Thoracic Cancer

Open Access

Thoracic Cancer ISSN 1759-7706

ORIGINAL ARTICLE

KIF4A enhanced cell proliferation and migration via Hippo signaling and predicted a poor prognosis in esophageal squamous cell carcinoma

Xiaozheng Sun, Pengxiang Chen, Xue Chen, Wenjing Yang, Xuan Chen, Wei Zhou, Di Huang & Yufeng Cheng

Department of Radiation Oncology, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan, China

Keywords
Biomarker; esophageal squamous cell carcinoma; KIF4A; potential biological functions; prognosis.

Correspondence
Yufeng Cheng, Department of Radiation Oncology, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan 250012, China.
Tel: +86 153 1827 7520
Fax: +86 (0531) 8216 9820
Email qlcyf@sdu.edu.cn

Received: 27 October 2020;
Accepted: 30 November 2020.

doi: 10.1111/1759-7714.13787

Thoracic Cancer **12** (2021) 512–524

Abstract

Background: In this study, we aimed to explore and clarify the function of KIF4A in esophageal squamous cell carcinoma (ESCC).

Methods: The microarray data were extracted from the Gene Expression Omnibus (GEO) database. We then used the database for Annotation, Visualization, and Integrated Discovery (DAVID) to perform the gene ontology function (GO) and KEGG Orthology-Based Annotation System (KOBAS) to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differentially expressed genes (DEGs). The six core candidate genes were identified using protein–protein interaction (PPI) network analysis and Cytoscape software. Among them, the expression of KIF4A were validated by UALCAN database from the Cancer Genome Atlas (TCGA) ($P < 0.05$). Western blotting, qRT-PCR and IHC were used to detect the expression of KIF4A in tissues. Cell experiments (transwell migration assays, wound healing assay, CCK8 assay, and clone formation experiment) were utilized to verify the roles of KIF4A on the ESCC cells. Western blotting was used to explore the mechanism of KIF4A in ESCC.

Results: The expression level of KIF4A was upregulated in ESCC samples compared to those in paracancerous tissues. Transwell migration and wound healing assay showed overexpression of KIF4A significantly promoted the migration of ESCC cells. CCK8 assay and clone formation experiment analysis showed that overexpression of KIF4A promoted proliferation of ESCC cells. Western blot detection found that KIF4A could affect the phosphorylation level of Hippo signaling pathway related proteins.

Conclusions: In summary, KIF4A promotes ESCC cell proliferation and migration by regulating the biological function of ESCC cells through the Hippo signaling pathway.

Key points

Significant findings of the study: We found that high KIF4A expression was associated with poor overall survival in esophageal squamous cell carcinoma. KIF4A expression also promoted the proliferation and migration of ESCC cells in vitro.

What this study adds: Our experimental results explain the role of KIF4A in ESCC, and provide a new biomolecular target for the treatment of ESCC.

Introduction

Esophageal cancer is a common malignant tumor of the digestive tract, and its prognosis is poor, ranking sixth in cancer-related causes of death worldwide.[1] In China, esophageal squamous cell carcinoma (ESCC) is the main histological type, accounting for about 90% of esophageal cancer.[2,3] With the emergence of a combination of surgical treatments, supplemented by radiotherapy and chemotherapy, the survival rate of ESCC patients has improved, but the overall five-year survival rate is still low.[4,5] Therefore, further study of the pathogenesis of ESCC to establish effective methods of prevention, diagnosis, and treatment is urgently required.

Kinesin superfamily 4 (kinesin family member 4, KIF4), a kind of interaction with microtubules, which also provides power for components in the cell movement of motor, which can make the microtubules to the cells at the pole movement, has many physiological functions.[6,7] KIF4A spinning cone in the late adjustment form and plays a key role in the process of mitosis; the anomaly can lead to abnormal mitosis checkpoint and DNA damage repair, and chromosome instability and the formation of aneuploidy, cause abnormal cell proliferation, differentiation and cause tumor formation.[8,9] Some scholars have found that intracellular KIF4A can lead to mitotic defects, such as high chromosome concentration and chromosome aneuploidy, while the Hippo signaling pathway plays a key role in limiting the transformation of chromosomes from biploid to polyploid/aneuploid.[10,11] Previous studies have shown that inactivation of LATS1/2, a key kinase in the Hippo signaling pathway, or overexpression of YAP, a target gene downstream of the Hippo signaling pathway, can lead to substantial accumulation of E3 ubiquitin ligase Skp2 in the cytoplasm and decrease of Skp2 level in the nucleus, leading to cell mitosis arrest and polyploid formation.[12,13] However, the relationship between KIF4A and the Hippo signaling pathway in ESCC is unclear.

In this study, we selected three microarray data sets: GSE20347, GSE29001 and GSE17351. Using GEO2R online tools and Venn diagram software for three datasets DEGs, DAVID Database and KOBAS 3.0 analyzes the Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genome Elements (KEGG) approaches of screened DEGs, respectively.[14,15] In addition, we established a protein-protein interaction (PPI) network and selected the most meaningful modules and hub genes and analyzed the GO and KEGG of these genes. Next, we designed a series of basic experiments to see whether KIF4A affects the malignancy of ESCC cells and the specific mechanisms of its action, providing important evidence for studying the role of KIF4A in ESCC.

