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ORIGINAL ARTICLE

Survivin/BIRC5 as a novel molecular effector at the crossroads of glucose metabolism and radioresistance in head and neck squamous cell carcinoma

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Abstract

Background: Metabolic reprogramming and abnormal glucose metabolism are hallmarks of head and neck squamous cell carcinoma (HNSCC). Certain oncogenes can promote cancer-related metabolic changes, but understanding their crosstalk in HNSCC biology and treatment is essential for identifying predictive biomarkers and developing target therapies.

Ester Benaiges and Victòria Ceperuelo-Mallafré contributed equally to this study.

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Methods: We assessed the value of survivin/BIRC5 as a radioresistance factor potentially modulated by glucose for predicting therapeutic sensitivity and prognosis of HNSCC in a cohort of 32 patients. Additionally, we conducted in vitro experiments to explore the role of survivin/BIRC5 in glucose metabolism concerning radiation response.

Results: Tumoral BIRC5 expression is associated with serum glucose and predicts locoregional disease-free survival and lower BIRC5 mRNA levels are associated with better outcomes. Upregulation of BIRC5 by radiation depends on glucose levels and provokes a pro-tumoral and radioresistant phenotype in surviving cells.

Conclusions: Survivin/BIRC5 might be independently associated with the risk of recurrence in patients with HNSCC.

KEYWORDS
glucose metabolism, HNSCC, radioresistance, radiotherapy, survivin

1 | INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) comprises approximately 4% of all cancers globally and ranks as the sixth most prevalent malignancy. Roughly half of the patients diagnosed with HNSCC have advanced-stage disease when they are diagnosed. These individuals typically face a 5-year survival rate of approximately 50%.[1] Despite the existing treatment modalities, which often involve a combination of invasive surgery, radiotherapy, and/or chemotherapy, the overall long-term survival rate remains relatively low due to the persistence or recurrence of the disease. The low survival rate observed in patients experiencing both locoregional and distant recurrences underscores the urgent need for novel clinical tools for diagnosis and treatment. Currently, there is a lack of efficient prognostic factors capable of accurately predicting treatment outcomes.[2] The identification of a specific deregulated gene/protein network—for example a metabolic pathway—may provide tools for early detection, for therapeutic intervention, or for monitoring HNSCC disease progression. It could also be used to explore disease mechanisms and to develop personalized treatments that maximize survival while minimizing morbidity.

Glucose metabolic reprogramming is a hallmark of tumor progression and treatment resistance.[3,4] In the context of HNSCC, hyperglycemia has been reported to promote pro-tumoral phenotypes and chemoresistance,[5] to increase lactate production, leading to an acidification of the tumor microenvironment,[6,7] and to reduce patient survival rates.[8,9] Although tumorigenesis has historically been viewed as a disorder of proliferation, accumulating evidence indicates that it can also be considered as a metabolic disease.[10] Indeed, many oncogenes and tumor suppressors are known play key roles in the cancer-associated changes in metabolism. In this context, survivin, encoded by *BIRC5* (Baculoviral IAP Repeat Containing 5) gene, a proto-oncogene associated with both inhibition of apoptosis and activation of cell proliferation,[11,12] has been proposed as a diagnostic tumor marker because of its elevated expression in tumoral tissues.[13,14] It is unclear, however, whether there is a relationship between survivin/BIRC5 and glucose metabolism. Some studies have reported that survivin/BIRC5 is regulated by glucose in mature adipocytes[15] and in neuroblastoma.[16] It also appears necessary for glucose uptake and for restoring normal metabolism in CD4 + T cells,[17] and it also suppresses mitochondrial oxidative phosphorylation and enhances glycolysis in cancer cells.[18] In the context of HNSCC, survivin/BIRC5 has been proposed as a predictive factor of tumor progression,[19–22] and as a potential therapeutic target.[23] By contrast, its relationship with radioresponse is more controversial.[24–26] It has been previously described that hyperglycemia might induce tumor resistance to chemotherapy[27]; however, whether this could also apply in the context of radiotherapy is unknown. In the present study, we aimed to explore the involvement of survivin/BIRC5 in the progression, treatment and metabolic status of patients with HNSCC. Additionally, we investigated survivin/BIRC5 as a radioresistance factor potentially modulated by glucose.

2 | MATERIALS AND METHODS

2.1 | Study design and patients

The study enrolled a total of 32 consecutive patients diagnosed with pathologically confirmed HNSCC. This patient cohort included individuals with untreated advanced tumors at stages III to IV. The study was conducted at Hospital Universitari Joan XXIII in Tarragona, Spain. Tumor boards reviewed and assessed all patients, following the hospital's standard protocols and guidelines, to determine the appropriate treatment approach, including radiotherapy, chemoradiotherapy, or surgery. Generally, patients received chemoradiotherapy as part of their treatment. For individuals diagnosed with oropharyngeal carcinoma ($n = 5$), their human papillomavirus (HPV) status was known and considered during the decision-making process. HPV status was detected by a multiplex polymerase chain reaction assay.

Regarding treatment, external-beam radiotherapy was administered by continuous-course radiotherapy 5 days a week, using 2 Gy per session in normo-fractionated treatments, and using 1.2 Gy twice daily in hyper-fractionated treatments. Treatment was administered in a total dose of 65–74 Gy to the primary site, 50 Gy to the neck in all patients with N0 node, and 70 Gy to the neck in patients with clinical metastatic neck nodes (N+). Chemoradiotherapy consisted of radiotherapy at the same doses plus 3 cycles of cisplatin at a dose of 100 mg/m^2 on Day 1 every 3 weeks.

