



## High expression of H2AX/ $\gamma$ -H2AX is associated with distinct biological pathway alterations and shorter survival in oropharyngeal squamous cell carcinoma

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### ABSTRACT

**Background:** The histone gene *H2AX* and its phosphorylated protein  $\gamma$ -H2AX play a crucial role in the DNA damage response. This study investigates the expression of *H2AX* mRNA and its phosphorylated  $\gamma$ -H2AX protein in oropharyngeal squamous cell carcinoma (OPSCC), its association with distinct biological pathway alterations and its potential as a biomarker.

**Materials and Methods:** Expression of *H2AX* mRNA in 76 OPSCC from The Cancer Genome Atlas (TCGA) cohort was analyzed. Patients were stratified into *H2AX*<sup>high</sup> and *H2AX*<sup>low</sup> OPSCC based on a survival-associated cutoff. Differentially expressed genes were identified using DESeq2, followed by pathway enrichment analyses. Immunohistochemical staining of  $\gamma$ -H2AX protein expression was performed on an independent cohort of 209 OPSCC, followed by survival and Cox regression analyses.

**Results:** High *H2AX* mRNA expression was a significant prognostic factor associated with shorter OS in the TCGA OPSCC cohort (HR 4.77,  $p = 0.04$ ). In *H2AX*<sup>high</sup> tumors, differential gene expression analysis revealed upregulation of genes regulating DNA repair and cell cycle (*CDK1*, *CCNB1*, *ZWINT*). High  $\gamma$ -H2AX protein expression was significantly associated with HPV-negative OPSCC ( $p = 0.005$ ), and remained an independent predictor of poor survival in the total OPSCC cohort (HR 2.24,  $p = 0.03$ ) and particularly in HPV-negative patients (HR 3.67,  $p = 0.007$ ).

**Conclusion:** H2AX/ $\gamma$ -H2AX expression is a potential prognostic biomarker in OPSCC, with elevated levels indicating poor survival, especially in HPV-negative cases. These findings suggest distinct molecular behaviors in OPSCC based on H2AX expression and highlight the need for further investigation into its therapeutic implications.

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## Introduction

Oropharyngeal squamous cell carcinoma (OPSCC), a subtype of head and neck squamous cell carcinoma (HNSCC), has historically been linked to alcohol and tobacco consumption, primarily affecting older individuals [1]. Although the global incidence of tobacco-related OPSCC has declined in recent years, the incidence of human papillomavirus (HPV)-associated OPSCC (HPV-OPSCC) has increased, particularly among younger men with high-risk sexual behavior [2,3]. HPV-OPSCC make up an estimate of 30 % of all OPSCC worldwide and are more prevalent in developed countries, including Europe and North America where HPV positive tumors currently account for more than 70 % of all OPSCC [4,5]. Importantly, HPV-positive OPSCC tends to have a better prognosis than HPV-negative OPSCC, largely due to an enhanced response to chemoradiotherapy [6,7].

Despite the improved survival rates for HPV-positive OPSCC, therapeutic approaches remain largely similar for both HPV-positive and HPV-negative cases. These include surgery or radiation for early-stage cancers and multimodal treatment strategies for locally advanced tumors [8]. However, standard treatments can lead to significant toxicities, and efforts to de-escalate treatment intensity in eligible patients are still under investigation in clinical trials, with cisplatin combined with radiotherapy remaining the gold standard at this time [8–12]. While HPV-positive OPSCC is generally associated with better outcomes, it remains a heterogeneous tumor group and the molecular drivers of its comparatively better prognosis are not fully understood [8].

Histone protein H2AX plays a key role in maintaining genomic stability through its involvement in the DNA repair process. [13,14]. When DNA double-strand breaks occur, H2AX is rapidly phosphorylated to form  $\gamma$ -H2AX, which localizes to the damage site and recruits DNA repair proteins [15,16]. Although  $\gamma$ -H2AX is critical in DNA repair, its broader biological functions remain unclear. In studies with H2AX-deficient mice, genomic instability led to severe phenotypes such as growth retardation, immunodeficiency, and radiation sensitivity, underscoring its role in maintaining cellular integrity [17]. Interestingly, elevated levels of  $\gamma$ -H2AX have been observed in cancer cells, indicating that DNA damage is a key factor in tumorigenesis [18]. For instance, high  $\gamma$ -H2AX expression has been associated with poorer outcomes in pancreatic ductal adenocarcinoma [15].

The role of H2AX/ $\gamma$ -H2AX in OPSCC, however, remains largely unexplored. Given its involvement in DNA damage response and its potential prognostic value in other cancers, this study aims to investigate the expression of H2AX mRNA and  $\gamma$ -H2AX protein in OPSCC. By evaluating their impact on survival and uncovering associated molecular pathways, we seek to determine the prognostic significance of H2AX/ $\gamma$ -H2AX in OPSCC and identify potential therapeutic targets.

## Material and Methods

### TCGA cohort of oropharyngeal squamous cell carcinoma patients

Data retrieval, processing, visualization, and statistical analyses were performed using R (v.4.2.2) and RStudio (v. 2022.12.0 + 353) with publicly available packages, including survival (v.3.4-0), survminer (v.0.4.9), and ggplot2 (v.3.4.0). Raw counts, normalized counts (transcript per million, RSEM-normalized), and clinical data were received from the Broad Institute Firehose GDC portal (<https://gdac.broadinstitute.org/>) [19]. We selected n = 76 patients with oropharyngeal squamous cell carcinoma of the base of the tongue, the tonsils, and the oropharynx (Supplementary Table S1). To exclude artificially increased H2AX mRNA levels due to radiation/chemotherapy, patients who received neoadjuvant treatment were excluded. To account for post-surgical complications, patients with an overall survival (OS) < 30 days (1 month) were excluded.

### Cohort dichotomization and DESeq2 analysis

The OPSCC cohort was dichotomized based on the mean mRNA expression and median mRNA expression of H2AX (TPM, RSEM-normalized). Additionally, a survival-associated cutoff value for H2AX expression was determined using the Cutoff Finder tool, as previously described [20]: This cutoff enabled significant stratification of OPSCC patients by overall survival (OS) using the log-rank test, with a significance threshold of  $p \leq 0.05$  [21,22]. The OPSCC cohort was further analyzed using the DESeq2 BioConductor package (v.3.16). Differentially expressed genes compared to benign tissue (n = 47) were identified using a Wald test with Bonferroni correction for multiple comparisons SPS:refid::bib23 [23]. For noise reduction and further normalization, a log fold change shrinkage method (apeglm package v.3.16) was used [24]. An adjusted p-value of  $\leq 0.05$  was considered significant. For data visualization, the Enhanced Volcano package and openly available packages (Complex Heatmap) were used [25]. Genes with a log2 fold change (log2FC) of  $\geq 2.0$  were deemed upregulated, genes with a log2FC of  $\leq -2.0$  were considered downregulated.

### Identification of exclusive clusters and hub genes

Exclusively up- and downregulated genes in H2AX<sup>high</sup>- and H2AX<sup>low</sup> OPSCC were followed by gene set and pathway enrichment analysis: Closely related genes were identified using Cytoscape v.3.10.2 with the GENEMANIA plugin [26]. Clusters were identified using the MCODE function (Degree cutoff: 2, Node Score Cutoff: 0.2, K-Core: 2, maximal depth: 100). Hub genes of the most prominent clusters were identified with the Cytohubba plugin [27]. Subsequently, the gene clusters with the highest Cluster Score in the MCODE analyses were allocated to specific pathways using the Enrichr database [28].