Methods

Bioinformatic analysis

The GSE20347, GSE29001, and GSE17351 were obtained from NCBI-GEO which included 17 ESCC tissues and 17 normal adjacent esophageal tissues, 21 ESCC tissues and 24 normal adjacent esophageal tissue, and five ESCC tissues and five normal adjacent esophageal tissues, respectively. The GEO2R method analysis of differentially expressed genes (DEGs) in ESCC tissues, we set "adjusted *P*-value <0.05 and |logFC|≥2" as the cutoff criterion. The application of Venn diagrams (http://www.interactivenn.net) to find common differentially expressed genes, three datasets used DAVID (http://david.abcc.ncifcrf.gov/) for screening the differentially expressed genes GO enrichment analysis and KOBAS (3.0, http://KOBAS.cbi.pku.edu.cn) for KEGG pathway enrichment analysis. A Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (http://string-db.org) was used to conduct a protein-protein interaction (PPI) analysis on the screened differentially expressed genes. The PPI network was then visualized by Cytoscape. The MCODE plugin in Cytoscape was applied to look for regulatory nodes-proteins in the occurrence and development of ESCC. The expression of KIF4A were validated by UALCAN (http://UALCAN.path.uab.edu/) data from the Cancer Genome Atlas (TCGA) ($P < 0.05$).

Human ESCC specimens

A total of eight pairs of fresh ESCC tissues matched normal adjacent specimens and 107 paraffin-embedded tumor tissue samples from patients who had undergone esophagectomy were collected between January 2008 and October 2008 from Qilu Hospital, Cheeloo College of Medicine, Shandong University. The normal tissue adjacent to the cancer was the noninvasive tissue more than 5 cm away from the cancerous tissue. The diagnosis of all specimens was clear and the pathological data was complete. No chemotherapy was administered preoperatively. All samples were approved for use by the Ethics Committee of the Qilu Hospital.

IHC analysis

The samples were prepared in liquid nitrogen, the thickness of the tissue sections was 5 μm, and they were baked in an incubator at 58°C for two hours, dewaxed with xylene for 30 minutes, soaked in gradient ethanol for five minutes each, and washed with sterile distilled water for five minutes. Sodium citrate antigen repair solution was added and bathed in boiling water for 20 minutes, then

cooled to room temperature and rinsed with PBS solution three times, five minutes each time. Drops of KIF4A antibody (1:200) were added and stored overnight in a wet box at 4°C. After removal, the product was reheated for one hour, and the film washed with PBS solution three times, for five minutes each time. Diaminobenzidine (1:50) was then added for color development for one minute, and finally distilled water was added to terminate the reaction. After the addition of hematoxylin and the differentiation of hydrochloric acid ethanol, the tablets were dehydrated with gradient ethanol and washed with xylene three times until transparent. Immunohistochemical staining was observed under the microscope after neutral resin had been used to seal the slices.

Five fields were randomly selected for each specimen under the lens, and all pathological sections were confirmed by more than two experienced physicians using the double-blind method. Score according to the degree of staining and percentage of stained cells (6): 0 for nonstaining, 1 for light yellow, 2 for yellow and 3 for brownish yellow. The number of pigmented cells ≤5% 0.6%–25% 1.26%–50% 2, ≥51% 3; Negative (−) score ≤ 1, weak positive (+) score 2–3; moderate positive (++) score 4–5 points; strong positive (+++) score ≥6 points.

Cell culture and transfection

A total of 10% percent fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) was added to RPMI-1640 culture medium (Gibco; Thermo Fisher Scientific, Inc.). ESCC cells Eca150 and Eca109 were placed in the above culture medium, and then cultured in an incubator containing 5% CO_2 at 37°C. The fresh culture medium was changed daily. The logarithmic growth cells were taken for the experiment.

KIF4A silencing in Eca109 cells was achieved by transfection with KIF4A siRNA (GenePharma China). To transiently transfect cells with KIF4A siRNA, EndoFecti MAX (GeneCopoeia Inc.) transfection Reagent was used. Media was removed 48 hours after transfection. KIF4A-specific siRNA oligonucleotides were synthesized according to the following target sequences: siKIF4A#1, Sense sequence 5′-GGAAUGAGGUUGUGAUCUUTT-3′, Anti-sense sequence 5′-AAGAUCACAACCUCAUUCCTT-3′; and siKIF4A #2, Sense sequence 5′-GGUCCAGACUACUACUCU ATT-3′, Antisense sequence 5′-UAGAGUAGUAGUCU GGACCTT-3. Nonsilencing siRNA (siNC, forward: 5′-UUCUCCGAACGUGUCACGUTT-3′, reverse: 5′-AC GUGACACGUUCGGAGAATT-3′ was used as a negative control for unspecific effects of transfection.

To observe the effect of KIF4A, EX-A3631-Lv105 (OE-KIF4A, GeneCopoeia, Inc.) was transfected into Eca150 cells. The following shRNA sequences were used: for the targeting shRNA, 5′-GCGGTAGGCGTGTACGGT-3′ (sense) and 5′-ATTGTGGATGAATACTGCC-3′ (antisense); and for the negative control scrambled shRNA (Vector), 5′-GCGGTAGGCGTGTACGGT-3′ (sense); 5′-ATTGTGGATGAATACTGCC-3′ (antisense).

Western blot assay

The treated cells were collected, lysed on liquid ice for 30 minutes, and the supernatant removed by centrifugation. Protein samples were prepared by BCA protein quantification then transferred to polyvinylidene fluoride (PVDF) membrane by SDS-POLYacrylamide gel electrophoresis (PAGE). After sealing by 3% BSA, primary antibody against KIF4A (1:1000; cat. no. ab124903; Abcam) and β-actin (1:1000; cat. no. ab8226; Abcam) was added overnight at 4°C, and corresponding peroxidase-labeled secondary antibodies (1:5000; Affinity Biosciences) was added. The levels of KIF4A protein were evaluated using chemiluminescence detection system (EMD Millipore, Billerica, MA, USA) according to the Electrochemical luminescence (ECL) color development kit.