Routine follow-up was done at 2-month intervals during the first year, 3-month intervals in the second year, and 4-month intervals over years 3–5, consisting of an evaluation of symptoms and locoregional examinations. The mean follow-up of the patients included in the study was 43.6 months (SD 34.8, median 35.9).

Peripheral blood was collected from all patients at the diagnostic visit after an overnight fasting before any treatment was instituted. Blood samples were collected using 10-mL vacutainer tubes from the antecubital vein. Plasma was separated within 1 h of drawing (1500*g* for 15 min at 4°C) and samples were stored at −80°C until analytical measurements were performed.[28]

2.2 | Cell culture and treatments

The human Pharynx Squamous Cell Carcinoma (FaDu, RRID: CVCL_1218) cell line was purchased from the ATCC (Rockville, MD). Cells were routinely maintained in glucose-free Dubelcco's modified Eagle's medium (DMEM) (11966-025, Gibco; Thermo Fisher Scientific Inc., Waltham, MA), supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and three distinct glucose concentrations (3.5, 6 and 10 mM). To obtain each working concentration, filtered glucose was added to culture media. Once FaDu cells reached 80% confluence, they were treated or not (non-irradiated control cells) with 3 Gy radiation every 2–3 days in a medical linear accelerator (Varian 2100 CD; Varian Medical Systems Inc., Palo Alto, CA) until an accumulative dose of 60 Gy (after 20 cycles) had been delivered. Control and irradiated cells underwent 1, 2, 3, 4, or 6 passages. Non-irradiated control cells and surviving FaDu cells after 3, 9, 15, 30, and 60 Gy were harvested and subsequently frozen for the purpose of gene expression analysis and apoptosis assessment. All experiments were performed with mycoplasma-free cells. All human cell lines have been authenticated using STR profiling within the last 3 years.

2.3 | Gene expression analysis

Tissue specimens were taken from the primary site, and adjacent and distal to the tumor. Macroscopically healthy tissue from the adjacent mucosa, located 1 cm away from the tumor lesion, was obtained. Part of the sample was used for the pathologic diagnosis of the malignancy, and another part was immediately stabilized by inclusion in RNAlater preservative (Qiagen GmbH, Hilden, Germany) and stored at −80°C until processing. Total RNA was extracted from 30 mg of tissue using the RNeasy Mini Kit from Qiagen.[28]

For cell culture experiments, RNA was extracted from FaDu cells using TRI Reagent (Molecular Research Center, Cincinnati, OH). Quantification of nucleic acid was performed at 260 nm and purity was assessed using the OD260/OD280 ratio. cDNA was prepared by reverse transcribing 2 μg RNA from tissue samples and 1 μg RNA from cell samples using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was conducted in duplicate on a 7900 HT Fast Real-Time PCR platform using commercially available TaqMan Gene Expression Assays from Applied Biosystems (Table S1, Supporting Information). Cycle threshold (Ct) values for each sample were normalized against the reference gene *RPLP0* (Hs99999902_m1) in tissue samples and against *18S* (Hs03928985_g1) in cell samples. Results were calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) and expressed relative to a calibrator (a mix of nine samples from normal, adjacent, and tumoral mucosa) in tissue samples, and expressed relative to the control condition set to 1 in cell samples. Two technical duplicates were performed for each biological replicate.

2.4 | Apoptosis assessment

The Cell Death Detection ELISA Plus kit (Roche Applied Science, Mannheim, Germany) was used to evaluate apoptosis. Briefly, surviving FaDu cells after radiation were seeded at 10 000/well in a 96-well microplate and were incubated for 48 h with or without the cytotoxic agent AZD5582 (Ref 5141/10; R&D Systems, Minneapolis, MN) used at a previously reported IC_{50} concentration.[29] After incubation, cells were lysed in 200 μL of lysis buffer for 30 min at room temperature and centrifuged at 200*g* for 10 min. Supernatants were collected and incubated in a streptavidin-coated microplate together with an immunoreagent consisting of anti-histone-biotin and anti-DNA-peroxidase for a duration of 2 h. The DNA fragments of histones in the cytoplasm were detected photometrically at a wavelength of 405 nm. The results were normalized to the 0-h time point to measure basal apoptosis and AZD-induced apoptosis.

2.5 | Statistical analysis

In vitro experiments were performed three times and pooled for statistical analysis. Data are presented as mean ± SEM and represent the number of biologically independent samples.

Differences between groups were assessed using an unpaired Student's *t* test for comparing two groups (2-tailed, 95% confidence interval). For comparing three groups, one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was employed. GraphPad Prism 8.0.2 software from GraphPad Software Inc., La Jolla, CA was used for conducting the analyses. A *p*-value less than 0.05 was considered statistically significant. The relationship between categorical variables was analyzed using the chi-square test.

In patients, we evaluated the outcome in terms of locoregional control with a follow-up of at least 2 years. Disease-free survival was defined as the period of time from the completion of the primary treatment of the tumor to any local, regional, or distant recurrence. The survivin/BIRC5 categories were defined according to the control of the disease (local–regional–distant recurrence) using the classification and regression tree (CRT) method. Considering the number of subjects presenting the event, and in order not to incur overfitting errors, two clinically relevant variables were chosen to build the multivariate model (Cox proportional hazards models): the presence of lymph node disease and local extension as well as the profile according to *BIRC5* mRNA levels in the tumor mucosa. The disease-free survival according to the survivin/BIRC5 profile was calculated using the Kaplan–Meier method. The log-rank test was employed to compare differences in survival rates. All statistical analyses were performed using SPSS software version 20.0 (IBM, Madrid, Spain).

