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Beta-adrenergic pathway activation enhances aggressiveness and inhibits stemness in head and neck cancer

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ABSTRACT

Chronic stress leads to the activation of the beta-adrenergic pathway. Its activation has been implicated in the progression of different types of cancer but its role on head and neck squamous cell carcinomas (HNSCCs) remains undefined. The aim of this study was to investigate the influence of the beta-adrenergic pathway activation in the progression of HNSCCs and offer a panel of potential treatments for patients with the active beta-adrenergic pathway. Five hundred and twenty TCGA patients with primary HNSCCs were divided in two groups: $ADRB2^{low}$ / $SLC6A2^{low}$ and $ADRB2^{high}$ / $SLC6A2^{high}$. Differentially expressed genes (DEGs) were identified through differential expression analysis. The association of clinicopathological and genomic features between the groups was analyzed using a bioinformatic approach. Potential drugs for treatment of HNSCC were identified based on the DEGs. There was association between ADRB2 and SLC6A2 expressions with age, race, tumor site, histologic grade, perineural invasion, and HPV p16 status. It was identified 898 DEGs between the groups. High ADRB2/SLC6A2 expression stimulated HNSCC proliferation, adhesion, invasion, and angiogenesis. On the other hand, genes related to cell stemness were downregulated in patients with activation of the beta- adrenergic pathway. Finally, 56 FDA-approved antineoplastic and immunotherapeutic drugs were identified as potential targets for the personalized treatment.

Introduction

Psychological chronic stress has been linked to the onset or to the worst outcome of several diseases such as depression, anxiety [1], cardiovascular [2], gastrointestinal [3] illnesses, and cancer [4]. In individuals under chronic stress, higher levels of the hormones norepinephrine (NE) and epinephrine (E) are released, respectively, by the terminal sympathetic nerve fibers locally and by the adrenal gland systemically [5]. Then NE and E binds to the alpha- or beta-adrenergic (ADRB) receptors, that are thoroughly expressed across the body, and activate the adrenergic pathway. Interestingly, clinical studies have found association between the activation of the alpha- or beta-adrenergic signaling pathway and the worst prognosis [6], perineural invasion [7], and metastasis [8] in different types of cancer. Furthermore, *in vitro* studies have shown that the overactivation of the adrenergic pathway plays a role in the pathogenesis of several malignant tumor types including skin [9], pancreas [10], and brain [11] by increasing tumoral progression, dissemination, and proliferation. The local activation of the adrenergic receptors has also been shown to play an important role in the alteration of the tumoral microenvironment by inducing the epithelial-mesenchymal transition (EMT) [12], and angiogenesis [13].

Conflicting evidences about the effects of chronic stress on head and neck squamous cell carcinoma (HNSCC) progression have been discussed over the years. While some *in vitro* studies have shown that the activation of the beta-adrenergic pathway seems to be implicated in increased proliferative activity [14], invasion, and EMT [15] of head and neck cancer cells, other studies have had opposing findings. The stimulation of HNSCC cells with agonists of the beta-adrenergic receptors has resulted in decrease in HNSCC cell migration, invasion [16,17], and EMT [18]. Clinical studies have obtained dissonant findings as well. Beta-adrenergic receptor 2 (ADRB2) has been found as a positive predictive factor for oral cancer in one study [15] or to do not influence the prognosis of oral cancer in other investigation [19]. These apparently opposing pieces of evidence raise doubts about the function of the beta-adrenergic pathway activation in the biology of HNSCC.

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Thus, this study was designed to address the enigma about how the beta-adrenergic pathway activation affects the development of HNSCC and the impact it has on the patients. For that purpose, it was performed a comprehensive clinical and genomic analysis of 520 patients with HNSCC using a bioinformatic approach.

Material and Methods

Access to the data

Clinical and molecular data of patients with head and neck cancer were retrieved through the National Cancer Institute's (NCI's) Genomic Data Commons (GDC - www.portal.gdc.cancer.gov) Legacy Archive from The Cancer Genome Atlas (TCGA). The database was accessed using TCGAbiolinks package [20] in the RStudio software (Integrated Development for RStudio Inc., Boston, MA, USA - http://www.rproject.org). The selection criteria were: 1) patients with primary head and neck squamous cell carcinoma; and 2) patients with clinical and genomic information available. A total of 520 patients fulfilled the inclusion criteria. **All bioinformatic approach is listed in Supplementary file 1.**

Clinical data

Clinical information was downloaded and imported into RStudio using the TCGAbiolinks package [20]. Socio-demographic (gender, age, and race), clinical (tumor site, TNM clinical, and tumor stage), and microscopical (lymphovascular invasion, perineural invasion, histologic grade, and HPV p16 status) information were analyzed. The histologic grade and TNM clinical followed the American Joint Committee on Cancer system [21].

Fisher test was used to verify the association of the adrenergic pathway activation with gender, tobacco consumption, alcohol consumption, lymphovascular invasion, perineural invasion, and HPV p16 status. Chi-square test was used to assess the association of the adrenergic pathway activation with age, ethnic group, tumor site, T, N, M clinical, tumor stage, histologic grade. Data analysis was carried out using IBM Statistical Package for the Social Sciences (SPSS) Version 20 (Chicago, USA). $P<0.05$ was considered statistically significant.

RNA expression and mutation data

RNA-Seq data (Illumina HiSeq RNAseqv2) were downloaded from the GDC Data Portal and imported into RStudio. RNA expression data were obtained using the functions GDCquery, GDCdownload, and GDCprepare of TCGAbiolinks package [20]. The TCGAnalyze_Preprocessing function, which calculates the Array Intensity correlation for each sample, was used to detect possible outliers. The cut was set at 0.6 to define the samples with low correlation (outliers). As we did not find any outliers, we continued with the analysis of the 520 HNSCCs. Afterwards, we normalized the RNA expression among the samples (TCGAnalyze_Normalization) using the EDASeq package [22]. Finally, we filtered (TCGAnalyze_Filtering) those RNAs with low signal across the samples (cut = 0.25) and obtained a final number of 14,899 RNAs.

The gene mutation data of HNSCCs samples were retrieved from cBioPortal [23]. Five-hundred and twelve patients had this information available. To calculate the stemness index [24] of the HNSCCs, the argument TCGAanalyze_Stemness of the TCGAbiolink package [20] was used.