Real-time reverse transcription PCR (qRT-PCR)

Reverse transcription was carried out according to the instructions of PrimeScriptTMRT reagent Kit (Takara), and SYBR Premix Ex TaqII (Takara) was used for qRT-PCR. We used several sequences: KIF4A forward primer 5′-TGGTGTGGAAACAAGCAGTGTG-3′ and reverse primer 5′-GGAATCCTGGGTCCGTTCA-3′; β-actin forward primer 5′-CCAGACAGCACTGTGTTGGCATA-3′, and reverse primer 5′-CCCTCGTAGATGGGCACAGT-3′.

CCK8 assay

Cell proliferation was assessed using the cell counting kit-8 (CCK-8) description (Beyotime Technology, China). At one, two, three, four and five days after transfection, 10 μL CCK-8 reagent was added to each well, and the absorbance was measured at 450 nm. The mean value of five wells was calculated, and each experiment was repeated three times.

Clone formation experiment

After cell transfection for six hours, the cells were digested with trypsin and resuspended with the cell suspension, which was inoculated into a six-well plate with 2000/well, and 2 mL complete medium was added. The six-well plate was placed in the incubator for further cultivation. After 14 days, methanol was fixed for 30 minutes, 0.1% crystal violet was dyed for 20 minutes, and the tap water side was rinsed and photographed for counting.

Wound healing assay

Cells at logarithmic growth stage were taken and placed in a six-well plate with a cell density of 5×10^5/well. Three multiple wells were set for each group. When the cells were adherent to the wall in a single layer, a pipetting tip of 200 μL was used to vertically scratch the six-well plate to avoid tilting. The suspension cells were cleaned and removed with PBS and cultured in an incubator with 5% CO_2 at 37°C. Photographs were taken at 0 and 48 hours under the microscope, and the experiment was repeated three times.

Transwell migration assay

The treated ESCC cells were diluted to 10 × 104/mL with serum-free RPMI-1640 medium, and 200 μL cell suspension was added to the upper transwell chamber, and 600 μL medium containing 20% fetal bovine serum was added to the lower chamber, respectively. The upper chamber was carefully immersed in the lower chamber liquid with sterile forceps. The 24-well plate with transwell chamber was incubated in a 37°C incubator for 24 hours. After 24 hours, the liquid was removed from the upper chamber, and placed in a hole containing 600 μL PBS three times. After crystal violet staining, the upper chamber was observed under electron microscope and photographed. Each experiment was repeated three times.

Statistical analysis

The difference in KIF4A protein level between cancerous and paired paracancerous tissues were compared by paired Student's *t*-test using the SPSS 25.0. The data is presented as the mean ± standard deviation. Individual experiments were repeated three times. Differences between groups were considered significant for $P < 0.05$. Kaplan-Meier analysis calculated the overall survival of the two subgroups (the KIF4A overexpression group and the KIF4A underexpression group). A Chi-squared test was employed to analyze important independent prognostic factors relevant to ESCC.

Results

Identification of DEGs in ESCC

All DEGs were identified by comparing ESCC and paracancerous esophageal tissue samples by GEO database. Adjusting the criteria for adjusted *P*-value <0.05 and |log FC | ≥2, there were 286 differentially expressed genes, among which 84 were upregulated genes and 202 downregulated genes in GSE20347 (Fig 1a). A total of 574 DEGs were identified from GSE29001, including 310 upregulated genes and 264 downregulated genes (Fig 1b). From GSE17351, 247 DEGs were identified, including 135 upregulated genes and 112 downregulated genes (Fig 1c). The results of these three studies were then analyzed using the Venn diagram tool. There were 20 upregulated and 33 downregulated DEGs among them (Fig 1d–f).

KEGG and GO enrichment analyses of DEGs

To explore the potential biological functions of differentially expressed genes, we performed GO enrichment analyses, including molecular functions (MF), cellular components (CC), and biological processes (BP) by DAVID. Results showed that changes in MF of DEGs were significantly enriched in monooxygenase activity, actin binding, iron ion binding, RNA polymerase II transcription corepressor activity and aromatase activity. For CC, DEGs were mainly enriched in cell cortex, midbody, mitotic spindle, proteinaceous extracellular matrix and centralspindlin complex. Within the BP category, collagen catabolic process, extracellular matrix organization, mitotic cytokinesis, ductus arteriosus closure and liver development were predominant (Fig 1g). KEGG enrichment analysis showed that transcriptional misregulation in cancer, drug metabolism — cytochrome P450, protein digestion and absorption, axon guidance, apoptosis — multiple species etc were significantly enriched in DEGs (Fig 1h).

PPI network construction and module analysis

PPI networks of DEGs were constructed using STRING and visualized in cytoscape (Fig 2a), and densely connected protein clusters were identified using the graph theoretical clustering algorithm MCODE (six nodes, 15 edges) (Fig 2b). The most significant genes were ATAD2, ECT2, KIF23, KIF4A, AURKA, and MCM2. The functional analysis was then performed in DAVID to establish the dominant functional nature of genes contained in this module. GO analysis results showed that the genes in this module were mainly enriched in ATP binding, microtubule motor activity, histone binding, ATPase activity in MF; midbody, mitotic spindle, centralspindlin complex, nucleoplasm, microtubule in CC; mitotic spindle midzone assembly, mitotic cytokinesis, positive regulation of cytokinesis, cytokinesis, microtubule-based movement in BP (Fig 2c); KEGG ontology terms enriched in DNA replication, progesterone-mediated oocyte maturation, cell cycle, oocyte meiosis, microRNAs in cancer (Fig 2d).