3 | RESULTS

3.1 | Characteristics of the patients included in the study

A total of 32 patients with HNSCC were included in a follow-up prospective study. At the end of the study, 11 patients (34%) presented with locoregional recurrence. The main clinical and pathological characteristics of the patients, as well as the locoregional disease-free survival rate stratified by each variable, are summarized in Table 1. HPV status was evaluated in five patients with oropharyngeal carcinoma, of whom four (80%) presented with HPV-negative tumors.

3.2 | Survivin/BIRC5 is predominantly expressed in tumoral mucosa and positively associates with glucose metabolism in HNSCC

We assessed the gene expression profile of *BIRC5* in tumoral, non-tumoral adjacent tissue and normal mucosa from same patient. The analysis results showed a significant increase in *BIRC5* mRNA levels in tumoral mucosa compared to peri-tumoral and healthy tissue (adjacent and normal mucosa) (Figure 1A), with differences between the latter two also significant. We found that high *BIRC5* expression in tumor tissue was associated with high expression in healthy tissue (Figure 1B). Further analysis of tumor tissue revealed a positive association between the expression of *BIRC5* and hypoxia-inducible factor 1 alpha (*HIF1A*) (Figure 1C), a transcription factor that regulates hypoxic stress and glucose metabolism in tumor tissues and is involved in cancer progression and resistance to chemotherapy and radiotherapy.[30–32] Of note, *BIRC5* expression in tumoral tissue positively correlated with the expression of several genes involved in glucose metabolism, including glucose transport (GLUT1 [*SLC2A1*] and GLUT3 [*SLC2A3*]), glycolysis (hexokinase [*HK2*] and phosphofructokinase [*PFK*]), lactate metabolism (lactate dehydrogenase [*LDHA*], monocarboxylate transporters MCT1 [*SCL16A1*], and MCT3 [*SCL16A8*]) and with the tricarboxylic acid cycle (pyruvate dehydrogenase kinase 1 [*PDK1*], pyruvate dehydrogenase catalytic subunit 1 [*PDH*], succinate dehydrogenase A and B [*SDHA* and

TABLE 1 Characteristics of the patients and the locoregional disease-free survival rate stratified by each variable.

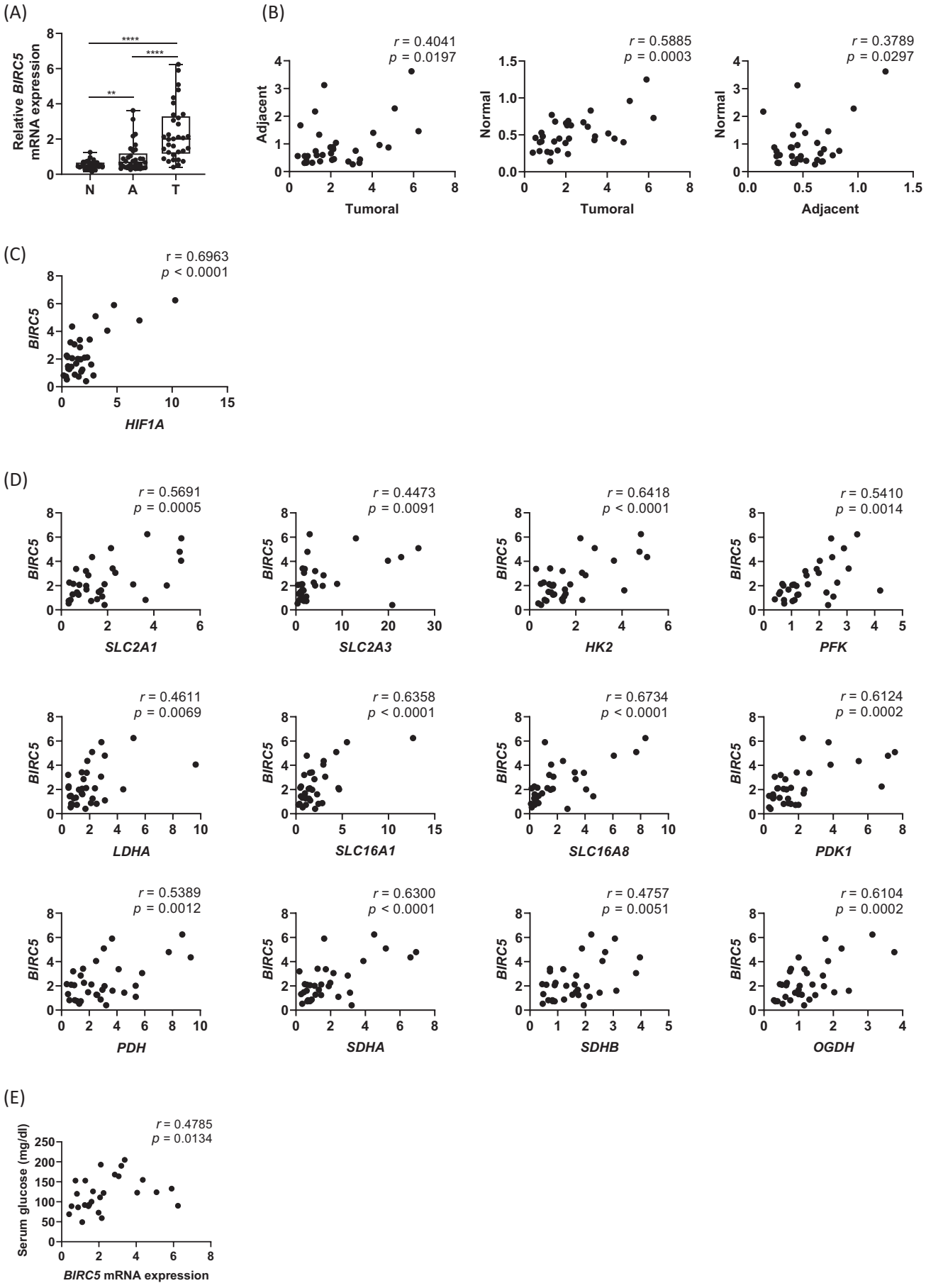
Characteristics	Number of patients (%)	Disease-free survival % (95 CI%)	*p*-value
Age (years)			
<50	2 (6.3)	50.0 (9.4–98.7)	0.690
50–60	11 (34.4)	54.5 (28–78.7)	
60–70	12 (37.5)	33.3 (13.8–60.9)	
>70	7 (21.9)	57.1 (25–84.1)	
Sex			
Male	30 (93.8)	43.3 (27.3–60.8)	0.212
Female	2 (6.3)	100 (34.2–100)	
Tobacco consumption			
Never	7 (21.9)	71.4 (35.8–91.7)	0.209
<20 cigarettes per day	0 (0)	0 (0–100)	
>20 cigarettes per day	25 (78.1)	40 (23.4–59.2)	
Alcohol consumption			
Never	14 (43.8)	42.8 (21.3–67.4)	0.458
Mild–moderate	2 (6.2)	0 (0–65.7)	
Severe	16 (50)	56.2 (33.1–76.9)	
ECOG index			
0	16 (50)	31.2 (12.1–55.5)	0.431
>0	16 (50)	62.5 (38.6–81.5)	
Tumor location			
Oral cavity–oropharynx	7 (34.4)	71.4 (35.8–91.7)	0.149
Larynx–hypopharynx	25 (78,1)	40 (23.4–59.2)	
T category			
1–2	11 (34.4)	54.5 (28–78.7)	0.398
3–4	21 (68.8)	42.8 (24.4–63.4)	
N category			
N0	20 (62.5)	35 (18.1–56.7)	0.085
N+	12 (37.5)	66.6 (39–86.1)	

