

Research Article

Dihydroartemisinin Suppressed Cell Migration and Promoted Autophagy through *RALB* in Tongue Squamous Cell Carcinoma

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Abstract

Background: Overexpression of RAS-like proto-oncogene B (*RALB*), a member of the RAS-like Guanosine triphosphatase (GTPase) family, is significantly associated with poorer overall survival in patients with head and neck squamous cell carcinoma (HNSCC). However, potential drugs that inhibit *RALB* remain unknown. **Methods:** Differentially expressed genes (DEGs) in HNSCC were identified using the The Cancer Genome Atlas - Head and Neck Squamous Cell Carcinoma (TCGA-HNSC) dataset. Genes associated with the Rat sarcoma virus (RAS) signaling pathway were retrieved from the GeneCards database, the National Center for Biotechnology Information (NCBI) Gene resource, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. Subsequently, experimental validation was conducted using Cal-27 human tongue squamous cell carcinoma cells. First, wound-healing and colony-formation assays were performed, followed by treatment with dihydroartemisinin (DHA). Finally, an orthotopic xenograft model was established by implanting sh*RALB*-Cal-27 cells into the tongues of BALB/c nude mice, which were then treated with DHA. **Results:** We found that *RALB* expression was correlated with N stage and closely related to patient survival in HNSCC. *RALB* knockdown promoted autophagy in Cal-27 cells. Moreover, DHA significantly inhibited the proliferation and migration in Cal-27 cells. DHA increased the expression levels of P62 and LC3B in the tongues of nude mice implanted with sh*RALB*-Cal-27 cells. **Conclusion:** DHA inhibited migration and promoted autophagy through *RALB* in tongue squamous cell carcinoma (TSCC).

Keywords: head and neck squamous cell carcinoma; Ral GTP-binding proteins; ras genes; dihydroartemisinin; autophagy

1. Introduction

Tongue squamous cell carcinoma (TSCC), one of the main subtypes of head and neck squamous cell carcinoma (HNSCC) [1], is a highly aggressive and heterogeneous malignancy posing significant therapeutic challenges [2]. The incidence of TSCC-associated diseases is on the rise within the population [3], and its mortality rate remains high [4]. Patients with HNSCC are often diagnosed at a locally advanced stage, at which point the tumor demonstrates more aggressive biological behavior, thereby contributing to persistently elevated risks of recurrence and distant metastasis [5]. The effectiveness of conventional therapeutic agents, such as cisplatin, anti-programmed cell death protein 1 (anti-PD-1) monoclonal antibodies, and anti-epidermal growth factor receptor (anti-EGFR) drugs, is frequently compromised by the emergence of drug resistance [6,7,8,9,10]. Consequently, the identification of novel molecular targets and responsive drugs is of paramount importance.

The Ras family of small GTPases, particularly the frequently mutated oncogenes harvey rat sarcoma virus (HRAS), Neuroblastoma RAS viral oncogene homolog

(NRAS), and Kirsten rat sarcoma virus (KRAS), represents a promising avenue for molecular targeting [11,12]. Because of the consecutive failure in discovering Ras inhibitors, it was once referred to as an undruggable target [13]. The Ral GTPases, RalA and RalB, which function as downstream effectors of RAS, have emerged as promising therapeutic targets [14,15]. Both RalA and RalB are activated by oncogenic RAS signaling, irrespective of the specific RAS isoform involved [14]. Notably, RalB has been particularly associated with processes such as cell motility, invasion, and autophagy [16,17]. The overexpression of *RALB* is implicated in the pathogenesis and progression of various cancers [18,19] and is significantly correlated with poorer overall survival in patients with HNSCC [20].

Dihydroartemisinin (DHA) is an active metabolite from artemisinin compounds (artemisinin, artesunate, artemether) [21,22]. DHA exerts pleiotropic anticancer effects through Matrix metalloproteinase-9 (MMP-9) down-regulation [23] and c-Myc [24]. This study utilized the TCGA database to assess *RalB* expression levels in HNSC patients. Moreover, DHA may function as a *RalB* inhibitor, which inhibits migration and promotes autophagy.



2. Materials and Methods

2.1 Identification of HNSC/RAS Pathway-Associated Genes

The TCGA_HNSC dataset was acquired from the TCGA program (accessible at <https://portal.gdc.cancer.gov/>). The ‘limma’ package was employed to identify differentially expressed genes in HNSC, with a false discovery rate threshold set at 0.05 and an $|\log\text{fold change (FC)}| > 1$. Additionally, genes associated with the RAS pathway were curated from the GeneCards database, the NCBI gene function module, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Only genes with a relevance score of 20 or higher were selected, specifically focusing on Homo sapiens entries in the NCBI data. The ‘KEGGREST’ package in R was utilized to retrieve RAS pathway-related genes from the KEGG database.

2.2 Identification of HNSC and RAS Signal Pathway-Associated Genes

To explore the correlation between RAS pathway-related genes and survival status in HNSC, survival analysis was conducted. Univariate Cox proportional hazards regression was used to screen genes significantly correlated with overall survival ($p < 0.05$). Multivariate Cox regression was further adopted to obtain candidate genes closely linked to patient survival.

2.3 Analysis of Genes Associated With Patient Survival

To evaluate the association between gene expression and critical clinical parameters—namely age, sex, survival status, metastasis, and stage-comparative analyses were conducted and visualized utilizing the Beeswarm package in R.

2.4 Cell Line and Treatment

Cal-27 cells, which serve as a model for human tongue squamous cell carcinoma, were procured from the American Type Culture Collection located in Manassas, Virginia, USA. The shCont- and shRALB Cal-27 cell lines were derived from the preliminary investigations conducted by our research team [25]. All cell cultures were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), under conditions of 5% CO₂ and 100% humidity at 37 °C. All cell lines were validated by STR profiling and tested negative for mycoplasma.

2.5 Colony Formation Assay

Log-phase Cal-27 cells were seeded into 6-well plates at a density of 600 cells per well (yielding an actual count of 300–500 cells per well). Following cell attachment, the cells were treated with either 24.5 μM dihydroartemisinin (DHA, Tci, Tokyo, Japan) or 0.1% Dimethyl sulfoxide (DMSO, Solarbio, Beijing, China) for 24 hours. Each condition was performed in triplicate. After treatment, the cells

were maintained in complete growth medium for 14 days to allow colony formation, and subsequently stained with crystal violet solution.