### Patient cohort of OPSCC for immunohistochemistry

For immunohistochemical analysis of H2AX protein expression we obtained patient tissue from 209 cases of OPSCC for which histopathological data and follow-up data were available (Supplementary Table S2). All patients were treated between 2005 and 2020 at the University Hospital of Cologne, Germany. The study protocol was approved by the Ethics committee of the University of Cologne (study number 19-1288).

### Tissue microarray generation and immunohistochemical staining

Tissue microarrays (TMAs) from formalin-fixed, paraffin-embedded, surgically resected tumor specimens as reported before [29]: In brief, two 1.2 mm tissue cores from each patient were transferred to a recipient paraffin block with a self-constructed, semi-automated precision instrument. Immunohistochemical staining for histone protein  $\gamma$ -H2AX was performed according to the manufacturer's instructions using the antibody anti-H2AX (ab81299, Abcam, Cambridge, UK, 1:200, EDTA). The  $\gamma$ -H2AX expression was quantified using the H score method: We divided the staining intensity into negative (= 0), weak (= 1), moderate (= 2) and strong (= 3) staining intensity. The H-score was calculated as:

$$\text{H Score} = (\text{cells}^{\text{negative}} * 0) + (\text{cells}^{\text{weak}} * 1) + (\text{cells}^{\text{moderate}} * 2) + (\text{cells}^{\text{strong}} * 3)$$

The resulting H-score ranged from 0 (100 % negative cells) to 300 (100 % strong positive cells).

### Statistical analysis and survival analysis

Data processing, survival analyses, and visualizations were conducted in R (v4.2.2) and RStudio (v2022.12.0 + 353), utilizing open-source packages survival (v3.4-0), survminer (v0.4.9), and ggplot2

(v3.4.0). Associations between clinical, histopathological data, and H2AX expression were evaluated using Chi-square tests, Fisher's exact test, and Spearman's correlation test. OS was calculated from the date of primary diagnosis to the date of death (from any cause), and Kaplan-Meier curves were generated with significance determined by the log-rank test. Patients without events or lost to follow-up were censored at the last known date. Patients with survival times < 30 days were excluded due to the likelihood of death from post-surgical complications. Univariate and multivariable Cox regression analyses were performed to assess the impact of patient characteristics and histopathological parameters. A p-value < 0.05 was considered statistically significant in all analyses.

## Results

### *High expression of H2AX is associated with shorter overall survival in the TCGA cohort of OPSCC*

The TCGA cohort of OPSCC consisted of  $n = 76$  patients with oropharyngeal squamous cell carcinoma of the base of the tongue, the tonsils, and the oropharynx (Supplementary Table S1).

When dichotomized by the mean mRNA expression and median mRNA expression of H2AX, no significant difference in overall survival (OS) was observed (Fig. 1A, 1B). When H2AX mRNA expression was stratified using the Cutoff Finder tool, a cutoff of 2523 counts (TPME, RSEM) revealed a statistically significant difference in survival outcomes. Patients with H2AX<sup>high</sup> OPSCC survived significantly shorter compared to H2AX<sup>low</sup> OPSCC (log-rank test;  $p = 0.022$ , Fig. 1C). High levels of H2AX mRNA expression were a significant adverse prognostic factor for reduced OS in univariate analysis (HR 3.28, 95 % CI 1.12–9.60,  $p = 0.030$ ). In multivariable Cox regression analysis, high H2AX expression remained an independent predictor of shorter survival, despite the limited sample size of patients with elevated H2AX expression ( $n = 14$ ) (Table 1). No significant associations were found between high H2AX expression and patients' age, sex, or other clinicopathological parameters (Tumor stage (T), lymph node status (N), tumor grade (G)). 25/76 tumors (32.9 %) were HPV-negative, 51/76 tumors (67.1 %) were HPV-positive. No significant correlation between H2AX expression levels and HPV status was observed.

When subdivided into HPV-positive- ( $n = 51$ ) and HPV-negative ( $n = 25$ ) OPSCC, H2AX<sup>high</sup> OPSCC showed a significant difference in OS ( $p = 0.004$ ) (Fig. 1D). High H2AX mRNA expression was associated with reduced OS in univariate analysis (HR 19.22, 95 %CI 1.98 – 1.87e2,  $p = 0.011$ ). Multivariable analysis was not conducted due to sample size limitations. No significant difference in OS was observed in the HPV-negative OPSCC subgroup (Fig. 1E).

### *DeSeq analysis of H2AX<sup>high</sup>- and H2AX<sup>low</sup> OPSCC reveals distinct biological behavior*

We performed DESeq analyses on H2AX<sup>high</sup>- and H2AX<sup>low</sup> OPSCC, stratified by the H2AX mRNA expression cutoff (TPM, RMSE-normalized). H2AX<sup>high</sup>- and H2AX<sup>low</sup> OPSCC showed only few differently expressed genes compared to benign tissue (Fig. 1F). In accordance with this finding, in the PCA plot, the variance in gene expression was not explained by H2AX status (Fig. 1G).

H2AX<sup>high</sup> OPSCC showed a dysregulation of 16,970 identified genes compared to benign tissue, with 9788 being significantly dysregulated, while H2AX<sup>low</sup> OPSCC showed 17,460 dysregulated genes, 10,565 of which being significantly altered (Fig. 1H, 1I; Supplementary Table S3).

Exclusively up- and downregulated genes were allocated to closely related genes, and gene clusters were identified with Cytoscape (MCODE function, see Methods). The most prominent gene clusters with the highest cluster scores were then analyzed using the Enrichr database. As expected, compared to benign tissue, H2AX<sup>high</sup> OPSCC demonstrated an

exclusive upregulation of genes involved in DNA repair, cell cycle regulation, checkpoint control, and chromosome maintenance, as revealed in Enrichr pathway analysis (Fig. 2A). Exclusively upregulated key Hub genes in these pathways were *Cyclin dependent kinase 1 (CDK1)*, *Cyclin B1 (CCNB1)*, *Cyclin A2 (CCNA2)* and *ZWINT*. Additionally, H2AX<sup>high</sup> OPSCC showed an upregulation of several inflammatory and immunomodulatory pathways with *CD27*, *CD79B* and *LIMD2* identified as prominent Hub genes (Fig. 2B).

In contrast, H2AX<sup>low</sup> OPSCC displayed a distinct biological profile characterized by upregulation of pathways related to extracellular matrix (ECM) remodeling and cell adhesion (Fig. 2C). Key upregulated hub genes included genes for various collagens (*COL1A2*, *COL1A1*) and matrix metalloproteinases (*MMP2*, *MMP14*). In H2AX<sup>high</sup> tumors, exclusively downregulated genes were involved in calcium signaling, muscle contraction, the endothelin pathway, and cytoskeletal regulation (Fig. 3A, 3B). Downregulated hub genes in these clusters included *ACTA2* (*Actin Alpha 2*), *CNN1* (*Calponin 1*), *FHL1*, and *PDLIM3*. In contrast, H2AX<sup>low</sup> OPSCC exhibited downregulation of the *IL24* pathway.

