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Research Article

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The neuropeptide Adrenomedullin Promoted the Progression of Head and Neck Squamous Carcinoma by Negatively Regulating Tumour Immunity

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Abstract

Objective: *To investigate the putative regulatory mechanisms and functions of the neuropeptide gene adrenomedullin (ADM) in head and neck squamous cell carcinoma (HNSC).*

Methods: *Publicly available data from the TCGA-HNSC database were utilized to explore the involvement of ADM single gene in HNSC by analyzing RNA expression levels of ADM. The involvement of ADM in tumor immunity was particularly investigated from the aspect of tumor-infiltrating immune cells, immune modulator genes, immune checkpoint genes, Estimate-Immune-Stromal score, and immune clusters' prognostic values. Statistical analysis of the data pertaining to cancer and non-cancerous samples was performed using R software packages. Many web servers were used in the present analyses, including cBioportal, TIMER, STRING, GeneMANIA, and GEPIA.*

Results: *ADM was found to be significantly upregulated in HNSC tumor samples and associated with worse prognostic outcomes. ADM-significantly correlated genes in HNSC were enriched in several tumor promoting pathways, including cytokine-cytokine receptor interaction, HIF-1 signaling, MAPK signaling, chemokine signaling, AGE-RAGE signaling, Relaxin signaling, viral protein interaction with cytokine and cytokine receptor, and NF-kappa B signaling. ADM is involved in tumor immunity of HNSC by being negatively correlated with several tumor-infiltrating immune cells, including tumor macrophages, T cells, B cells, Treg cells, T helper cells, Th17 cells, NK cells, mast cells, and dendritic cells. The negative correlation was observed between ADM expression and the majority of immunomodulator genes. ADM was also found to be negatively correlated with the Estimate-Stromal-Immune score in HNSC, indicating its involvement in the tumor immune microenvironment.*

Conclusions: *ADM gene may play a significant role in promoting the progression of HNSC by negatively regulating immune cells, mediating cytokine and chemokine signaling, HIF-1 signaling, MAPK signaling, and NF-kappa B signaling. Therefore ADM can be regarded as a valuable candidate biomarker and holds promising prospects in treating* ***head and*** *neck cancer.*

Keywords: Neuropeptide, Adrenomedullin, Bioinformatics Analysis, Head and Neck Squamous Cell Carcinoma, Survival, Tumor Immunity.

Introduction

Adrenomedullin (ADM), as a known vasoactive neuropeptide, has gained much attention due to its various functions, such as peripheral vasodilatory actions, immunological function, cardiovascular and lymphatic development, as well as tumor progression [1]. ADM has been found to be abundantly expressed in many types of cancers, for instance, breast cancer, lung carcinoma, clear cell renal cell carcinoma, and prostate cancer [2]. Previous literature has strongly supported that by means of using either an ADM-specific antibody or an ADM-antagonist could block the production of ADM protein in cancer cells and tumor-associated macrophages (TAMs) and thereby further inhibit the progression of cancers [3].

The potential role of ADM in cancers was manifested in many aspects, including promoting angiogenesis, inhibiting apoptosis, and involving immunology. Chen et al. Have ever found that ADM can promote the angiogenic process in epithelial ovarian carcinoma by upregulating the expression level of vascular endothelial growth factor (VEGF) [4]. In addition, ADM was demonstrated to be a survival factor in inhibiting hypoxic-induced apoptosis of

osteosarcoma cells, with the underlying mechanisms of activating the MEK/ERK1/2 signaling pathway [5]. Furthermore, tumor-associated macrophage as a type of tumor-infiltrating immune cells involved in tumor immunosuppression, has been found to secret ADM and further promote the proliferation of tumor cells, thereby indicating the involvement of ADM in tumor immunology [6].

Head and neck cancer belongs to be the sixth most commonly occurring malignant tumor in the world. The majority type of head and neck cancer belongs to head and neck squamous cell carcinomas (HNSC) [7]. Several neuropeptide genes have been demonstrated well in the head and neck squamous cell carcinoma (HNSC) because of their potential prognostic values, for instance, neuropeptide Y (NPY), somatostatin (SST), hypocretin neuropeptide precursor (HCRT), tachykinin precursor 1 (TAC1), as well as galanin (GAL) [8]. However, to date, the clinical significance and the regulating role of ADM in HNSC remain unclear. In order to integratively and comprehensively investigate the role of the ADM single gene in HNSC, the current study performed bioinformatics techniques to achieve this research gap.

Materials and Methods

Protein expression of ADM in Cancers

The Human Protein Atlas (HPA) database provided access to tissue and cell distribution information and was available for public inquiries. The ADM protein expression in HNSC tumor tissue was searched firstly by selecting Pathology Atlas. Subsequently, normal tissue was selected to identify the expression of ADM in the nasopharyngeal and oral mucosa.

Patient datasets of TCGA-HNSCC data set

The mRNA expression data (546 samples including 502 HNSC samples and 44 adjacent healthy control samples, Workflow Type: HTSeq-FPKM (Fragments Per Kilobase per Million)) and their respective clinicopathological characteristics data were procured from the TCGA (The Cancer Genome Atlas) database (URL: https://cancergenome.nih.gov). The samples with almost zero expression value or without enough survival information were removed from the subsequent analysis. As a result, 502 HNSC samples and their respective clinicopathological characteristics were included to be analyzed in the current research. The RNA sequencing data were grouped depending on the expression levels of ADM[ENSG00000148926]. In other words, the sample data for which the ADM expression level was higher than the median values were grouped as the ADM-high expression group, while the sample data for which the ADM expression level was lower than the median values were grouped as the ADM-low expression group.

The correlation relationship between ADM expression and clinical features

The relationship between ADM expression and clinical features was investigated by using the logistics regression analysis based on the binary logistics model. During the analysis process of logistics regression, the ADM gene expression level was regarded as the independent variable, while the clinicopathological variables were regarded as the dependent variables. The independent variable-ADM was divided into two different categories, including low expression of ADM and high expression of ADM, among which the low expression of ADM was used as the reference. The dependent variable-characteristics were also divided into two different categories, for example, T3&T4&T2 stage and T1 stage; N1&N2&N3 stage and N0 stage; M1 stage and M0 stage; Stage III&Stage IV and Stage I&Stage II; With radiation therapy and without receiving radiation therapy; partial response (PR) & complete response (CR) and progression disease (PD)&stable disease(SD); male and female; white and other races (Asian, Black/African American); patients older than 60 and patients younger than 60; histologic grade G3&G4 and G1&G2; smokers and non-smokers; with alcohol history and without alcohol history; with lymphovascular invasion and without lymphovascular invasion; having received lymph node neck dissection and without having received lymph node neck dissection. Among these characteristics, the latter ones were considered as the reference.

Survival analysis

Among the tumor samples of the TCGA-HNSC data set, only cancer samples with sufficient survival information were used for carrying out the survival analysis. Kaplan-Meier plots were used for visualizing the difference in survival probability between the high and low ADM gene expression groups. The prognostic values of the ADM gene in HSNCC were calculated by using the p-value, which was examined by the log-rank test. The prognostic value of ADM expression in HNSC patients was analyzed by applying the survival package (version 3.2-13) in the R program (version 3.6.3), and the Kaplan Meier plots were drawn by employing the survminer package (version 0.4.9) in the R program (version 3.6.3). Three prognostic types of survival were analyzed, including overall survival (OS), progress-free interval (PFI), as well as disease-specific survival (DSS).

Univariate/multivariate Cox analysis

The relationship between pathological parameters and tumor prognosis was evaluated by single-gene or prognostic models. The method can be implemented in R through the survival package. Nomogram and calibration plot was also constructed.