Abbreviations: CI, confidence interval; ECOG, Eastern Cooperative Oncology Group.

SDHB], and alpha-ketoglutarate dehydrogenase [*OGDH*]) (Figure 1D). *BIRC5* expression also positively correlated with serum glucose levels in patients (Figure 1E).

3.3 | Survivin/BIRC5 expression in tumoral mucosa is associated with tumor stage and patient outcome

We then examined the expression of *BIRC5* based on tumor stage and locoregional control. Tumoral *BIRC5* expression was significantly higher in patients with advanced stage disease (stage III-IV) compared to those with early stage disease (I-II) (Figure 2A). Moreover, it was found to be elevated in patients with poor locoregional control (Figure 2B). To assess the predictive value of *BIRC5* expression in tumoral tissue for locoregional disease-free survival, the CRT method was utilized. It identified a cut-off value of 2.547 for *BIRC5*, enabling discrimination of disease-free survival. Based on this method, a categorized variable "profile" was generated, classifying patients according to their *BIRC5* expression in tumoral tissue (Low *BIRC5* < 2.547; High *BIRC5* > 2.547). Table 2 presents the outcomes of the multivariate analysis, with disease-free survival as the dependent variable. The survivin/BIRC5 profile significantly correlated with disease control. When considering patients with "Low *BIRC5*" as the reference category, those with "High *BIRC5*" exhibited a higher risk of unfavorable outcomes following treatment (Table 2), even



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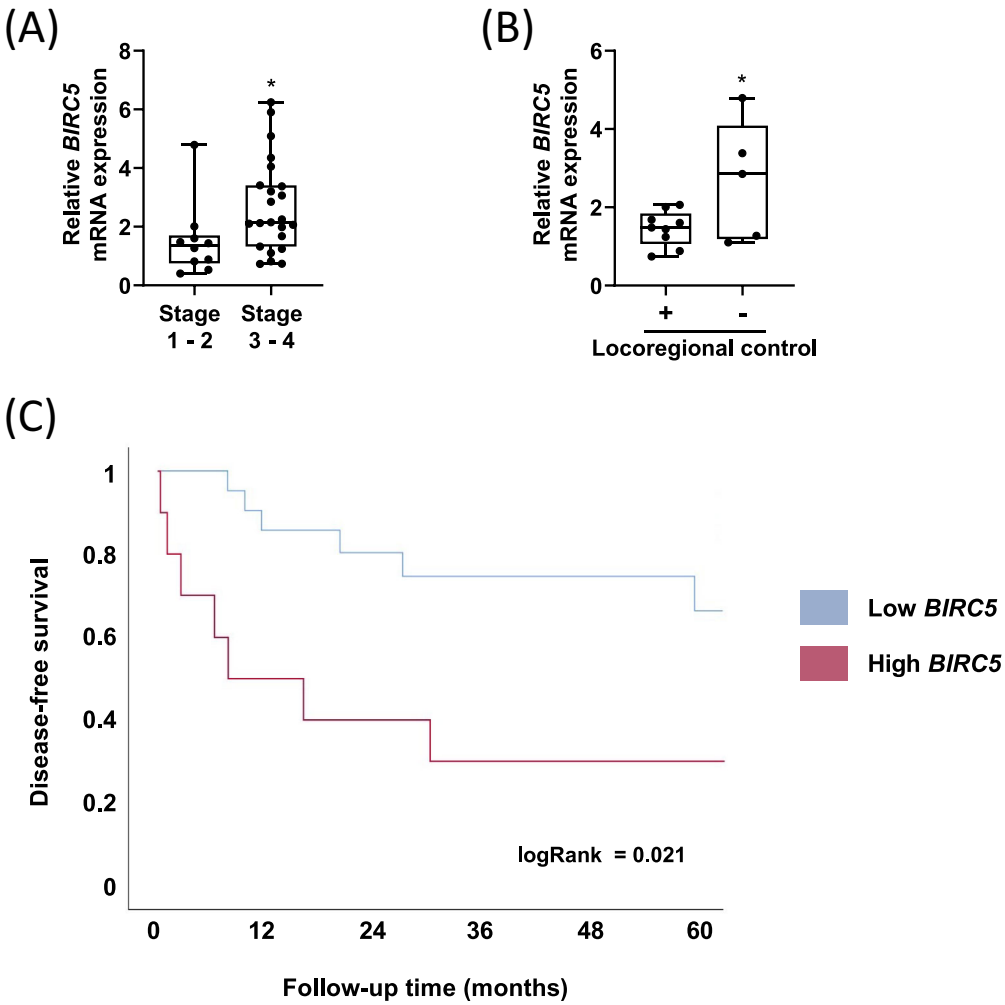