Beta-adrenergic pathway activation

A hallmark of the beta-adrenergic pathway activation is the overexpression of the adrenergic receptors [5]. They bind to the hormones epinephrine and norepinephrine, which are secreted in response to stress. Among the upstream beta-adrenergic RNAs, only ADRB2 were expressed in HNSCC. Both ADRB1 and ADRB3 presented low signal and did not pass the filter. Solute Carrier Family 6 Member 2 (SLC6A2) encodes the norepinephrine transport (NET) protein, which is responsible for the reuptake of NE into presynaptic nerve terminals and is a regulator of norepinephrine homeostasis [25]. SLC6A2 is mostly expressed in neural tissue however we observed that most of our sample was positive for SLC6A2. Considering that this gene is upregulated when there is higher concentration of NE, SLC6A2 was to paired with ADRB2 and their overexpression was defined as a readout for the beta-adrenergic pathway activation. The patients were divided into two groups according to the ADRB2 and SLC6A2 expression in $ADRB2^{low}/SLC6A2^{low}$ and $ADRB2^{high}/SLC6A2^{high}$.

Differentially expressed genes

Differentially expressed genes (DEGs) between the HNSCCs with $ADRB2^{low}/SLC6A2^{low}$ and $ADRB2^{high}/SLC6A2^{high}$ expression were determined with the TCGAbiolinks package [20] using the function TCGAanalyze_DEA. RNA expression data were batch-corrected using the tissue source sites (TSS) method and also submitted to voom transformation. Genes with false discovery rate (FDR)<0.01 and log fold-change (logFC)≥1 or ≤-1 were considered DEGs. DEGs were upregulated when logFC≥1 and downregulated, when logFC≤-1. DEGs between the groups were represented using ComplexHeatmap package [26].

Gene Ontology and pathway enrichment analysis

Gene Ontology (GO) resource is one of the most comprehensive and widely used database with the functions of thousands of genes [27]. GO group genes related to the same function in processes and classified them in: biological process (BP), cell components (CC), and molecular function (MF). In this study, we performed the GO enrichment analysis to verify the relationships among the DEGs using the packages Cluster Profiler [28] and GOplot [29]. Only the processes with a minimum of 5 genes and $p<0.05$ were considered enriched. Of note, in our analysis, the logFC and FDR values for each DEG were used to define if the gene was down- or upregulated.

Protein-protein interaction network analysis

The Search Tool for the Retrieval of Interacting Genes (STRING) is a database used to predict protein-protein interactions (PPI) [30]. We queried the STRING database with the DEGs names, logFC, and FDR values to build a protein-protein interaction network, which was analyzed in the Cytoscape [31].

Survival analysis

Overall survival rates were calculated using Kaplan-Meier method and the comparison of the survival curves was performed using log-rank test. Data analysis was carried out using IBM SPSS Version 20 (Chicago, IL, USA). P-values below 0.05 were considered statistically significant.

Drug-gene interaction analysis

To determine which drugs might be potentially effective against the DEGs identified between the $ADRB2^{low}/SLC6A2^{low}$ and $ADRB2^{high}/SLC6A2^{high}$ groups, the Drugs Gene Interaction Database (DGIdb) [32] was consulted. The DGIdb compiles information of other four databases: 1) DrugBank; 2) Therapeutic target database (TTD); 3) PharmGKB; and, 4) ClinicalTrial.gov. Using DGIdb in September 2020, we evaluated pharmacological data of components potentially effective towards our collection of DEGs. Only anti-neoplastic and immunotherapeutic drugs that were Food and Drugs Administration (FDA)-approved were analyzed.

Results

Beta-adrenergic pathway activation in HNSCC is mediated by ADRB2 and SLC6A2

The first step to study the role of the beta-adrenergic pathway in HNSCC was to define the key genes that were representative of its activation. We have selected the genes that encode the beta-adrenergic receptors – namely ADRB1, ADRB2, and ADRB3 - which bind to NE and E. In addition, we included in the analysis the gene SLC6A2, that encodes the NET protein. NET is responsible for the reuptake of extracellular NE into the cell in neuronal tissues. NET expression has also been observed in cancer [33,34]. After determining the global gene expression of all 520 HNSCCs and filtering those genes with low signal across the samples, it was obtained a final number of 14,899 RNAs that were expressed in HNSCC. ADRB1 and ADRB3 did not pass the filter and were excluded from the analysis. On the other hand, ADRB2 and SLC6A2 exhibited expression in HNSCC and were used as a readout for the activation of the beta-adrenergic pathway in this study. To further confirm if ADRB2 and SLC6A2 were simultaneously expressed in HNSCC, Spearman's rank correlation coefficient was calculated. Notably, the expression of ADRB2 was positively correlated with the expression of SLC6A2 ($p < 0.0001$, R = 0.2896, **Supplementary** figure 1**A).**

As the overexpression of some genes in cancer can be driven by their mutation, we also searched for genomic alterations in ADBR2 and SLC6A2 in HNSCCs. **Supplementary figure 1B** shows that only 0.2% and 2.4% of the 520 HNSCC patients presented at least one type of mutation in ADRB2 and SLC6A2, respectively. These data confirm that the activation of the adrenergic pathway was mediated by the overexpression of the ADRB2 and SLC6A2 genes rather than by any type of mutation.

Next, we classified the HNSCCs into two categories based on the average value of ADRB2 (882.03) and SLC6A2 (235.99) expressions: ADRB2 ^{low} / SLC6A2^{low} and ADRB2^{high} / SLC6A2^{high}. Tumors with high expression of ADRB2 and low expression of SLC6A2 or *vice-versa* were excluded. After the application of the exclusion criteria, the total sample included 343 patients: 256 (74.6%) with ADRB2^{low} / SLC6A2^{low} expression and 87 (25.4%) patients with ADRB2^{high} / SLC6A2^{high} expression.