Protein (PPI) and Gene (GGI) interplay networks

The 3D pinball PPI diagram was arranged from the STRING database by selecting "homo sapiens" using the differential proteins analyses. The results after retrieving ADM in GeneMania were reasonably layout for GGI analysis.

Identification of the highly correlated genes of ADM

The gene correlation analysis was carried out in order to explore the genes which were correlated with ADM in HNSC by using TCGA data, and the Pearson correlation coefficient was calculated. The genes which were significantly positively and negatively cor-

related with the ADM gene were identified by using the stat package in the R program. The heatmap showing the expression pattern of the top 10 ADM-positively correlated protein-coding genes was visualized by using the ggplot2 package in the R program.

Functional enrichment analysis of top 100 ADM-correlated genes

The top 100 ADM-positively correlated genes were obtained by ranking the descending order of the Pearson correlation coefficient r value. The top 100 ADM-negatively correlated genes were obtained by ranking the ascending order of the Pearson correlation coefficient r values. The functional enrichment analysis was performed to identify the GO terms and KEGG pathways, which were significantly enriched by the top 100 ADM-positively and negatively correlated genes. Such analysis was performed by clusterProfiler package in the R program, and the bubble charts were used to visualize the enrichment results by using the ggplot2 package in the R program. In addition, gene set enrichment analysis (GSEA) was also investigated to identify the significantly enriched pathways of ADM-highly correlated genes.

Evaluation about if ADM gene expression was correlated with tumour immunity

The research question regarding if ADM gene expression was involved in tumor immunity by impacting the tumor-infiltrating immune cells (TIICs) was addressed by investigating the correlation between the ADM gene and immune cells based on the statistical analysis of Pearson correlation analysis. Such analysis was performed by applying the GSVA package (version 3.14) in the R program (version 3.6.3). The ssGSEA algorithm in the GSVA package was used for calculating the correlation between TIICs and ADM gene expression. The analyzed 24 immune cells consisted of activated dendritic cells, B cells, CD8 T cells, cytotoxic cells, dendritic cells, eosinophils, immature dendritic cells, macrophages, mast cells, neutrophils, NK CD56bright cells, NK CD56dim cells, natural killer cells, Plasmacytoid dendritic cells, T cells, T helper cells, T central memory cells, T effector memory cells, T follicular helper cells, T gamma delta cells, Th1 cells, Th17 cells, Th2 cells, as well as T regulatory cells.

The 15 immune checkpoint genes (ICGs) were selected from the systematic reviews regarding the summary of ICGs in cancer [9, 10], for example, CD274, CTLA4, LAG3, HAVCR2, TIGIT, VSIR, CD276, VTCN1, BTLA, IDO1, CD70, CD40, CD47, TNFRSF18, and TNFSF14. The immune modulator genes, including immunoinhibitor genes and immunostimulator genes, were selected from the TISIDB database (URL: http://cis.hku.hk/TISIDB/), which is an integrated repository portal for tumor-immune system interactions. The correlation between ADM expression and any immune gene's expression in HNSC was evaluated by using Spearman statistical method. The correlation coefficient value "r" less than zero and p-value less than 0.05 represents that the ADM expression was significantly negatively correlated with the immune gene expression in HNSC; conversely, the correlation coefficient value "r" more than zero and p-value less than 0.05 represents that the ADM expression was significantly positively correlated with the immune gene expression in HNSC.

The correlation between ADM and Estimate-Stromal-Immune score was investigated by the ESTIMATE (Estimation of Stromal and Immune cells in Malignant Tumor tissues Using Expression Data) algorithm. TIMER 2.0 webserver was also used for exploring the prognostic values of clusters divided by the low- and high-expression of ADM and several tumor-infiltrating immune cells in HNSC.

Results

Expression of ADM in cancers

Most cancers showed moderate to strong cytoplasmic positivity. Additional membranous positivity was observed in several cancers, e.g., colorectal, liver, pancreatic, skin, and urothelial cancers (Fig 1A). Noticeably, more than 70% of HNSC patients showed high/medium ADM expression. Figure 1B displayed the expression of ADM in different healthy tissue. Fig 1C shows that the level of ADM protein in HNSC tissue was higher than that in normal tissue (Antibody HPA068955).

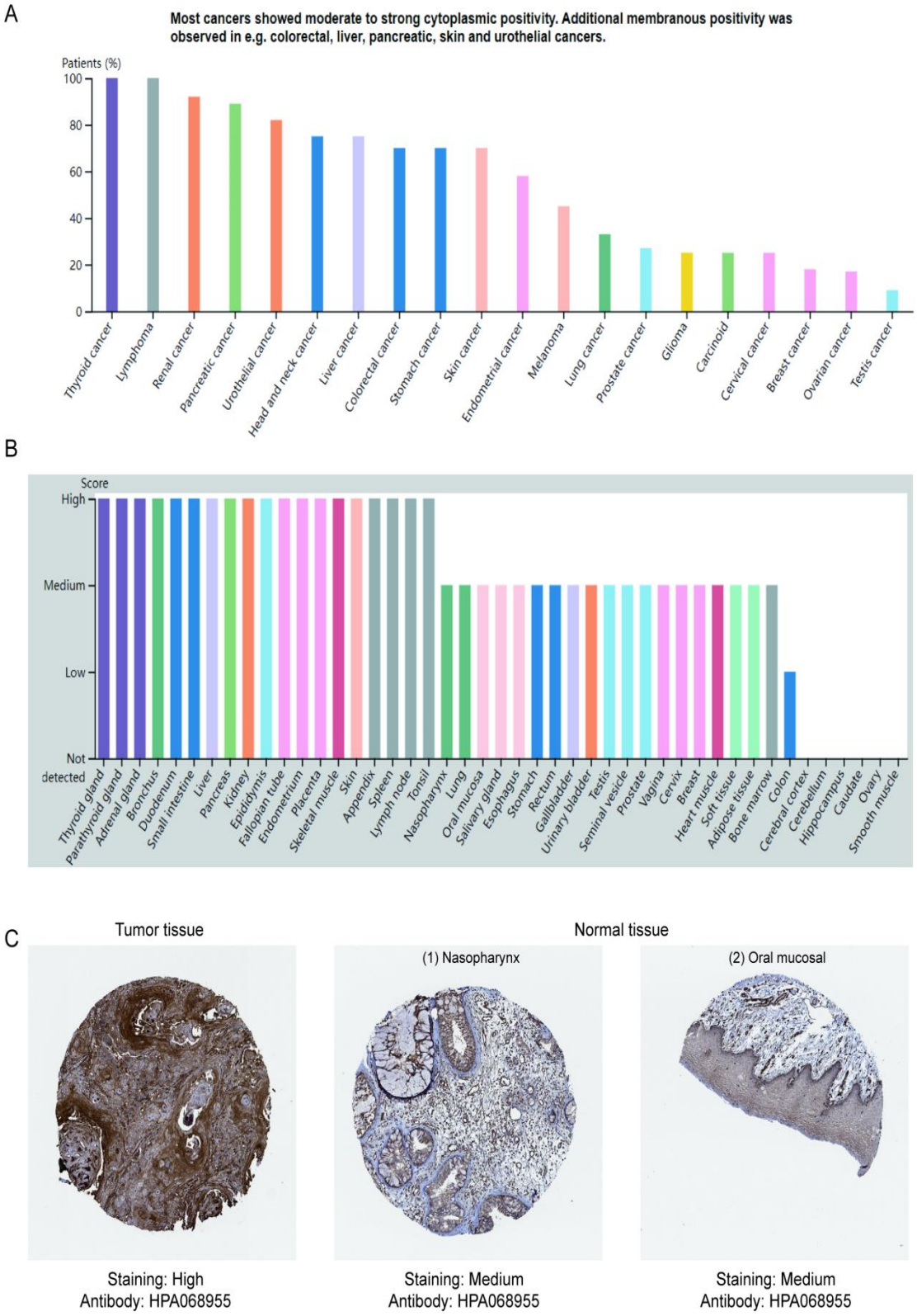

Figure 1: The protein expression of ADM in cancers. (A) Percentage form of ADM expression distribution in pan-cancer patients. (B) Score form of ADM expression distribution in healthy tissues. (C) Immunohistochemical profiles showed the expression of ADM in HNSC and healthy tissues at the protein level.