### *High $\gamma$ -H2AX protein expression is associated with HPV-negativity and shorter survival*

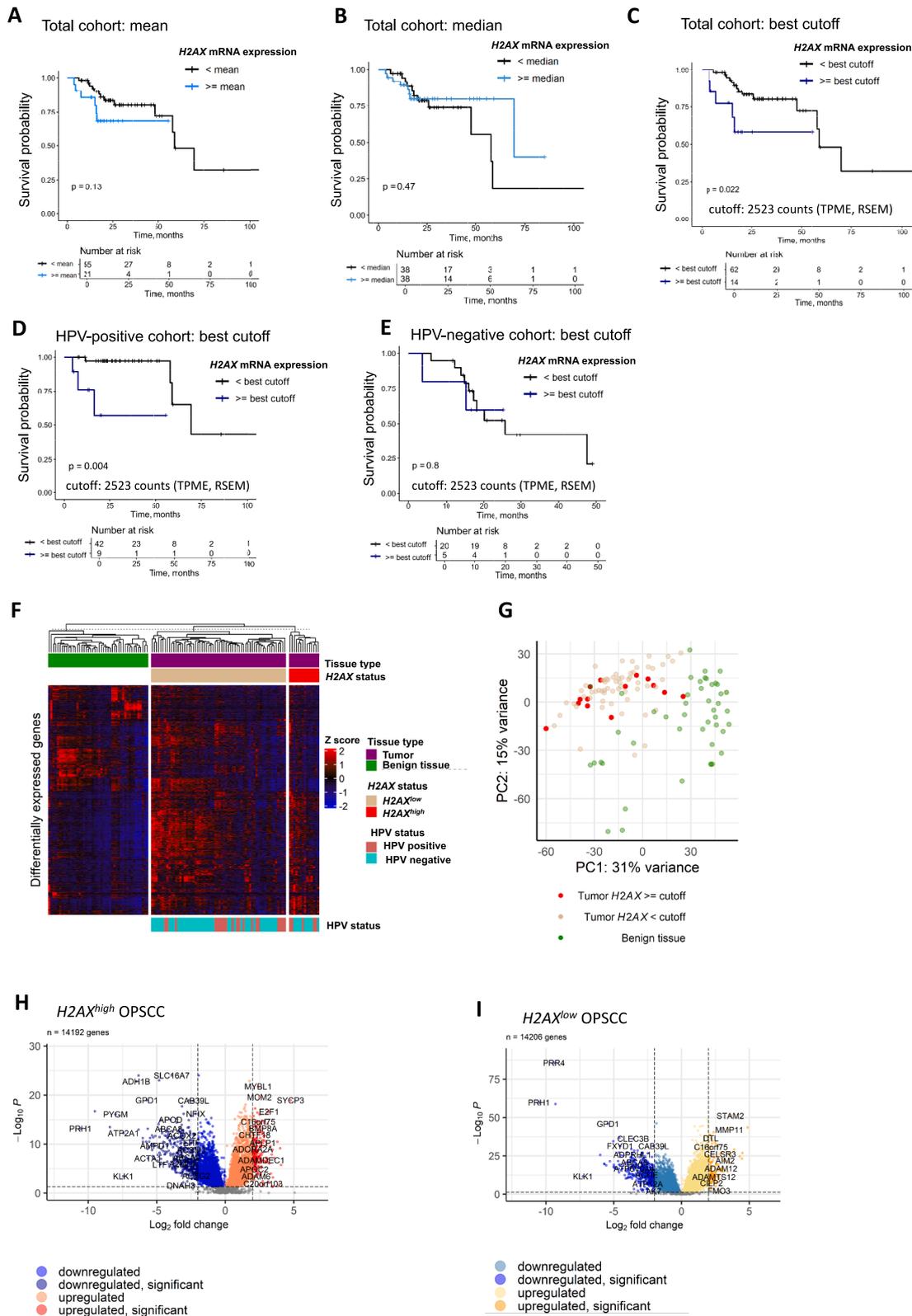
In the Cologne University Hospital OPSCC cohort comprised 209 patients. 102 OPSCC were HPV-positive (49.0 %), 106/208 OPSCC (51.0 %) were HPV-positive. The  $\gamma$ -H2AX expression was assessed using the H-Score method. When dichotomized by median and mean  $\gamma$ -H2AX expression, no significant difference in overall survival (OS) was observed (Fig. 1A, 1B). A subset of patients ( $n = 21$ , 10.0 %) exhibited  $\gamma$ -H2AX expression levels exceeding the 90th percentile (H-Score > 240). When dichotomized by this high H-Score threshold, 188 cases (90.3 %) belonged to the  $\gamma$ -H2AX<sup>low</sup> OPSCC subgroup and 21 tumors (9.7 %) to the  $\gamma$ -H2AX<sup>high</sup> subgroup.  $\gamma$ -H2AX expression was significantly associated with HPV status: 98/188  $\gamma$ -H2AX<sup>low</sup> OPSCC (52.1 %) were HPV-positive, whereas only 4/21  $\gamma$ -H2AX<sup>high</sup> OPSCC (19.0 %) were HPV-positive (Fisher's exact test,  $p = 0.005$ ) (Fig. 4). In the full cohort, high  $\gamma$ -H2AX expression levels were not associated with sex, age, or alcohol/nicotine use (data not shown). No significant correlation was found between  $\gamma$ -H2AX expression and clinicopathological parameters, or with the type of primary treatment (radiochemotherapy or surgery).

In the full cohort, patients with high  $\gamma$ -H2AX expression had significantly shorter OS compared to those with lower  $\gamma$ -H2AX levels (log-rank test,  $p = 0.002$ ). In univariate analysis, high  $\gamma$ -H2AX expression was a significant adverse prognostic factor (HR 2.44, 95 % CI 1.38–4.32,  $p = 0.002$ ). Multivariable Cox regression analysis confirmed that high  $\gamma$ -H2AX levels remained an independent prognostic factor associated with worse survival outcomes (Table 2).

### *High $\gamma$ -H2AX expression predicts shorter OS in HPV-negative OPSCC*

The cohort was further stratified into HPV-positive and HPV-negative OPSCC. In HPV-positive OPSCC, there was no significant difference in OS between tumors with high and low  $\gamma$ -H2AX expression ( $p = 0.73$ ) (Fig. 3F). However, in the HPV-negative cohort, patients with high  $\gamma$ -H2AX expression (H-Score  $\geq 240$ ) had significantly shorter OS compared to those with low  $\gamma$ -H2AX expression (log-rank test,  $p = 0.004$ ) (Fig. 3G).

Univariate analysis showed that high  $\gamma$ -H2AX expression was significantly associated with worse outcomes in HPV-negative OPSCC (HR 2.49, 95 % CI 1.34–4.60,  $p = 0.004$ ) (Table 3). Multivariable Cox regression analysis further confirmed that high  $\gamma$ -H2AX levels were an independent prognostic factor for poor survival in HPV-negative OPSCC patients (Table 3).



