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Rab8a Serves as a Valuable Biomarker of Esophageal Squamous Cell Carcinoma

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Abstract

Esophageal carcinoma (ESCA) is a digestive tract malignancy with high morbidity and mortality in China, among which esophageal squamous cell carcinoma accounts for 90% of the confirmed cases. Rab8a is a member of the Ras small GTPase superfamily, and it has been shown to play an important role in endometrial cancer and hepatocellular carcinoma. However, the expression and function of Rab8a in esophageal squamous cell carcinoma (ESCC) are currently unclear.Our study demonstrated that Rab8a is upregulated in ESCC and may promote its proliferation and migration by activating mitochondrial respiration. This study provides a rationale for clinical diagnosis and screening of new therapeutic targets for ESCC.

Keywords: Esophageal Squamous Cell Carcinoma; Rab8a; Proliferation; Migration; Biomarker

Introduction

Esophageal carcinoma (ESCA) is one common malignant tumor of digestive system with the seventh incidence and sixth mortality worldwide [1]. And the main histologic subtype is esophageal squamous cell carcinoma (ESCC) in China, which accounting for about 90% of all diagnosed cases [2]. Although there has been improvement of therapeutic approaches, the outcome of ESCC patients remains poor because of the late diagnosis [3]. Therefore, it is of great significance to find a valuable biomarker for ESCC diagnosis.

Rab8a protein is a member of RAS superfamily which participates in membrane traffic processes [4-7]. Previous study showed that Rab8a was up-regulated in endometrial cancer and might be a new biomarker [8]. In addition, it was reported that the enhanced expression of Rab8a could improve the immunotherapy efficacy of hepatocellular carcinoma, which suggested that it might be a novel therapeutic target [9]. However, the expression and biological functions remain unknown in other human cancers including ESCC.

In the current study, we first analyzed the expression of Rab8a in many cancer types and their normal tissues by conducting pan-cancer analysis from some public databases. And according to the result of the analysis, we then investigated the expression and biological function of Rab8a in ESCC progression and at last explored the potential molecular mechanism of Rab8a in ESCC.

Materials and Methods

Rab8a Expression and Survival Analysis in Different Cancers

The normalized transcripts per million (nTPM) levels of Rab8a in different types of tumors were identified based on the data of TIMER, Gene Expression Profiling Interactive Analysis (GEPIA) and UALCAN online resources. The relationship of Rab8a mRNA expression and the pathological features were available from UALCAN online databases. Also, we analyzed the correlation between Rab8a expression and overall survival (OS) outcomes of the patients with different cancers using the web database of Kaplan-Meier plotter.

Tissue Specimens, Cell Lines and Transfection

62 cases of formalin-fixed paraffin-embedded and 12 cases of fresh ESCC samples and their matched adjacent normal tissues were collected from the Department of pathology, Third Affiliated Hospital of Xinxiang Medical University (Xinxiang City, Henan Province, China) from June 2016 to July 2022. The fresh tissues were frozen in liquid nitrogen before extracting the total RNA. None of the patients underwent chemotherapy, radiotherapy or immunotherapy before surgery. Prior approval for the study had been obtained from Xinxiang Medical University Institutional Board (Xinxiang, China).

ESCC cell lines of ECA109 and ECA9706 were obtained from the Cell Bank of the Chinese Academy (Shanghai, China). All ESCC cells were cultured in RPMI-1640 with 10% FBS at 37°C in a humidified atmosphere with 5% CO2.

The overexpression and knockdown plasmids of Rab8a were the products of Public Protein/Plasmid Library (PPL). Lentivirus was produced by HEK293FT cells using the calcium phosphate method. The transfected cells were selected in medium containing puromycin.

Immunohistochemistry (IHC)

The formalin-fixed paraffin-embedded sample tissues were cut into 4-μm sections and baked at 60°C for 2 h. IHC was conducted by the SP kits (ZSGB-BIO, Beijing, China). The sections were incubated overnight at 4°C with the rabbit polyclonal antibody of Rab8a (1:100, Proteintech, USA). PBS was used as the negative control. The sections were stained with 3,3-diaminobenzidine (DAB) and sealed with neutral balsam. Finally, we observed the sections by two pathologists used immunoreactive score (IRS) which was mainly based on the proportion and intensity of the stained tumor cells [10].

Real-time Quantitative PCR (qPCR)

TRIzol reagent (Invitrogen, USA) was used to extract the total RNA from human tissues and cultured cells. qPCR was carried out according to the manufacturer's protocol with SYBR Green I. The data were normalized to the the housekeeping gene of GAPDH, and calculated as $2^{-\Delta\Delta CT}$. The follows were primer sequences: GAPDH (F: ACA GTC AGC CGC ATC

TTC TT, R: GAC AAG CTT CCC GTT CTC AG), Rab8a (F: ACG CCT TCA ACT CCA CTT; R: ACC AGC ATG ATG CCC ATT).

Western Blot

The protein was extracted from the corresponding cells with the sample buffer lysate solution. Then the lysates were subjected to SDS-PAGE and transferred to PVDF membranes. 5% non-fat dry milk were used to block the membranes and incubated overnight at 4°C with the primary antibodies of polyclonal rabbit anti-Rab8a and mouse anti-GAPDH (Proteintech, USA). The appropriate secondary antibodies were used to incubate the membranes at room temperature for 1 h and then exposed for autoradiography.