2.6 Cell Migration Assay

Cal-27 cells, in their exponential growth phase, were introduced into 6-well plates with a density of 5×10^6 cells per well. After the cells adhered and formed a monolayer, scratches were made on the bottom of the wells using a 10 μL pipette tip. Subsequently, the cells were treated with 24.5 μM DHA, 0.1 μM rapamycin (Sigma-Aldrich, USA), 1 mM 3-MA (Sigma-Aldrich, USA), or 0.1% DMSO for 24 hours. Microscopic images were taken to observe wound healing at the start and 24 hours after treatment commenced. The cell migration ability = (scratch area at 0 h – scratch area at 24 h) / scratch area at 0 h $\times 100\%$.

2.7 Orthotopic Nude Mice Model of TSCC and Tumor Treatment

BALB/c nude mice (male, 6 weeks old) were obtained from GemPharmatech (Jiangsu, China) and SiPeiFu (Beijing, China). Animals were housed in a specific pathogen-free barrier facility. All animal experiments were approved by the Animal Experiment Ethics Committee of Hebei University of Chinese Medicine with the approval number: No. DWLL202409007. To generate orthotopic TSCC models, 1×10^6 Cal-27 cells stably expressing shCont- or shRALB were injected into the tongues of recipient mice ($n = 10$ per group). Then, according to the dosage of 25 mg/kg DHA [26], DHA or DMSO was administered to the mice with

Table 1. Univariate Cox proportional hazards regression analysis of HNSC combined with RAS pathway genes.

Symbol	HR	HR.95L	HR.95H	p-value
<i>RASGRF1</i>	2.6783	1.5674	4.5767	3.1×10^{-4}
<i>RRAS2</i>	1.039	1.0222	1.0561	4.0×10^{-6}
<i>PDGFA</i>	1.0364	1.0141	1.0592	1.3×10^{-3}
<i>AURKA</i>	1.0282	1.0103	1.0465	2.0×10^{-3}
<i>RALB</i>	1.0079	1.003	1.0129	1.7×10^{-3}
<i>ITGA5</i>	1.0076	1.0031	1.0121	8.9×10^{-4}
<i>PSMD2</i>	1.0049	1.0023	1.0074	1.8×10^{-4}
<i>ITGA3</i>	1.0047	1.0022	1.0072	2.1×10^{-4}
<i>IL1A</i>	1.0036	1.0016	1.0055	2.7×10^{-4}
<i>PLAU</i>	1.0026	1.0011	1.004	8.1×10^{-4}
<i>APP</i>	1.0022	1.001	1.0035	6.0×10^{-4}
<i>TYK2</i>	0.9328	0.9004	0.9663	1.1×10^{-4}
<i>LCK</i>	0.9302	0.892	0.97	7.1×10^{-4}
<i>JAK3</i>	0.9043	0.8542	0.9574	5.4×10^{-4}
<i>PIK3C2B</i>	0.857	0.7844	0.9362	6.3×10^{-4}
<i>SGSM2</i>	0.8515	0.7736	0.9371	1.0×10^{-3}
<i>EZH1</i>	0.8282	0.7372	0.9303	1.5×10^{-3}
<i>ZAP70</i>	0.8006	0.7187	0.8918	5.4×10^{-5}
<i>GNG7</i>	0.6528	0.5031	0.8471	1.3×10^{-3}
<i>ABCB1</i>	0.401	0.2415	0.6657	4.1×10^{-4}