ADRB2 and SLC6A2 expression is associated with histologic grade, perineural invasion, and hpv p16 status in HNSCC

The clinicopathological features of the HNSCCs included in this study are shown in Table 1. The great majority of HNSCC patients were male ($p = 0.257$) and older than 55 years-old. The group ADRB2^{high} / SLC6A2^{high} presented higher number of young individuals than the group ADRB2^{low} / SLC6A2^{low} (14.94% vs 4.69%, $p = 0.006$). Regarding the race, most patients were white in both groups. There were more Asian individuals in the ADRB2^{high} / SLC6A2^{high} group ($p = 0.024$). The most frequent tumor site was the larynx (30.8%) in the ADRB2^{low} / SLC6A2^{low} group and the oral tongue (39.08%) in the ADRB2^{high} / SLC6A2^{high} group ($p = 0.001$).

In both groups, moderately differentiated (G2) HNSCCs were more frequent. However, when it came to less differentiated states, ADRB2^{low} / SLC6A2^{low} group exhibited higher number of tumors graded as G3 and G4 ($p = 0.020$). Tumors of the ADRB2^{low} / SLC6A2^{low} group also presented less perineural invasion (40%) than tumors of the ADRB2^{high} / SLC6A2^{high} group (57.14%, $p = 0.026$). Almost all cases in the group ADRB2^{high} / SLC6A2^{high} were negative for HPV p16 expression (93.33%), contrasting with the 35.48% of positive cases in the ADRB2^{low} / SLC6A2^{low} group ($p = 0.031$). There was no difference between the groups regarding gender, tobacco and alcohol consumption, T, N, and M clinical, tumor stage, and lymphovascular invasion ($p > 0.05$).

Overview of DEGs in ADRB2^{low} / SLC6A2^{low} vs ADRB2^{high} / SLC6A2^{high} HNSCC

To understand better the molecular mechanisms regulated by the activation of the beta-adrenergic pathway in HNSCC, we performed the differentially expression analysis. A total of 898 DEGs were identified between ADRB2^{low} / SLC6A2^{low} and ADRB2^{high} / SLC6A2^{high} groups based on RNA-Seq data: 258 upregulated and 640 downregulated DEGs (Fig. 1A and **Supplementary File 2**). The heatmap in Fig. 1B shows the top 50 downregulated and top 50 upregulated DEGs between the groups ADRB2^{low} / SLC6A2^{low} and ADRB2^{high} / SLC6A2^{high}.

GO analysis links ADRB2^{high} / SLC6A2^{high} HNSCC to angiogenesis, cell proliferation, and ECM plasticity

To assess how the DEGs were related and to identify potential mechanisms that might distinguish the ADRB2^{low} / SLC6A2^{low} HNSCC from ADRB2^{high} / SLC6A2^{high} HNSCC, we performed the over-representation enrichment analysis using the GO database. We divided the DEGs between the groups ADRB2^{low} / SLC6A2^{low} and ADRB2^{high} / SLC6A2^{high} in upregulated and downregulated genes according to their logFC values. The GO analysis revealed that there were 768 downregulated (Fig. 2A) and 528 upregulated processes in the ADRB2^{high} / SLC6A2^{high} group (Fig. 2B). The downregulated processes were divided in 671 biological processes, 18 cell components, and 78 molecular functions. Among the upregulated processes, 410 were biological processes, 37 cell components, and 80 molecular functions (**Supplementary File 3**).

The enrichment maps of the top 30 biological processes (Fig. 2C-D) show that the ADRB2^{high} / SLC6A2^{high} presented multiple processes related to the tumoral progression that were upregulated: keratinocyte differentiation, extracellular matrix (ECM) metabolism, and cell adhesion. On the other hand, processes associated with the development of different types of tissue, and cellular metabolism were downregulated in the ADRB2^{high} / SLC6A2^{high} group.

Next, the processes closely related to the tumor progression were manually curated and grouped according to their main role. These grouped biological processes accompanied by their involved genes are represented in Fig. 3. One can observe that processes associated with angiogenesis, proliferation, and microenvironment are upregulated in ADRB2^{high} / SLC6A2^{high} tumors.

PPI network analysis in ADRB2^{high} / SLC6A2^{high} vs ADRB2^{low} / SLC6A2^{low} HNSCC

PPI network is a widely used tool to map predicted physical and functional interactions among proteins in a large scale. In this study, the STRING was chosen once this is the most comprehensive and up-to-date PPI database. To visualize the interactions among the DEGs, we firstly constructed the PPI network inputting all DEGs names, logFC values, and FDR. The resulting network included 868 nodes and 3060 edges. Each node in the network represented one protein. The edges represented the relationships between the proteins. Because the network was too large to obtain additional important information, we manually selected the most altered DEGs and/or DEGs that are related to known tumoral processes (starting node). From the starting node, the first neighbors were automatically retrieved from the STRING database successively and clean networks were generated. The network of the Fig. 4A includes the proteins that were most closely related to ADRB2 and SLC6A2 and contains 146 nodes and 701 edges. In the Fig. 4B, the findings of this study are confirmed: proteins that regulate the tumor invasion and spread in HNSCC are shown to be overexpressed. Upregulation of MMP-1, 3, 10, and 13, and CSF2 and downregulation of VCAM1 and ICAM4 are implicated in the cancer cells migration and stromal invasion. Additionally, the overexpression of VEGF-C, and NGF and underexpression of LEF1 are indicatives of the local influence of

Table 1
Clinicopathological characteristics of ADRB2^{low} / SLC6A2^{low} and ADRB2^{high} / SLC6A2^{high} head and neck squamous cell carcinomas.