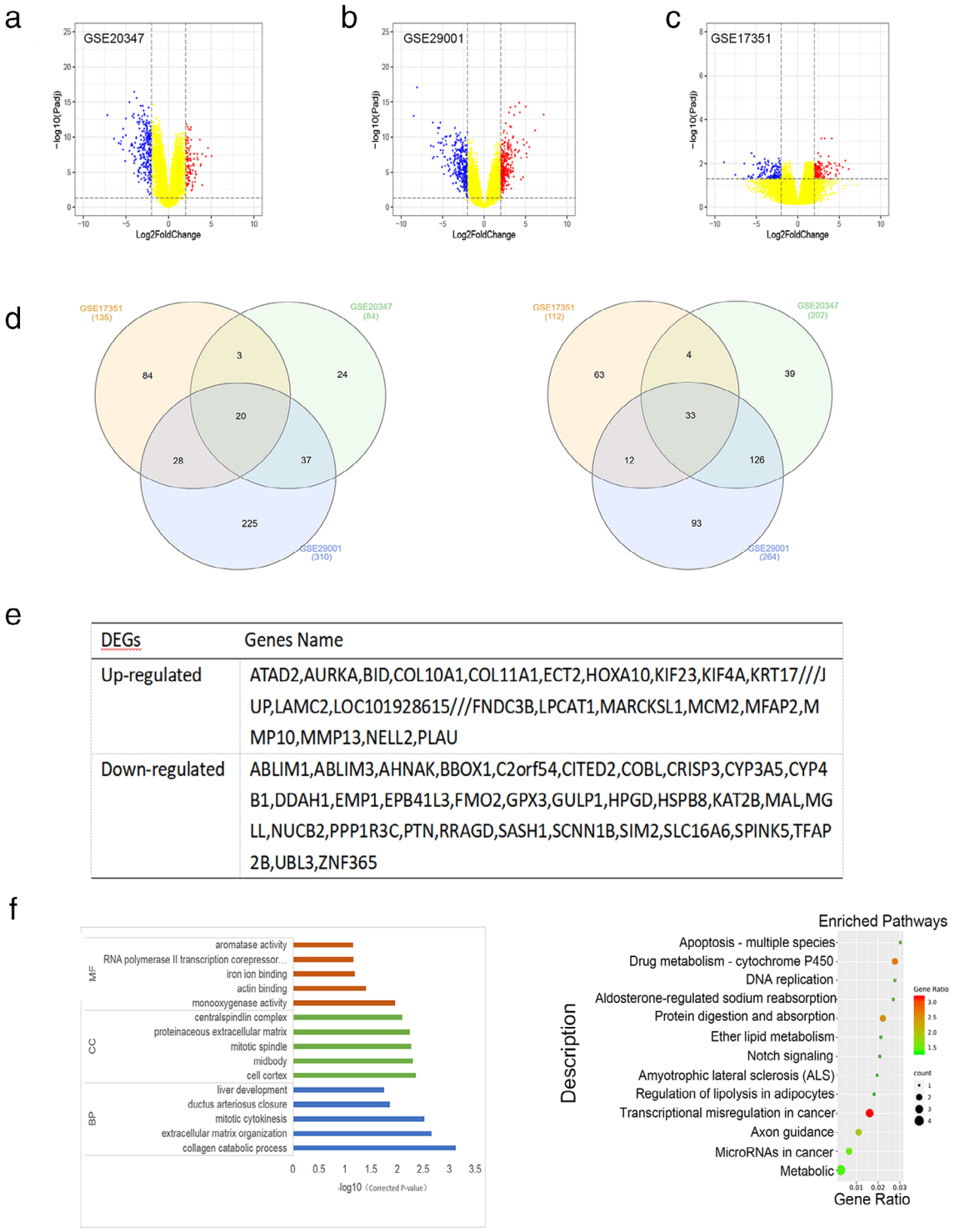

Figure 1 Identification and Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) enrichment analyses of differentially expressed genes (DEGs) in esophageal squamous cell carcinoma (ESCC). (**a**–**c**) The DEGs of GSE20347, GSE29001, and GSE37351. The blue and red dots represent DEGs filtered based on the cutoff criteria of adjusted |log2 (fold change)| ≥2 and *P*-value < 0.05. While the yellow dots represent genes that do not satisfy the cutoff criteria. (**d**–**f**). The up- and downregulated DEGs in the three datasets. (**g** and **h**). Enriched GO terms and KEGG pathways of the DEGs. (**a**) (●) DOWN; (●) NOT; (●) UP; (**b**) (●) DOWN; (●) NOT; (●) UP; (**c**) (●) DOWN; (●) NOT; (●) UP; (**g**) (■) BP; (■) CC; (■) MF.