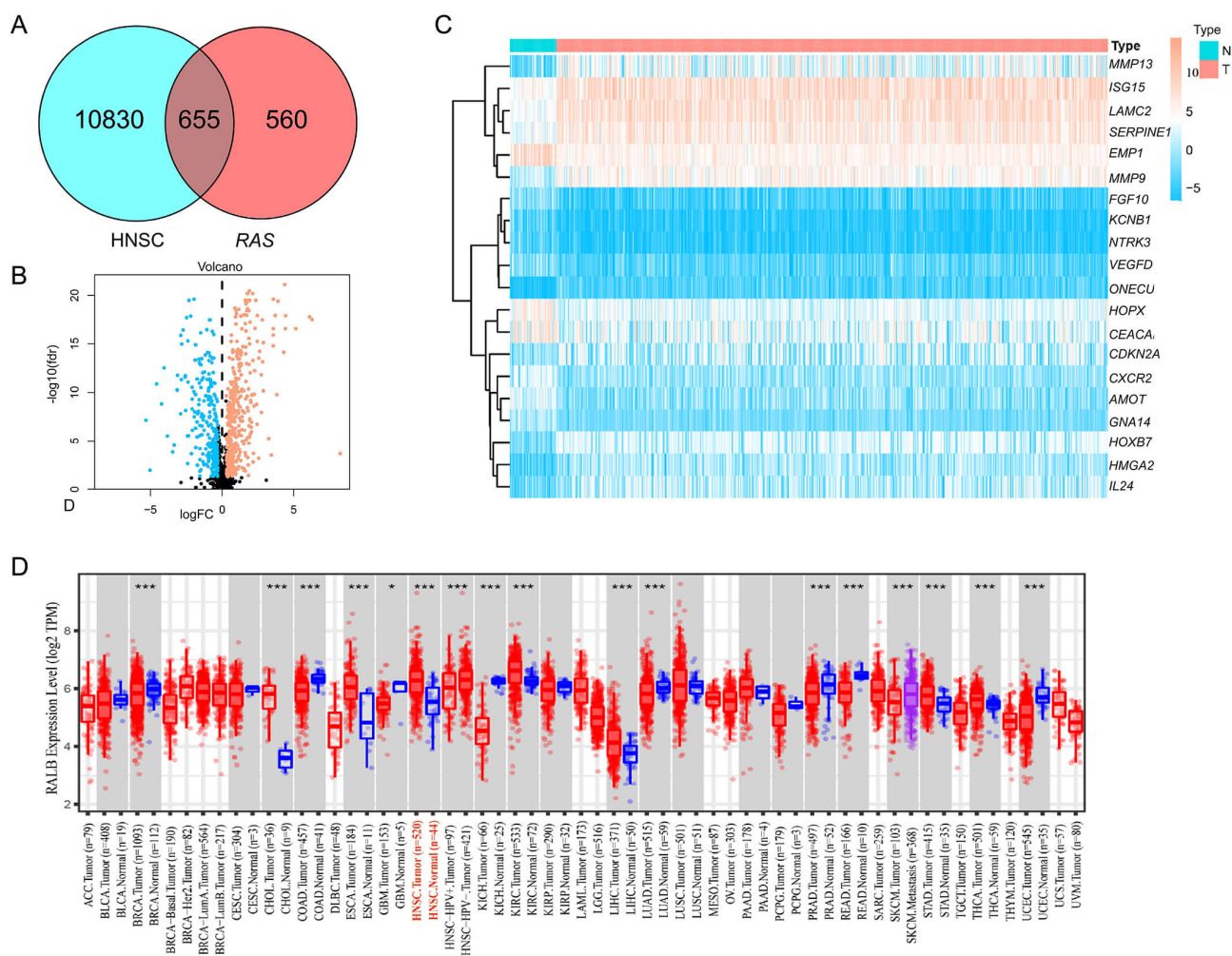


Fig. 1. Identification of Head and Neck Squamous Cell Carcinoma (HNSCC) targets and RAS pathway targets. (A) 655 intersection genes were identified based on the Venn diagram in the HNSC/RAS pathway. (B) A volcano plot showing differentially expressed genes (DEGs) in the datasets KEGG, NCBI, and GeneCards. (C) Heat map of the top 20 differentially expressed genes. (D) The messenger RNA expression levels of *RALB* in different types of human cancers by the Tumor Immune Estimation Resource database (* $p \leq 0.05$, *** $p \leq 0.001$).

Table 2. Multivariate Cox proportional hazard regression analysis.

Symbol	coef	HR	HR.95L	HR.95H	<i>p</i> -value
<i>RASGRF1</i>	0.97	2.65	1.53	4.59	4.95×10^{-4}
<i>EZH1</i>	0.24	1.28	1.08	1.51	4.5×10^{-3}
<i>PIK3R2</i>	-20.01	2.04×10^{-9}	7.81×10^{-16}	5.3×10^{-3}	7.9×10^{-3}
<i>GAST</i>	9.7×10^{-3}	1.01	1.00	1.02	2.7×10^{-2}
<i>PIK3C2B</i>	-9.6×10^{-2}	0.91	0.82	1.00	5.5×10^{-2}
<i>TYK2</i>	4.0×10^{-2}	0.96	0.92	1.00	6.0×10^{-2}
<i>RALB</i>	6.3×10^{-3}	1.01	1.00	1.01	6.8×10^{-2}
<i>ABCB1</i>	-4.0×10^{-1}	0.67	0.41	1.08	0.10
<i>PSMD2</i>	2.3×10^{-3}	1.00	1.00	1.01	0.12
<i>APP</i>	1.1×10^{-3}	1.00	1.00	1.00	0.13
<i>ZAP70</i>	9.4×10^{-2}	0.91	0.80	1.03	0.14
<i>SGSM2</i>	7.9×10^{-2}	0.92	0.83	1.03	0.16

shCont- or sh*RALB*- Cal-27 cells (n = 5) via intraperitoneal injection once daily for 10 consecutive days. After the treat-

ment period, blood was collected by eye enucleation, and mice were euthanized by cervical dislocation. Then, all