FIGURE 2 Higher *BIRC5* expression in tumoral tissue is related to a worse tumor stage and locoregional control, and poor survival. (A) *BIRC5* mRNA expression in tumoral mucosa classified according to the tumoral stage of patients. (B) *BIRC5* mRNA expression in tumoral mucosa of patients treated with radiotherapy, classified according to the achievement of locoregional control. In (A, B), an unpaired t test was performed to determine significant statistical differences between both groups. *$p < 0.05$. (C) Kaplan–Meier survival analysis showing locoregional recurrence-free survival according to *BIRC5* expression in tumoral tissue. Low BIRC5 profile: *BIRC5* mRNA expression in tumoral tissue <2.547. High BIRC5 profile: *BIRC5* mRNA expression in tumoral tissue >2.547. [Color figure can be viewed at wileyonlinelibrary.com]

after adjusting for nodal disease and T-stage. Specifically, patients with "High *BIRC5*" had a 13-fold increased risk of tumor recurrence (95% confidence interval [CI]: 2.52–67.3, $p = 0.002$). Subsequently, we proceeded to assess the prognosis of locoregional disease-free survival using the Kaplan–Meier method. The locoregional recurrence-free survival curves were generated based on the *BIRC5* expression profile in tumoral tissue, as shown in Figure 2C.

The disease-free survival rate was 72.5% (95% CI: 56.4–88.5%) for patients classified as "Low *BIRC5*," and 30.9% (95% CI: 9.7–52%) for those classified as "High *BIRC5*," indicating a lower rate of locoregional recurrence-free survival for the latter patients. We also found significant differences in the disease-free survival as a function of the BIRC5 profile ($p = 0.021$).

3.4 | Radiation-induced survivin/BIRC5 expression is dependent on glucose concentration and is associated with a pro-tumoral phenotype

To investigate the role of survivin/BIRC5 in glucose metabolism in the context of radiation response, we cultured the HNSCC cell line FaDu in low (3.5 mM), normal

FIGURE 1 *BIRC5* gene levels are increased in tumoral tissue and are associated with serum glucose and with glucose-related metabolism genes. (A) *BIRC5* mRNA expression in normal (N), adjacent (A), and tumoral mucosa (T). Gene expression differences between tissue samples were calculated using one-way ANOVA with Tukey's multiple comparisons test. **$p \leq 0.01$, ****$p < 0.0001$ between tissues. (B) Correlations between *BIRC5* expression in normal, adjacent, and tumoral mucosa. (C) Correlation between gene expression of *HIF1A* and *BIRC5* in tumoral tissue. (D) Correlations between gene expression of metabolic markers and *BIRC5* in tumoral tissue. (E) Correlation between *BIRC5* expression in tumoral mucosa and serum glucose. All associations between variables were calculated using Pearson's rank-order correlation test. Rho coefficients were considered statistically significant at $p < 0.05$.

Variables	Categories	HR	95%CI	*p*-value
Multivariate analysis				
T-stage	T3-T4 vs. T1-T2	22.16	2.61–187.9	**0.004**
Nodal disease	N+ vs. N0	2.04	0.55–7.56	0.285
Survivin/BIRC5	High vs. Low	13.04	2.526–67.35	**0.002**