**Fig. 1.** H2AX mRNA expression in the TCGA Firehose cohort of OPSCC. **A – C**) For the mean expression and median expression of H2AX, a significant difference in overall survival was not observed; When the Cutoff Finder Tool was applied, a cutoff of 2523 counts (TPM, RSEM-normalized) was identified: Patients with an H2AX mRNA expression  $\geq$  cutoff lived significantly shorter (log rank test,  $p = 0.022$ ). **D, E**) Patients with HPV-positive OPSCC ( $n = 51$ ) and an H2AX mRNA expression  $\geq$  cutoff lived significantly shorter (log rank test,  $p = 0.012$ ). This was not observed in the HPV-negative subgroup ( $n = 25$ ). **F**) This heatmap displays significantly dysregulated genes in H2AX<sup>high</sup> and H2AX<sup>low</sup> OPSCC ( $n = 14$ ;  $n = 62$ ) and benign tissue ( $n = 47$ ). A clustering between tumor tissue and benign tissue was observable; Between tumors with high and low H2AX expression, larger clustering was not observed. **G**) In the PCA plot, the variance in gene expression was not explained by H2AX status or tissue type. **H, I**) Volcano plots of OPSCC with high and low H2AX expression. TCGA = The Cancer Genome Atlas; OPSCC = oropharyngeal squamous cell carcinoma.

**Table 1**  
Multivariable Cox regression analysis for *H2AX* expression in TCGA patients with OPSCC.

	HR	95 %CI	p
<i>H2AX</i> expression > cutoff	4.77	1.04–21.86	<b>0.04</b>
age	1.07	1.01–1.14	<b>0.02</b>
sex	1.89	0.53–6.76	0.33
tumor stage (T)	0.93	0.49–1.78	0.83
lymph node stage (N)	3.18	0.68–14.91	0.14
tumor grade (G)	1.40	0.41–4.76	0.59
HPV status (positive)*	0.09	0.01–0.49	<b>0.006</b>

\* Verification of HPV DNA; HPV = human papilloma virus, OPSCC = oropharyngeal squamous cell carcinoma.

## Discussion

In this study, we evaluated the mRNA- and protein expression of histone protein *H2AX*/ $\gamma$ -H2AX in oropharyngeal squamous cell carcinoma (OPSCC), revealing its potential both as a prognostic marker as well as its implications in distinct molecular and biological alterations in OPSCC.

In the TCGA cohort (n = 79), patients with high *H2AX* expression survived significantly shorter than patients with low *H2AX* expression. To investigate this, we identified differentially expressed gene clusters and hub genes between *H2AX*<sup>high</sup>- and *H2AX*<sup>low</sup> OPSCC. Tumors with elevated *H2AX* expression displayed an upregulation of pathways related to DNA repair and cell cycle regulations, with key hub genes including *CDK1*, *CCNB1* and *CCNA2*. These genes are known to promote rapid cell division by facilitating the cell cycle checkpoint transitions (G1/S, G2/M), and their overexpression has been linked to poor survival and tumor progression in various cancers, such as breast cancer, pancreatic cancer and adrenocortical carcinoma [30–32]. Additionally, *ZWINT*, another hub gene upregulated in *H2AX*<sup>high</sup> OPSCC, encodes a kinetochore protein involved in chromosome segregation during mitosis, while its exact function remains unknown [33]. However, *ZWINT* upregulation has been observed in other cancers, including lung adenocarcinoma, ovarian cancer, and prostate cancer, where high *ZWINT* expression correlates with tumor progression and reduced survival [34–36]. *H2AX*<sup>high</sup> OPSCC showed downregulated expression levels of *ACTA2* and *CCN1*. *ACTA2* encodes a vascular smooth muscle cell-specific isoform of actin, and its dysregulation has been implicated in various cancers, including non-small lung cancer, bladder cancer, breast cancer and colorectal cancer, with mutations of *ACTA2* promoting metastatic disease [37]. Additionally, *CCN1* (*CYR61*) a gene involved in promoting adhesion, endothelial cell integrity, angiogenesis, and tissue repair, was downregulated [38]. Its role in cancer appears to be complex: While *CCN1* promotes epithelial-mesenchymal transition, invasion, and metastasis in breast cancer, prostate cancer, and pancreatic ductal adenocarcinoma [39–41], other studies demonstrated that *CCN1* acts as a tumor suppressor gene in other cancer entities, including non-small lung cancer, endometrial cancer, and melanoma [42–44]. In summary, *H2AX*<sup>high</sup> tumors showed altered expression of key pathways involved in cell cycle regulation, chromosomal stability, and molecular mechanisms associated with aggressive tumor behavior. However, the precise molecular mechanisms by which elevated *H2AX*/ $\gamma$ -H2AX expression contributes to poorer survival in OPSCC remain unclear and is a limitation to address in this study: While  $\gamma$ -H2AX as a central component of the complex DNA damage response network, mainly as an initiator of DNA repair foci, has been well investigated, its direct functions in the DNA repair process remain unknown. Furthermore, a linkage between DNA damage and/or high *H2AX* expression and the distinct dysregulated pathways described above has yet to be established.

Interestingly, low levels of  $\gamma$ -H2AX protein expression were associated with a positive HPV status. While  $\gamma$ -H2AX expression did not significantly impact survival in the HPV-positive cohort, in the HPV-negative subgroup patients with high  $\gamma$ -H2AX expression had a

significantly shorter survival. Specifically, in the HPV-negative cohort, high levels of  $\gamma$ -H2AX were independently associated with worse survival (HR 3.67, 95 %CI 1.43–9.41; p = 0.007). Conversely, we observed no significant difference in overall survival based on *H2AX* mRNA expression (high or low) in HPV-negative OPSCC. However, in the HPV-positive cohort, high *H2AX* mRNA expression was significantly associated with reduced overall survival (p = 0.004).

These findings highlight the distinct mechanisms of carcinogenesis between HPV-positive and HPV-negative OPSCC: HPV-positive tumors primarily arise through the overexpression of viral oncoproteins E6 and E7, which drive tumorigenesis by reduced apoptosis, degradation of p53 protein and promotion of cell cycle [45,46]. Most HPV positive OPSCC occur in the palatine and lingual tonsils, presumptively due to their reticulated epithelium that allows for viral deposition without mucosal trauma and enables lymphocytes to access the crypts [3,47]. Conversely, the mechanisms underlying HPV-negative OPSCC remain poorly understood. HPV-negative OPSCC often arises from genomic instability associated with environmental mutagens, such as tobacco and alcohol exposure [48]. This suggests that HPV-negative OPSCC may rely more heavily on functional DNA repair pathways, including those involving *H2AX*, for survival and proliferation. Our findings that high  $\gamma$ -H2AX expression is associated with worse survival in HPV-negative OPSCC may be explained by high baseline levels of  $\gamma$ -H2AX indicating pre-existing DNA damage, which may contribute to genomic instability and more aggressive tumor behavior. Alternatively, it could suggest impaired DNA repair efficiency despite high  $\gamma$ -H2AX levels, possibly due to defects in downstream repair pathways. These hypotheses warrant further investigation to fully elucidate the mechanisms underlying the prognostic value of *H2AX*/ $\gamma$ -H2AX in HPV-negative OPSCC.

The observed discrepancy between *H2AX* mRNA and  $\gamma$ -H2AX protein expression in our study underscores the intricate relationship between transcription and post-translational modifications of *H2AX* in OPSCC. While high *H2AX* mRNA levels were associated with reduced overall survival in HPV-positive OPSCC,  $\gamma$ -H2AX protein expression demonstrated prognostic significance in HPV-negative tumors. Such a lack of direct correlation between mRNA and protein levels is documented in cancer research. For example, studies in breast cancer have shown that *H2AX* knockdown reduces both *H2AX* and  $\gamma$ -H2AX protein expression, indicating a potential direct relationship between transcription and protein abundance [49]. However, this correlation is highly context-dependent and can vary across cancer types and cellular conditions [50]. In our study, the discrepancy between mRNA and protein levels may stem from differences in post-translational modifications, protein stability, or the specific influence of HPV status on *H2AX* phosphorylation. These findings highlight the need for further in-depth investigations to elucidate the molecular mechanisms underlying these complex interactions.