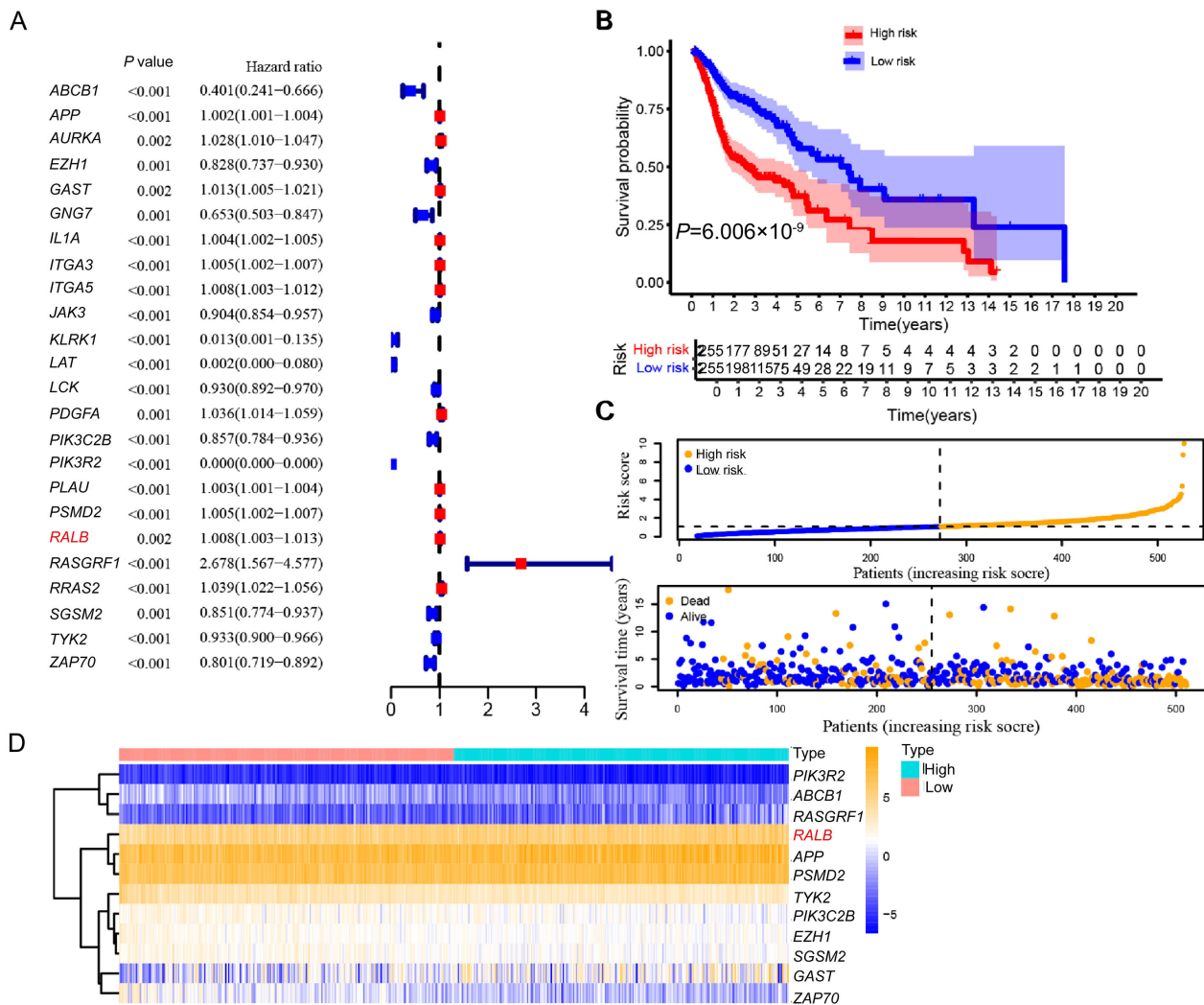


Fig. 2. Identification of survival-related genes associated with RAS in HNSC. (A) Univariate Cox analysis of RAS-associated genes significantly correlated with survival in HNSC ($***p \leq 0.001$) (B) Survival analysis of overall survival between high- and low-risk groups. The red and blue curves represent the high-risk group and low-risk group, respectively ($***p \leq 0.001$). (C) The risk curve of each sample is reordered. The scatter plot of the sample survival overview. The blue dots represent survival, and the yellow means death, respectively. (D) Heatmap showed expression levels of genes in the high-risk and low-risk groups. The pink and blue bars represented the low-risk group and the high-risk group. And the evolution from blue to yellow represented the -5 to 5 level of gene expression.

mouse tongues and cervical lymph nodes were collected, fixed in 4% paraformaldehyde. The study was performed and reported in line with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines [27].

2.8 Hematoxylin and Eosin (H&E) Staining

The tongue tissues needed for the experiment were swiftly excised, fixed in 4% formaldehyde, embedded in paraffin, cut into 5 μ m thick slices. After hematoxylin and eosin staining, histopathological changes of the tongue tissues were observed under a microscope (Leica DM2500, Germany).

2.9 Immunohistochemistry (IHC) Staining

Tongue tissues were sectioned into 5 μ m thick slices. After dewaxing and rehydration, antigen retrieval was performed under high pressure, followed by inactivation of endogenous enzymes and incubation with primary and secondary antibodies. The primary antibodies were rabbit anti-P62 antibody (CST, #5114, dilution 1:500) and anti-LC3B (CST, #2775, dilution 1:200). The steps for the second antibody incubation, coloring with 3,3-diamethoxybenzidine (DAB), and restaining were then completed consecutively. The images were captured with the help of a microscope (DM4B, Leica, Germany). Quantitative analysis of positive staining areas was performed using ImageJ software (National Institutes of Health, USA) to evaluate the IHC staining results.

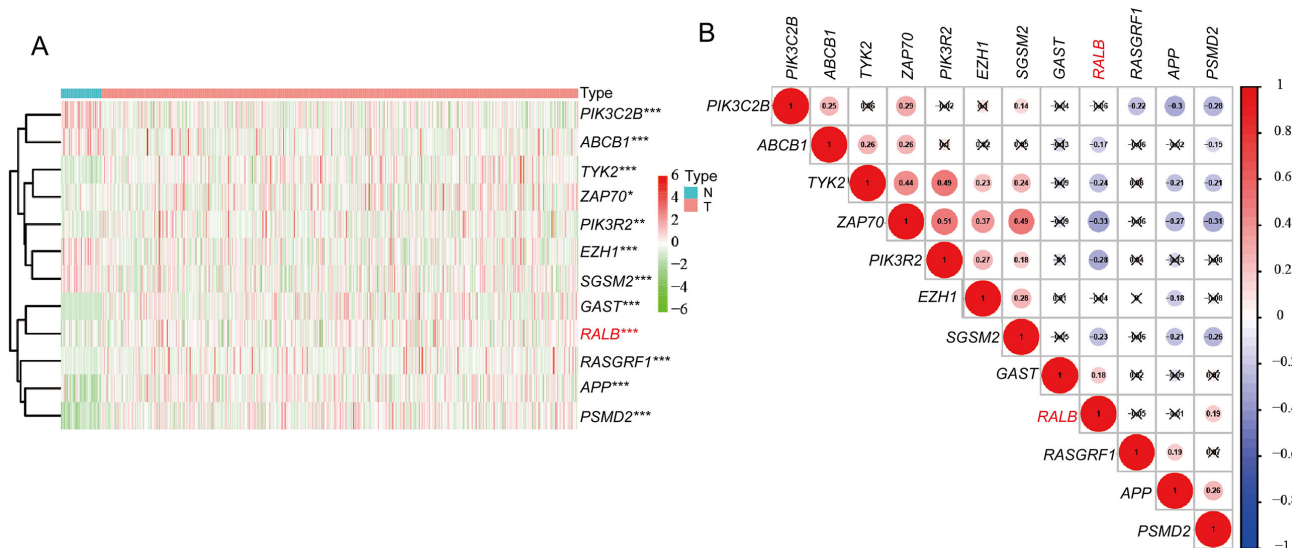


Fig. 3. *RALB* was significantly correlated with other differentially expressed genes. (A) The heatmaps of 12 genes in tumor tissues and normal tissues. N, normal tissues; T, tumor tissues. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). (B) Spearman correlation analysis of the 12 differentially expressed genes in HNSC.

Table 3. Clinical correlation analysis.