CCK-8 Assay

Cells with stable over- or knockdown expression of Rab8a were seeded in 96-well plates at the density of 1×10^3 cells per well and cultured for 24 h. Then, the culture medium was discarded and 10 μL of CCK-8 reagent and 100 μL of medium were added to every well. After 2 h incubation, the absorbance at 450 nm was detected. The experiment was conducted repeatedly for three times.

Colony Formation Assay

Cells with stable over- or knockdown expression of Rab8a were plated on 6-well plates (200 cells/well) and cultured for 2 weeks. The colonies were stained with Hematoxylin for 30 min after fixation with 4% paraformaldehyde for 10 minutes. The number of colonies, defined as >50 cells/colony, were counted. Three independent experiments were performed.

Transwell Migration Assay

Boyden chambers were used to conducted Transwell migration assays. Cells (1×10^5) in culture medium without FBS were seeded to the upper chamber, and culture medium containing 20% FBS as a chemoattractant was added to the lower chamber. After incubation for 48 h, the chamber was fixed in 4% paraformaldehyde and stained with Hematoxylin. Cells on the upper sides of the filters were removed with cotton swabs. Cells that migrated to the lower sides were stained with Hematoxylin. The migratory cells on the lower surfaces of the filters were counted. Three independent experiments were performed.

Wound-Healing Assay

Cells were seeded to 6-well plates and incubated under permissive conditions until the cells reached 90% confluence. After serum starvation for 24 h, wounds were generated in the confluent cells using a pipette tip. Wound healing within the scrape line was then observed and photographed at indicated time points. Each experiment was repeated at least three times.

Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) is a knowledge-based approach for interpreting genome-wide expression differences between two biological states. We performed GSEA to explore the potential biological functions affected by Rab8a expression in ESCC.

Statistical Analysis

All statistical analyses were performed using SPSS20.0 for Windows. The data are expressed as mean ± standard deviations (s.d.) from at least three independent experiments. The two-tailed paired Student's t-test was conducted for the analysis of two groups. Mann-Whitney U test was conducted to analyze the relationship between Rab8a expression and the clinicopathologic characteristics of ESCC. $p<0.05$ was considered as statistically different. * represented $p<0.05$; **represented $p<0.01$.

Results

Rab8a Expression in Pan-cancer

We analyzed the expression of Rab8a by applying the TIMER2.0 and found that it was significantly increased in multiple tumor types compared with those in normal tissues, including ESCA, bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cholangio carcinoma (CHOL), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), liver hepatocellular carcinoma (LIHC), stomach adenocarcinoma (STAD) and uterine corpus endometrial carcinoma (UCEC) (Figure 1A). The results of GEPIA online database analysis demonstrated

that the expression levels were obviously increased in ESCA, BRCA, STAD, glioblastoma (GBM), acute myeloid leukemia (LAML), lower grade glioma (LGG), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD) and testicular germ cell tumors (TGCT) than those in their normal tissues (Figure 1B). Also, the expression of Rab8a was analyzed across 24 types of human cancer samples compared with their normal controls in UALCAN online database and revealed that it was increased in the following cancers: ESCA, CHOL, BLCA, HNSC, KIRC, LIHC, STAD, UCEC, BRCA and cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC)(Fig 1C). Summarized the above results, it was found that the expression of Rab8a was significantly up--regulated in the common solid cancers of BRCA, STAD and ESCA.

Expression and Prognostic Significance of Rab8a in BRCA, STAD and ESCA

Then we explored the Rab8a expression with different histological subtypes and the OS significance in BRCA, STAD and ESCA. It was revealed that Rab8a expression was significantly increased in all of the histological subtypes of BRCA, STAD and ESCA (Figure 2A,C,E). And the survival analysis revealed that Rab8a expression had no prognostic value in BRCA and STAD (Figure 2B,D). But in ESCA, Rab8a was the valuable prognostic marker. It was found that the high expression of Rab8a revealed poor prognosis of ESCC patients but good prognosis of esophageal adenocarcinoma (EAC) (Figure 2F,G). So, the above analytical results implied that Rab8a might be an important diagnostic and prognostic marker of ESCC.

Rab8a Expression and its Correlation with the Clinicopathological Characteristics in Human ESCC Tissues

We then detected Rab8a mRNA expression in 12 cases of ESCC and their paired adjacent normal tissues by qPCR. It was found Rab8a mRNA was obviously increased (T/N>0.5) in 9 cases (Figure 3A,B). The IHC results showed that the rate of high expression of Rab8a protein was obviously increased in ESCC (77.42%) than that in their paired adjacent normal tissues (8.06%) (Figure 3C,D). Further analysis of the correlation of Rab8a expression with ESCC clinicopathological characteristics demonstrated that the expression of Rab8a was significantly associated with the size and infiltration depth of ESCC (Table 1). Therefore, the higher expression of Rab8a implied greater ability of proliferation and invasion.