Characteristics of patients in TCGA-HNSCC data set

A total of 502 HNSC sample data with the respective clinicopathological features were procured from the TCGA database. The clinicopathological features depending on the expression level of ADM genes are exhibited in Table 1.

Table 1. Clinical characteristics of the HNSC patients grouped by the expression level of ADM.

Characteristic	Low expression of ADM	High expression of ADM	p	statistic	method
n	251	251			
T stage, n (%)			< 0.001	23.48	Chisq.test
T1	27 (5.5%)	6 (1.2%)			
T2	82 (16.8%)	62 (12.7%)			
T3	55 (11.3%)	76 (15.6%)			
T4	76 (15.6%)	103 (21.1%)			
N stage, n (%)			0.913		Fisher.test
N0	119 (24.8%)	120 (25%)			
N1	37 (7.7%)	43 (9%)			
N2	77 (16%)	77 (16%)			
N3	4 (0.8%)	3 (0.6%)			
M stage, n (%)			1.000		Fisher.test
M0	235 (49.3%)	237 (49.7%)			
M1	2 (0.4%)	3 (0.6%)			
Clinical stage, n (%)			< 0.001	16.73	Chisq.test
Stage I	16 (3.3%)	3 (0.6%)			
Stage II	56 (11.5%)	39 (8%)			
Stage III	41 (8.4%)	61 (12.5%)			
Stage IV	128 (26.2%)	144 (29.5%)			
Radiation therapy, n (%)			0.020	5.44	Chisq.test
No	89 (20.2%)	65 (14.7%)			
Yes	131 (29.7%)	156 (35.4%)			
Primary therapy outcome, n (%)			0.010		Fisher.test
PD	11 (2.6%)	30 (7.2%)			
SD	3 (0.7%)	3 (0.7%)			
PR	4 (1%)	2 (0.5%)			
CR	190 (45.5%)	175 (41.9%)			
Gender, n (%)			0.480	0.5	Chisq.test
Female	71 (14.1%)	63 (12.5%)			
Male	180 (35.9%)	188 (37.5%)			
Race, n (%)			0.940		Fisher.test
Asian	5 (1%)	5 (1%)			
Black or African American	25 (5.2%)	22 (4.5%)			
White	216 (44.5%)	212 (43.7%)			
Age, n (%)			0.562	0.34	Chisq.test

<=60	126 (25.1%)	119 (23.8%)			
>60	124 (24.8%)	132 (26.3%)			
Histologic grade, n (%)			0.196		Fisher.test
G1	34 (7%)	28 (5.8%)			
G2	141 (29.2%)	159 (32.9%)			
G3	65 (13.5%)	54 (11.2%)			
G4	2 (0.4%)	0 (0%)			
Anatomic neoplasm subdivision, n (%)			0.109	16.97	Chisq.test
Alveolar Ridge	10 (2%)	8 (1.6%)			
Base of tongue	17 (3.4%)	6 (1.2%)			
Buccal Mucosa	8 (1.6%)	14 (2.8%)			
Floor of mouth	29 (5.8%)	32 (6.4%)			
Hard Palate	5 (1%)	2 (0.4%)			
Hypopharynx	4 (0.8%)	6 (1.2%)			
Larynx	46 (9.2%)	65 (12.9%)			
Lip	2 (0.4%)	1 (0.2%)			
Oral Cavity	31 (6.2%)	41 (8.2%)			
Oral Tongue	71 (14.1%)	55 (11%)			
Oropharynx	5 (1%)	4 (0.8%)			
Tonsil	23 (4.6%)	17 (3.4%)			
Smoker, n (%)			0.564	0.33	Chisq.test
No	58 (11.8%)	53 (10.8%)			
Yes	185 (37.6%)	196 (39.8%)			
Alcohol history, n (%)			0.071	3.26	Chisq.test
No	89 (18.1%)	69 (14.1%)			
Yes	157 (32%)	176 (35.8%)			
Lymphovascular invasion, n (%)			0.044	4.04	Chisq.test
No	116 (34%)	103 (30.2%)			
Yes	50 (14.7%)	72 (21.1%)			
Lymphnode neck dissection, n (%)			0.028	4.8	Chisq.test
No	55 (11%)	35 (7%)			
Yes	195 (39.1%)	214 (42.9%)			
OS event, n (%)			0.038	4.29	Chisq.test
Alive	154 (30.7%)	130 (25.9%)			
Dead	97 (19.3%)	121 (24.1%)			
DSS event, n (%)			0.390	0.74	Chisq.test
Alive	180 (37.7%)	167 (35%)			
Dead	61 (12.8%)	69 (14.5%)			
PFI event, n (%)			0.233	1.42	Chisq.test

Alive	161 (32.1%)	147 (29.3%)			
Dead	90 (17.9%)	104 (20.7%)			
Age, meidan (IQR)	60 (54, 69)	61 (53, 68)	0.862	31656	Wilcoxon

Expression pattern of ADM in pan-cancer and HNSC

Fig 2A shows that the genetic mutation of ADM occurred in 0.3% of HNSC patients. Fig 2B shows that there was a significant correlation between ADM copy-number value and mRNA expression in HNSC samples (capped relative linear copy-number values: r=0.18, p=2.14e-7 (Pearson); Log2 copy-number values: r=0.17, p=7.537e-5 (Pearson)). This result indicated that the alteration of ADM copy numbers was the mechanism that caused the upregulation of ADM among HNSC patients.

The transcription levels of the ADM gene in pan-cancers were evaluated based on the TCGA RNA-seq data and shown in Fig 2C-D. As seen from Fig 2C-D, ADM mRNA expression was remarkably dysregulated in several cancers (e.g., BRCA (Breast invasive carcinoma), ESCA (Esophageal carcinoma), GBM (Glioblastoma multiforme), HNSC (Head and Neck squamous cell carcinoma), KICH (Kidney Chromophobe), KIRP (Kidney renal papillary cell carcinoma), LIHC (Liver hepatocellular carcinoma), LUSC (Lung squamous cell carcinoma), PCPG (Pheochromocytoma and Paraganglioma), THCA (Thyroid carcinoma), and UCEC (Uterine Corpus Endometrial Carcinoma)), compared with healthy control samples. With similar results of pan-cancer analysis, the ADM gene was significantly upregulated in HNSC samples compared with healthy control oral samples.

The results of unpaired sample analysis (Fig 2E) indicated that the ADM gene expression was significantly higher in HNSC samples than in healthy control samples. The median difference between 502 HNSC tumor samples and 44 adjacent healthy control head and neck samples was 1.175(0.813 - 1.536), with statistically significant differences ($t = 6.385$, $P < 0.001$). The paired sample t-test (Fig 2E) showed that the expression of ADM in HNSC tumor samples was significantly higher than that in healthy control samples. The median difference between 43 HNSC tumor samples and their matched 43 adjacent healthy control head and neck samples was 1.489(1.019 - 1.96), with statistically significant differences ($t = 6.386$, $P < 0.001$). In addition, Fig 2F shows the three-dimensional protein structure of ADM visualized by the cBioportal web server.

