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The Expression and Prognostic Value of TERF2 in Head and Neck Squamous Cell Carcinoma

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Abstract: ***TERF2 is identified as a potential biomarker in many cancers. However, its role in Head and neck squamous cell carcinoma (HNSC) remains poorly understood. TERF2 levels among HNSC patients were studied by TIMER and UALCAN. The overall survival and clinical features of HNSC patients were analyzed by Kaplan-Meier (KM) method. Potential relation of TERF2 with HNSC was explored by using cBioPortal, MEXPRESS and TIMER. Meanwhile, TERF2-related genes were subject to GO and KEGG analyses. Higher TERF2 expression predicted a poor prognosis in HNSC patients. In addition, TERF2 was associated with genetic alterations, methylation and immune infiltration in HNSC. TERF2 was crucially involved in biological processes such as protein processing in the endoplasmic reticulum, ubiquitin-mediated proteolysis, insulin signaling pathway, and thyroid hormone signaling pathway and so on. The expression of TERF2 was closely related to HNSC survival. This probably provided new visions for TERF2 as a possible diagnostic and prognostic indicator for HNSC.***

Keywords: TERF2, Head and neck squamous cell carcinoma, Bioinformatics, Prognosis**.**

1. Introduction

Head and neck squamous cell carcinoma (HNSC) ranks the 6th place among cancers globally, which occupies over 90% among all head and neck cancers [1]. HNSC mainly originates from the mucosa of the upper respiratory and upper gastrointestinal tracts, mainly involving oral cavity, larynx together with pharynx. HNSC is a cancerous epidermal-derived tumor that poses a major danger to human being and health. As we known, about 58% of patients with HNSC were already in the advanced stage of the disease (stage III-IV) when they were diagnosed. Therefore, HNSC patients had a five-year survival rate of only about 50% [3]. As a result, it's critical to dig deeper into the molecular mechanisms of oncogenes linked to HNSC.

TERF2 is one of the telomere binding protein subunits, which specifically attaches to double-stranded TTAGGG repeats and locates in all human chromosomal telomeres, and is a key factor in maintaining telomere length [5]. Apart from telomere maintenance, TERF2 has been found to be localized in the telomere region and influence the expression of several target genes [6-7]. In addition, TERF2 can affect angiogenesis balance by regulating platelette-derived growth factor receptor (GFR)-β within endothelial cells (ECs), thereby affecting tumorigenesis. TERF2 expression has been found to be high in other solid tumors, like breast cancer (BC), colorectal cancer (CRC), and hepatoma, among others [8-10]. Furthermore, TERF2 exhibits features that promote environmental tumor, which suppresses microenvironment killing and immune recognition, impacts peripheral blood vessel formation, while maintaining cancer cell stemness by acting on several cell proliferation-related proteins involved in signaling pathways [11]. This suggests that TERF2 performs an important effect on cancer genesis and development [12-13]. Nonetheless, no study has assessed the effect of TERF2 in development of HNSC.

The present work analyzed TERF2 gene's biological effect on HNSC. TERF2 expression was studied using TIMER and UALCAN. The connection between survival rate and clinical characters of patients with HNSC was studied using the Kaplan Meier plotter. Moreover, we utilized a variety of bioinformatics approaches for analyzing potential relationship of TERF2 with HNSC. This study indicated that TERF2 may have potential biological function and prognostic value in HNSC.

2. Materials and Methods

2.1 Expression Analysis of TERF2

We contrast TERF2 levels within HNSC and non-carcinoma samples from TCGA-HNSC cases based on UALCAN database (http://ualcan.path.uab.edu/index.html). We will also analyze the differential expression of TERF2 from different angles such as tumor stage, sex, age, race, tumor subtype, and so on.

2.2 Survival Curve Analysis

This work also utilized Kaplan-Meier (KM) plotter (https://kmplot.com/analysis/) for investigating overall survival (OS) as well as recurrence-free survival (RFS) of TERF2 among HNSC cases. Clinical factors, including the stage, gender, race, grade and mutation burden were also considered in the subgroup analysis.

2.3 Analysis of Genetic Alteration

cBio Cancer Genomics Portal (http://cbioportal.org) has been designed as the free, open-access database to explore multiple cancer genomics data using TCGA datasets. It was employed in the present work for investigating TERF2 genetic variation for TCGA-HNSC cohort and visualized the frequency and type of variation of the data by the "OncoPrint" module and

the "Mutation" module to display the mutation information of the genes.