Symbol	Fustat	Age	Gender	Stage	T	N
<i>ABCB1</i>	3.303 (0.001)	-0.866 (0.387)	-0.937 (0.350)	1.927 (0.056)	2.825 (0.005)	0.193 (0.847)
<i>APP</i>	-2.669 (0.008)	-0.582 (0.561)	2.544 (0.012)	0.235 (0.814)	-1.007 (0.314)	0.209 (0.834)
<i>EZH1</i>	3.713 (2.346×10^{-4})	-1.244 (0.214)	-2.167 (0.031)	-1.09 (0.277)	2.748 (0.006)	-1.592 (0.112)
<i>GAST</i>	-1.175 (0.241)	1.689 (0.093)	-1.248 (0.213)	-4.547 (7.091×10^{-6})	-2.036 (0.042)	-0.715 (0.475)
<i>PIK3C2B</i>	1.771 (0.077)	0.907 (0.365)	0.803 (0.423)	2.202 (0.029)	2.154 (0.032)	1.869 (0.063)
<i>PIK3R2</i>	5.007 (8.079×10^{-7})	0.949 (0.343)	-0.045 (0.965)	0.872 (0.384)	1.016 (0.310)	0.77 (0.442)
<i>PSMD2</i>	-2.451 (0.015)	-1.188 (0.236)	-0.92 (0.359)	-1.559 (0.121)	-1.685 (0.093)	-1.276 (0.203)
<i>RALB</i>	-1.724 (0.086)	0.41 (0.682)	-0.53 (0.596)	-1.448 (0.149)	-1.904 (0.058)	1.968 (0.050)
<i>RASGRF1</i>	-2.579 (0.011)	0.906 (0.366)	-2.251 (0.025)	-0.811 (0.418)	1.975 (0.049)	-1.822 (0.069)
<i>SGSM2</i>	3.75 (2.03×10^{-4})	-1.275 (0.203)	-2.194 (0.029)	-1.413 (0.159)	0.995 (0.320)	0.299 (0.765)
<i>TYK2</i>	4.612 (5.373×10^{-6})	-3.256 (0.001)	-2.785 (0.006)	-0.148 (0.882)	1.748 (0.082)	-1.051 (0.294)
<i>ZAP70</i>	4.387 (1.445×10^{-5})	-1.068 (0.286)	-0.989 (0.323)	-0.596 (0.552)	2.518 (0.012)	-1.613 (0.107)
riskScore	-4.17 (4.715×10^{-5})	0.985 (0.326)	-1.467 (0.143)	-3.895 (1.148×10^{-4})	-2.014 (0.045)	-0.326 (0.745)

2.10 Western Blot

Total protein from the tumors in the tongue was extracted after different treatments using a Cell Protein Extraction Kit (EpiZyme, China) and quantified via BCA assay (Solarbio, China). The primary antibodies were rabbit anti-P62 (#5114, CST, diluted at 1:1000), rabbit anti-LC3B antibody (#2775, CST, diluted at 1:400), rabbit anti-GAPDH antibody (ab0037, Abways, 1: 5000). Goat anti-rabbit IgG-HRP (ZB-2301, ZSGB-BIO, 1: 5000) served as the secondary antibody. The bands were found using the ECL detection system (Vilber, Fusion FX5, Spectra, France).

2.11 Statistical Analysis

R version 3.6.1 was used to evaluate all statistical data. The data were displayed as mean $\pm$ SD. If they were reg-

ularly distributed, the *t*-test was used for both groups, or ANOVA was employed when there were more than two groups included after the homogeneity of variance was determined. *p* values ≤ 0.05 were considered statistically significant.

3. Results

3.1 Identification of HNSC Targets and RAS Pathway Targets

To explore the expression of genes associated with the RAS pathway in HNSC, we initially screened pertinent genes across three independent databases. This analysis identified 655 genes that are integral to the RAS pathway in HNSC (Fig. 1A). The differentially expressed genes (DEGs) between HNSC tumor and normal tissues are shown in the volcano plot (Fig. 1B). The top 20 genes ex-