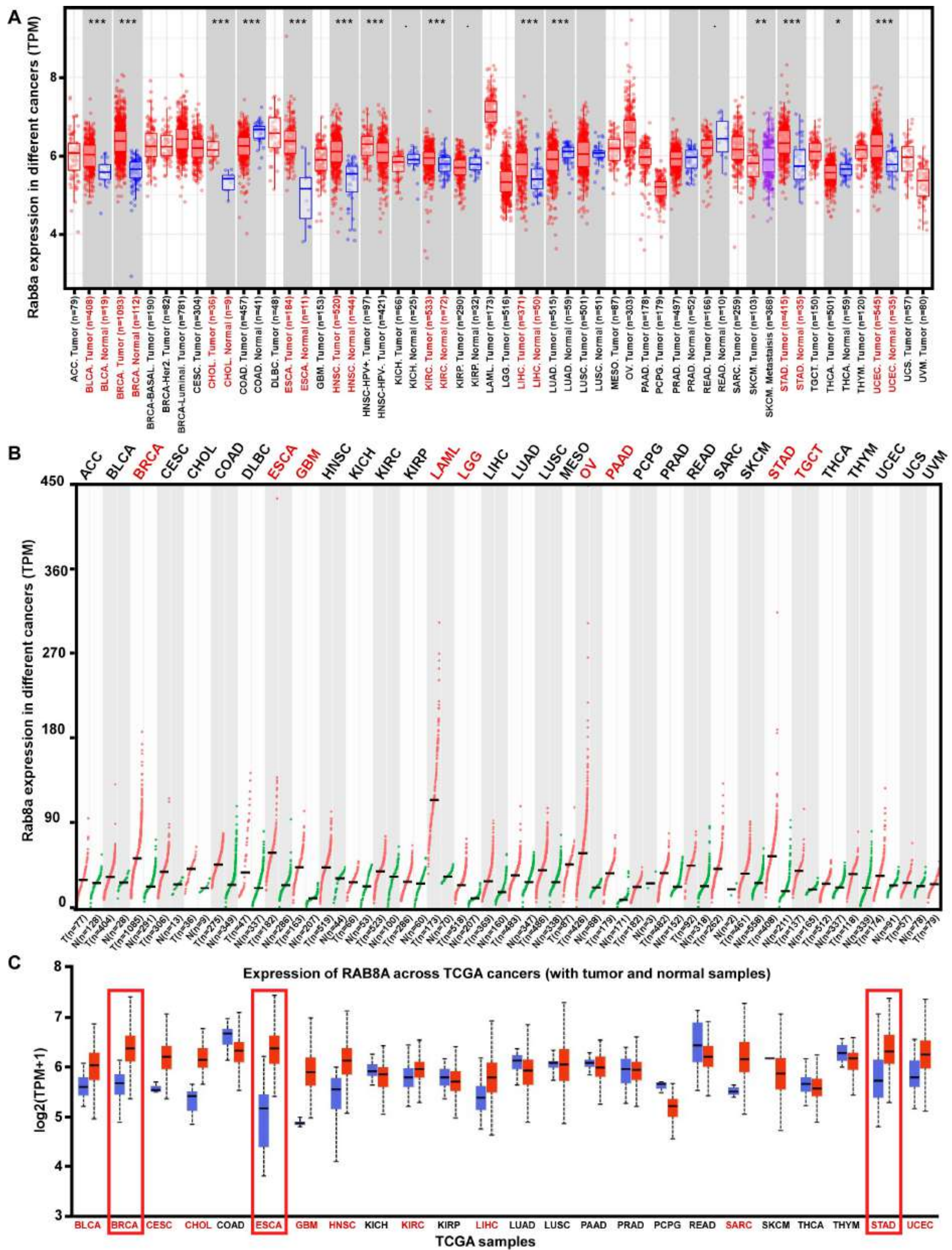

Figure 1: Rab8a expression in pan-cancer. (A)Expression levels of Rab8a in TCGA cancers were analyzed by TIMER2.0 database.(B) Expression levels of Rab8a were analyzed by GEPIA.(C) Expression levels of Rab8a in TCGA cancers were analyzed by UALCAN.