In a recent study by our group, we observed that high  $\gamma$ -H2AX protein expression was associated with a more aggressive, basal-like subtype of pancreatic ductal adenocarcinoma and was linked to worse prognosis [15]. This further demonstrates that elevated *H2AX*/ $\gamma$ -H2AX expression is a feature of aggressive subtypes in several cancers. While additional validation in larger cohorts is necessary both for this subtype of pancreatic cancer as well as HPV-negative OPSCC, assessing *H2AX*/ $\gamma$ -H2AX expression could help stratify patients into different risk categories. Moreover, it may serve as a potential molecular target that could enhance tumor radiosensitivity and improve survival outcomes.

Incorporating *H2AX*/ $\gamma$ -H2AX expression into current prognostic models for OPSCC may improve their predictive power and guide personalized treatment strategies.

Another important topic to address is the intricate and context-dependent relationship between the proteins *H2AX* and  $\gamma$ -H2AX. While  $\gamma$ -H2AX is widely recognized as a sensitive marker for DNA double-strand breaks, its induction is not limited to ionizing radiation and can occur in response to various other cellular stressors. The conversion of *H2AX* to  $\gamma$ -H2AX is influenced by numerous factors and is not

## A *H2AX<sup>high</sup>* OPSCC: Cluster 1 (Cluster-Score: 57.695)

### Enrichr pathway analysis

Cell cycle (WP179), p adj. < 0.001

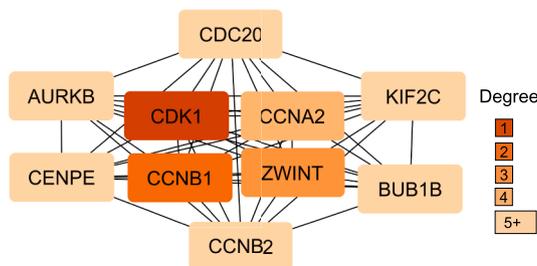
DNA damage response (WP707), p adj. < 0.001

G1 to S Cell Cycle Control (WP45), p adj. < 0.001

Regulation of Sister Chromatid Separation (WP4240), p adj. < 0.001

IL 24 Signaling Pathway (WP5413), p adj. < 0.001

### Hub genes



## B *H2AX<sup>high</sup>* OPSCC: Cluster 2 (Cluster-Score: 9.636)

### Enrichr pathway analysis

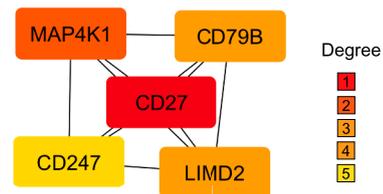
Modulators of TCR signaling and T cell activation (WP5072), p adj. = 0.01

B Cell receptor signaling pathway (WP23), p adj. = 0.01

TNF alpha signaling pathway (WP23), p adj. = 0.01

MAPK signaling pathway (WP382), p adj. = 0.049

### Hub genes



## C *H2AX<sup>low</sup>* OPSCC: Cluster 1 (Cluster-Score: 31.312)

### Enrichr pathway analysis

Focal adhesion (WP306), p adj. < 0.001

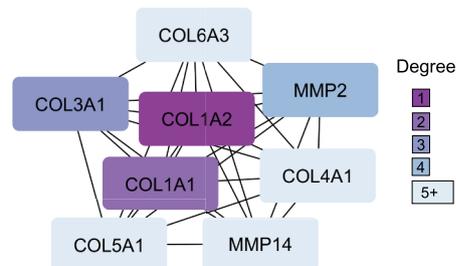
PI3K Akt signaling pathway (WP4172), p adj. < 0.001