Variables	Head and neck squamous cell carcinoma				*p*
	ADRB2^{low}/SLC6A2^{low}		ADRB2^{high}/SLC6A2^{high}		
	N	Percentage (%)	N	Percentage (%)	
Gender					
Male	194	75.78	60	68.97	0.257
Female	62	24.22	27	31.03	
Age					
<45	12	4.69	13	14.94	0.006
46–55	58	22.66	20	22.99	
>55	186	72.66	54	62.07	
Ethnic group*					
White	222	89.16	69	81.18	0.024
Black	25	10.04	11	12.94	
Asian	1	0.40	4	4.70	
Indian or Alaska native	1	0.40	1	1.18	
Tumor site					
Alveolar ridge	5	1.95	4	4.60	0.0001
Base of tongue	18	7.03	1	1.15	
Buccal Mucosa	11	4.30	4	4.60	
Floor of mouth	33	12.90	10	11.49	
Hard palate	3	1.17	1	1.15	
Hypopharynx	5	1.95	1	1.15	
Larynx	77	30.08	13	14.94	
Lip	3	1.17	0	0	
Oral cavity	30	11.72	15	17.24	
Oral tongue	42	16.40	34	39.08	
Oropharynx	6	2.34	2	2.30	
Tonsil	23	8.99	2	2.30	
Tobacco consumption*					
Smoker	144	56.25	48	55.17	0.901
Not reported	112	43.75	39	44.83	
Alcohol consumption*					
No	81	32.53	35	40.70	0.189
Yes	168	67.47	51	59.30	
T clinical*					
T1	17	6.64	6	6.98	0.679
T2	72	28.12	24	27.91	
T3	64	25.00	26	30.23	
T4	93	36.33	29	33.72	
TX	10	3.91	1	1.16	
N clinical*					
N0	109	42.58	41	47.65	0.723
N1	46	17.97	18	20.93	
N2	86	33.59	23	26.74	
N3	6	2.34	1	1.16	
NX	9	3.52	3	3.49	
M clinical*					
M0	238	93.33	82	95.34	0.371
M1	3	1.18	2	2.33	
MX	14	5.49	2	2.33	
Tumor stage*					
I	8	3.23	3	3.53	0.872
II	43	17.34	17	20.00	
III	50	20.16	19	22.35	
IV	147	59.27	46	54.12	
Histologic grade*					
G1	26	10.24	13	14.94	0.020
G2	136	53.54	59	67.82	
G3	75	29.53	13	14.94	
G4	6	2.36	0	0	
GX	11	4.33	2	2.30	
Lymphovascular invasion*					
No	97	59.15	40	65.57	0.443
Yes	67	40.85	21	34.43	
Perineural invasion*					
No	102	60.00	27	42.86	0.026
Yes	68	40.00	36	57.14	
HPV p16 status*					
Negative	40	64.52	14	93.33	0.031
Positive	22	35.48	1	6.67	
TOTAL	256	100	87	100	

* Patients with unknown information excluded. Fisher test used for gender, Tobacco consumption, Alcohol consumption, Lymphovascular invasion, Perineural invasion, HPV p16 status. Chi-square test used for Age, Ethnic group, Tumor site, T, N, M clinical, Tumor stage, Histologic grade. $p < 0.05$ was considered statistically significant.

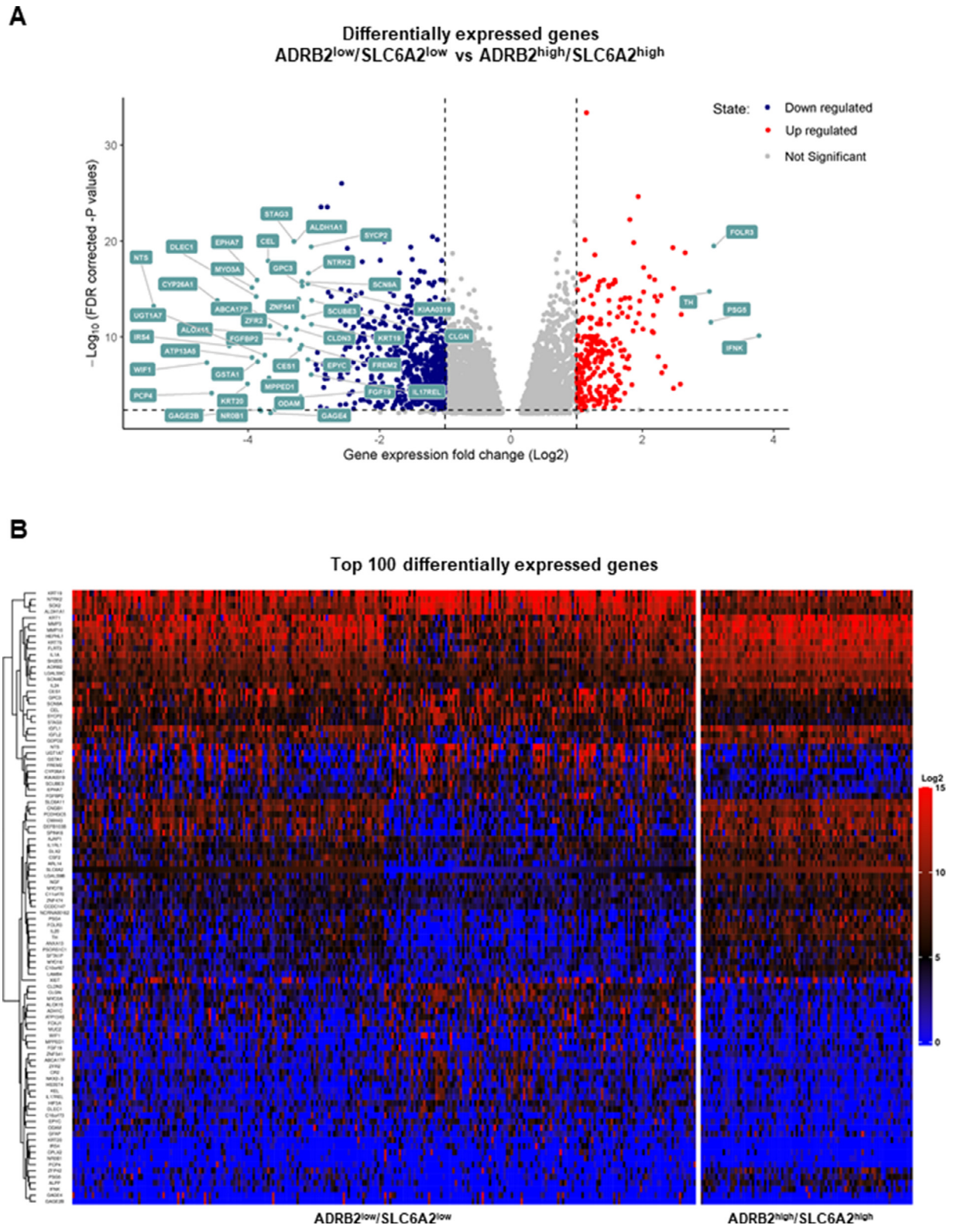

Fig. 1. DEGs based on ADRB2 and SLC6A2 expression in HNSCC. (A) Volcano plot of the 898 DEGs between ADRB2^{low} / SLC6A2^{low} versus ADRB2^{high} / SLC6A2^{high} tumors. Genes in green had logFC>±3. (B) Heatmap showing the top 100 DEGs (50 upregulated and 50 downregulated) between ADRB2^{low}/SLC6A2^{low} versus ADRB2^{high}/SLC6A2^{high} tumors. Complete data in **Supplementary file 2.**