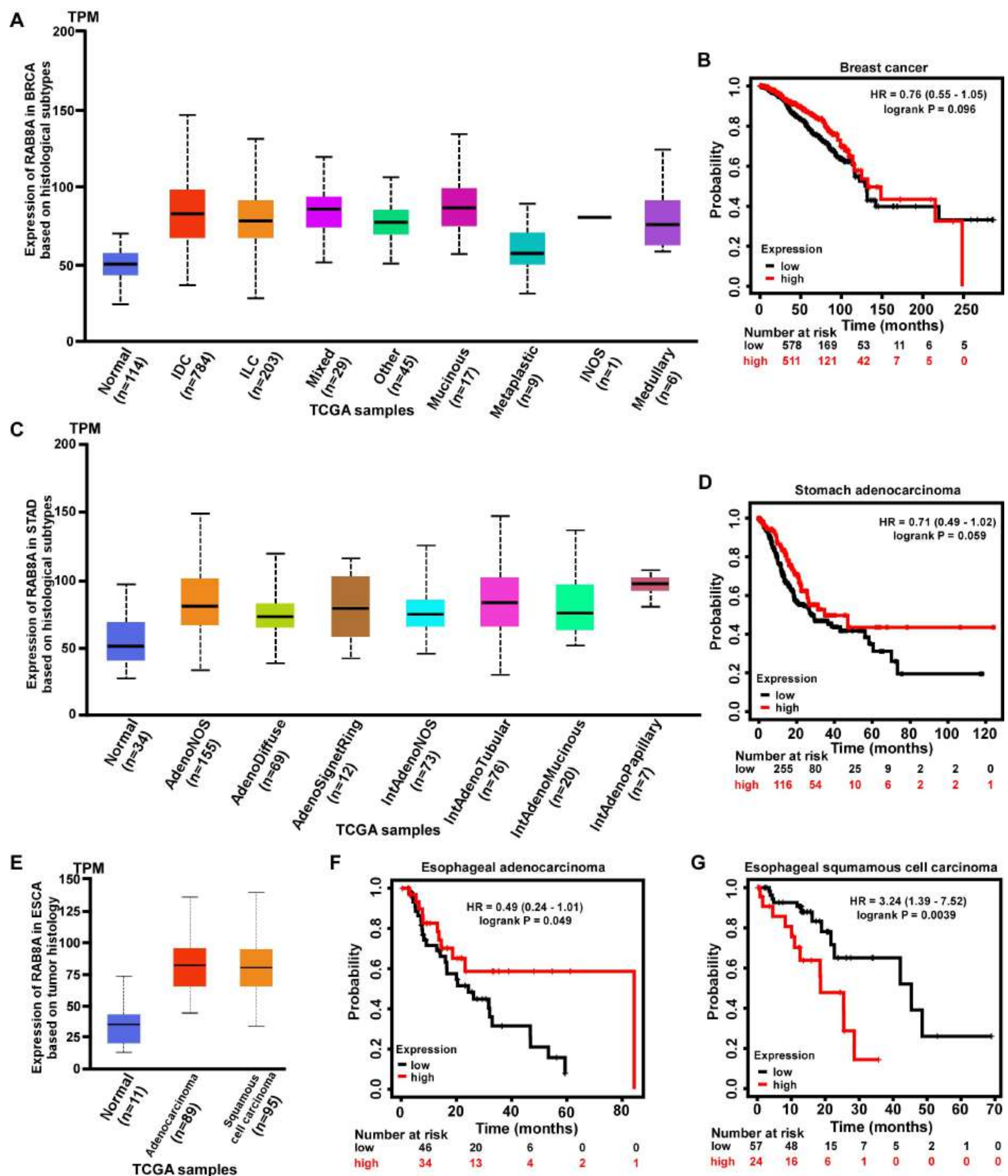

Figure 2: Expression and prognostic significance of Rab8a in BRCA, STAD and ESCA.(A) Rab8a expression in all of the histological subtypes of BRCA, STAD and ESCA.(B) KM survival curves showed that Rab8a expression was highly associated with clinical outcomes in BRCA.(C) Rab8a expression in all of the histological subtypes of STAD.(D) KM survival curves showed that Rab8a expression was highly associated with clinical outcomes in STAD.(E) Rab8a expression in all of the histological subtypes of ESCA.(F,G) KM survival curves showed that Rab8a expression was highly associated with clinical outcomes in EAC and ESCC.

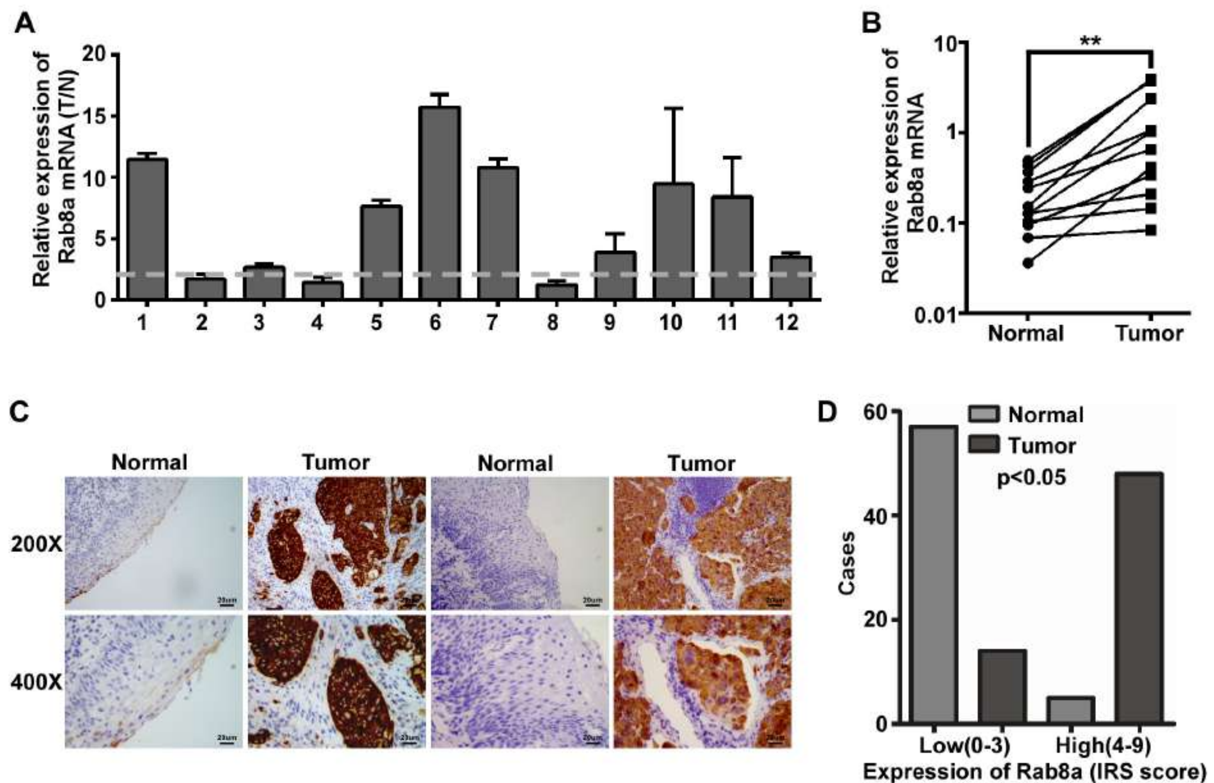

Figure 3: Rab8a expression and its correlation with the clinicopathological characteristics in human ESCC tissues.(A,B) qPCR of Rab8a in 12 cases of fresh human ESCC and normal tissues ($2^{-\Delta\Delta Ct}$, T/N, dotted line represents two-fold difference.(C,D) IHC of Rab8a in ESCC and normal tissues. ** p<0.01.