Matrix metalloproteinases (WP129), p adj. < 0.001

Inflammatory response pathway (WP453), p adj. < 0.001

miR 509 3P alteration of YAP1 ECM axis (WP3967), p adj. < 0.001

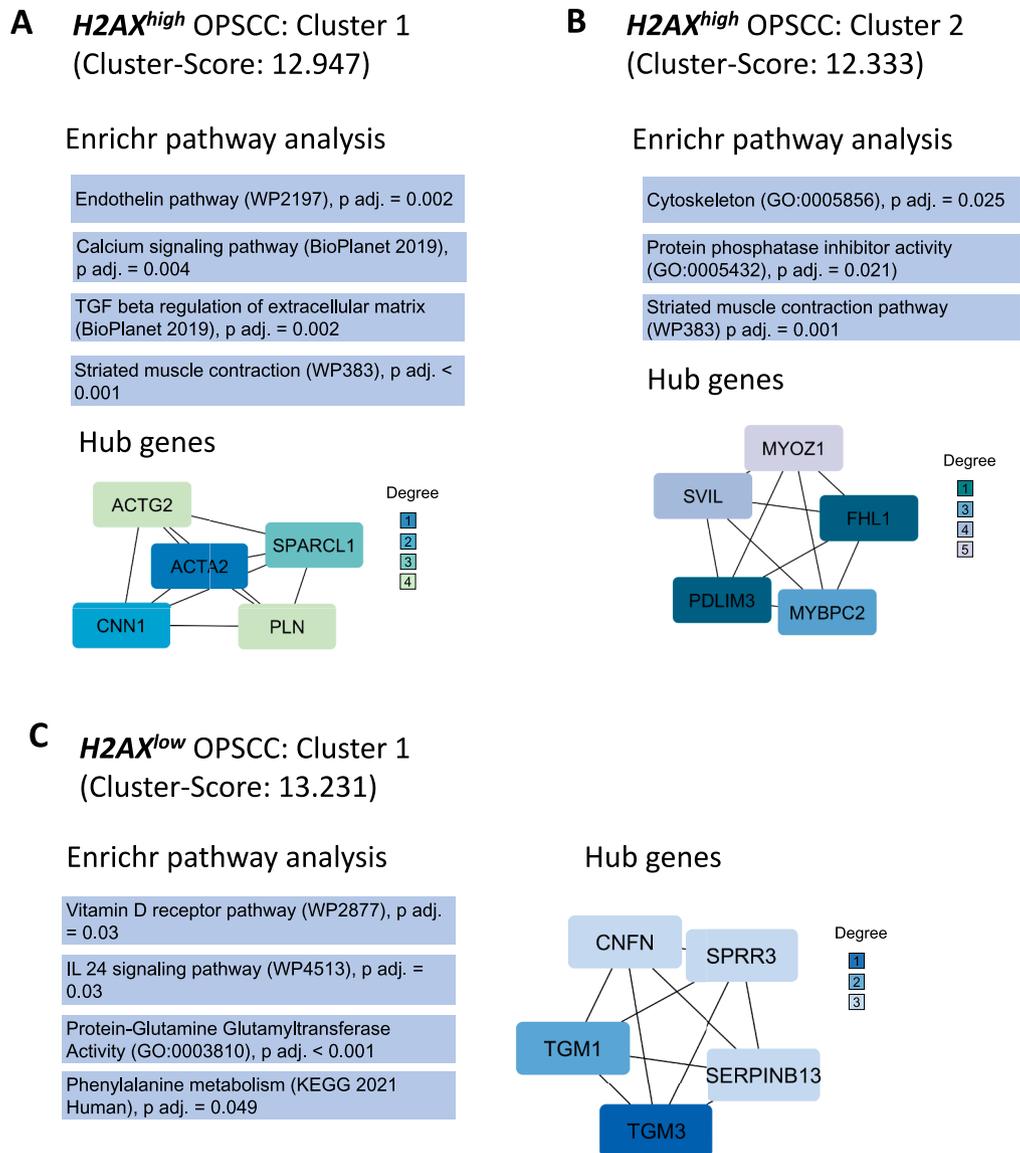
### Hub genes



**Fig. 2.** Upregulated pathways and Hub genes in *H2AX<sup>high</sup>*- and *H2AX<sup>low</sup>* OPSCC. **A)** Exclusively upregulated genes in *H2AX<sup>high</sup>* OPSCC and their closely related genes, identified by the GENEMANIA plugin in Cytoscape v3.10.2, were clustered using the MCODE tool. Cluster 1, analyzed using the Enrichr data repository, was involved in regulation of cell cycle, DNA damage response, cell cycle progression, and chromatid separation. Additionally, an upregulation of the IL24 pathway was observed. *CDK1*, *CCNB1*, *CCNA2* and *ZWINT* were identified as central Hub genes using the CytoHubba plugin. **B)** Additionally, *H2AX<sup>high</sup>* OPSCC exhibited upregulated pathway expression of TCR and T cell signaling, B cell receptor signaling, TNF alpha signaling and the MAPK pathway, with *CD27* and *MAP4K1* being central Hub genes. **C)** in *H2AX<sup>low</sup>* OPSCC, exclusively upregulated genes were allocated to adhesion, PI3K signaling and matrix metalloproteinases and inflammatory response; Several Hub genes involved in extracellular matrix modelling, including *COL1A2*, *COL1A1* and *COL3A1* were identified. WP = WikiPathways 2023 database; OPSCC = oropharyngeal squamous cell carcinoma. A p-value adj.  $\leq 0.05$  was considered significant (Bonferroni correction).

always straightforward. For instance,  $\gamma$ -H2AX foci can vary significantly in size and in the extent of H2AX phosphorylation, reflecting the complexity of the underlying processes. [51,52] This variability in the phosphorylation of H2AX to form  $\gamma$ -H2AX may contribute to the nuanced prognostic implications we observed in OPSCC. Although

$\gamma$ -H2AX holds significant potential as a practical biomarker, its detection sensitivity is influenced by several factors, including the background levels of nuclear foci present in unstressed cells. These background levels, associated with normal DNA replication and mitotic progression, add another layer of complexity when interpreting  $\gamma$ -H2AX as a marker



**Fig. 3.** Downregulated pathways and Hub genes in *H2AX*<sup>high</sup> and *H2AX*<sup>low</sup> OPSCC. **A)** Exclusively downregulated genes in *H2AX*<sup>high</sup> OPSCC and their closely related genes, identified by the GENEMANIA plugin in Cytoscape v3.10.2, were clustered using the MCODE tool. In *H2AX*<sup>high</sup> tumors, exclusively downregulated genes were allocated to the endothelin pathway, pathways mediating calcium signaling, TGF beta and striated muscle contraction. *ACTA2*, *CNN1* and *SPARCL1* were central Hub genes. **B)** Cluster 2 contained exclusively downregulated genes which were allocated to the cytoskeleton formation, protein phosphatase inhibitor activity and striated muscle contraction as well in Enrichr analysis, with *FHL1* and *PDLIM3* being the most prominent Hub genes. **C)** In *H2AX*<sup>low</sup> OPSCC, exclusively downregulated genes were involved in vitamin D receptor signaling, IL24 signaling and metabolic pathways; *TGM3* and *TGM1* were identified as central Hub genes. WP = WikiPathways 2023 database; GO = Gene Ontology database; OPSCC = oropharyngeal squamous cell carcinoma. A p-value adj.  $\leq 0.05$  was considered significant (Bonferroni correction).

in the context of cancer [53,54].

## Conclusion

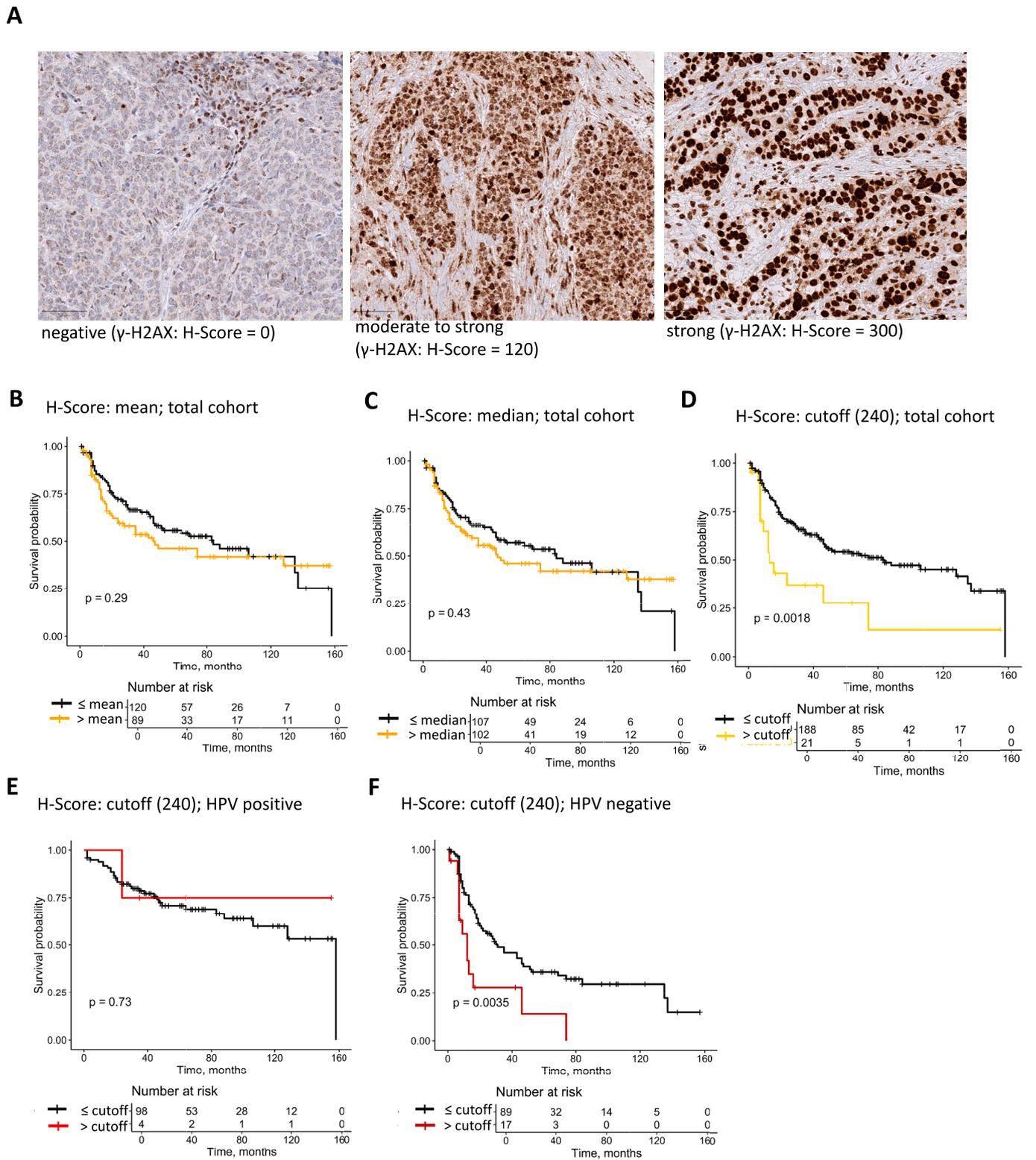
In summary, our study demonstrates that *H2AX*/ $\gamma$ -*H2AX* expression in OPSCC is associated with significant alterations in pathways related to DNA repair, cell cycle regulation, and chromosomal stability, and is a potential prognostic biomarker and therapeutic target, particularly in HPV-negative OPSCC. These findings provide new insights into the molecular biology of OPSCC, but might also be a step towards a more precise prognostic risk stratification for optimized treatment. Understanding the underlying biology of *H2AX*/ $\gamma$ -*H2AX* and its complex functions might additionally allow the identification of new potential therapeutic targets.

## Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of the manuscript the authors used ChatGPT v.4.0 (OpenAI, San Francisco, USA) for improvement of readability, grammar check, and spelling check. After usage, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

## CRediT authorship contribution statement

**Su Ir Lyu:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Caroline Fretter:** Writing – review & editing, Formal analysis. **Hans Nikolaus Caspar Eckel:** Data curation. **Karl Knipper:** Resources.



**Fig. 4.** Protein expression of  $\gamma$ -H2AX in the Cologne University Hospital OPSCC cohort. **A)** Spectrum of  $\gamma$ -H2AX protein expression in the Cologne University Hospital OPSCC cohort; **B, C)** When dichotomized by the mean and median expression, no significant difference in overall survival (OS) was observed for the total OPSCC cohort. **D)** When dichotomized by the 90<sup>th</sup> percentile (H-Score > 240), OPSCC with high expression of  $\gamma$ -H2AX showed a significantly shorter OS ( $p = 0.002$ , log-rank test). **E, F)** When subdivided by the HPV status, HPV positive OPSCC did not show a difference in OS, while HPV-negative OPSCC with high levels of  $\gamma$ -H2AX lived significantly shorter ( $p = 0.004$ , log-rank test). OPSCC = oropharyngeal squamous cell carcinoma.

**Table 2**

Multivariable Cox regression analysis for  $\gamma$ -H2AX expression: total OPSCC cohort.

	HR	95 % CI	p
$\gamma$ -H2AX: high expression (H-Score > 240)	2.24	1.09–4.61	<b>0.03</b>
HPV positive*	0.32	0.19–0.54	<b>&lt; 0.001</b>
age	1.03	1.01–1.05	<b>0.03</b>
sex	0.75	0.38–1.47	0.40
tumor stage (T)	1.30	0.98–1.72	0.07
lymph node stage (N)	0.79	0.48–1.32	0.38
distant metastasis (M)	1.69	0.49–5.79	0.41
tumor grade (G)	0.86	0.53–1.41	0.55
primary surgery	2.93	1.50–5.71	<b>0.001</b>

\* Verification of HPV DNA; HPV = human papilloma virus, OPSCC = oropharyngeal squamous cell carcinoma.

**Table 3**

Multivariable Cox regression analysis for  $\gamma$ -H2AX expression: HPV-negative OPSCC.

	HR	95 % CI	p
$\gamma$ -H2AX: high expression (H-Score > 240)	3.67	1.43–9.41	<b>0.007</b>
age	1.05	1.02–1.08	<b>&lt; 0.001</b>
sex	0.34	0.11–1.034	0.06
tumor stage (T)	1.53	1.08–2.16	<b>0.017</b>
lymph node stage (N)	0.99	0.52–1.88	0.97
distant metastasis (M)	1.79	0.38–8.34	0.46
tumor grade (G)	1.20	0.64–2.23	0.58
primary surgery	3.87	1.57–9.55	<b>0.003</b>

HPV = human papilloma virus, OPSCC = oropharyngeal squamous cell carcinoma.

**Anne Maria Schultheis:** Resources. **Reinhard Büttner:** Resources. **Alexander Quaas:** Resources. **Jens Peter Klussmann:** Supervision, Resources, Data curation. **Adrian Georg Simon:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

### Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the University of Cologne (ethics committee number: 19-1288).

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### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.oraloncology.2024.107171>.

### Data Availability Statement:

The dataset for the TCGA cohort is openly available at the Broad

Institute Firehose TCGA depository (<https://gdac.broadinstitute.org/>). The dataset for the Cologne University Hospital cohort is available from the corresponding author on reasonable request.