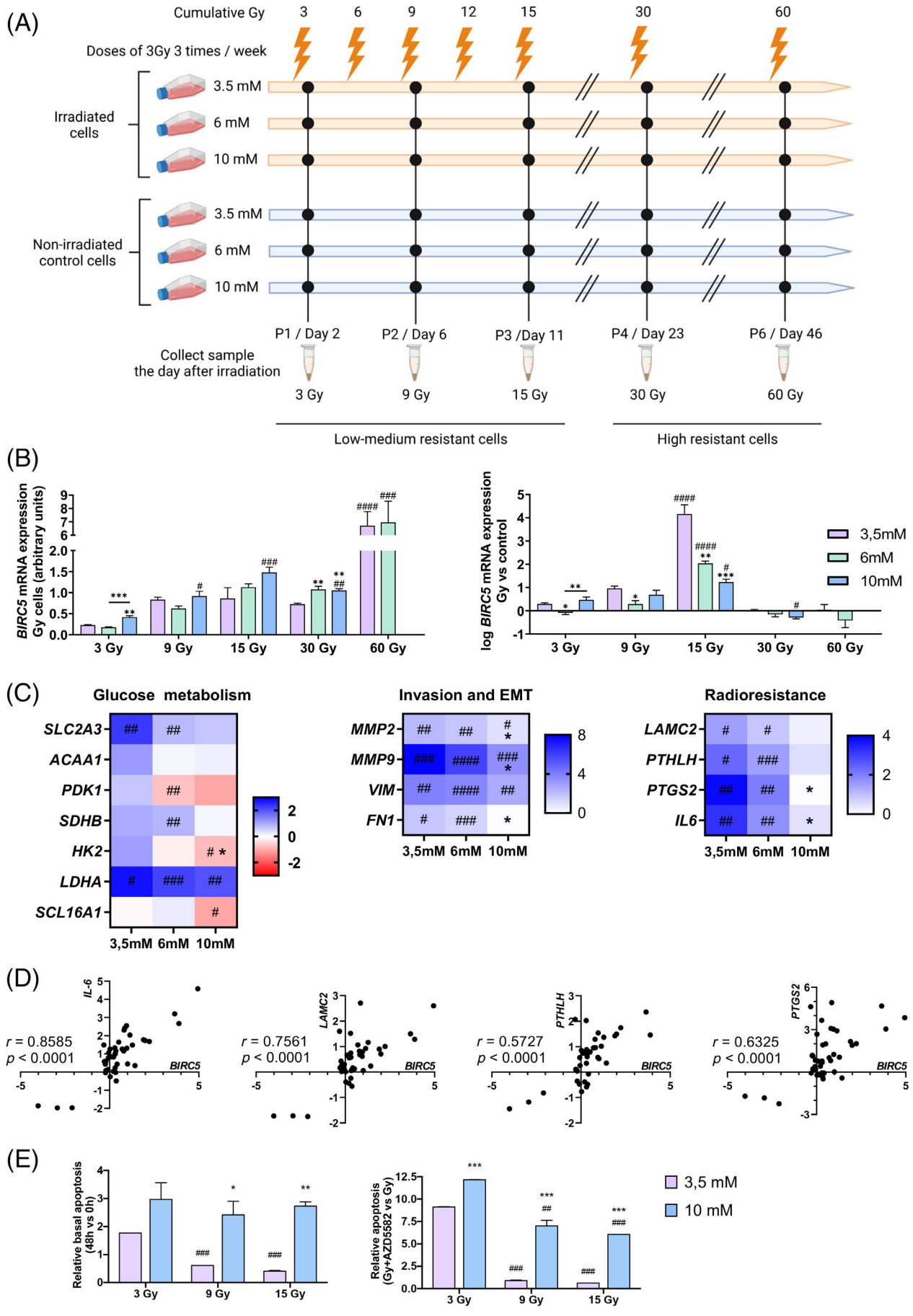
Note: A p-value < 0.05 was considered statistically significant.
Abbreviations: CI, confidence interval; HR, hazard ratio.

TABLE 2 Prognostic factors of disease-free survival in multivariate Cox regression analyses.

(6 mM) and high (10 mM) levels of glucose and exposed them to a fractionated irradiation cycle of 3 Gy until reaching a total irradiation dose of 60 Gy in 20 cycles. We collected non-irradiated control cells and the irradiated cells after 1 passage (1 cycle, 3 Gy), 2 passages (3 cycles, 9 Gy), 3 passages (5 cycles, 15 Gy), 4 passages (10 cycles, 30 Gy), and 6 passages (20 cycles, 60 Gy). Cells were classified as "low/medium-resistant" and "high-resistant" populations based on the irradiation protocol (1–5 cycles and 10–20 cycles, respectively) (Figure 3A). The expression analysis of the cells revealed a significant increase in *BIRC5* expression with radiation exposure and glucose concentration in the surviving cells after irradiation (Figure 3B, left panel). However, given that the radiation protocol also includes multiple rounds of culture expansion, which can potentially alter the transcriptional characteristics of the cells, we conducted an analysis to determine whether the observed effect might be attributed to expansion rather than radiation. As depicted in Figure S1A, there is an upregulation of *BIRC5* gene expression during long-term culture under basal conditions. When we compared the *BIRC5* expression in irradiated cells to their respective control (non-irradiated cells) within each condition, we observed a significant increase in *BIRC5* fold-change in irradiated cells, particularly following exposure to a 15 Gy dose. Notably, we did not detect further transcriptional regulation of *BIRC5* expression in the "high-resistant populations" (Figure 3B, right panel) suggesting that the impact of radiation on *BIRC5* expression is cycle-dependent, with a discernible saturation point in its response. Indeed, this upregulation becomes evident as surviving cells transition to a more resistant state after the initial radiation treatments Interestingly, this upregulation was attenuated when cells were cultured in high-glucose environment, as illustrated in Figure 3B (right panel). A parallel trend was evident under hypoxic conditions (Figure S1B), a known factor potentially influencing radiotherapy response.[33] Concurrently, elevated glucose levels mitigated the radiation-induced upregulation of genes associated with glucose and energy metabolism (e.g., GLUT3 and LDHA) (Figure 3C, left panel). Moreover, we investigated the phenotype of surviving cells post-medium-dose ionizing irradiation, examining mRNA expression of genes linked to invasiveness (matrix metalloproteinase-2 and 9 [MMP2 and MMP9]), epithelial-to-mesenchymal transition (vimentin [VIM] and fibronectin [FN1]), and radioresistance (interleukin-6 [IL6], laminin subunit gamma-2 [LAMC2], parathyroid hormone-like hormone [PTHLH], and prostaglandin-endoperoxide synthase-2 [PTGS2]). Notably, all these genes exhibited increased expression following radiation, but this effect was dose-dependently hindered by glucose (Figure 3C, middle and right panels).

In the context of radioresistance markers, we confirmed a positive correlation between *BIRC5* expression and several relevant markers, including IL6, LAMC2, PTHLH, and PTGS2 (Figure 3D). Furthermore, our assessment of glucose's impact on these radioresistance genes across radiation doses from 3 to 30 Gy revealed a consistent pattern akin to BIRC5 behavior (Figure S1C). Specifically, the most notable radiation-induced effects on radioresistance markers materialized at a 15 Gy radiation dose, resulting in a consistent decrease at 30 Gy, mirroring BIRC5's behavior. Importantly, our analysis underscored that while specific glucose effects surfaced as early as 9 Gy for select markers such as PTGS2 and IL6, the most pronounced effects persisted at the 15 Gy radiation dose, closely paralleling BIRC5's response pattern.