Table 1: Clinicopathologic Characteristics of Rab8a Expression in ESCC Patients

Characteristics	**Rab8a Expression**		**χ^2 Value**	***p* Value**
	Low	**High**		
Age				
<60	9	19	2.671	0.102
≥60	5	29		
Gender				
Male	8	32	0.429	0.512
Female	6	16		
Differentiation				
Well-Moderate	11	34	0.326	0.568
Poor	3	14		
Tumor Size				
<2 cm	7	8	6.566	0.010
≥2 cm	7	40		
T classification				
T1+T2	9	15	4.986	0.026
T3+T4	5	33		

N classification				
N0	4	16	0.112	0.737
N1	10	32		

Overexpression of Rab8a Increased the Proliferation and Migration of ESCC

To elucidate the biological roles of Rab8a in ESCC progression, we established the ESCC cells of ECA109-Rab8a and ECA9706-Rab8a with stable Rab8a overexpression (Figure 4A,B). Then, we conducted a series of in vitro biological experiments with these cells. The CCK-8 and colony formation assays showed that the overexpression of Rab8a obviously promoted the abilities of ESCC proliferation (Figure 4C-F). Transwell migration and wound-healing assays revealed that the migrated abilities of these ESCC cells were significantly increased compared with their vector cells (Figure 4G-J).

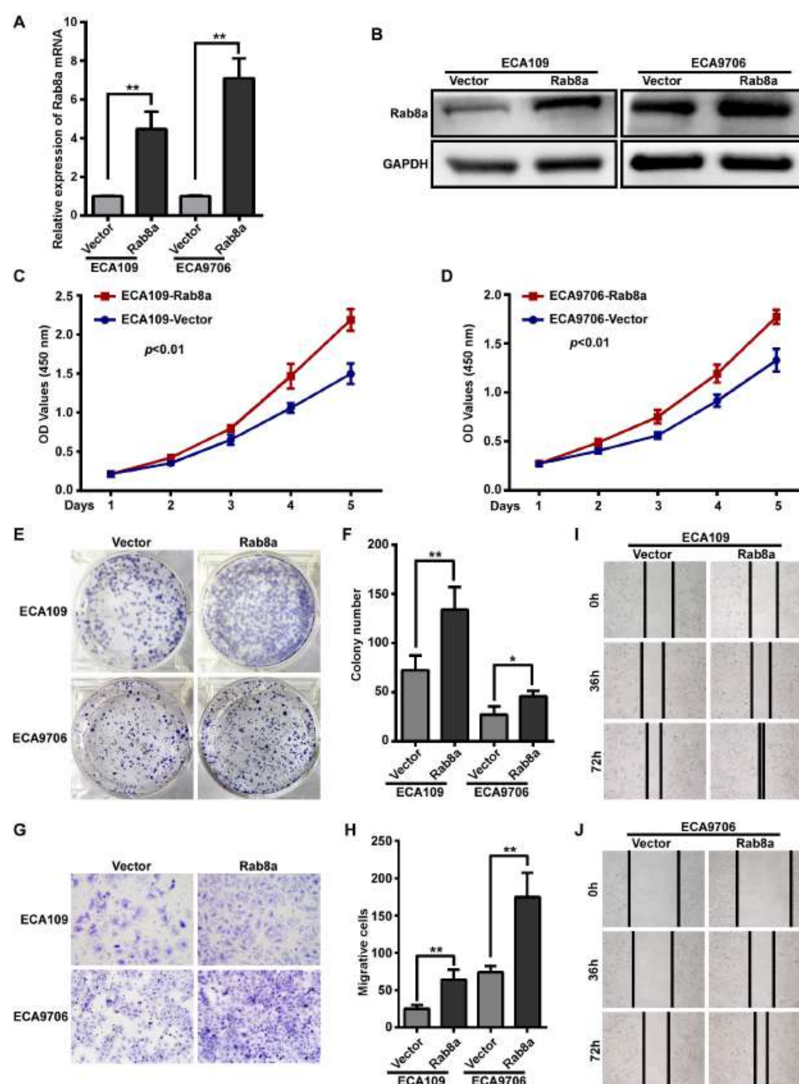

Figure 4: Overexpression of Rab8a increased the proliferation and migration of ESCC. (A, B) The confirmation of Rab8a overexpressed ESCC cells by qPCR and Western blot analysis.(C-F)The proliferative ability of the indicated cells detected by CCK-8 assays and colony formation assays.Histograms represent the average number of the colonies from three independent experiments. Error bars represent mean±s.d.(G-J)The migration ability of the indicated cells detected by Transwell migration and wound-healing assays.** p<0.01.