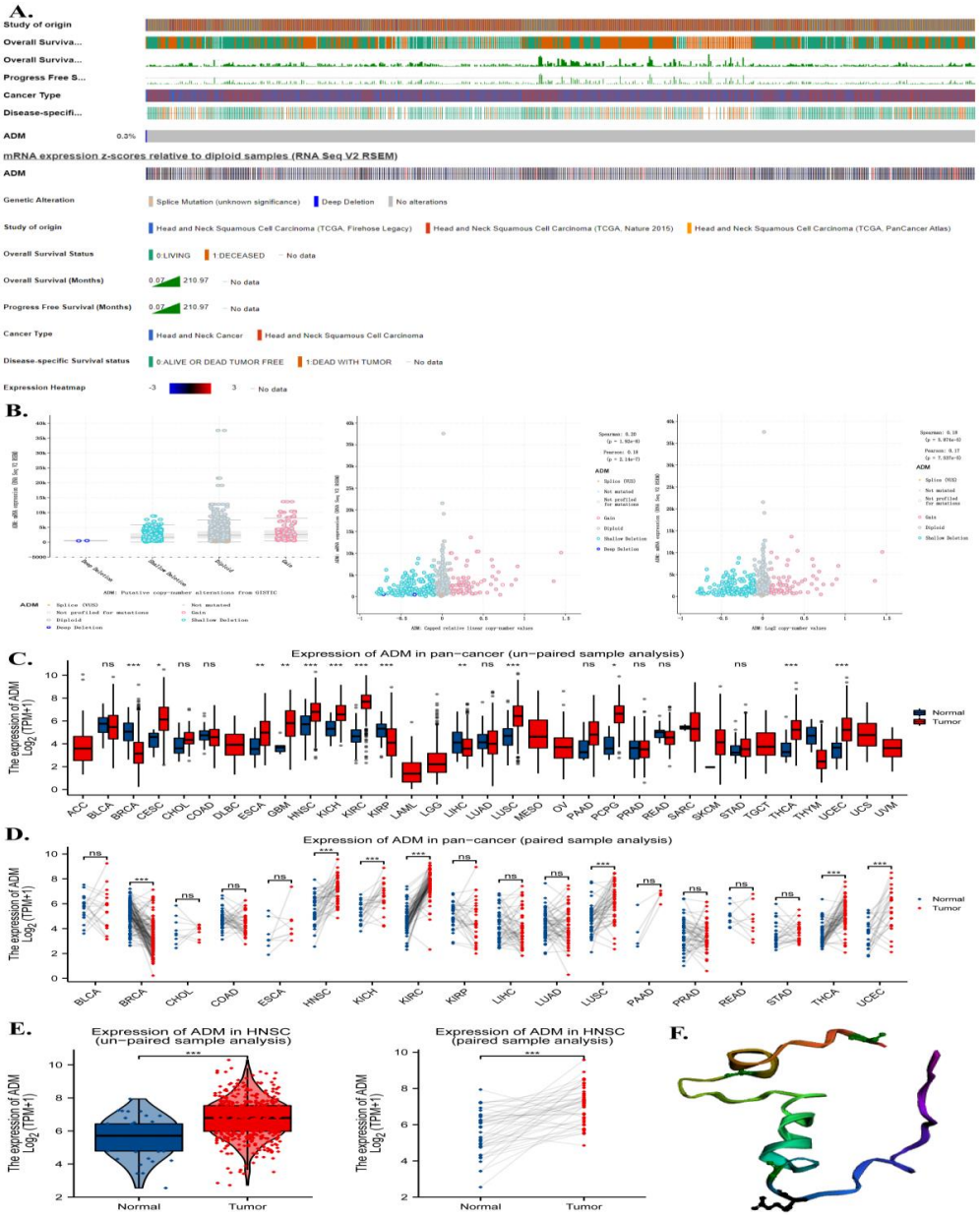

Figure 2: The expression pattern of ADM gene in pan-cancers including HNSC. (A) The Oncoprint shows the genetic mutations of ADM in HSNC patients; (B) The correlation between the ADM expression and copy number alterations in HNSC; (C) The un-paired sample analyses showing the expression level of ADM mRNA in multiple pan-cancers; (D) The paired sample analyses showing the expression level of ADM mRNA in multiple pan-cancers; (E) The unpaired and paired sample analyses showing the expression level of ADM mRNA in HNSC; (F) The three-dimensional protein structure of ADM.

Evaluation of the relationship between ADM expression level and clinicopathological variables

Table 2 shows the association between ADM expression level and various clinicopathological variables in HNSC data. Each row in the table represents a binary Logistics regression model. The independent variable is the gene ADM, and the low expression of ADM is the reference of the independent variable; the dependent variable is the clinicopathological characteristics, and the characteristics on the right side of vs. in brackets is the reference of the dependent variable. Results of Table 2 show that ADM expression is significantly related to several clinicopathological characteristics, including T stage ($p < 0.001$); clinical stage ($p = 0.004$); radiation therapy ($p = 0.026$); primary therapy outcome ($p < 0.001$); alcohol history ($p = 0.037$); lymph node neck dissection ($p = 0.012$); and lymphovascular invasion ($p = 0.036$).

Table 2: Logistic regression results show the association between ADM expression and clinical features.

Characteristics	Total(N)	Odds Ratio(OR)	P value
T stage (T3&T4 vs. T1&T2)	487	2.190 (1.505-3.205)	<0.001
N stage (N1&N2&N3 vs. N0)	480	0.920 (0.643-1.316)	0.647
M stage (M1 vs. M0)	477	0.655 (0.086-3.990)	0.645
Clinical stage (Stage III&Stage IV vs. Stage I&Stage II)	488	1.889 (1.235-2.914)	0.004
Radiation therapy (No vs. Yes)	441	0.639 (0.429-0.946)	0.026
Primary therapy outcome (PD&SD vs. PR&CR)	418	3.197 (1.652-6.600)	<0.001
Gender (Male vs. Female)	502	1.277 (0.860-1.903)	0.226
Race (Asian&Black or African American vs. White)	485	0.984 (0.564-1.713)	0.954
Age (>60 vs. <=60)	501	1.164 (0.820-1.653)	0.397
Histologic grade (G3&G4 vs. G1&G2)	483	0.862 (0.570-1.301)	0.479
Smoker (Yes vs. No)	492	1.348 (0.883-2.067)	0.168
Alcohol history (Yes vs. No)	491	1.502 (1.027-2.204)	0.037
Lymphnode neck dissection (No vs. Yes)	499	0.548 (0.340-0.871)	0.012
Lymphovascular invasion (Yes vs. No)	341	1.617 (1.035-2.543)	0.036

Survival analysis showing ADM to be an independent risk factor of HNSC

Fig 3A shows that the high expression of ADM indicated worse overall survival outcomes in several cancer types, including CESC, GBM, HNSC, LGG, LIHC, LUAD, MESO, and UCS. Fig 3B shows that the high expression of ADM indicated worse disease-free survival outcomes in GBM, KICH, LGG, LIHC, SARC, and UCS.

The survival analysis visualized by Kaplan-Meier curves showed that ADM should be regarded as an independent risk factor of HNSC (p = 0.04) (Fig 3C). The high expression of the ADM gene represented the worse prognosis; in contrast, the low expression of the ADM gene represented the better prognosis. ADM expression was not associated with disease-specific survival (p=0.22) and progress-free survival (p=0.08).