2.4 Analysis of DNA Methylation

MEXPRESS (https://mexpress.be/) has been developed as the data visualization approach that makes it simple to see clinical data, methylation levels, gene expression, and correlations in the TCGA database. This work employed MEXPRESS for analyzing DNA methylation status of TERF2 in the TCGA-HNSC cohort. Further, we applied Pearson's test in determining relation of TERF2 gene level with DNA methylation.

2.5 Immune Infiltration Analysis

TIMER (http://timer.cistrome.org/) has been designed as the integrative analysis site to analyze immune infiltration in different cancer types in a systematic manner. The expression of TERF2 and its connection with immune cell infiltration in HNSC were investigated using TIMER in this study. The association of TERF2 level and immunocyte immune markers within HNSC was calculated using GEPIA2.0.

2.6 Construction of Related Genes Network

CBioPortal, UACLAN and LinksedOmics were used to obtain TERF2 co-expressed genes. The absolute value of Spearman correlation coefficient was set as >0.5. The intersection gene set was obtained using Venn diagram online implement (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

2.7 Gene Ontology (GO) as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

To better understand genes' biological significance, the Database for Annotation, Visualization, and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) offers comprehensive approaches for functional enrichment. On DAVID, pathway enrichment analysis functions of TERF2 changes and frequently altered neighbor genes were subject to GO and KEGG analyses. $P<0.05$ stood for statistical significance.

3. Result

3.1 Expression Feature of TERF2

Based on the TCGA-HNSC cohort, we looked at the levels of TERF2 expression in HNSC tissues and normal tissues (Figure 1). Figures 1B-G present elation of TERF2 with clinicopathological features among HNSC cases. In subgroups of race, gender, age, tumor stage, and grade of patients, TERF2 expression considerably increased compared with control, indicating its effect on the genesis and development of HNSC.

3.2 Survival Analysis of TERF2

Possible link of TERF2 level with HNSC prognostic outcome was explored. RFS was not significantly linked with TERF2 mRNA expression levels (P=0.096). Patients with HNSC who had high TERF2 mRNA levels were predicted to have a poor prognosis (HR=1.53(1.1-2.12), P=0.01). Based on the above results, TERF2 was possibly the novel biomarker used to predict HNSC prognosis.

3.3 Genetic Alteration of TERF2

Using cBioPortal, we investigated at the genetic alterations in TERF2 in the TCGA-HNSC cohorts. It was found that the TERF2 gene had a 4% chance of genetic alteration in all TCGA-HNSC cohorts. The genetic alterations of TERF2 in HNSC such as "splice mutation", "missense mutation", "amplification", "truncating mutation", "mRNA high" and "deep deletion". Among them, "missense mutation" is the most common type of TERF2 gene alterations.

3.4 TERF2-Related Immune Cell Infiltration

More and more studies have shown the interaction between immune response and pathophysiological processes. As a consequence, we tried to analyze the effect of TERF2 gene on HNSC genesis via immune cell infiltration. We evaluated the relationship between TERF2 and immune markers in HNSC using the TIMER database. The degree of TERF2 expression in HNSC was found to be substantially correlated with the level of immune marker expression, such as the expression level of CD4+T cell, Neutrophils (CD66b/ITGAM/CCR7) and Macrophage (NOS2/IRF5/PTGS2) were positively related to TERF2 level within HNSC. CD8+T cell (CD8A/CD8B/CD27), B cell (CD19/CR2/CD79A) levels were negatively linked with the expression level of TERF2 in HNSC, and the difference was statistically significant. We applied the GEPIA2.0 database for validation and found that the results were similar in the TIMER database. Therefore, TERF2 may be involved in the immune escape process in HNSC microenvironment.