Repressed Expression of Rab8a Decreased the Proliferation and Migration of ESCC

To further verify the biological roles of Rab8a in ESCC progression, we also established the ESCC cells of ECA109-

shRab8a and ECA9706-shRab8a with stable knockdown expression of Rab8a (Figure 5A,B). Then we conducted in vitro biological experiments with these cells. The CCK-8 and colony formation assays demonstrated that the knockdown expression of Rab8a could inhibit the proliferation of ESCC (Figure 5C-F). Transwell migration and wound-healing assays confirmed that the migrated abilities of these ESCC cells were markedly repressed compared with their control cells (Figure 5G-J). So, the above data verified that the increased expression of Rab8a could facilitate ESCC progression.

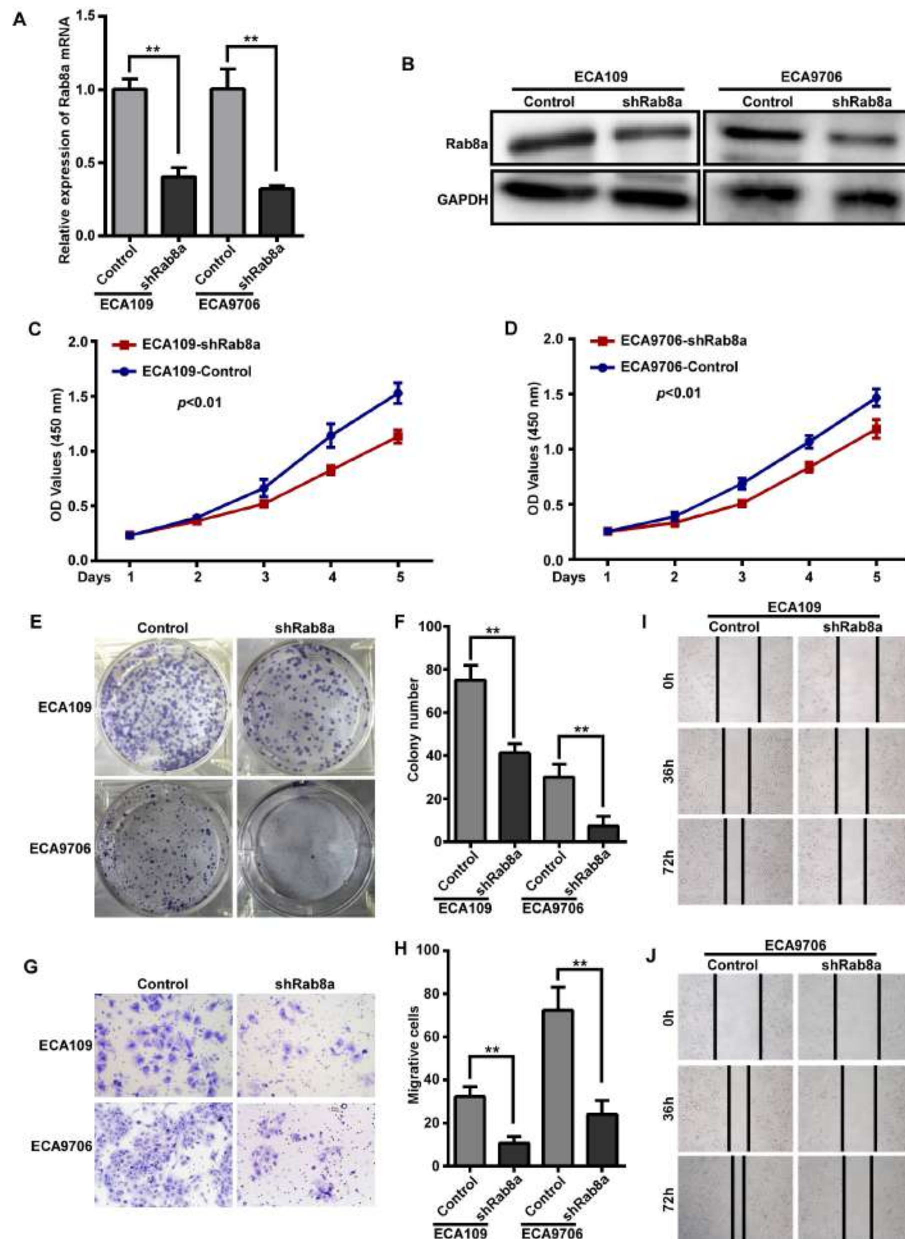

Figure 5: Repressed expression of Rab8a decreased the proliferation and migration of ESCC. (A, B) The construction and confirmation of stable knockdown ECA109 and ECA9706 cells of Rab8a by qPCR and Western blot analysis. (C-F)The proliferative ability of the indicated cells detected by CCK-8 assays and colony formation assays.Histograms represent the average number of the colonies from three independent experiments. Error bars represent mean±s.d. (G-J)The migration ability of the indicated cells detected by Transwell migration and wound-healing assays.** p<0.01.

Rab8a Promoted ESCC Progression by Activating Mitochondrial Mitochondrial Respiration

To investigate the molecular mechanism of Rab8a in promoting ESCC progression, we conducted the GSEA using the publicly available portal of LinkedOmics. The results showed that the mitochondrial respiration pathway-related genes were significantly enriched, including the mitochondrial gene expression, mitochondrial respiratory chain complex assembly and NADH dehydrogenase complex assembly pathways (Figure 6A-D). NADH ubiquinone oxidoreductase subunit A1 (NDUFA1) and Cytochrome C1 (CYC1) had been identified as key genes of these pathways. So, we analyzed the correlation between the expression of Rab8a and NDUFA1, CYC1 in GSE21293, GSE33426 and GSE38129 and found the expression of Rab8a was positively correlated with NDUFA1 and CYC1 (Figure 6E-G). These data implied that Rab8a promoted ESCC progression by activating mitochondrial mitochondrial respiration.