Both univariate and multivariate Cox analyses further confirmed that high ADM expression was an independent risk factor for OS in HNSC patients (Fig 3D). Other clinicopathological features were also associated with poorer prognosis in HNSC, including N stage, radiation therapy, and primary therapy outcome. Fig 3E shows the nomogram plot for evaluating the survival probability according to the points scored by clinical features. Fig 3F shows the calibration plot for assessing the prediction accuracy of the model constructed in the nomogram plot.

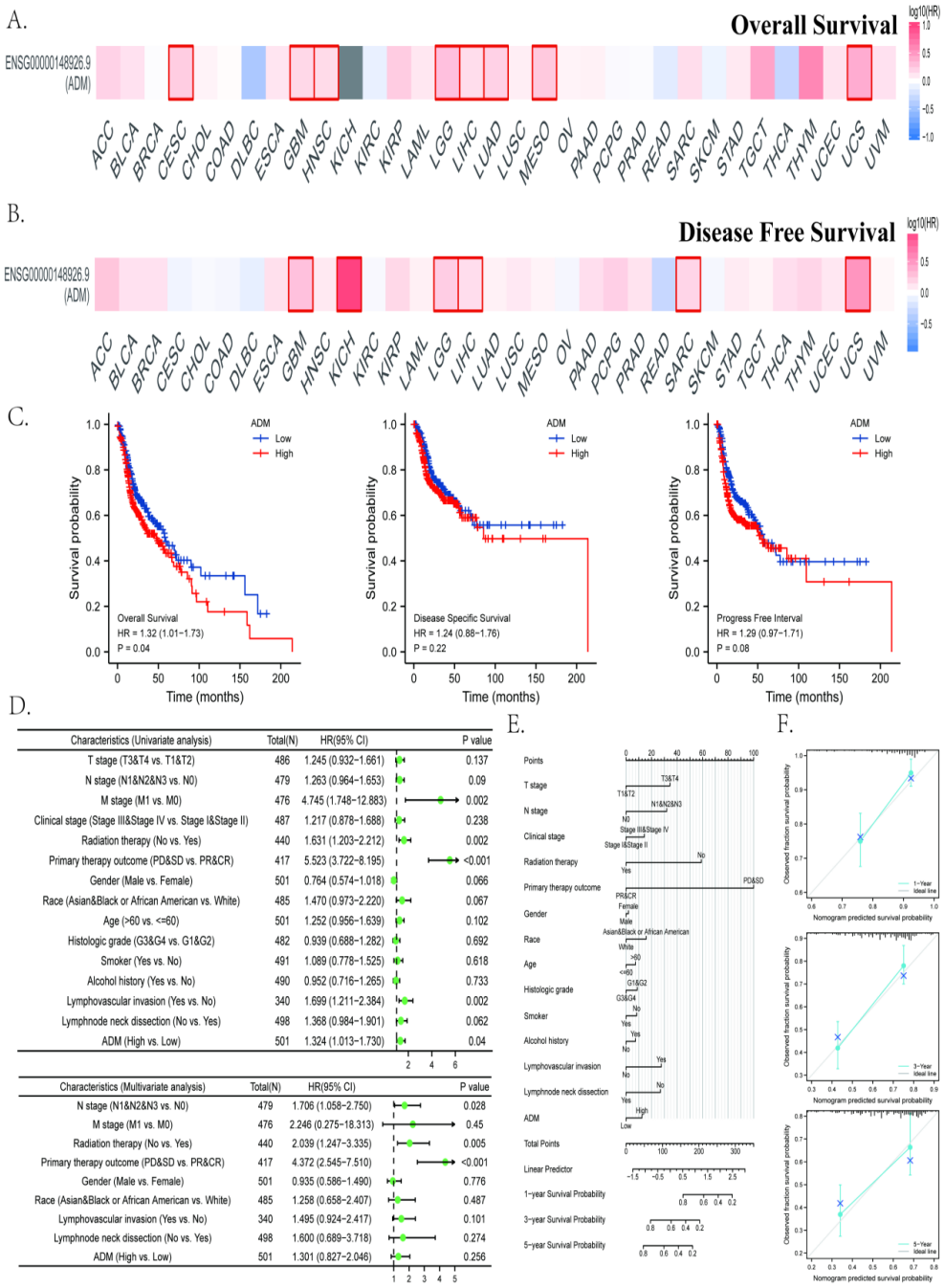

Figure 3: Survival analysis results regarding ADM. (A) The survival map showing the prognostic values of ADM in overall survival of multiple pan-cancer; (B) The survival map showing the prognostic values of ADM in disease-free survival of multiple pan-cancers; (C) The association between ADM expression and different survival outcomes (overall survival, disease-specific survival, progress-free survival) in HSNC; (D) The results of univariate and multivariate analyses; (E) The nomogram plot used for predicting the survival probability; (F) The calibration plot used for predicting the accuracy of the model constructed in nomogram plot.

ROC curves analyses results

The diagnostic value of ADM expression was evaluated by the ROC curve. Fig 4 shows the diagnostic capability of ADM mRNA expression in predicting different clinical variables in HNSC. The predictive ability of ADM gene expression has acceptable accuracy in distinguishing the HNSC tumor samplers from the healthy control samples (AUC = 0.757>0.7). However, the predictive ability of ADM expression has poor accuracy in distinguishing the other clinicopathological variables (AUC<0.7).