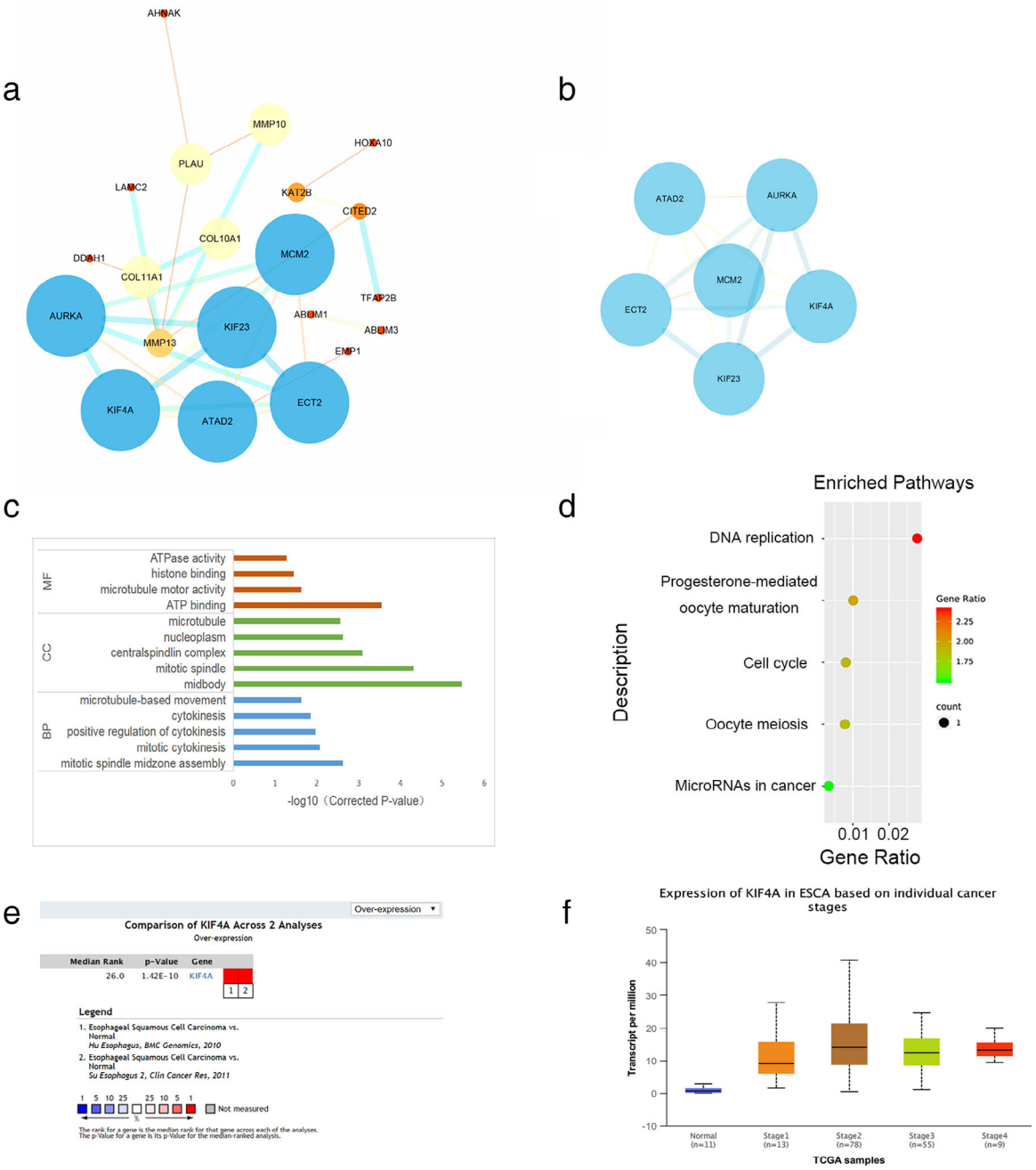

Figure 2 Protein-protein interaction (PPI) network and modular analysis of selected genes and the identification of the significance of KIF4A in esophageal squamous cell carcinoma (ESCC). (**a**) PPI network constructed by differentially expressed genes (DEGs). The diameter and color of the nodes represent, respectively, the value and the degree and logFC. (**b**) The degree of protein interaction ranks to determine six hub genes in protein-protein Interaction. (**c** and **d**) gene ontology (GO) analysis and biological pathway enrichment analyses of the module. (**e**) KIF4A mRNA expression in ESCC tissues is higher than in normal tissues from the Oncomine database. (**f**) The KIF4A mRNA expression differences among the successive stages of esophageal carcinoma were analyzed in UALCAN. (**c**) (■) BP; (■) CC; (■) MF.

Identification of the significance of KIF4A in ESCC

The Oncomine dataset (https://www.Oncomine.org/resource/login.html) suggested that KIF4A was highly expressed in ESCC samples compared to normal samples (Fig 2e). The differences in expression of KIF4A mRNA between stages of esophageal carcinoma were also analyzed in UALCAN (http://UALCAN.path.uab.edu/) (Fig 2f).