Finally, we evaluated whether glucose levels could modulate both the basal apoptosis and apoptosis sensitivity of "low/medium resistant" populations treated with AZD5582, an antagonist of the inhibitor of apoptosis family proteins (IAP), which have been previously described as candidates for combination therapy with irradiation.[29] We observed that radiation suppressed basal apoptosis (Figure 3E, left panel) and the sensitivity to AZD5582-induced apoptosis (Figure 3E, right panel) of surviving FaDu cells, in accord with a previous study.[33] However, high glucose counteracted this effect (Figure 3E). Overall, these data indicate that high-glucose levels impair the upregulation of *BIRC5* induced by radiation and increase the basal apoptosis and the sensitivity of irradiated cells to cytotoxic agents.



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4 | DISCUSSION

The present study reveals that the high tumoral expression of survivin/BIRC5, a proto-oncogene associated with cellular sensitivity to radiotherapy, is related to glucose metabolism in HNSCC. We found that high tumoral *BIRC5* expression is indicative of unfavorable locoregional disease-free survival in patients. Moreover, we establish that the transcriptional regulation of *BIRC5* by radiation in HNSCC cells is dependent on glucose levels and provokes a pro-tumoral and radioresistant phenotype in surviving cells.

HNSCC is one of the most frequent types of cancer and is characterized by poor prognosis and a high relapse rate.[2] Despite improvements in radiotherapy, there remains a high rate of tumor radioresistance and recurrence in patients with HNSCC,[34,35] which might be related to dysregulated glucose metabolism.[9] Indeed, it has been proposed that glucose homeostasis in HNSCC might be targeted to improve the prognosis of patients.[9,36,37] Nonetheless, the molecular drivers involved in the metabolic shift of cancer cells, and how glucose levels might affect the sensitivity of cancer cells to radiotherapy or cytotoxic molecules, are not known.

Survivin/BIRC5 expression is associated with the aggressiveness and grade of metastasis, and with reduced survival.[11,38] Overexpression of survivin/BIRC5 has been observed in various cancer types, including HNSCC, and has been linked to increased cell proliferation, invasion, metastasis, and enhanced cell survival, protecting tumor cells from apoptosis.[20,39–42] Similar to previous studies,[13,43] we show that *BIRC5* expression is higher in tumoral tissue than in adjacent tissue. Interestingly, we also demonstrate, for the first time to our knowledge, that *BIRC5* expression is higher in peri-tumoral tissue than in healthy tissue, and we found a positive association between the three tissue types from the same patient. Several clinical studies have proven the relationship between increased survivin/BIRC5 expression and tumor aggressiveness in HNSCC,[20,44] and it is also well documented that survivin/BIRC5 overexpression promotes chemo- and radio-therapy resistance in a wide type of cancers.[26,45,46] Although contentious,[24] it is generally accepted that survivin/BIRC5 acts as a radioresistance factor in many cancers,[47,48] and its downregulation correlates with lower radioresistance and a better outcome.[45,49] Indeed, we show here that patients with higher levels of *BIRC5* in tumoral tissue have worse locoregional control after radiotherapy, and that higher *BIRC5* tumoral expression is observed in advanced-stage HNSCC. We also found that *BIRC5* tumoral expression was positively associated with serum glucose levels and with the expression of several genes related to glucose metabolism in tumoral tissue, pointing to a potential role for survivin/BIRC5 in reprogramming cancer metabolism.

At the cellular level, our data demonstrate that radiation exposure induces *BIRC5* expression in an HNSCC cell line, which is in line with previous research.[26] However, this regulation seems to be dependent on the dose of irradiation, as the expression of *BIRC5* was not affected by high irradiation doses (>30 Gy), which might explain the polemic about survivin/BIRC5 as a radioresistance factor.[19,24] Notably, high glucose levels in vitro inhibited radiation-induced *BIRC5* expression, suggesting that in terms of radiotherapy, a hyperglycemic environment might favor a better outcome. This glucose-dependent effect was also observed at the metabolic gene level. Metabolic switching from oxidative phosphorylation to aerobic glycolysis, also known as the Warburg effect, is the

FIGURE 3 *BIRC5* expression induced by radiation is dependent on extrinsic glucose levels and is associated with a pro-tumoral phenotype. (A) Schematic representation of the workflow performed with HNSCC FaDu cells. Both control and irradiated cells were cultured at the same time and under the same conditions, except that the treated group was irradiated three times weekly with doses of 3 Gy. Cells were collected at a cumulative dose of 3, 9, 15, 30, and 60 Gy. (B) *BIRC5* mRNA expression in FaDu cells cultured with 3.5, 6, or 10 mM glucose in the medium and treated with 3, 9, 15, 30, or 60 Gy of radiation. Results are expressed in arbitrary units of irradiated cells (left panel) or as logarithmic expression versus control cells (right panel). For statistical analysis, one-way ANOVA was performed. * shows statistical differences between glucose concentrations at the same radiation dose. # shows statistical differences between radiation doses (versus 3 Gy) cultured at the same glucose concentration. * or # $p \leq 0.05$; ** or ## $p \leq 0.01$; *** or ### $p \leq 0.001$; **** or #### $p \leq 0.0001$. (C) Heatmaps of the logarithmic expression of markers related to metabolism (left), pro-tumoral phenotype (middle) or radioresistance (right) in FaDu cells cultured with 3.5, 6, or 10 mM of glucose in the medium and treated with 15 Gy of radiation versus their control condition. For statistical analysis, an unpaired *t* test was performed. * shows statistical differences versus 3.5 mM glucose concentration. # shows statistical differences versus each control condition. * or # $p \leq 0.05$; ** or ## $p \leq 0.01$; *** or ### $p \leq 0.001$; **** or #### $p \leq 0.0001$. (D) Correlations between *BIRC5* and radioresistance markers (*IL-6*, *LAMC2*, *PTHLH*, *PTGS2*) logarithmic expression in all conditions. (E) Relative apoptosis of FaDu cells cultured with low- or high-glucose, irradiated with 3, 9, or 15 Gy and exposed or not to the cytotoxic agent AZD5582 for 48 h versus 0 h (left panel) and versus non-exposure to AZD5582 (right panel). * shows statistical differences between different glucose concentrations. # shows statistical differences between doses of radiation at the same glucose concentration. * or # $p \leq 0.05$; ** or ## $p \leq 0.01$; **** or #### $p \leq 0.0001$. [Color figure can be viewed at wileyonlinelibrary.com]