### References

- [1] Fonsêca TC, et al. Global prevalence of human papillomavirus-related oral and oropharyngeal squamous cell carcinomas: a systematic review and meta-analysis. *Clin Oral Invest* 2023;28(1):62.
- [2] Gillison ML, et al. Epidemiology of Human Papillomavirus-Positive Head and Neck Squamous Cell Carcinoma. *J Clin Oncol* 2015;33(29):3235–42.
- [3] Shinomiya H, Nibu KI. Etiology, diagnosis, treatment, and prevention of human papilloma virus-associated oropharyngeal squamous cell carcinoma. *Int J Clin Oncol* 2023;28(8):975–81.
- [4] de Martel C, et al. Worldwide burden of cancer attributable to HPV by site, country and HPV type. *Int J Cancer* 2017;141(4):664–70.
- [5] Chaturvedi AK, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol* 2011;29(32):4294–301.
- [6] Fakhry C, et al. Improved Survival of Patients With Human Papillomavirus-Positive Head and Neck Squamous Cell Carcinoma in a Prospective Clinical Trial. *JNCI: Journal of the National Cancer Institute* 2008;100(4):261–9.
- [7] Gillison ML, et al. Distinct Risk Factor Profiles for Human Papillomavirus Type 16-Positive and Human Papillomavirus Type 16-Negative Head and Neck Cancers. *JNCI: Journal of the National Cancer Institute* 2008;100(6):407–20.
- [8] Lim YX, et al. Clinical, morphologic and molecular heterogeneity of HPV-associated oropharyngeal cancer. *Oncogene* 2023;42(40):2939–55.
- [9] Gillison ML, et al. Radiotherapy plus cetuximab or cisplatin in human papillomavirus-positive oropharyngeal cancer (NRG Oncology RTOG 1016): a randomised, multicentre, non-inferiority trial. *Lancet* 2019;393(10166):40–50.
- [10] Yom SS, et al. Reduced-Dose Radiation Therapy for HPV-Associated Oropharyngeal Carcinoma (NRG Oncology HN002). *J Clin Oncol* 2021;39(9):956–65.
- [11] Mehanna H, et al. Radiotherapy plus cisplatin or cetuximab in low-risk human papillomavirus-positive oropharyngeal cancer (De-ESCALaTE HPV): an open-label randomised controlled phase 3 trial. *Lancet* 2019;393(10166):51–60.
- [12] Rischin D, et al. Prognostic stratification of HPV-associated oropharyngeal cancer based on CD103(+) immune cell abundance in patients treated on TROG 12.01 and De-ESCALaTE randomized trials. *Ann Oncol* 2022;33(8):804–13.
- [13] Contreras L, et al. DNA damage signalling histone H2AX is required for tumour growth. *Cell Death Discov* 2024;10(1):99.
- [14] Fernandez-Capetillo O, et al. H2AX: the histone guardian of the genome. *DNA Repair (Amst)* 2004;3(8–9):959–67.
- [15] Knipper K, et al. Upregulation of the histone  $\gamma$ -H2AX correlates with worse patient survival and basal-like subtype in pancreatic ductal adenocarcinoma. *J Cancer Res Clin Oncol* 2024;150(3):137.
- [16] Paull TT, et al. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol* 2000;10(15):886–95.
- [17] Celeste A, et al. Genomic instability in mice lacking histone H2AX. *Science* 2002;296(5569):922–7.
- [18] Sedelnikova OA, Bonner WM. GammaH2AX in cancer cells: a potential biomarker for cancer diagnostics, prediction and recurrence. *Cell Cycle* 2006;5(24):2909–13.
- [19] Broad GDAC Firehose. 04-03-2023; Available from: <https://gdac.broadinstitute.org/>.
- [20] Simon AG, et al. The tight junction protein claudin 6 is a potential target for patient-individualized treatment in esophageal and gastric adenocarcinoma and is associated with poor prognosis. *J Transl Med* 2023;21(1):552.
- [21] Budczies J, et al. Cutoff Finder: a comprehensive and straightforward Web application enabling rapid biomarker cutoff optimization. *PLoS One* 2012;7(12):e51862.
- [22] Li B, Dewey CND. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinf* 2011;12(323).
- [23] Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15(12):550.
- [24] Zhu A, Ibrahim JG, Love MI. Heavy-tailed prior distributions for sequence count data: removing the noise and preserving large differences. *Bioinformatics* 2019;35(12):2084–92.
- [25] Blighe, K., S. Rana, and M. Lewis. EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling. R package version 1.16.0, 2022.
- [26] Warde-Farley, D., et al., The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res*, 2010. **38**(Web Server issue): p. W214-20.
- [27] Chin CH, et al. cytoHubba: identifying hub objects and sub-networks from complex interactome. *BMC Syst Biol* 2014;8 Suppl 4(Suppl 4):S11.
- [28] Xie Z, et al. Gene Set Knowledge Discovery with Enrichr. *Curr Protoc* 2021;1(3):e90.
- [29] Quaas A, et al. Sex-specific prognostic effect of CD66b-positive tumor-infiltrating neutrophils (TANs) in gastric and esophageal adenocarcinoma. *Gastric Cancer* 2021;24(6):1213–26.
- [30] Alam MS, et al. Identification of Hub of the Hub-Genes From Different Individual Studies for Early Diagnosis, Prognosis, and Therapies of Breast Cancer. *Bioinform Biol Insights* 2024;18:11779322241272386.
- [31] Dong S, et al. Overexpression of BUB1B, CCNA2, CDC20, and CDK1 in tumor tissues predicts poor survival in pancreatic ductal adenocarcinoma. *Biosci Rep* 2019;39(2).

- [32] Xu WH, et al. Screening and Identification of Potential Prognostic Biomarkers in Adrenocortical Carcinoma. *Front Genet* 2019;10:821.
- [33] Starr DA, et al. HZWint-1, a novel human kinetochore component that interacts with HZW10. *J Cell Sci* 2000;113(Pt 11):1939–50.
- [34] Cao S, et al. Identification of the cell cycle characteristics of non-small cell lung cancer and its relationship with tumor immune microenvironment, cell death pathways, and metabolic reprogramming. *Front Endocrinol (Lausanne)* 2023;14:1147366.
- [35] Tang Q, et al. Comprehensively analyzing the genetic alterations, and identifying key genes in ovarian cancer. *Oncol Res* 2023;31(2):141–56.
- [36] Liu D, et al. Telomerase related molecular subtype and risk model reveal immune activity and evaluate prognosis and immunotherapy response in prostate cancer. *Cancer Cell Int* 2024;24(1):294.
- [37] Suresh R, Diaz RJ. The remodelling of actin composition as a hallmark of cancer. *Transl Oncol* 2021;14(6):101051.
- [38] Kim KH, et al. The matricellular protein CCN1 in tissue injury repair. *J Cell Commun Signal* 2018;12(1):273–9.
- [39] Haque I, et al. Cyr61/CCN1 signaling is critical for epithelial-mesenchymal transition and stemness and promotes pancreatic carcinogenesis. *Mol Cancer* 2011; 10:8.
- [40] Sun ZJ, et al. Involvement of Cyr61 in growth, migration, and metastasis of prostate cancer cells. *Br J Cancer* 2008;99(10):1656–67.
- [41] Xie D, et al. Breast cancer. Cyr61 is overexpressed, estrogen-inducible, and associated with more advanced disease. *J Biol Chem* 2001;276(17):14187–94.
- [42] Tong X, et al. Cyr61, a Member of CCN Family, Is a Tumor Suppressor in Non-Small Cell Lung Cancer \*. *J Biol Chem* 2001;276(50):47709–14.
- [43] Chien W, et al. Cyr61 Suppresses Growth of Human Endometrial Cancer Cells \*. *J Biol Chem* 2004;279(51):53087–96.
- [44] Dobroff AS, et al. Silencing cAMP-response element-binding protein (CREB) identifies CYR61 as a tumor suppressor gene in melanoma. *J Biol Chem* 2009;284 (38):26194–206.
- [45] Munger K, White Elizabeth A. What are the essential determinants of human papillomavirus carcinogenesis? *MBio* 2024:e00462–524.
- [46] Vogt M, et al. Inhibition of Bax activity is crucial for the antiapoptotic function of the human papillomavirus E6 oncoprotein. *Oncogene* 2006;25(29):4009–15.
- [47] Lim YX, D’Silva NJ. HPV-associated oropharyngeal cancer: in search of surrogate biomarkers for early lesions. *Oncogene* 2024;43(8):543–54.
- [48] Gillison ML, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst* 2000;92(9):709–20.
- [49] Katsuta E, et al. H2AX mRNA expression reflects DNA repair, cell proliferation, metastasis, and worse survival in breast cancer. *Am J Cancer Res* 2022;12(2): 793–804.
- [50] Grusso T, et al. Chronic oxidative stress promotes H2AX protein degradation and enhances chemosensitivity in breast cancer patients. *EMBO Mol Med* 2016;8(5): 527–49.
- [51] Collins PL, et al. DNA double-strand breaks induce H2Ax phosphorylation domains in a contact-dependent manner. *Nat Commun* 2020;11(1):3158.
- [52] Podhorecka M, Skladanowski A, Bozko P. H2AX Phosphorylation: Its Role in DNA Damage Response and Cancer Therapy. *J Nucleic Acids* 2010. 2010..
- [53] Ichijima Y, et al. Phosphorylation of histone H2AX at M phase in human cells without DNA damage response. *Biochem Biophys Res Commun* 2005;336(3): 807–12.
- [54] McManus KJ, Hendzel MJ. ATM-dependent DNA damage-independent mitotic phosphorylation of H2AX in normally growing mammalian cells. *Mol Biol Cell* 2005;16(10):5013–25.