from the kinetochores. However, there is no evidence about associations of *AURKAPS1*, *HERC2P2* and *SDHAP1* with response to radiotherapy provided by other researchers. Our study is the first one to examine that issue, and should be verified by independent study.

Conclusions

To summarize, it is the first report about *AURKAPS1*, *HERC2P2* and *SDHAP1* in HNSCC. We showed that *AURKAPS1* is diagnostic and, potentially, could be used as a predictive biomarker in the case of response to radiotherapy. Moreover, *AURKAPS1,* together with *HERC2P2* pseudogene, shows potential as prognostic biomarker in HNSCC patients. It should be noted that, this study is the first to indicate the important role of *AURKAPS1*, *HERC2P2* and *SDHAP1* pseudogenes in biology of HNSCC and *AURKAPS1, in particular,* is associated with changes in DNA repair, which should be more deeply analyzed in the future.

Authors' contributions

Authors' individual contributions: conceptualization — T.K., P.M.; methodology — T.K.; investigation — M.D., K.K., M.C., A.L., T.K., K.G., J.K.M., P.P., K.D., N.G., K.R., P.M., A.F., M.J.P., U.K., P.M.; data curation — M.D., M.C., K.K., A.L., T.K.; writing — original draft preparation — M.D., M.C., K.K., A.L., T.K.; writing — review and editing — T.K., Z.C., A.T., P.M., P.G., B.S.; visualization — M.D., K.K., M.C., A.L., T.K.; supervision — T.K.,

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Raw data are available on the XenaBrowser, Ualcan and cBioportal databases.

Ethics approval

Study is based on analysis of freely available data sets and does not need any ethics committee's agreement, nor does it violate any rights of other persons or institutions.

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