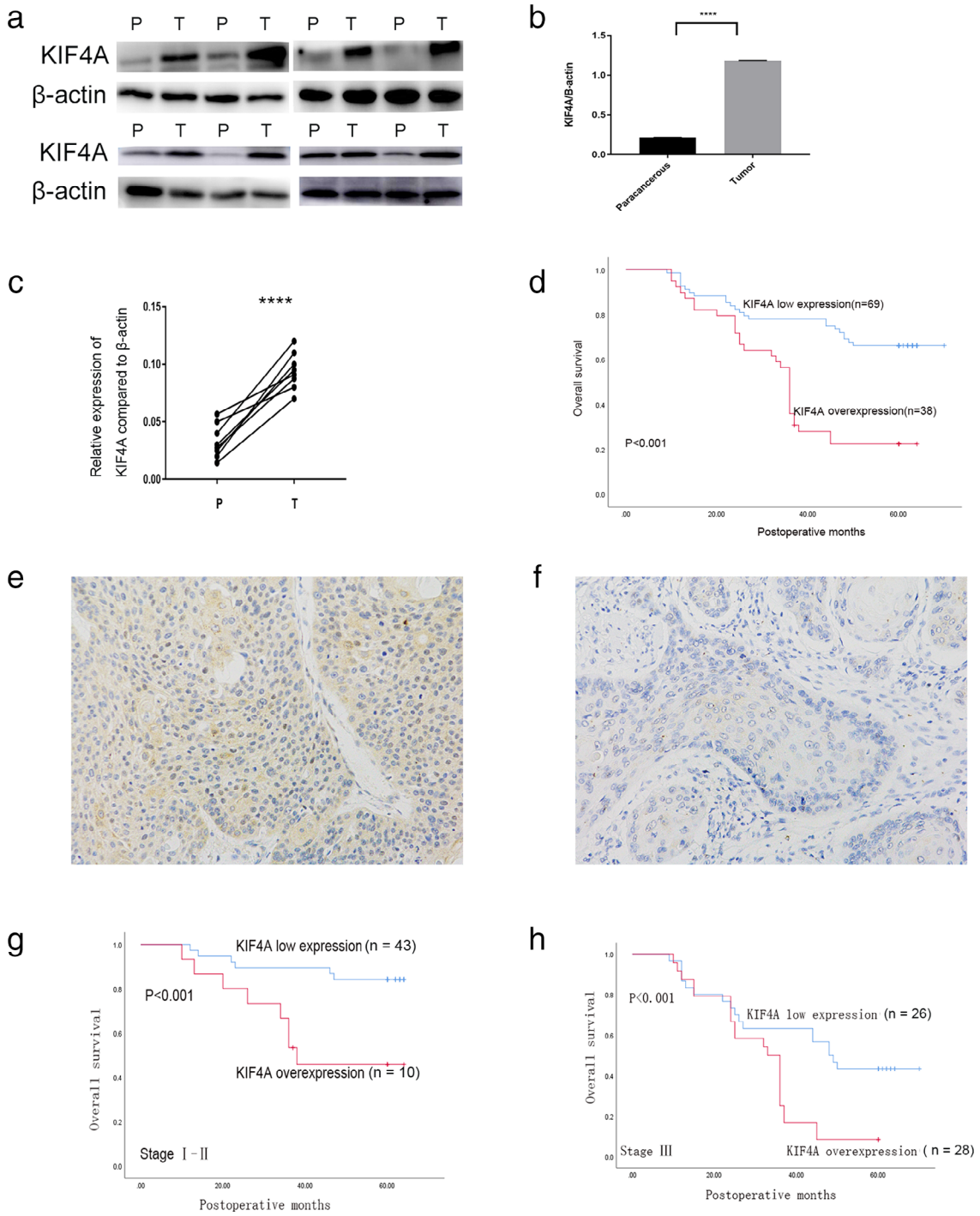

Figure 3 KIF4A expression increases in esophageal squamous cell carcinoma (ESCC) tissues and upregulation of KIF4A is associated with poor prognosis in ESCC. (**a**–**c**) The protein levels of KIF4A in ESCC tissues and matched noncancerous tissues from eight patients with ESCC were determined by western blotting and qRT-PCR assay. P, paracancerous; T, tumor. (**d**) Overexpression and (**e**) low expression levels of immunohistochemical staining of KIF4A in esophageal squamous cell cancer tissues. KIF4A expression was localized to the cytoplasm of the cells. Magnification, x400. (**f**) KIF4A overexpression significantly predicted decreased OS by Kaplan-Meier analysis and log-rank test. (**g** and **h**) Kaplan-Meier curves for OS stratified according to the pTNM stage. KIF4A overexpression was associated with OS in patients at stages I, II, and III ESCC (OS, overall survival; $****P < 0.0001$).

Expression level of KIF4A in human ESCC tissues

Western blotting and qRT-PCR were used to detect the protein and mRNA expression of KIF4A in eight ESCC tissues and adjacent tissues. The results showed that KIF4A expression in ESCC tissues was significantly higher than that in adjacent tissues ($P < 0.05$) (Fig 3a–c).

KIF4A is associated with poor prognosis in ESCC

The patient demographics, clinicopathological features, and tumor characteristics of 107 ESCC patients are listed in Table 1. Among the patients, there were 55 women (51.4%) and 52 men (48.6%) aged between 32 and 84 years. Among them, a total of 56 (52.3%) smoked and 55 (51.4%) drank alcohol. Postoperative pathological stage classification showed at TNM stage I there were 20 cases (18.7%), 32 cases (29.9%) were at TNM stage II, and at TNM stage III there were 55 cases (51.4%). KIF4A expression level in the tumor tissues was investigated using IHC staining and appear brown (Fig 3d,e). The results revealed that although KIF4A was expressed positively mainly in the cytoplasm, it was also observed in the nucleus. KIF4A was overexpressed in 35.5% (38/107) of the sample and its expression was low in 64.5% (69/107) of the sample. We further investigated the association of KIF4A expression with clinicopathological characteristics of patients to explore its potential role in ESCC progression. The results showed that KIF4A expression was significantly correlated with age ($P = 0.014$), T stage ($P = 0.027$), and pathological stage ($P < 0.001$) (Table 2).