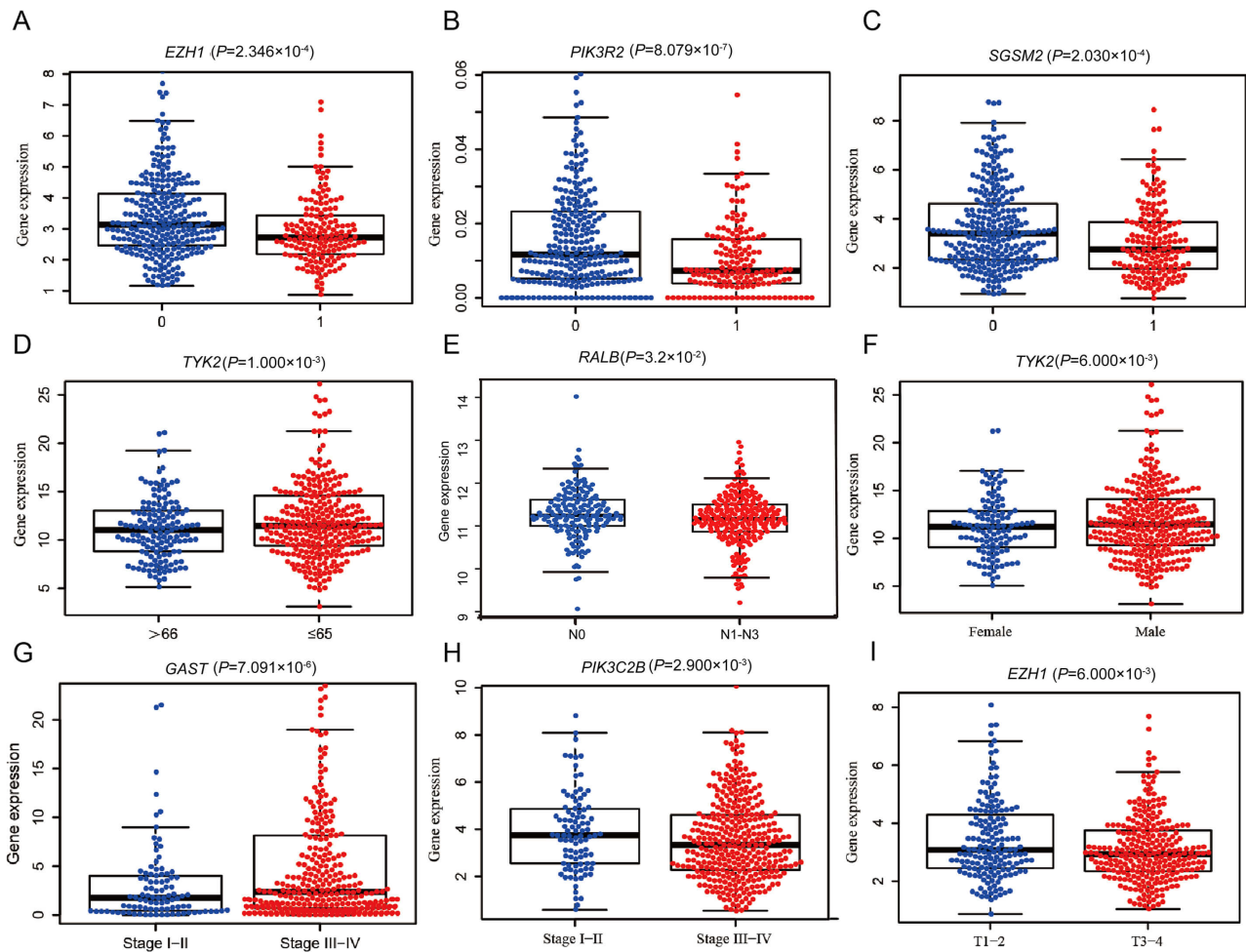


Fig. 4. *RALB* was associated with tumor lymphatic metastasis in HNSC patients. (A–C) Association of gene expression with the survival status of the HNSC patient. The numbers 0 and 1 represented the living patients and the deceased. ($***p \leq 0.001$). (D) Association of gene expression with age in HNSC ($***p \leq 0.001$). (E,F) Association of gene expression with gender in HNSC ($*p \leq 0.05$, $**p \leq 0.01$). (G,H) Association of gene expression with the stage in HNSC ($*p \leq 0.05$, $***p \leq 0.001$). (I) Association of gene expression with the T-classification in HNSC ($**p \leq 0.01$).

hibiting the most significant fold changes were ranked according to the absolute value of their LogFC and are presented in Fig. 1C. *RalB*, one of the *Ral* GTPases, is a promising therapeutic target [14,15]. To further validate the expression of *RALB* in HNSC, we analyzed its expression using the TIMER database. The results indicated that *RALB* expression was significantly up-regulated in HNSC tumor tissues compared to normal tissues ($p < 0.05$) (Fig. 1D). These findings suggest that the *RALB* gene is highly expressed in HNSC.

3.2 12 Survival-Related Genes Associated With *RAS* Were Screened in HNSC

We conducted both univariate and multivariate Cox proportional hazards analyses on the expression profiles of 655 genes to investigate the relationship between these differential gene expressions and clinical survival outcomes. The univariate Cox analysis revealed that 24 genes were

significantly associated with *RAS* expression levels in head and neck squamous cell carcinoma (HNSCC) ($p \leq 0.002$) (refer to Fig. 2A and Table 1). Univariate Cox analysis identified 20 genes significantly associated with survival (Table 1). Multivariate Cox analysis further confirmed 4 candidate genes, while *RALB* ($p = 0.068$) and *TYK2* ($p = 0.060$) showed a trend toward significance (Table 2). Survival analysis showed that patients with elevated *RALB* expression presented poorer survival prognosis in HNSC ($p = 6.006 \times 10^{-9}$) in HNSC (Fig. 2B). Combined survival status analysis indicated obvious survival differences among different gene expression subgroups (Fig. 2C). The expression heatmap reflected distinct expression patterns of these 12 genes in different subgroups (Fig. 2D).