main feature of metabolic reprogramming and is one of the hallmarks of cancer.[50] It is also a major factor contributing to radioresistance.[3,51] Our data suggest that radiation might promote glucose uptake via the upregulation of GLUT3, which has been described to be elevated in many types of cancer including triple-negative breast cancer[52] and lung cancer.[53] However, similar to our findings for survivin/BIRC5, GLUT3 upregulation was blocked by high glucose levels. LDHA expression, which is associated with lactate production, behaved similarly. It is well known that lactate-induced acidification of the tumor microenvironment triggers resistance to apoptosis, induces epithelial-to-mesenchymal transition, promotes a greater invasive capacity of cells, and stimulates radioresistance.[6,54] Accordingly, radiation induced the upregulation of both pro-tumoral and radioresistance markers, but in a glucose-dependent manner. The possibility that survivin/BIRC5 might be involved in reprogramming cancer cells towards a pro-tumoral phenotype after radiation fits with our previous research in an obesity-related tumor microenvironment, in which high survivin/BIRC5 levels promoted a tumor-associated macrophage phenotype and enhances the malignancy of cancer cells.[55] Consistent with these results, hyperglycemia increases the sensitivity of irradiated cells to the cytotoxic agent AZD5582, described as a potential candidate for combination therapies.[29] Thus, while abnormal glucose metabolism has been related to tumor progression,[8,9,27] hyperglycemia might increase the sensitivity to radiation and cytotoxic agents once malignant cells are established.

In conclusion, our findings indicate that survivin/BIRC5 is independently associated with the risk of recurrence in patients diagnosed with HNSCC. Moreover, we found that there is an optimal cut-off value of survivin/BIRC5 expression, and lower levels were associated with a better outcome. Notably, our findings support the modulation of glucose levels as a potential therapeutic approach to improve treatment response in patients with HNSCC. Our study has, however, some limitations that should be mentioned including the relatively small sample size and the heterogeneity of the patient population, which may have limited the statistical power of our analysis. Nevertheless, we were able to gather data from a prospective cohort of patients and observed consistent results even after adjusting for confounding factors, thereby reinforcing the robustness of our findings. Regarding HPV, immunohistochemistry in oropharyngeal cases did not reveal any association with disease-free survival. Regarding the in vitro analysis, the presence of different subclones (radioresistant or radiosensitive phenotype) after irradiation is a limitation; however, it has previously been shown that a dose per fraction ranging from 2 to 6 Gy with 48 h recovery is sufficient to select for radioresistant clones.[29] Finally, although our data demonstrate a link between *BIRC5* expression and glucose metabolism in the context of HSCC, it does not allow us to determine causality. Nonetheless, our work might guide future molecular studies to facilitate a better diagnosis and treatment for HNSCC.

In summary, our study reveals an intricate relationship among glucose levels, survivin/BIRC5 expression, and radiation response. Although patient-data initially suggested a positive correlation between BIRC5 and glucose levels, indicating a worse prognosis, our in vitro experiments reveal that high glucose might negatively impact BIRC5 expression in the context of radiation, potentially leading to better therapy responses in hyperglycemic conditions. While cell cultures have their limitations, they provide a controlled environment for exploring the effects of radiation and glucose over time, shedding light on the complexity of these relationships. Additionally, the differences observed in patient data could be influenced by factors beyond tumor cells, such as immune cell composition within the tumor microenvironment, as indicated by our recent study identifying BIRC5 as a specific marker of tumor-associated macrophages.[55] Overall, our study underscores the multifaceted and dynamic nature of these interactions, paving the way for future investigations.

AUTHOR CONTRIBUTIONS

Study concept and design: F.X.A.-J. and S.F.-V. *Acquisition of data, analysis, and interpretation of data*: E.B., V.C.-M., F.X.A.-J., and S.F.-V. *Administrative, technical, or material support*: S.G., E.M.-M., A.M., D.G., V.H., I.V., C.M., X.L., J.V., and F.X.A.-J. *Drafting of the manuscript*: E.B., V.C.-M., F.X.A.-J., and S.F.-V. *Critical revision of the manuscript for important intellectual content*: All authors. *Statistical analysis*: E.B., V.C.-M., X.T., and F.X.A.-J. *Obtained funding*: V.C.-M., X.L., I.V., J.V., F.X.A.-J., and S.F.-V. *Study supervision*: F.X.A.-J. and S.F.-V.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The research study was reviewed and approved by the Institutional Review Board (Hospital Universitari Joan XXIII-Institut d'Investigació Sanitària Pere Virgili; code 15/04057). The investigation conformed to the principles outlined in the Declaration of Helsinki, and all patients gave written informed consent.

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