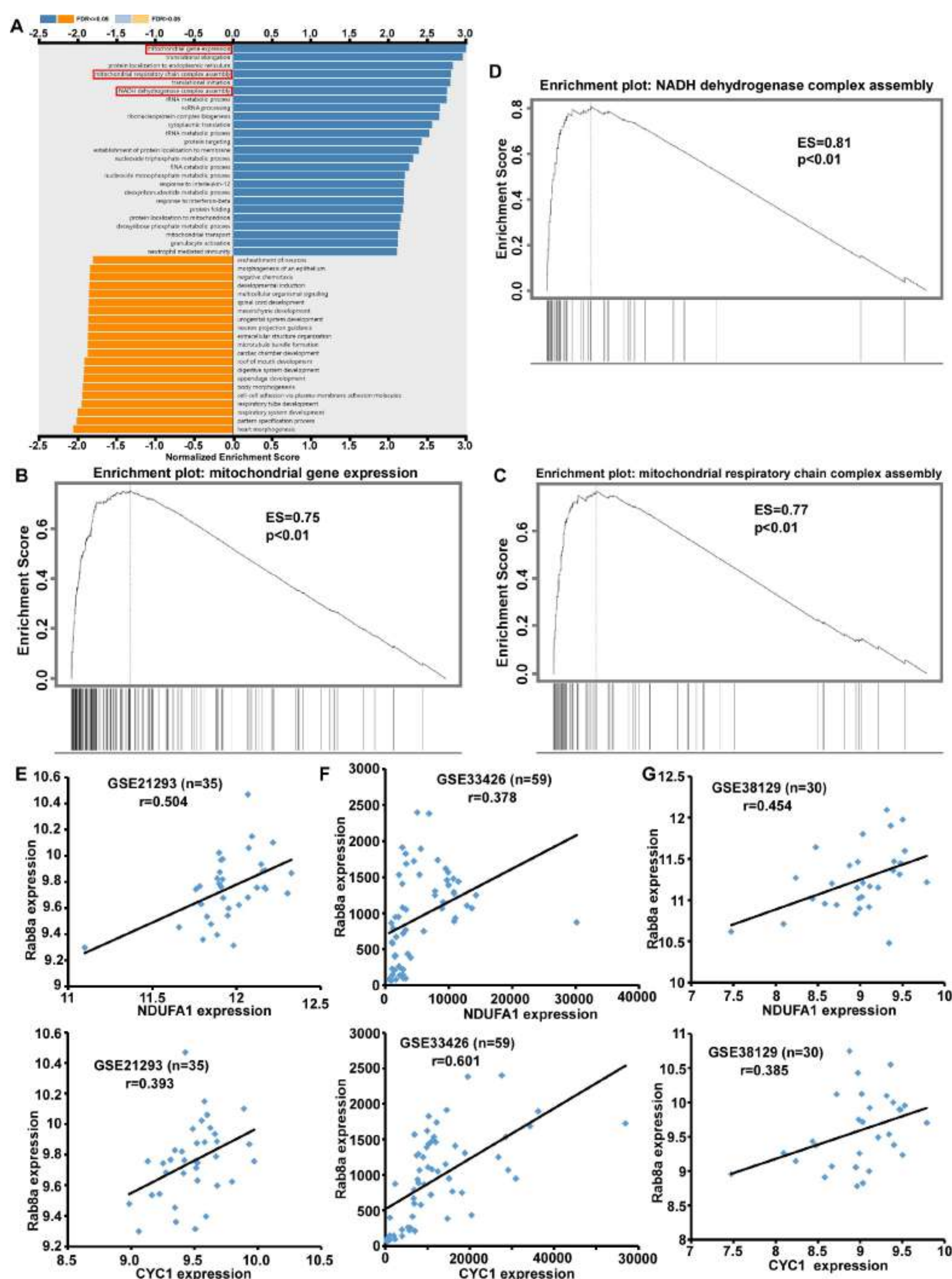

Figure 6: Rab8a promoted ESCC progression by activating mitochondrial mitochondrial respiration. (A-C) GO and GSEA analysis results in Linkedomics.(E-G) The expression correlation between Rab8a and NDUFA1,CYC1.

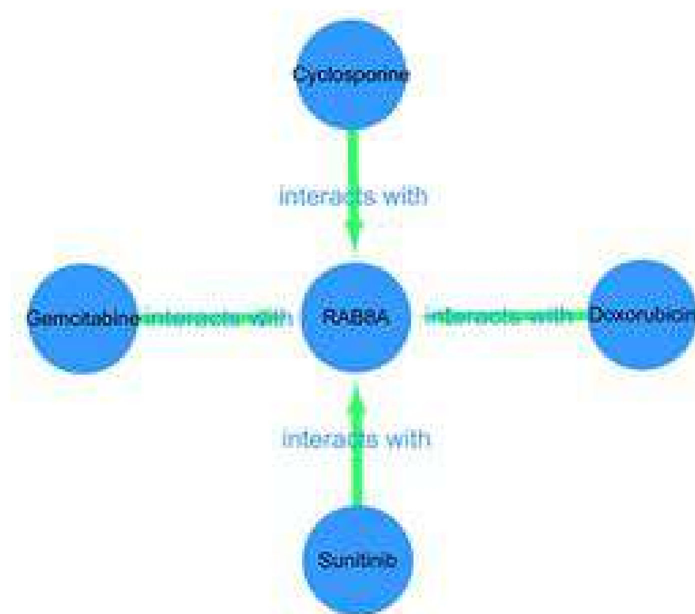

Figure 7: Rab8a-associated therapeutic drugs. blue arrows: Chemotherapeutic drugs relevant to lower Rab8a expression, and count of arrows: specific interaction supported numbers of studies in literature.