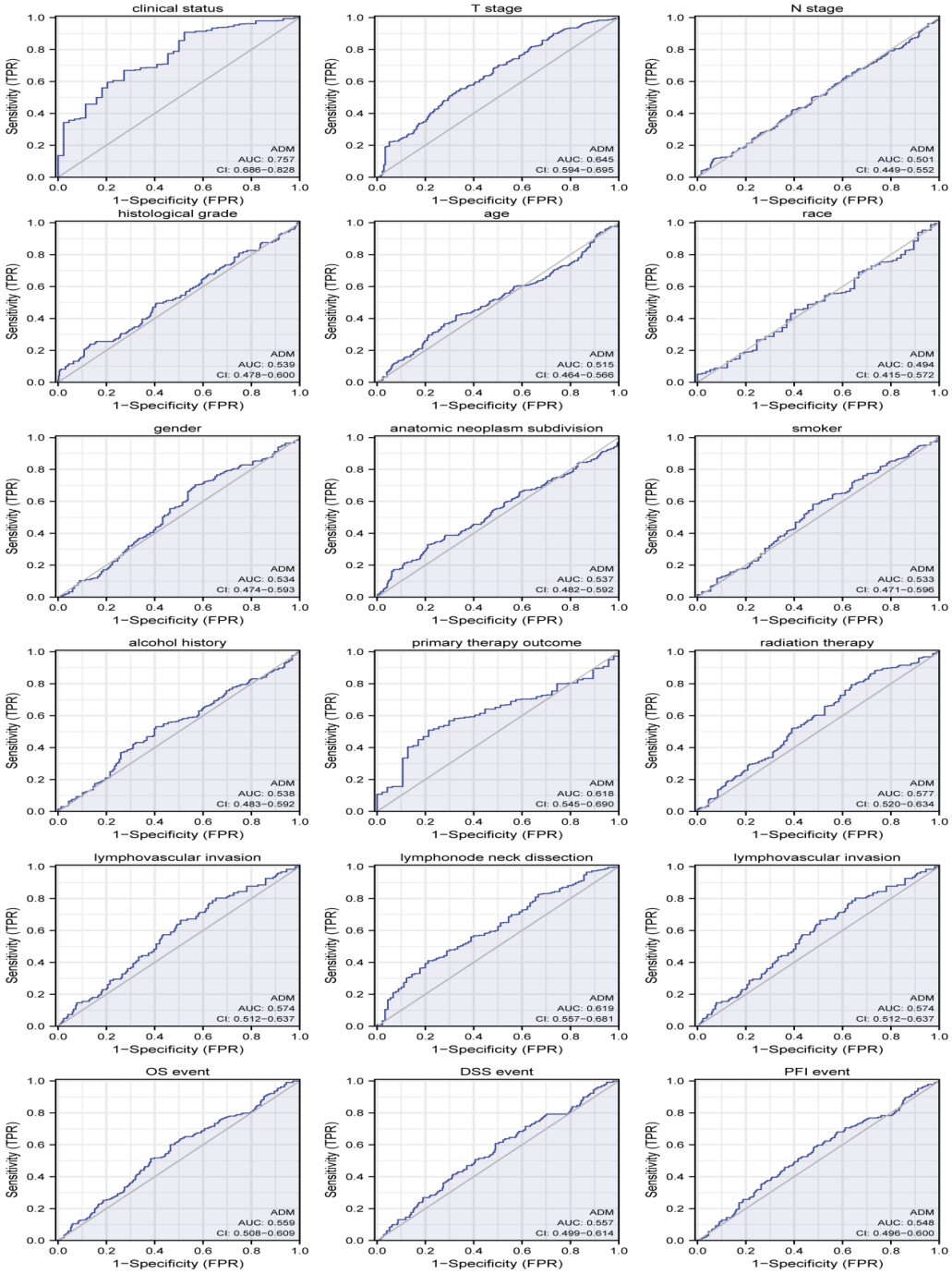

Figure 4: ROC curves were used for evaluating the diagnostic values of ADM in distinguishing various clinicopathological variables.

PPI and GGI networks

Fig 5A shows that ADM interacted with several hub genes, including VIP, GNAS, RAMP2, GNB1, CALCA, EDN1, ADM2, AVP, CALCRL, and IAPP. Fig 5B constructed the GGI network and shows that ADM interacted with 20 genes, for example, ADM2, IAPP, CALCB, CALCA, EDN1, HIF1A, VIPR1, RAMP2, RAMP3, RAMP2, et al. Several genes were overlapped for the interacted genes obtained from PPI and GGI network, for instance, ADM2, RAMP2, RAMP3, IAPP, CALCA, and CALCB.

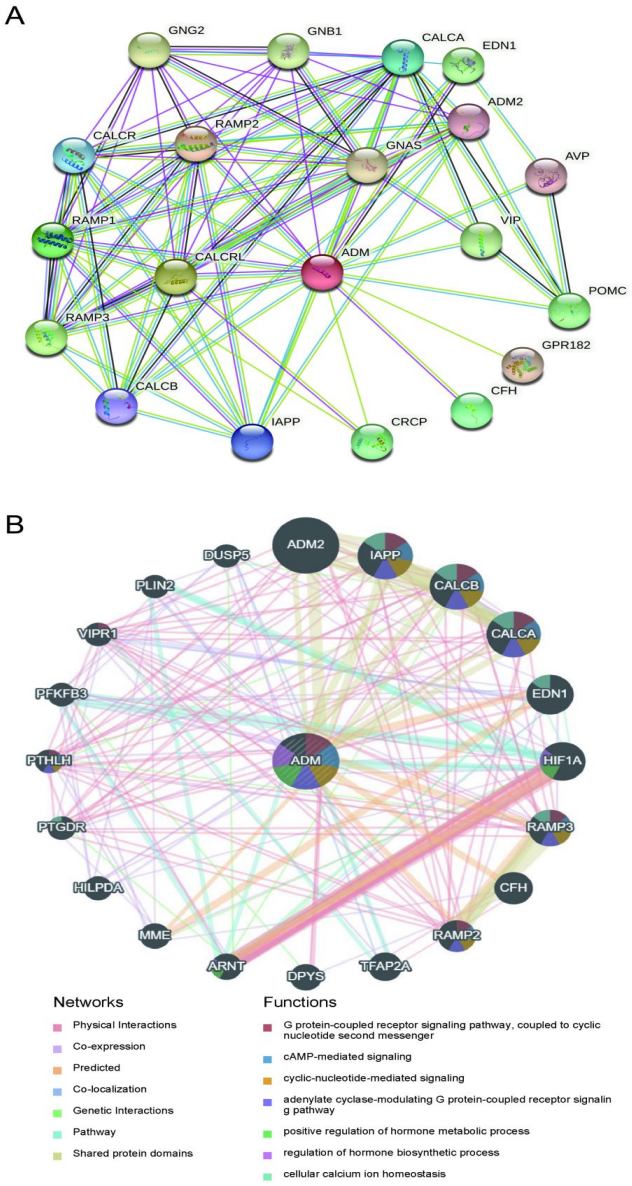

Figure 5: The ADM-interacted genes were identified by constructing interaction networks. (A) The ADM-involved protein-protein interaction network (PPI); (B) The ADM-involved gene-gene interaction (GGI) network

Heatmap showing the expression pattern of the top 10 positively- and top 10 negatively- correlated genes of ADM in HNSC

Among the ADM-significantly correlated genes, the top 10 positively- as well as the top 10 negatively- correlated genes were obtained. Fig 6A-B depicts a heatmap of the expression pattern of the top 10 positively- and the top 10 negatively- ADM correlated genes in HSNCC samples. The top 10 positively- correlated genes of ADM were found to be: DDIT4, HILPDA, PPP1R15A, ANGPTL4, ANKRD37, NDRG1, SLC2A1, KCTD11, VEGFA, and LDHA (Fig 6A). The top 10 negatively correlated genes of ADM were found to be: CCM2L, SHE, GIMAP1, CLEC3B, KCNA5, RAMP2, TIFAB, FAM110D, GIMAP7, and P2RY13 (Fig 6B).

Functions of top 100 ADM-positively and negatively correlated genes

Fig 6C shows that the top 100 positively- and top 100 negatively-ADM correlated genes were overrepresented in several biological processes, for example, regulation of angiogenesis, leukocyte migration, cellular response to hypoxia, response to oxygen levels, response to oxygen levels, and response to decreased oxygen levels. These genes were overexpressed in several molecular functions, for instance, cytokine activity, chemokine binding, and nucleotide receptor activity (Fig 6D). These genes were not overexpressed in any of the cellular component terms. Fig 6E shows that these genes were overexpressed in several signaling pathways, including cytokine-cytokine receptor interaction, HIF-1 signaling, MAPK signaling, chemokine signaling, AGE-RAGE signaling, Relaxin signaling, viral protein interaction with cytokine and cytokine receptor, and NF-kappa B signaling. The results obtained by gene set enrichment analysis show that ADM-correlated genes were enriched in several pathways, including collagen formation, calcium signaling pathway, biological oxidations, and signaling by Notch (Fig 6F).