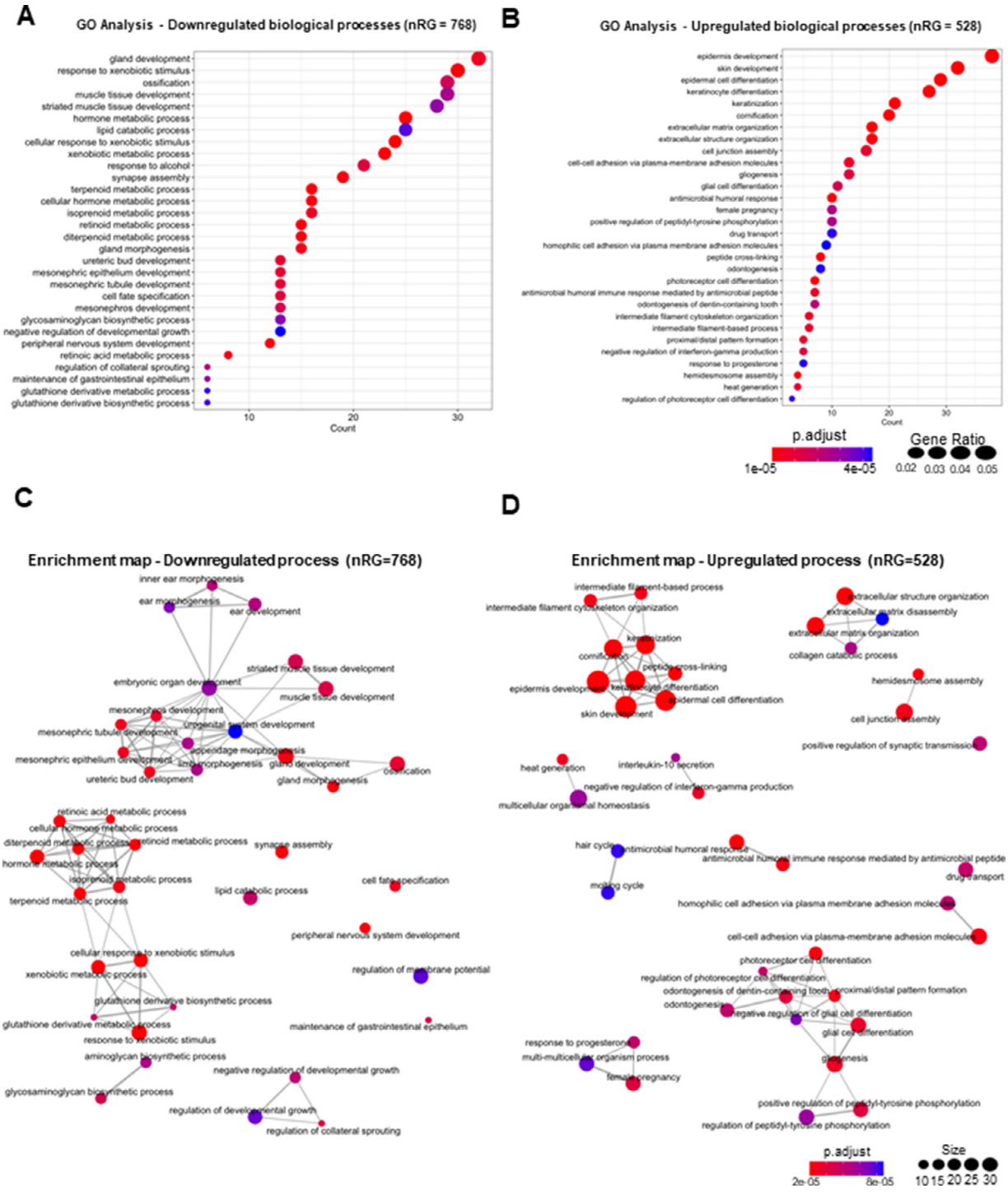

Fig. 2. GO and pathway enrichment analysis of DEGs in HNSCCs. (A) Downregulated and (B) Upregulated biological processes identified in ADRB2^{high}/SLC6A2^{high} tumors. (C-D) Enrichment maps showing the interactions among the processes from A and B. Complete data in **Supplementary file 3**.

the beta-adrenergic pathway activation on the vascular and nervous tissues.

Interestingly, proteins related to cancer cell stemness, namely SOX2, ALDH1A1, and EYA1 were underexpressed (Fig. 4C) in ADRB2^{high} / SLC6A2^{high} group. To further assess if ADRB2^{high} / SLC6A2^{high} group was associated to a lower degree of stemness, we calculated the stemness score [24] of both groups. Indeed, we found higher stemness index in ADRB2^{low}/ SLC6A2^{low} HNSCCs than in ADRB2^{high} / SLC6A2^{high} HNSCCs (p = 0.0417, Fig. 4D).

ADRB2^{high}/SLC6A2^{high} predicts worst prognosis of patients with pharyngeal and laryngeal squamous cell carcinoma

Clinical follow-up of the 343 patients with HNSCC ranged from 0 to 6417 days. Survival analysis indicated that the beta-adrenergic pathway activation did not influence the prognosis of the patients with HNSCCs (p = 0.173, Supplementary Figure 2A). When the tumors were analyzed according to their anatomic location, patients with larynx and pharynx squamous cell carcinomas presenting ADRB2^{high}/SLC6A2^{high}