Table 1 Baseline characteristics of the 107 esophageal squamous cell carcinoma patients

Characteristics	Value, *n* (%)
Sex	
Female	55 (51.4)
Male	52 (48.6)
Age	
Mean ± SD	62.20 ± 9.423
Median, *n* (range)	62 (32–84)
Smoking	
Yes	56 (52.3)
No	51(47.7)
Drinking	
Yes	55(51.4)
No	52(48.6)
Differentiation degree	
Well	32(29.9)
Middle	33(30.8)
Poor	41(38.8)
T stage	
T1	21(19.6)
T2	34(31.8)
T3	30(28.0)
T4	22(20.6)
N stage	
N0	51(47.7)
N1-3	56(52.3)
pTNM stage	
I	20(18.7)
II	32(29.9)
III	55(51.4)
KIF4A expression	
Low	38(35.5)
Over	69(64.5)
Adjuvant treatment	
None	28(26.2)
Radiotherapy	26(24.3)
Chemotherapy	22(20.6)
CRT	31(29.0)

CRT, radiochemotherapy; N stage, lymph node metastasis; pTNM, pathological TNM; T stage, invasion depth.

Table 2 Association between clinicopathological features of esophageal squamous cell carcinoma and KIF4A expression in tumor tissues

Clinicopathological features	KIF4A expression		*P*-value†
	Low, *n* = 69	Over, *n* = 38	
Age			
<60	35	10	0.014*
≥60	34	28	
Sex			
Male	32	20	0.536
Female	37	18	
Smoking			
No	30	21	0.234
Yes	39	17	
Drinking			
No	33	19	0.830
Yes	36	19	
Differentiation			
Well	21	12	0.497
Moderate	19	14	
Poor	29	12	
T stage			
T1-2	30	25	0.027*
T3-4	39	13	
N stage			
N0	34	17	0.842
N1-3	35	21	
pTNM			
I–II	43	10	0.000364*
III	26	28	
Adjuvant treatment			
None	21	7	0.245
Radiotherapy	14	12	
Chemotherapy	12	10	
CRT	22	9	

*$P < 0.05$. †χ^2 test. CRT, radiochemotherapy; N stage, lymph node metastasis; pTNM, pathological TNM; T stage, invasion depth.

Table 3 Univariate and multivariate analysis of prognostic variables for esophageal squamous cell carcinoma

Variables	Univariate analysis	Multivariate analysis		
	P-value	Hazard ratio	95% CI	*P*-value
Sex				
Male vs. female	0.979			
Age				
<60 vs. ≥60	<0.001*	4.312	2.173-8.559	<0.001*
Smoking				
Yes vs. No	0.883			
Drinking				
Yes vs. No	0.995			
T stage				
T1–2 vs. T3–4	0.001*			
N stage				
N0 vs. N1–3	0.137			
Differentiation				
Well vs. moderate vs. poor	0.612			
pTNM				
I–II vs. III	<0.001*	3.100	1.625-5.814	<0.001*
KIF4A expression				
Low vs. over	<0.001*	2.189	1.218-3.936	0.009*
CRT				
None vs. RT vs. CT vs. RT + CT	0.545			

*$P < 0.05$. CI, confidence interval; CRT, radiochemotherapy; CT, chemotherapy; HR, hazard ratio; RT, radiotherapy; N stage, lymph node metastasis; pTNM, pathological TNM; T stage, invasion depth.

Kaplan-Meier analysis showed that ESCC patients with a higher KIF4A expression had a significantly poorer survival when compared with ESCC patients who had a lower KIF4A expression ($P < 0.001$) (Fig 3f). Also, the prognostic significance of KIF4A expression in ESCC patient subgroups stratified by pTNM stage (I and II vs. III) was investigated, and the results showed that five-year overall survival was significantly lower in higher KIF4A expression cases than lower KIF4A expression cases both with early- and late-stage ESCC ($P < 0.001$) (Fig 3g,h). Both univariate and multivariate survival analyses were used to evaluate the effects of KIF4A expression and clinicopathological characteristics on prognosis in ESCC patients. Multivariate Cox regression analysis showed that age (HR = 4.312, $P < 0.001$), pTNM stage (HR = 3.100), KIF4A expression (HR =2.189, $P = 0.009$), were independent predictors of survival in ESCC patients (Table 3).

Determination of KIF4A expression in cell lines and transfection efficiency

Western blotting was used to detect the protein expression of KIF4A in ESCC cell lines, including Eca150, Eca109, and Eca9706. The results showed that the expression trend of KIF4A in ESCC cell lines was not uniform ($P < 0.05$) (Fig 4a,b). Next, Eca109 and Eca150were used for follow-up experiments. Eca109 cells were infected with either control or KIF4A siRNA and Eca150 cells were infected with negative control or EX-A3631-Lv105. Western blot analyses suggested that siRNA-KIF4A and EX-A3631-Lv105 could significantly inhibit and increase the expression of KIF4A in protein levels, respectively (Fig 4c,d).

KIF4A promotes proliferation of ESCC cells

CCK8 assay was applied to detect the proliferative ability of the ESCC cells. The results showed that the number of cells decreased significantly when Eca109 cells were treated with KIF4A siRNA ($P < 0.05$). Similarly, the number of cells increased when KIF4A was overexpressed ($P < 0.01$) (Fig 4e,f). The results of the clone formation experiment showed that the number of Eca109 cells in the siKIF4A group was significantly lower than that in the control group. In contrast, the number of Eca150 cell clones in the OE-KIF4A group increased significantly (Fig 4g,h). These results demonstrated that KIF4A may promote the proliferation of ESCC cells.