Rab8a-Associated Therapeutic Drugs

The results of gene-drug interaction network showed that Rab8a expression could be decreased by many popular clinical anticancer drugs (Figure 7A). The development of acquired resistance to therapy remains the current stumbling blocks in our fighting against cancer. Therefore, Rab8a might be a potential therapeutic target in combating ESCC.

Discussion

There are notable differences in the morbidity of ESCA among countries and more than half of the cases occur in China [11]. The carcinogenesis of ESCA is a complex process involving numerous gene alterations. And the two major histologic types of ESCA are ESCC and ECA. And they have different geographic patterns, causes and prognosis [12]. Patients with cancers have poor prognosis mostly because of the late-stage at diagnosis [13,14]. So it is very important to find the valuable diagnostic marker for combating cancers.

Rab8 is a member of Ras superfamily and key regulator of intracellular membrane trafficking [6,15,16]. Previous studies demonstrated that Rab8 played important roles in the cell migration, polarization and intracellular signal transduction [17,18]. Rab8a is one major subtype of Rab8 and is reported to play important roles in endometrial cancer and hepatocellular carcinoma and might be a potential tumor marker [19].

In this study, we first analyzed the expression of Rab8a by employing multiple public cancer genomics programs and found that Rab8a was obviously increased in some common solid cancers including ESCA. As more than 70% of ESCA cases occurred in China, and the main histology type was ESCC, we mainly explored the expression and significance of Rab8a in ESCC [2,20]. The analytical results based on the online web resource of UALCAN and Kaplan-Meier plotter showed that Rab8a was significantly up-regulated in ESCC and the high expression of Rab8a indicated poor prognosis of ESCC patients which implied that Rab8a may serve as a diagnostic and prognostic marker of ESCC. Therefore, we next detected the mRNA and protein expression of Rab8a in human ESCC tissues by qPCR and IHC and found that the expression Rab8a mRNA and protein was obviously increased in ESCC compared with their paired adjacent normal tissues. And the further analysis of the relationship of Rab8a protein expression and the clinicopathological characteristics showed that the high expression of Rab8a implied larger size and deeper invasion of ESCC. So, Rab8a could be a valuable marker for the diagnosis and prognosis of ESCC. In addition, we constructed the ESCC cells with stable over or knockdown expression of Rab8a and conducted the CCK-8, colony formation, transwell migration and wound-healing assays. The results identified Rab8a as a key promoter of ESCC proliferation and migra-

tion.

To further explore the molecular mechanisms of Rab8a in promoting ESCC progression, we conducted the analysis of GSEA and found that Rab8a the mitochondrial respiration pathway-related genes were enriched significantly. It became increasingly clear that the increased metabolic activity of mitochondria could promote the immortalization of tumor cells [21]. And the dysregulation of cellular metabolism is a hallmark of cancer, which is mainly to meet the bioenergetic demands of the high proliferation rates of cancer cells [22]. As we know, the normal bioenergetics of mitochondrial respiration is a vital process that produces ATP and provides energy to support the growth of cancers [23,24]. NDUFA1 and CYC1 are important genes of mitochondrial electron transport chain which had been identified to play important roles in the mitochondrial respiratory chain by transferring electrons [25,26]. Next, the further correlation analysis revealed the positive correlation between the expression of Rab8a and NDUFA1, CYC1 in public GEO databases. So, the data implied that Rab8a promoted ESCC progression by activating mitochondrial mitochondrial respiration.

As the excessive mitochondrial respiration could increase tumor cells oxygen consumption, which triggers hypoxia and irregular blood vessels formation and eventually results in the resistance to therapy [27]. To explore the role of Rab8a in cancer therapy, we conducted the analysis of gene-drug interaction network constructed via CTD and Cytoscape and found that Rab8a could be decreased by many popular clinical anticancer drugs. That is to say, the acquired resistance to these drugs might be overcome by the interference expression of Rab8a. Therefore, Rab8a might be a potential therapeutic target for ESCC therapy.

Conclusion

In conclusion, our study confirmed that Rab8a was up-regulated in ESCC and served as a key promoter in ESCC progression by activating mitochondrial respiration. Our current study uncovered a novel tumor-promoting function of Rab8a in ESCC and offered a potential diagnostic and therapeutic biomarker.

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Author Contributions

J.C.&Y.X.W.: Conceptualization, Methodology, Visualization, Funding acquisition. R. L.: investigation, formal analysis, investigation, writing-original draft. Z.T. K.: Methodology, Investigation. T. G. ,Y.R.N. W. & Z.Y. H.F.: Investigation, Visualization. All authors read and approved the final manuscript.

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Data Availability

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

Declarations

Conflict of Interest

The authors declare that they have no competing interests.

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