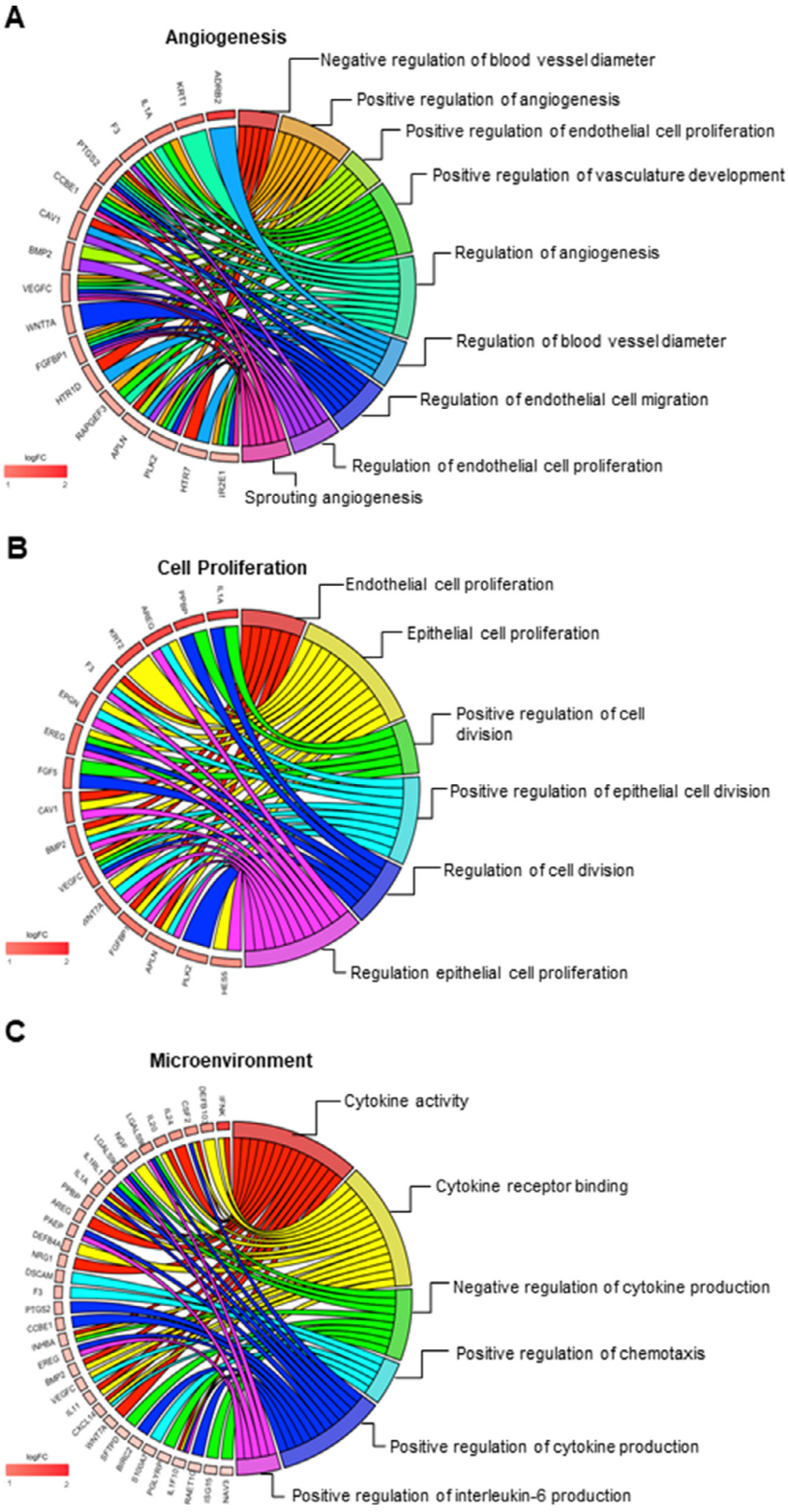

Fig. 3. Upregulated processes in ADRB2^{high} / SLC6A2^{high} HNSCC. DEGs and their relationship with the biological processes within the (A) angiogenesis, (B) cell proliferation, and (C) microenvironment classifications. The biological processes identified in the GO analysis were group according to their major role in HNSCC.