3.5 TERF2-Related Genes and Fuctional Erichment Analysis

As shown in Figure 5, 863 genes were identified as TERF2 related genes. DAVID employed GO and KEGG analyses to predict the functional enrichment of TERF2 associated genes. GO enrichment analysis was conducted to predict target genes' functions from 3 categories: biological processes (BPs), molecular activities (MFs) along with cellular components (CCs). These genes were mainly involved in 95 BPs, such as transcription, DNA-templated and protein phosphorylation, and so on. CCs showed the 55 most relevant pathways, for example, cytoplasm, nucleus, cytoplasm and cytofluid, etc. The above genes mostly participated in regulating 50 MFs, including cell composition, protein binding, ATP binding, and Poly (A) RNA binding. KEGG results showed that genes related to TERF2 expression were mainly focused on protein processing in endoplasmic reticulum, mRNA surveillance pathway, ubiquitin mediated proteolysis, thyroid hormone pathway, insulin pathway, insulin resistance and other processes.

4. Discussion

Although HNSC has been extensively studied, the mechanisms by which some aberrantly expressed factors in

the development of HNSC tumourigenesis are still not fully revealed. And to date, few clinically useful biomarkers are available. Therefore, it is a particularly urgent need to identify molecular biomarkers for predicting the progression, survival and response to therapeutic agents in HNSC [15]. For the diagnosis, management, and prognosis of HNSC, a thorough investigation of the key genes and mechanisms underlying its development is critical.

Telomere protection in mammals was discovered to be mediated by the crucial protein TERF2, which protected telomeres by suppressing different DNA repair activities, according to certain research. Whereas, cancer cells prevent replicative senescence by activating telomerase or alternative telomere lengthening (ALT) pathways, and increased TERF2 expression predisposes to telomere shortening and chromosome instability. TERF2 was establish to be overexpressed in a number of human malignancies, like BC, gastric cancer (GC), hepatoma and CRC [13]. TERF2 was involved in telomere stabilization by promoting t-loop folding, and stabilization of the t-loop structure restricts telomerase access to telomeres ultimately leading to telomere shortening and carcinogenesis in hepatocellular carcinoma [17]. Dong et al. reported that sp1 upregulates TERF2 expression and TERF2 inhibition reduces tumorigenesis of colorectal cancer [13]. In this study, we found that TERF2 was substantially upregulated in HNSC tissues. Meanwhile, the degree of TERF2 expression in HNSC patients was found to be linked to clinicopathological factors such as gender, race, and tumor stage. Furthermore, elevated TERF2 gene expression in HNSC patients was linked to a poor OS, suggesting the function of TERF2 in HNSC genesis and development.

Gene mutation, DNA methylation and immune infiltration were key factors in tumor genesis and development. Genetic alterations in HNSC were evaluated with regard to the oncogenie signaling pathway [21]. The DNA methylation profiles of HNSC metastases was association with stage progression of HNSC [22-23]. In addition, Luo et al., detected TERF2 was positively correlated with genome instability and methylation [24]. Zhang et al. discovered that HNSC were enriched in pathways connected to the immunosuppressive tumor microenvironment, and that most immune checkpoints were up-regulated [25]. For HNSC treatment, immune checkpoint inhibitors (ICIs) have become increasingly essential [26]. The high expression of TERF2 might inhibit a series of cytokines to enhance the anti-tumor immune reaction [10]. In this study, we found that TERF2 had a 4% mutation rate in the TCGA-HNSC. And the genetic alteration of TERF2 was linked to the overall survival prognosis of HNSC cases. We also found that the methylation levels of various probe sites in the TERF2 gene promoter region were likewise shown to be greater in primary HNSC tissues than in normal tissues. However, whether TERF2 gene mutation is linked to the occurrence and progression of HNSC remains to be further studied. We also found that TERF2 expression was positively related to CD4+T cells, neutrophils and macrophages, and negatively related to B cells and CD8+T cells. TERF2 level was markedly related to immune marker levels in HNSC. This research indicated that TERF2 might have an immune escape role in HNSC.

The regulation of biological functions of TERF2 expression related genes mainly focuses on DNA-templated, protein phosphorylation and ubiquitin mediated proteolysis, and so on. Previous studies have indicated that TERF2 differs from TERF1 due to the basic, but not acidic N-terminus [27]. SIRT6 deactetylates TERF2, and activated the ubiquitin-dependent proteolysis during oncogenesis and damage response [28]. TERF2/DREF complex inhibited notch signaling though chromatin activation [29]. TERF2 may directly control gene expression in telomere protection which contribute to a number of steps in tumor genesis, progression and metastasis [30].

5. Conclusions

In summary, TERF2 shows high expression within HNSC, which is associated with a variety of biological functions. TERF2 up-regulation predicts the dismal outcome of HNSC cases, suggesting that TERF2 could be used as potential prognostic biomarkers for HNSC patients.