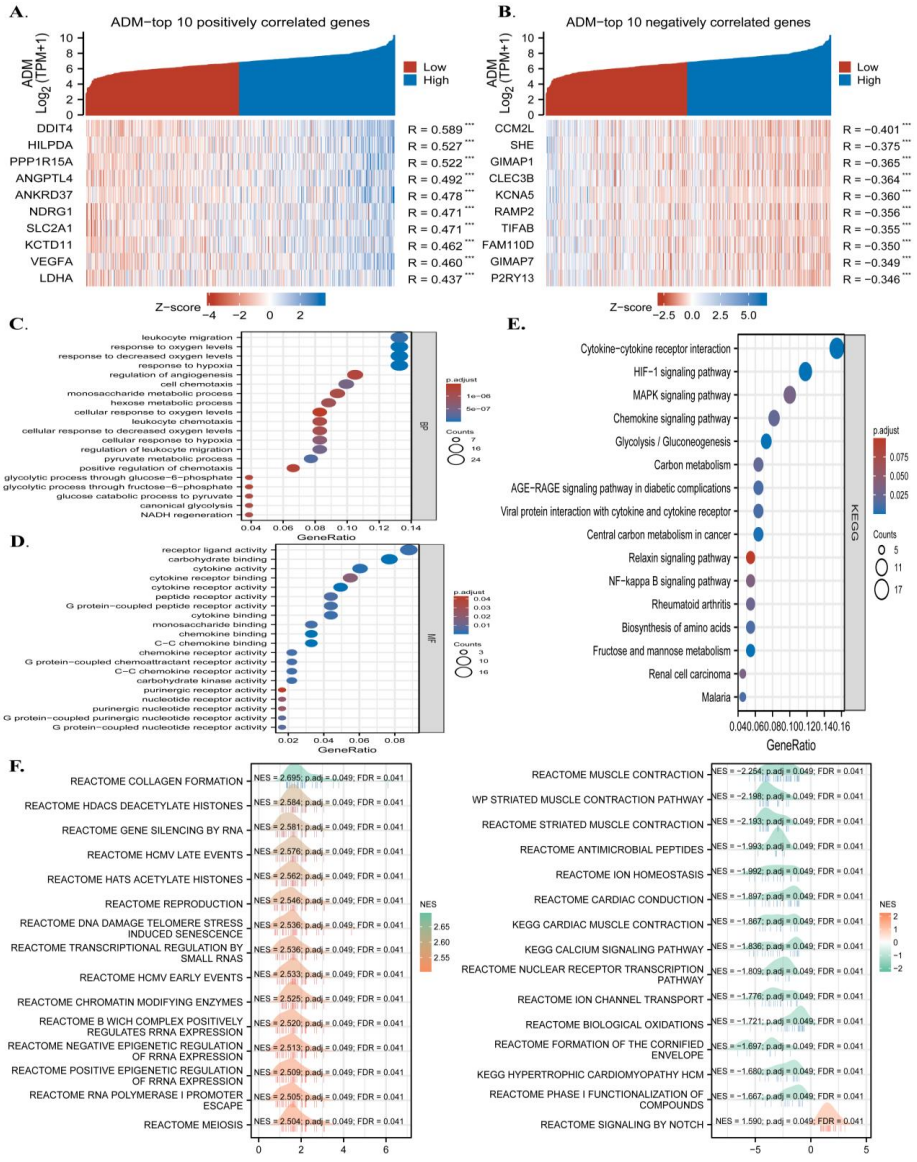

Figure 6: The correlated genes and their enriched biological functional terms. (A) Heatmap showing the expression pattern of the top 10 positively- ADM correlated genes in HNSC samples. (B) Heatmap showing the expression pattern of the top 10 negatively- ADM correlated genes in HNSC samples. (C) The involved biological processes of the top 100 positively- and negatively- ADM correlated genes. (D) The involved molecular functions of the top 100 positively- and negatively- ADM correlated genes. (E) The involved KEGG signaling pathways of ADM top 100 positively and negatively correlated genes. (F) The enriched top 30 pathways obtained by GESA analysis.

Correlation between ADM expression and tumor immunity in HNSC

Fig 7A shows that ADM was significantly negatively correlated with several TIICs, including macrophages, aDC, Th2 cells, Eosinophils, NK CD56dim cells, iDC, cytotoxic cells, T helper cells, Th17 cells, NK cells, mast cells, DC, TReg, Tem, T cells, TFH, B cells, and pDC. Scatter plots were used to depict the association between ADM expression and several representative cells, including macrophages (r=-0.093, p=0.038), T cells (r=-0.254, p<0.001), B cells (r=-0.301, p<0.001), T regulatory cells (r=-0.239, p<0.001), T helper cells (r=-0.170, p<0.001), Th17 cells (r=-0.177, p<0.001), NK cells (r=-0.199, p<0.001), mast cells (r=-0.213, p<0.001), and dendritic cells (r=-0.225, p<0.001).

Fig 7B shows that ADM was significantly negatively correlated with the majority of 15 classic ICGs (e.g., CTLA4, LAG3, HAVCR2, TIGIT, VSIR, BTLA, IDO1, and TNFSF14). Fig 7C-D shows that ADM was negatively correlated with the majority of immune modulator genes. Fig 7C shows that ADM was significantly negatively correlated with the majority of immunostimulatory genes, for example, CD27, CD28, CD40LG, CD48, CXCL12, CXCR4, ENTPD1, ICOS, IL2RA, KLRK1, LTA, TNFRSF13B, TNFRSF13C, TNFRSF14, TNFRSF17, TNFRSF4, TNFRSF8, TNFRSF9, TNFSF13, TNFSF14, TNFSF15, and TNFSF4. Fig 7D shows that ADM was significantly negatively correlated with the majority of immunoinhibitory genes, for example, ADORA2A, BTLA, CD160, CD244, CD96, CSF1R, CTLA4, HAVCR2, IDO1, IL10, KDR, KIR2DL3, LAG3, LGALS9, PDCD1, and TIGIT. Fig 7E shows that ADM was negatively correlated with each of the Stromal-Estimate-Immune scores in HNSC. Fig 7F shows the prognostic values of clusters divided by the low- and high-expression of ADM and several tumor-infiltrating immune cells in HNSC.