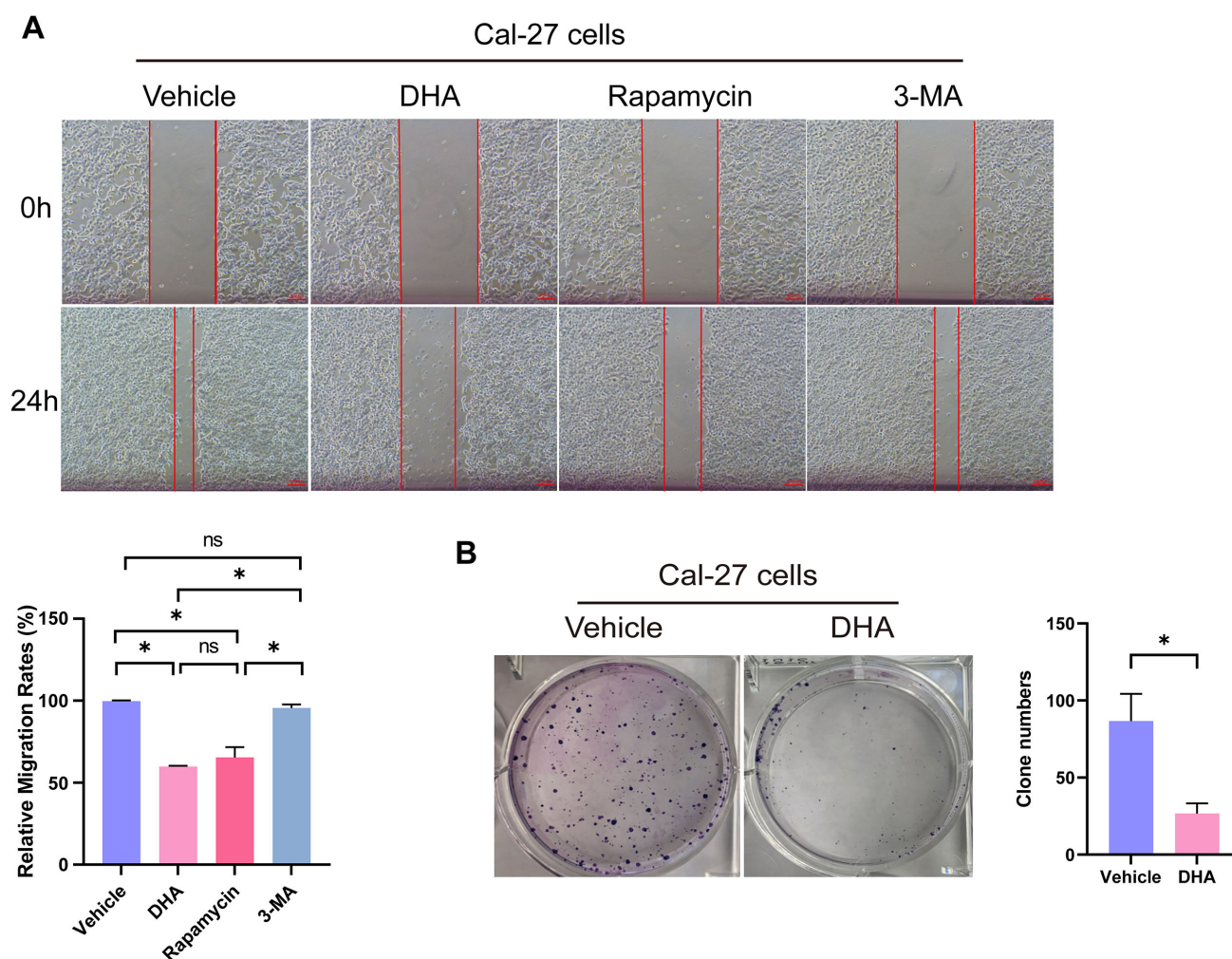


Fig. 5. DHA inhibited the proliferation and migration of Cal-27 cells. (A) Cal-27 cells were treated with DMSO (0.1%), DHA (24.5 μ M), 3-MA (1 mM), or rapamycin (0.1 μ M), respectively, for 24 h, and then all cells were photographed at 0 h and 24 h. The relative migration rates are shown in a bar graph (ns, not significant, $*p \leq 0.05$). (B) Cal-27 cells treated with DHA were cultured for 14 d. The relative clone numbers are shown in a bar graph ($*p \leq 0.05$). Scale bar = 100 μ m.

3.3 RALB Was Significantly Correlated With Other Differentially Expressed Genes

The heatmap analysis indicated a negative correlation between the genes RALB, TYK2, ZAP70, PIK3R2, GAST, RASGRF1, APP, and PSMD2 with normal samples, whereas PIK3C2B, ABCB1, EZH1, and SGSM2 exhibited a positive correlation with tumor samples (Fig. 3A). Further correlation analysis revealed associations between RALB and the genes ABCB1, ZAP70, EZH1, TYK2, SGSM2, GAST, and PSMD2 (Fig. 3B), suggesting that RALB is highly expressed and significantly correlated with other expressed genes in HNSC.

3.4 RALB Was Positively Associated With N Stage in HNSCC Patients

Utilizing clinical prognosis data, we explored the relationship between these 12 genes and clinical outcomes. The findings indicated a positive correlation between RALB ex-

pression and the N stage, implying an association with tumor lymphatic metastasis in HNSC patients (Table 3). Additionally, EZH1, PIK3R2, and SGSM2 were more highly expressed in living individuals compared to deceased ones (Fig. 4A–C). TYK2 expression was elevated in patients aged ≤ 65 years (Fig. 4D). RASGRF1 was predominantly expressed in female patients, in contrast to TYK2 (Fig. 4E,F). GAST expression was higher in stage III–IV, whereas PIK3C2B and EZH1 showed the opposite trend (Fig. 4G–I).

3.5 DHA Inhibited the Migration in Cal-27 Cells

Rapamycin, an autophagy activator [28], reduced the wound healing rate to $68.73\% \pm 0.89\%$ ($p < 0.05$). Similarly, treatment with DHA resulted in a decreased wound healing rate of $59.77\% \pm 0.40\%$ ($p < 0.05$) (Fig. 5A). There was no difference between the DHA group and the rapamycin group. 3-methyladenine (3-MA) is an autophagy