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Author Contributions

XWZ conceived and designed the experiments. YC performed the experiments and wrote the initial draft of the manuscript. YJL contributed to the statistical analysis. JFT, YC and YJL involved in reference collection and data management. XWZ and JFT reviewed and edited the manuscript. All authors reviewed and approved the final manuscript.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

Our study analyzed publicly available datasets, which can be found in The Cancer Genome Atlas (https://portal.gdc.cancer.gov), cbioportal (http://www.cbioportal.org), Oncomine database (https://www.oncomine.org/resource/main.html) and so on.

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Figure Legend

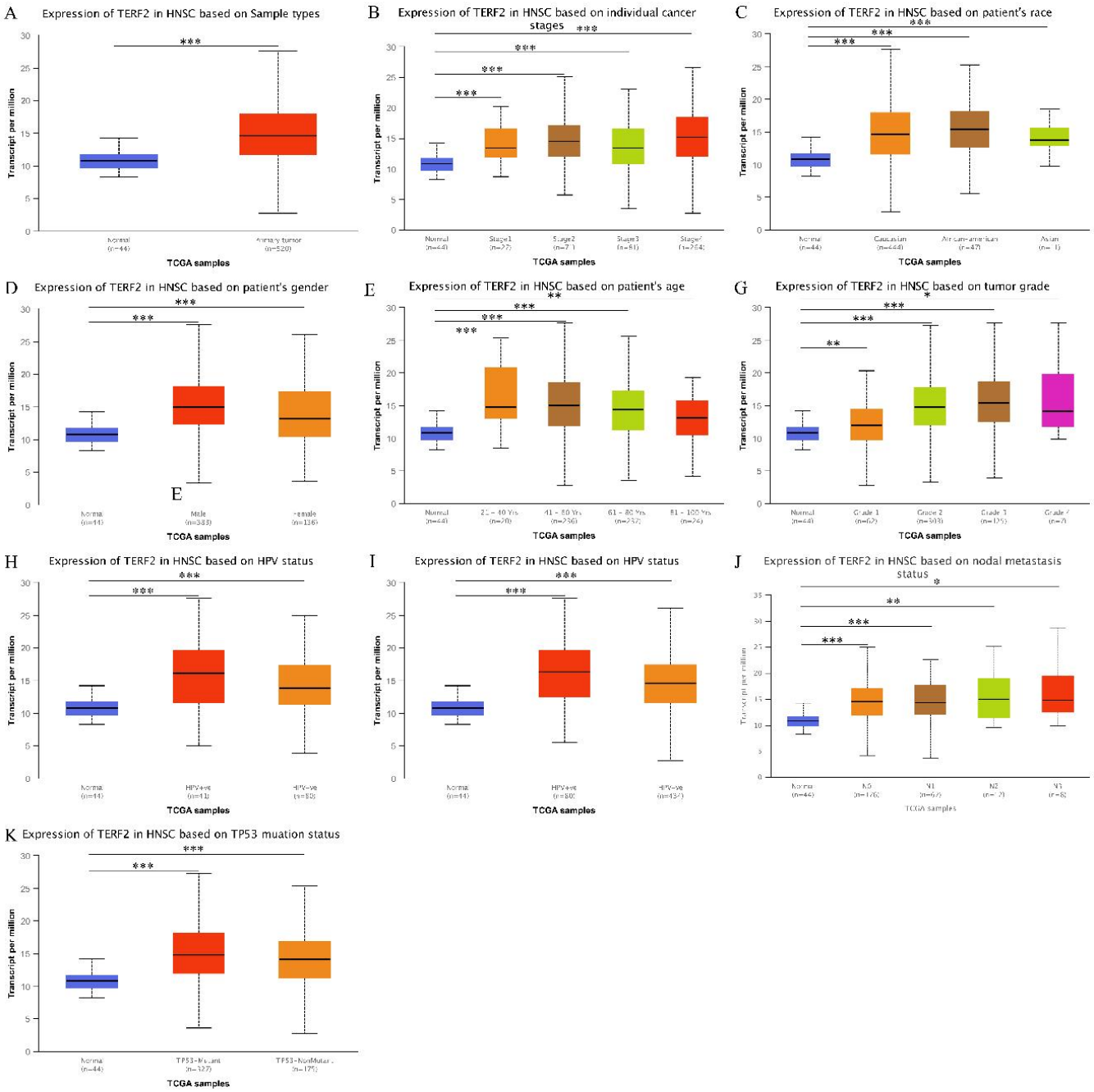


Figure 1: TERF2 expression levels in TCGA Normal and HNSC tissues. (A): The expression level of TERF2 in Normal and HNSC samples from TCGA. **(B-G):** TERF2 levels among healthy individuals and HNSC cases' cancer stage, race, gender, age, tumor grade. **(H&I):** TERF2 levels within HNSC according to HPV status. **(J):** TERF2 levels within HNSC according to lymph node metastasis (LNM). **(K):** TERF2 levels within HNSC according to TP53 mutation status. *p<0.05, **p<0.01, ***p<0.001.

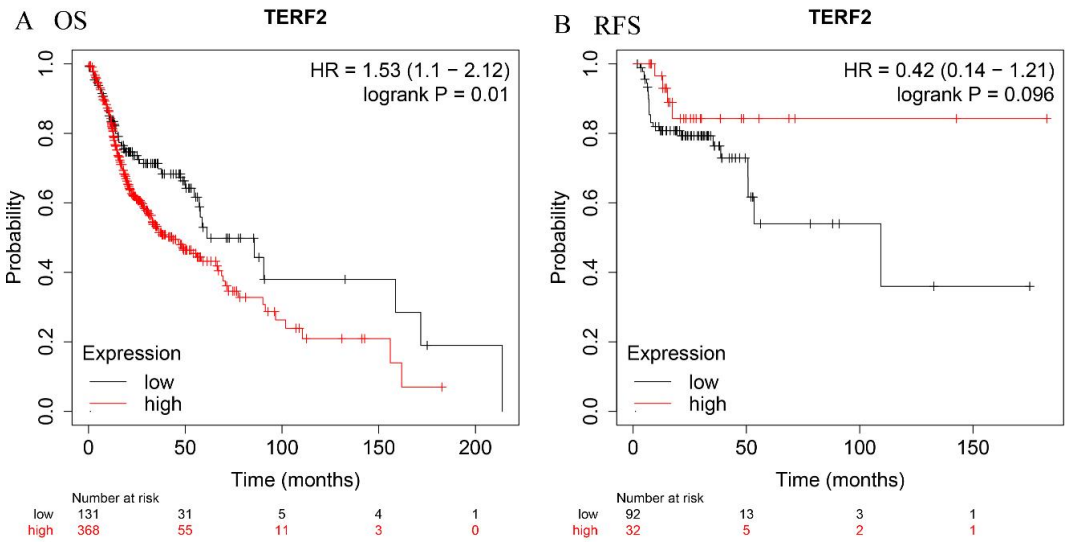


Figure 2: Relation of TERF2 expression with overall survival among patients with HNSC. (A&B): Performing OS and RFS analyses for cases within HNSC cohorts. The survival curve were shown.