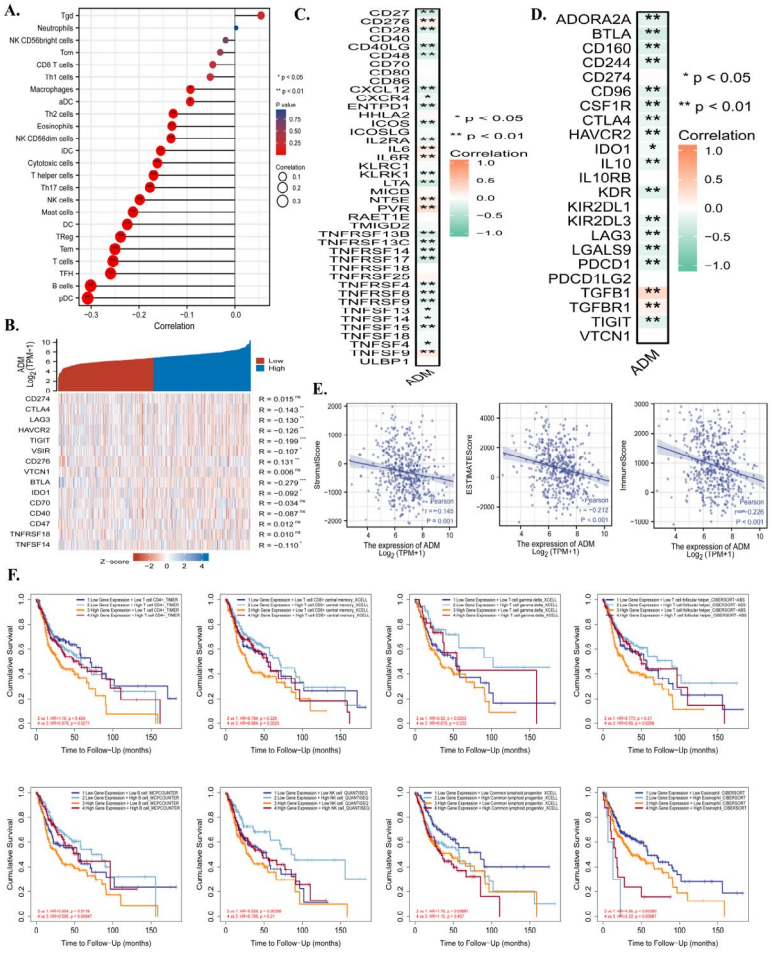

Figure 7: The involvement of ADM in tumor immunity. (A) The correlation between ADM expression and tumor-infiltrating immune cells. The lollipop plot showed the correlation between ADM expression and 24 TIICs in HNSC. (B) The correlation between ADM and 15 classic ICGs (i.e., CD274, CTLA4, LAG3, HAVCR2, TIGIT, VSIR, CD276, VTCN1, BTLA, IDO1, CD70, CD40, CD47, TNFRSF18, and TNFSF14) in HNSC. (C) The correlation between ADM expression and immunostimulatory genes in HNSC; (D) The correlation between ADM expression and immunoinhibitory genes in HSNC. (E) The correlation between ADM expression and Estimate-Stromal-Immune score in HNSC. (F) The TIMER web server was used for showing the prognostic values of clusters divided by the low- and high-expression of ADM and several tumor-infiltrating immune cells in HNSC.

Discussion

The results shown by bioinformatics analyses showed that the regulation of ADM in tumor biology of HNSC is mainly by several signaling pathways, including cytokine-cytokine receptor interaction, HIF-1 signaling, MAPK signaling, chemokine signaling, AGE-RAGE signaling, Relaxin signaling, viral protein interaction with cytokine and cytokine receptor, and NF-kappa B signaling. ADM is involved in tumor immunity of HNSC by being negatively correlated with several tumor-infiltrating immune cells, including tumor macrophages, T cells, B cells, Treg cells, T helper cells, Th17 cells, NK cells, mast cells, and dendritic cells. These findings can be well supported by either direct or indirect literature evidence, thereby indicating the prediction accuracy of such findings.

The overexpression of ADM has been well demonstrated in some HNSC-related studies. In accordance with the present results shown in HNSC, a previous study used an immunohistochemistry assay and found high ADM cytoplasmic expression in oral and oropharyngeal cancer [11]. Apart from the dysregulation of the ADM gene, its increased expression was also found to be significantly correlated with lymph node metastasis [11]. In agreement with the survival analysis results predicted by the present computational analysis, this previous also concluded that ADM expression could be regarded as an independent genetic marker in predicting the prognosis of oral and oropharynx cancer [11]. Another previous research investigating laryngeal cancer found that the expression level of ADM was significantly related to the TNM stage [12]. This research also found that the overexpression of ADM played a promotion role in the cancer progression of laryngeal cancer [12].

The regulation of ADM in tumor biology of HNSC is mainly by several signaling pathways, including cytokine-cytokine receptor interaction, HIF-1 signaling, MAPK signaling, chemokine signaling, AGE-RAGE signaling, Relaxin signaling, viral protein interaction with cytokine and cytokine receptor, and NF-kappa B signaling. Regarding the cytokine pathway, previous research regarding prostate cancer showed that human Adrenomedullin was able to upregulate a pleiotropic immune regulatory cytokine-Interleukin-13 (IL-13)'s receptor [13]. VEGF is a potent proangiogenic cytokine that is highly expressed in many cancers. The current research also identified the positive correlation between ADM and the cytokine VEGF. ADM was found to be a master regulator upstream of the VEGF pathway; thereby, ADM inhibitor could be regarded as an anti-angiogenic agent, which might be a promising alternative to VEGF factor treatment [2]. In addition, much evidence supported the involvement of ADM in Hypoxia-inducible factor-1 (HIF-1) signaling. Previous research conducted by Chen et al.found that ADM was able to promote the cell proliferation of human endothelial cells via HIF-1α [14]. Another research conducted by Garayoa et al. showed that the expression of the ADM gene was upregulated by the hypoxia-inducible factor-1 (HIF-1) in cancer cell lines, indicating that the HIF-1/ADM signaling axis might promote carcinogenesis by aiding angiogenesis and inhibiting apoptosis [15]. For another example of MAPK signaling, blocking ADM was found to suppress the tumor growth of sunitinib-resistant renal cell carcinoma by targeting the ERK/MAPK pathway [16]. Another research showed that the angiogenic role of ADM in tumor progression might be by activating the Akt signaling, mitogen-activated protein kinase (MAPK) signaling, and focal adhesion kinase signaling [17]. From another instance of NF-kappa B signaling, previous research conducted by Vijaya found that treatment of the human pancreatic adenocarcinoma cells with an ADM antagonist suppressed the cell proliferation and the activity of nuclear factor kappaB [18].

ADM is involved in tumor immunity of HNSC by being negatively correlated with tumor macrophages, T cells, B cells, Treg cells, T helper cells, Th17 cells, NK cells, mast cells, and dendritic cells. In contradiction with the results obtained by computational prediction, much previous research holds the opinion by arguing the positive correlation between ADM and these immune cells. A previous study found a positive correlation between ADM and macrophages by showing that tumor-associated macrophages were able to overproduce ADM protein and thus further enhance the tumor cell proliferation of melanoma by regulating angiogenesis [6]. Regarding mast cells, ADM has been shown to modulate the mast cell activation by inducing its degranulation, and thus further being involved in tumor promotion and progression [19]. Regarding dendritic cells, the exogenous ADM was demonstrated to modify the phenotype of dendritic cells and thus induce a semi-mature phenotype [20]. The previous literature evidence holds the same opinion with the negative correlation between ADM and Th17 cells by showing that ADM could decrease the presence and activation of encephalitogenic Th17 cells [21]. However, if and how ADM is involved in regulating T cells by influencing immune checkpoint genes is still a question and hasn't yet been investigated by previous cancer research.

The strengths and limitations that exist in the current research need to be emphasized. This research has performed traditional bioinformatics analyses to explore the implication of the ADM gene in HNSC as comprehensively as possible. The investigating aspects include expression patterns, prognostic values, significantly correlated genes, involved biological processes and KEGG signaling pathways, and the involvement in tumor immunity. Although the analyses carried out in the current research have been as comprehensive as we can, the limitation that existed in the current research also needs to be emphasized. Lacking experimental validation is the biggest limitation of the current research; however, validating the potential regulating mechanisms of ADM in HNSC, especially the implicated pathways, could be done as separate research by designing the gene transfection experiments. The ADM-mediated HNSC mechanisms identified in the current study could be regarded as the theoretical basis of such a research topic and provide experimental directions for future research.

Conclusion

In conclusion, ADM was significantly upregulated in HNSC tumor samples and associated with worse prognostic outcomes. ADM-significantly correlated genes in HNSC were enriched in several

tumor-promoting pathways, including cytokine-cytokine receptor interaction, HIF-1 signaling, MAPK signaling, chemokine signaling, AGE-RAGE signaling, Relaxin signaling, viral protein interaction with cytokine and cytokine receptor, and NF-kappa B signaling. ADM was involved in tumor immunity of HNSC by being negatively correlated with several tumor-infiltrating immune cells, including tumor macrophages, T cells, B cells, Treg cells, T helper cells, Th17 cells, NK cells, mast cells, and dendritic cells. All these results indicated that ADM could be a promising and novel therapeutic target in treating head and neck squamous cancer.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data Availability Statement

The data analyzed during the current study are available in the TCGA database with the accession numbers TCGA-HNSC. The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding authors.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SL conceptualized the research idea, performed the bioinformatics analyses, interpreted the results, wrote the manuscript, as well as administrated and supervised the whole research project.

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Nothing to declare.

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