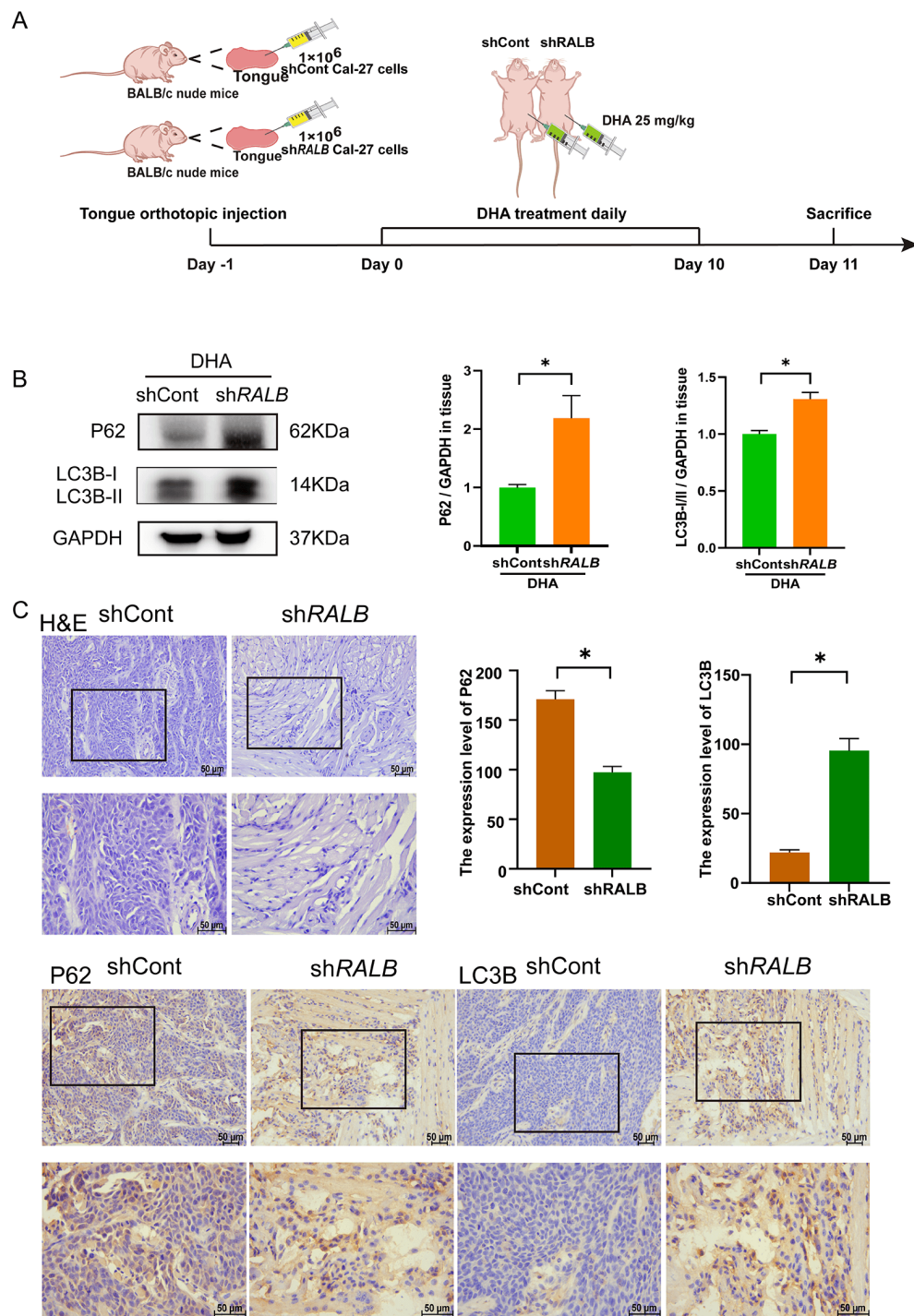


Fig. 6. DHA promoted autophagy through *RALB* in TSCC orthotopic nude mice. (A) The schedule of the orthotopic nude mice model of TSCC and treatment. (B) Western blot was used to detect the expression of P62 and LC3B in shCont or shRALB mouse tongue tumor tissues after DHA treatment ($*p \leq 0.05$). Gray value analysis of Western blot bands was performed using ImageJ software (Version 1.54, NIH, USA). GAPDH was used as the loading control, and the relative protein expression levels of target proteins (P62 and LC3B) were calculated as the ratio of the gray value of each target band to that of the corresponding GAPDH band. Data are represented as mean $\pm$ SD. vs. shCont. (C) Representative photos of histological H&E-stained, P62 and LC3B-stained tongue tissues are shown for each group, scale bar = 50 μ m. Magnification = 200 $\times$ and 400 $\times$, ($*p \leq 0.05$). Quantitative analysis of P62 and LC3B was performed using the Color Deconvolution plugin in ImageJ (Version 1.54, NIH, USA) by measuring the mean optical density (OD). Data are represented as mean $\pm$ SD, vs. shCont.

inhibitor [29]. Compared with the wound healing rate in the 3-MA group, both in the DHA group and the rapamycin group were decreased ($p < 0.05$) (Fig. 5A). These results suggested that DHA and rapamycin inhibited the migration in Cal-27 cells. The clone assay showed that the clone numbers in the DHA group were lower than those in the vehicle group ($p < 0.05$) (Fig. 5B). This result suggested that DHA inhibited the proliferation in Cal-27 cells.

3.6 DHA Promoted Autophagy Through *RALB* in TSCC Orthotopic Nude Mice

The earlier research showed that DHA inhibited *RalB* expression and promoted autophagy in tongue squamous cell carcinoma [30]. We have shown that DHA inhibited *RalB* expression in Cal-27 cells and the tongue of TSCC orthotopic nude mice. In this study, shCont and sh*RALB* Cal-27 cells were injected into the tongue of nude mice, and treated with DHA to verify whether DHA promotes autophagy through *RALB* (Fig. 6A). *P62*, as an autophagy substrate, is negatively correlated with autophagy activity [31]. Compared with the shCont group, the expression level of *P62* was decreased in the sh*RALB* group ($p < 0.05$) (Fig. 6C). DHA treatment increased the expression level of *P62* in mice with sh*RALB*-Cal-27 cells in the tongue ($p < 0.05$) (Fig. 6C). Microtubule-associated protein 1 light chain 3 beta (*LC3B*) is a marker of autophagy, which is positively correlated with autophagy [32]. *LC3B* expression level was increased in the sh*RALB* group compared with the shCont group ($p < 0.05$) (Fig. 6B,C). This result suggested that DHA no longer promoted autophagy in Cal-27 cells with *RALB* knockdown.

4. Discussion

The relationship between *RalB* and autophagy is still unclear in TSCC. Our study found that *RALB* was the target gene in HNSCC, and DHA acted as one of the *RalB* inhibitors to suppress cell migration and induce autophagy in Cal-27 cells.

The human proto-oncogene *RAS* is one of the mutated genes in cancers. The promising approach for treating cancer patients with *RAS* mutations is to target signaling networks downstream of *RAS*. Small G proteins known as Ras-like oncoprotein A (RALA) and Ras-like oncoprotein B (RALB) play an essential role in Ras effectors. *RALB* has played different roles in regulating autophagy. *RALB* maintained a balance between autophagy and apoptosis [33]. *R. intestinalis*/butyrate-elicited autophagy depend on *RalB* activate in irradiated CRC cells [34]. Despite its established role, the clinical and transcriptional profile of *RALB* in HNSCC remains uncharacterized. Our bioinformatic analysis firstly revealed that *RALB* is significantly upregulated in HNSC and its high expression correlates with an unfavorable prognosis.

Further, one study showed that DHA triggered autophagy and degraded *RALB* expression level [35], but the

relationship between *RALB* expression and autophagy did not reveal. Herein, *RALB* knockdown promoted autophagy in an orthotopic nude mouse model of TSCC. Additionally, *RALB* knockdown inhibited the proliferation and migration in Cal-27 cells [30]. Consistently, some studies reported that *RALB* promotes the invasion and migration in glioblastoma cells, lung cancer cells, and pancreatic cancer cells [36,37,38]. Our work highlighted an attractive target of *RALB* as an anti-cancer strategy.

DHA treatment showed a wide range of anticancer benefits, including triggering autophagy, reducing tumor growth, preventing tumor metastasis from triggering apoptosis, and angiogenesis [21]. DHA induced autophagy in cutaneous squamous cell carcinoma cells [39]. In multiple myeloma, DHA induced autophagy through P38/MAPK signaling pathways [40]. Our study found that DHA inhibited the expression of *RALB* to promote autophagy in Cal-27 cells.

5. Limitations

Further verification is required to determine whether DHA promotes autophagy by autophagy flux in Cal-27 cells and other TSCC cell lines such as SCC-9 cells. Besides *RALB* knockdown in Cal-27 cells, rescue experiments with *RALB* overexpression should be used to assess DHA-induced autophagy. The interpretation of DHA's therapeutic potential is constrained by several factors, including the lack of *in vivo* dose validation, the absence of pharmacokinetic analysis, and the lack of systematic off-target evaluation.

6. Conclusion

We showed that DHA inhibited cell migration and promoted autophagy through *RALB* in Cal-27 cells.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

KF Writing—Conception and design, Administrative support. YZ, JJ, WW Collection and assembly of data, Data analysis and interpretation. YG, KY Collection and assembly of data. Manuscript writing: All authors. Final approval of manuscript: All authors. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Hebei University of Chinese Medicine (Approval No.

DWLL202409007). All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines. All efforts were made to minimize animal suffering and reduce the number of animals used.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/IJP51935>.

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