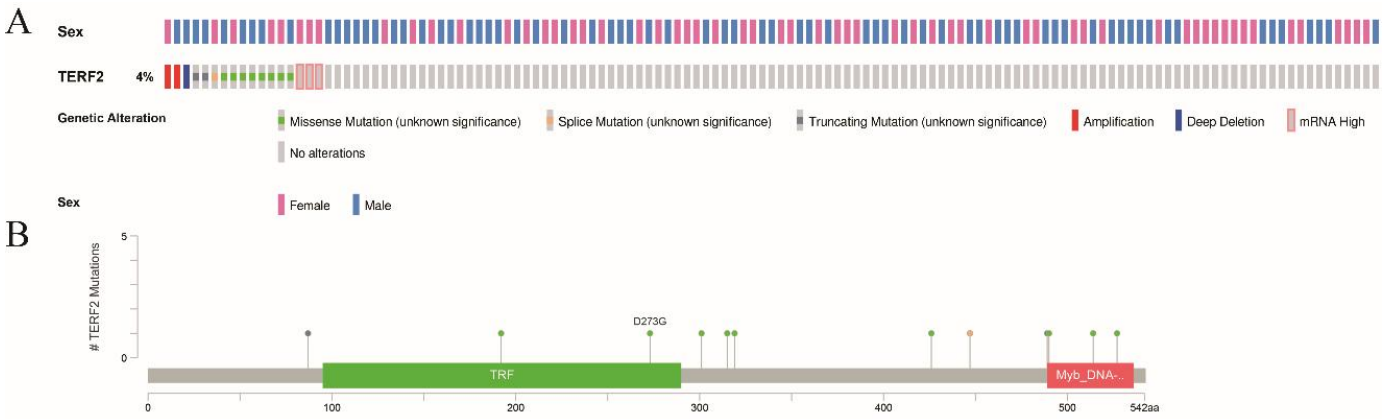


Figure 3: Genetic alteration analysis of TERF2. (A): TERF2 changes in HNSC OncoPrint. **(B):** TERF2 mutation locations in HNSC.

Figure 4: The DNA methylation status of TERF2 in HNSC cases of TCGA through MEXPRESS.

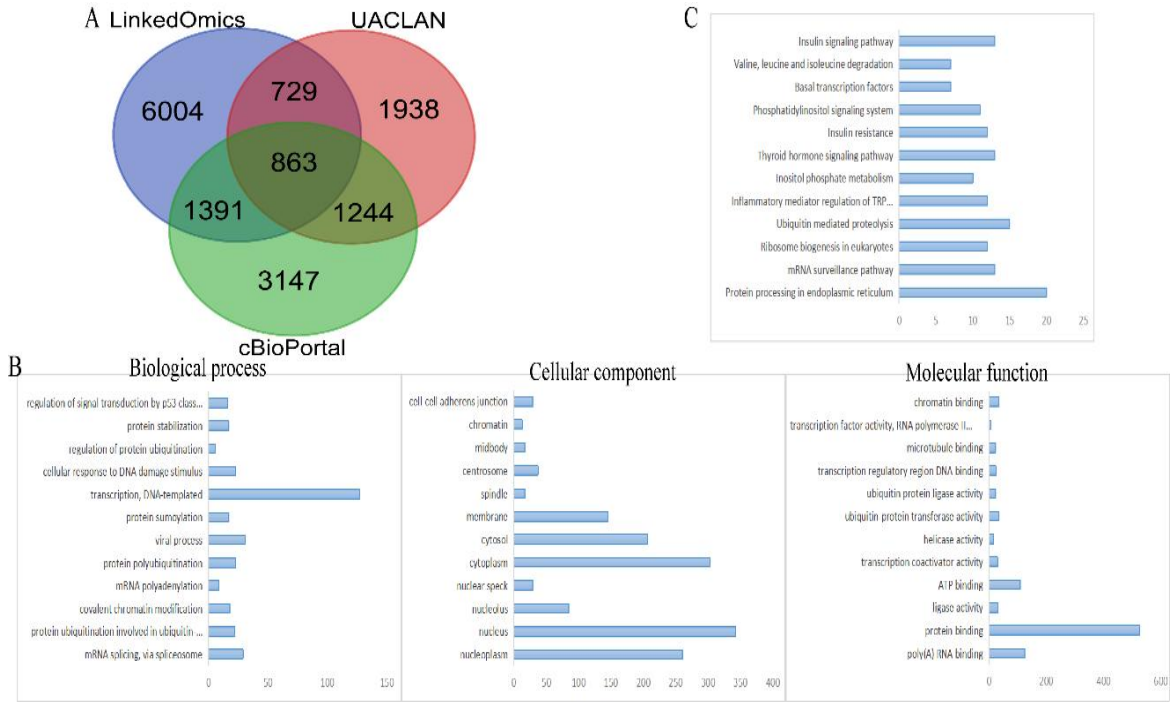


Figure 5: Analysis of TERF2-related genes' functional enrichment. A: The venn diagram of TERF2-expressed genes. **B:** GO functional annotation: BPs, CCs, MFs. **C:** KEGG pathway analysis. FDR<0.05 is considered statistically significant.