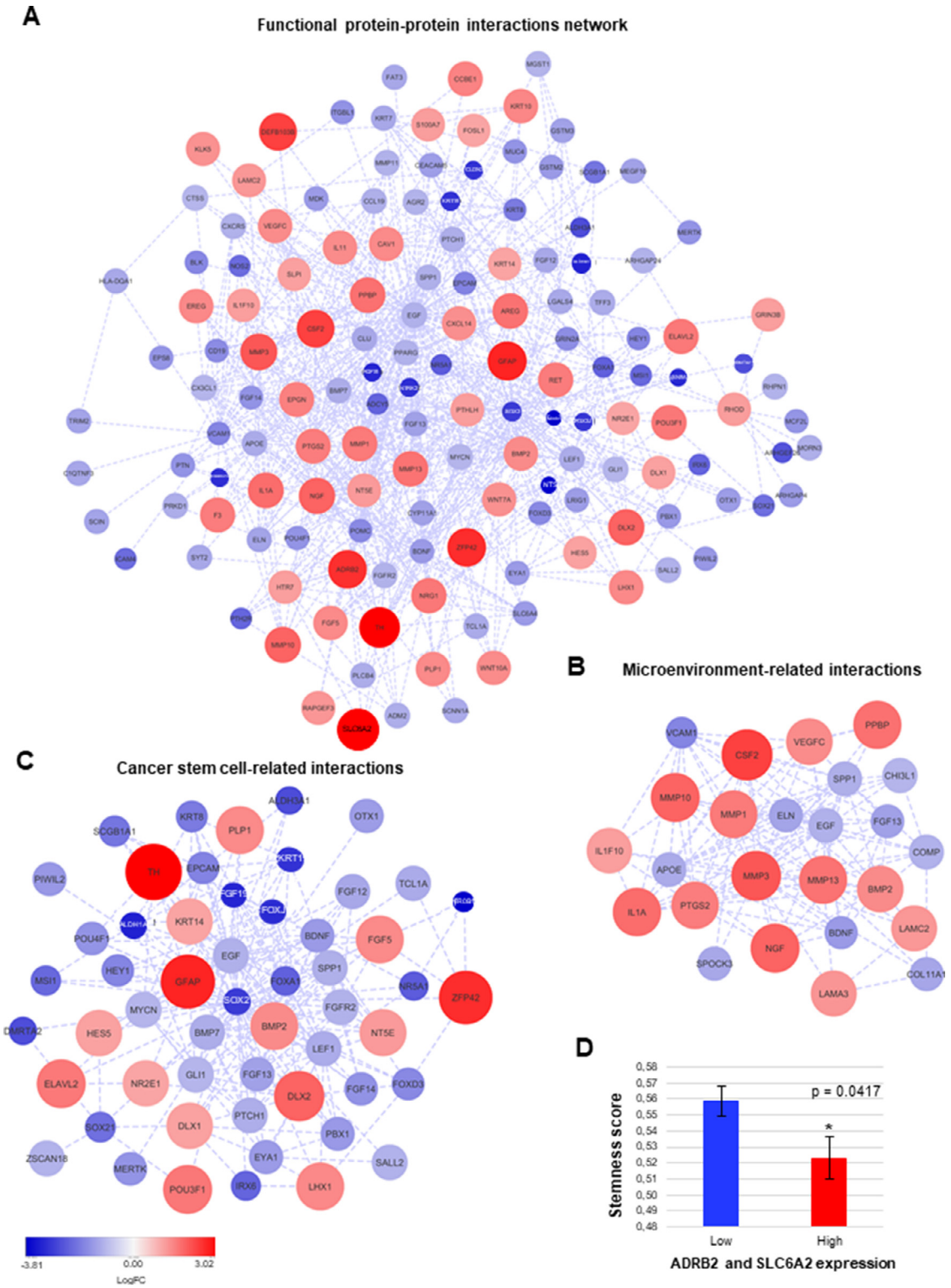

Fig. 4. PPI network of the DEGs in HNSCC. (A) Functional protein interactions network, (B) Microenvironment-related interactions, and (C) Cancer stem cell-related interactions in ADRB2^{high}/SLC6A2^{high} group. (D) Stemness score of ADRB2^{high}/ SLC6A2^{high} compared with ADRB2^{low}/ SLC6A2^{low} tumors. Student's t test.

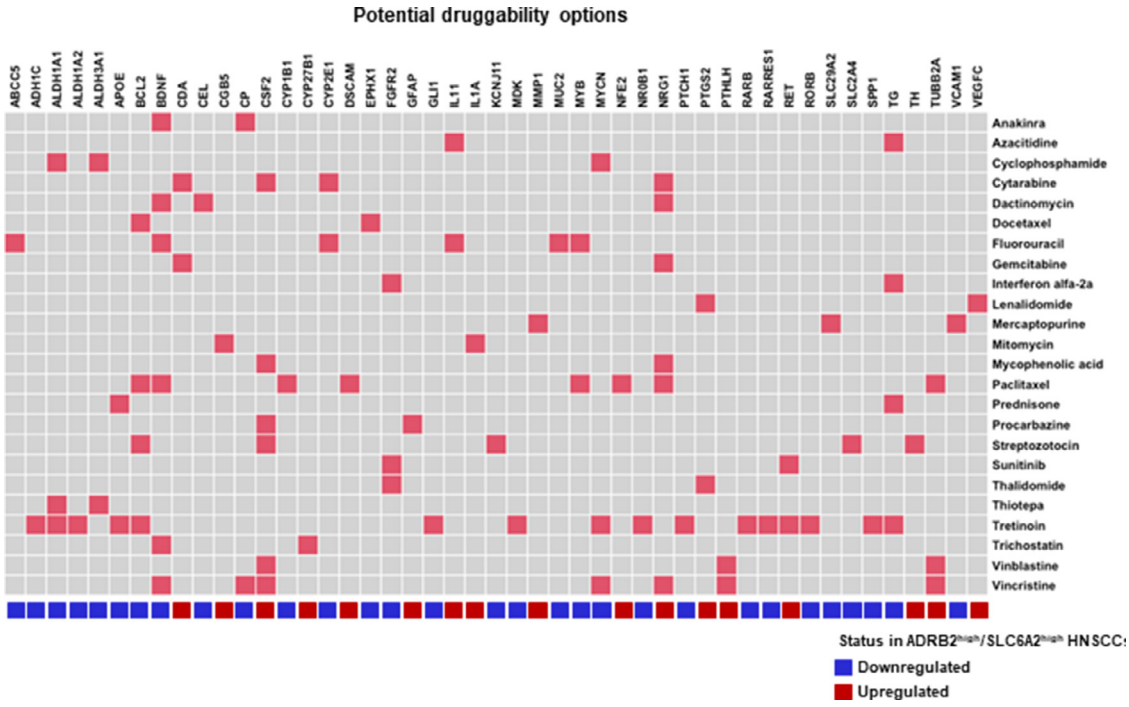

Fig. 5. Drug-gene interaction analysis and potential therapeutics for HNSCC. Heatmap showing the top-drugs and respective target-DEGs in HNSCC. Complete data in **Supplementary file 4.**

expression had lower survival rates than patients with HNSCC presenting ADRB2^{low}/SLC6A2^{low} expression (p=0.039, Supplementary Figure 2C). Beta-adrenergic pathway was not a prognostic predictor factor for oral cavity HNSCCs (p = 0.839, **Supplementary Figure 2B**).

Drug-gene interaction analysis reveals potential therapeutic options for HNSCC

The DEGs were inserted into the DGIdb database to explore the potential therapeutic options available for these genes. It was identified 56 FDA-approved antineoplastic and immunotherapeutic drugs that had 113 genes as targets **(Supplementary file 4)**. In the Fig. 5, drugs targeting two or more genes are displayed.

Among the listed drugs, docetaxel, fluorouracil, gemcitabine, and mitomycin are already used or in advanced phases of clinical trials for HNSCC therapy. Additionally, anakira, azacytidine, paclitaxel, sunitinib, and trichostatin are currently in pre-clinical or early clinical phases in studies of HNSCC therapy. Cyclophosphamide, lenalidomide, thalidomide, vinblastine, and vincristine have shown unsatisfactory results in HNSCC treatment in previous studies. Dactinomycin, mercaptopurine, mycophenolic acid, prednisone, procarbazine, streptozotocin, thiotepa, and tretinoin have not yet been tested as HNSCC therapy.

Discussion

Defining the role of chronic stress in HNSCC progression and outcome has been proven a challenging task. This might be explained by the heterogeneity among HNSCC from different anatomic sites and also by the complexity within the tumor bulk [35]. In an attempt to overcome this last limitation, we performed an integrated analysis of the clinical and molecular aspects of 520 HNSCC patients based on the beta-adrenergic pathway activation. Applying bioinformatics methods, we identified, for the first time, 898 DEGs between the ADRB2^{low} / SLC6A2^{low} and ADRB2^{high} / SLC6A2^{high} groups. By exploring these DEGs, several pathways related to cancer progression were shown to be altered and, ultimately, some DEGs were identified as potential targets for the personalized treatment of HNSCC.