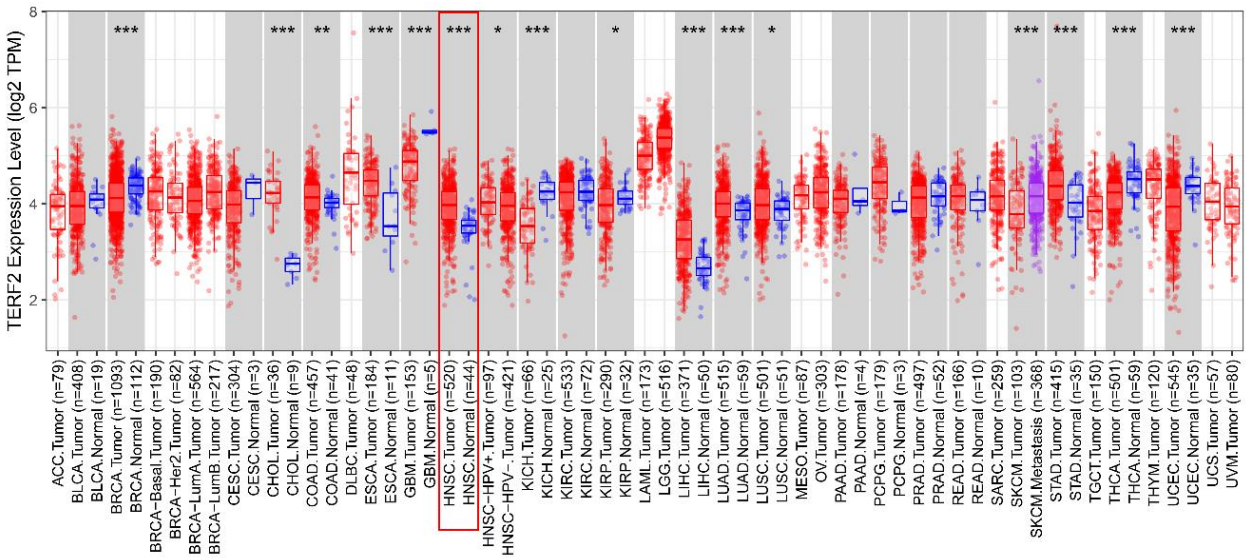
Table 1: Expression correlation between TERF2 and immune cell signatures.

Immune cells	Signature genes	HNSC	
		r	*p*
CD4+ T cell		0.241510828	5.83E-08
$CD8^+$ T cell	CD8A/CD8B/CD27	-0.274277153	6.12E-10
Neutrophils	CD66b/ITGAM/CCR7	0.214810837	1.52E-06
B cell	CD19/CR2/CD79A	-0.15814029	0.000429957
Macrophage	NOS2/IRF5/PTGS2	0.138103366	0.002139062
Myeloid dendritic cell	CD1C/ITGAX/CD83/HLA-DPB1/HLA-DRA	-0.03773663	0.403600973

Table 2: The association of TERF2 expression and immune markers in GEPIA2.0.

Description	Gene markers	HNSC					
		Tumor		Normal		GTEX	
		R	P	R	P	R	P
CD8+T cell	CD8A	-0.079	0.073	0.1	0.5	0.42	0.00095
	CD8B	-0.11	0.0097	0.009	0.95	0.36	0.0046
B cell	CD19	-0.022	0.61	-0.01	0.95	0.25	0.058
	CD79A	-0.024	0.58	0.099	0.52	0.17	0.19
	CR2	0.022	0.62	0.14	0.35	0.2	0.13
Neutrophils	CD66b	0.0094	0.83	0.24	0.12	0.041	0.75
	ITGAM	0.055	0.21	0.23	0.13	0.49	6.4E-05
	CCR7	0.0082	0.85	-0.078	0.61	0.62	1.6E-07
Myeloid dendritic cell	CD1C	0.036	0.41	0.1	0.51	-0.0057	0.97
	ITGAX	0.15	0.00044	0.09	0.56	0.57	1.76E-06
	CD83	0.17	0.00014	0.059	0.7	0.46	0.00023
	HLA-DPB1	-0.051	0.24	0.12	0.44	0.42	0.00098
	HLA-DRA	-0.017	0.7	0.26	0.092	0.4	0.0018
Macrophage	NOS2	0.047	0.28	0.055	0.72	0.47	0.00017
	IRF5	0.19	2E-05	0.45	0.0022	0.51	2.7E-05
	PTGS2	0.16	0.00017	0.2	0.2	0.54	8.7E-06

Bold values indicate P<0.05.



Supplementary Figure 1: TERF2 levels in several TCGA-derived cancers measured by TIMER. *P<0.05, **P<0.01, ***P<0.001.