Here we identified a group of alterations in the microenvironment of ADRB2^{high} / SLC6A2^{high} HNSCCs that are pivotal to the tumoral progression. Beta-adrenergic signaling significantly led to the enhancement of metalloproteinases (MMP1, MMP3, MMP10, and MMP13) and laminins (LAMC2 and LAMA3) expression, corroborating others' findings [14]. These molecules are directly implicated in the extracellular matrix (ECM) remodeling, facilitating tumor cell invasion, and contributing to the metastasis [36,37]. Furthermore, we have observed decreased expression of cell adhesion molecules (EPCAM, VCAM1, and ICAM4), what might potentiate the motility of cells of the tumoral microenvironment.

Besides dysregulating the ECM components, the activation of the beta-adrenergic pathway was shown to significantly stimulate the angiogenesis by the overexpression of VEGFC, as shown previously [14]. Additionally, ADRB2^{high} / SLC6A2^{high} HNSCCs presented higher expression of NGF, implicated in cancer cells dissemination and perineural invasion in HNSCC [38]. Indeed, our clinicopathological findings confirmed the molecular data: more patients with beta-adrenergic pathway overactivation displayed perineural invasion than patients with ADRB2^{low} / SLC6A2^{low} expression (p = 0.026, Table 1).

Inflammation has been shown to exert a dual role in HNSCC, and the activation of the beta-adrenergic receptors upregulated the expression of genes involved in inflammation [14,39]. In this study, we also found higher expression of pro-inflammatory genes (PTGS2/COX2 and IL1A), suggesting the close relationship between the beta-adrenergic pathway and inflammation. Notably, PTGS2 expression has been associated with poorer prognosis, and resistance to cisplatin in gastric cancer [40] and was significantly upregulated in ADRB2^{high} / SLC6A2^{high} HNSCCs.

Another notable facet of the activation of the beta-adrenergic pathway in HNSCC was that patients with ADRB2^{high} / SLC6A2^{high} expression presented underexpression of genes related to cancer cell stemness (SOX2, ALDH1A1, and EYA1) and lower stemness score. The genuine cancer stem cells comprise only about 5% [41] of the tumoral bulk, but the remaining cells present different grades of differentiation due to the

epithelial-mesenchymal transition. Thus, the term stemness refers to a less differentiated state of the malignant cell rather than the pure CSC [24]. Interestingly, our pathological findings demonstrated that less patients of the ADRB2^{high} / SLC6A2^{high} group presented tumor graded as less differentiated (G3 or G4, ADRB2^{low} / SLC6A2^{low}: 31.89% vs ADRB2^{high} / SLC6A2^{high}:14.94%, $p = 0.020$, Table 1). These findings related to stemness may seem contradictory to our data above: ADRB2^{high} / SLC6A2^{high} tumors present more aggressive and invasive behavior but have lower stemness score. However, the stemness is best defined as transitory rather permanent state [42]. Thus, those tumors in frankly expansion might exhibit a temporary phenotype related to the local invasion, proliferation, and angiogenesis. On the other hand, those tumors in a less active state may have lower metabolic activity, and increased stemness [43]. In this context, the plasticity of the cancer cells is essential to the success of the tumoral bulk to invade locally, metastasize, and be resistant to the therapy.

Finally, our results showed that the beta-adrenergic pathway activation did not influence the prognosis of HNSCC. However, when the lesions were split according to their anatomic location, patients with tumors arising in the larynx and pharynx that presented ADRB2^{high} / SLC6A2^{high} expression had lower survival rates than patients with ADRB2^{low} / SLC6A2^{low} HNSCCs. Curiously, the patients with ADRB2^{low} / SLC6A2^{low} expression showed greater number of patients with HPV p16 positive (35.48% vs 6.67%, $p = 0.031$). This finding may be explained by the higher percentage of tumors arising in the oropharyngeal region in ADRB2^{low} / SLC6A2^{low} than in ADRB2^{high} / SLC6A2^{high} tumors ($p = 0.0001$).

As the concomitant high expression of ADRB2 and SLC6A2 was a prognostic factor for larynx and pharynx squamous cell carcinomas ($p = 0.039$), we identified some drugs that may be an interesting option to the management of these tumors. Interestingly, some drugs that are already used in the management of HNSCC (docetaxel, fluorouracil, and gemcitabine) came up as an option. This can explain the high frequency of resistant tumors to these therapies: maybe the tumor heterogeneity among the patients may incite the drug resistance in some individuals and the successful treatment in others. Furthermore, we also identified other drugs that are currently under investigation and underscore that the results of these clinical trials should be analyzed carefully in the light of the personalized medicine: we may be missing drugs that would work properly in one patient but not in another.

This study has some limitations, as follows: 1) The lack of clinical information about the mental status of the patients. This study used the activation of the beta-adrenergic pathway as a read-out of for the chronic stress however, one cannot confirm if the patients were, indeed, with clinical chronic stress; 2) This study analyzed HNSCCs of different anatomic regions collectively. This may have masked individual features of the tumors as the behavior of HNSCC varies according to the where it arises. Thus, as future perspectives, we suggest a clinical study with information on the level of stress (psychological tests), and hormone dosage (plasma and/or saliva). Additionally, validation of our data in different databases may be interesting to verify if the DEGs identified in this study might be used as a molecular signature of a more aggressive HNSCC subtype.

In conclusion, this was the first study to provide a comprehensive panorama of the beta-adrenergic pathway activation repercussion in HNSCC. We have shown that beta- adrenergic pathway overexpression presents intimate relationship with the tumoral proliferation, invasion, migration, and angiogenesis. Concomitant high expression of ADRB2 and SLC6A2 was a prognostic factor for larynx and pharynx squamous cell carcinomas. Finally, we demonstrated a panel with possible treatment for patients with HNSCC.

Declaration of Competing Interest

The authors declare they do not have conflict of interest.

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Author Contribution

G.L.S Methodology, Data curation, Formal analysis, Investigation, and Writing the original draft. D.G.B Visualization, and Review and editing. G.I.M Visualization, and Review and editing. K.C.T Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Project administration, and Review and editing.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101117.

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