KIF4A enhances migratory capabilities of ESCC cells

To determine the effect of KIF4A on the migratory capabilities of ESCC cells, transwell migration, and wound

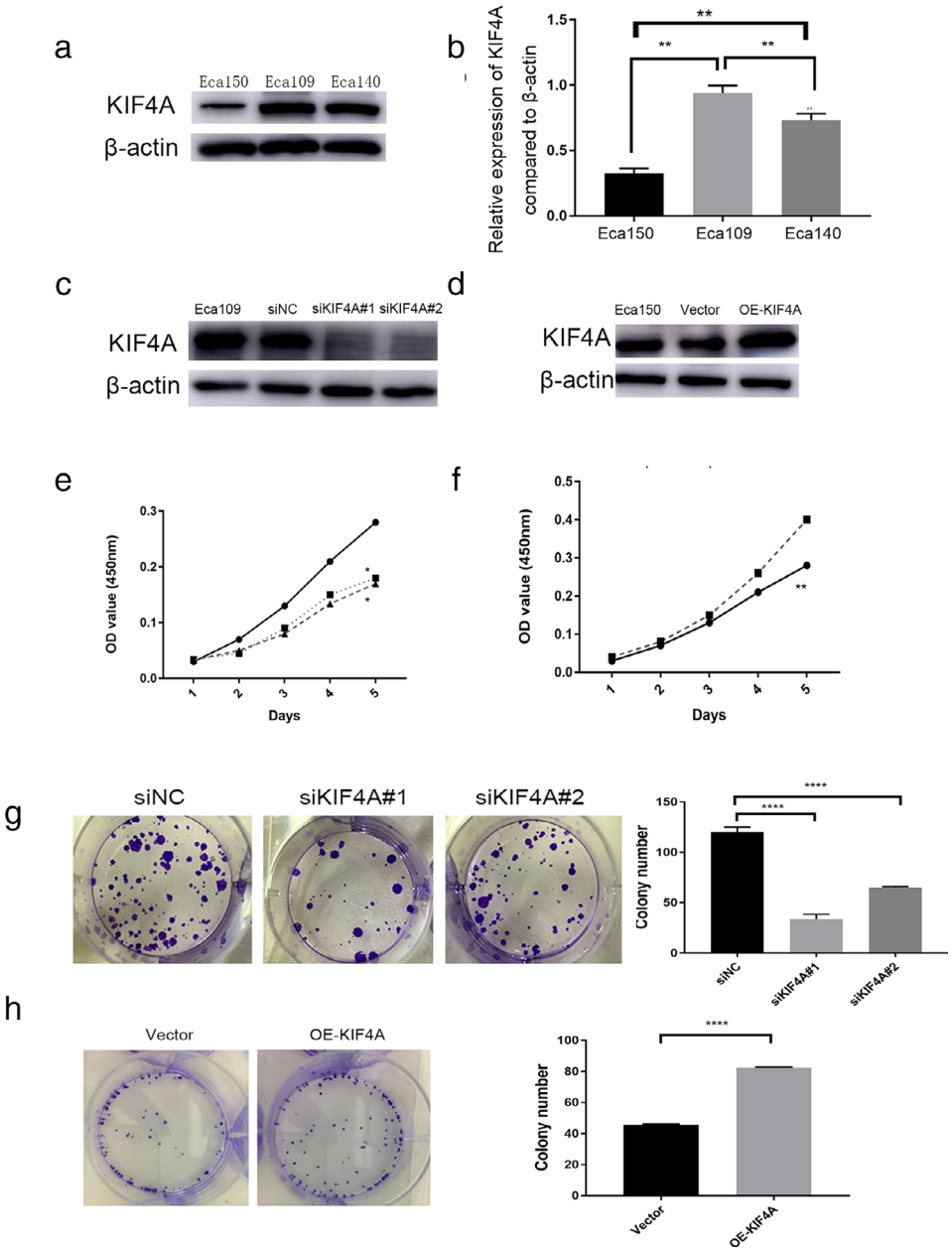

Figure 4 KIF4A promotes proliferation of esophageal squamous cell carcinoma (ESCC) cells. (**a** and **b**) KIF4A protein expressions in three hepatocellular carcinoma (HCC) cell lines examined by western blotting were not uniform ($P < 0.01$). (**c**) The effect of KIF4A knockdown with siRNAs was verified by western blotting. (**d**) The effect of KIF4A overexpression was verified by western blotting. (**e**,**f**) The cell number significantly decreased when the Eca109 cells were treated with KIF4A siRNA and increased when KIF4A was overexpressed. (**g**,**h**) The number of colony cells decreased significantly when the Eca109 cells were treated with KIF4A siRNA and increased when KIF4A was overexpressed. (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$). (**e**) (—●—) siNC, (--■--) siKIFA#1, (--▲--) siKIFA#2; (**f**) (—●—) Vector, (--■--) OE-KIF4A.

healing assays were performed. Transwell migration assays indicated that the migration of Eca109 cells infected with KIF4A siRNA decreased significantly compared with the control group (Fig 5a,b), whereas the migration of Eca150 cells infected with EX-A3631-Lv105 increased noticeably in comparison with the Vector group. Wound healing assays indicated that the rate of migration of Eca109 cells with KIF4A siRNA expression was 25 ± 5.01%, ie, lower than the 56 ± 1.32% rate of migration of Eca109 cells as compared with the control group. The rate of migration of Eca150 cells with OE-KIF4A expression was 79 ± 1.27%, higher than 31 ± 1.04% of Eca150 cells as compared with the Vector group, which suggested that KIF4A promoted the migration of ESCC cells (Fig 5c,d).

KIF4A promotes ESCC cell migration and proliferation by activating the Hippo-YAP signaling pathway

Western blotting was used to detect the protein expression levels of p-LATS1, LATS1, p-YAP, and YAP in ESCC cells after KIF4A silencing and overexpression to determine the effect of KIF4A on the Hippo pathway. Results are shown in Fig 5e, and after the transfection of KIF4A siRNA in

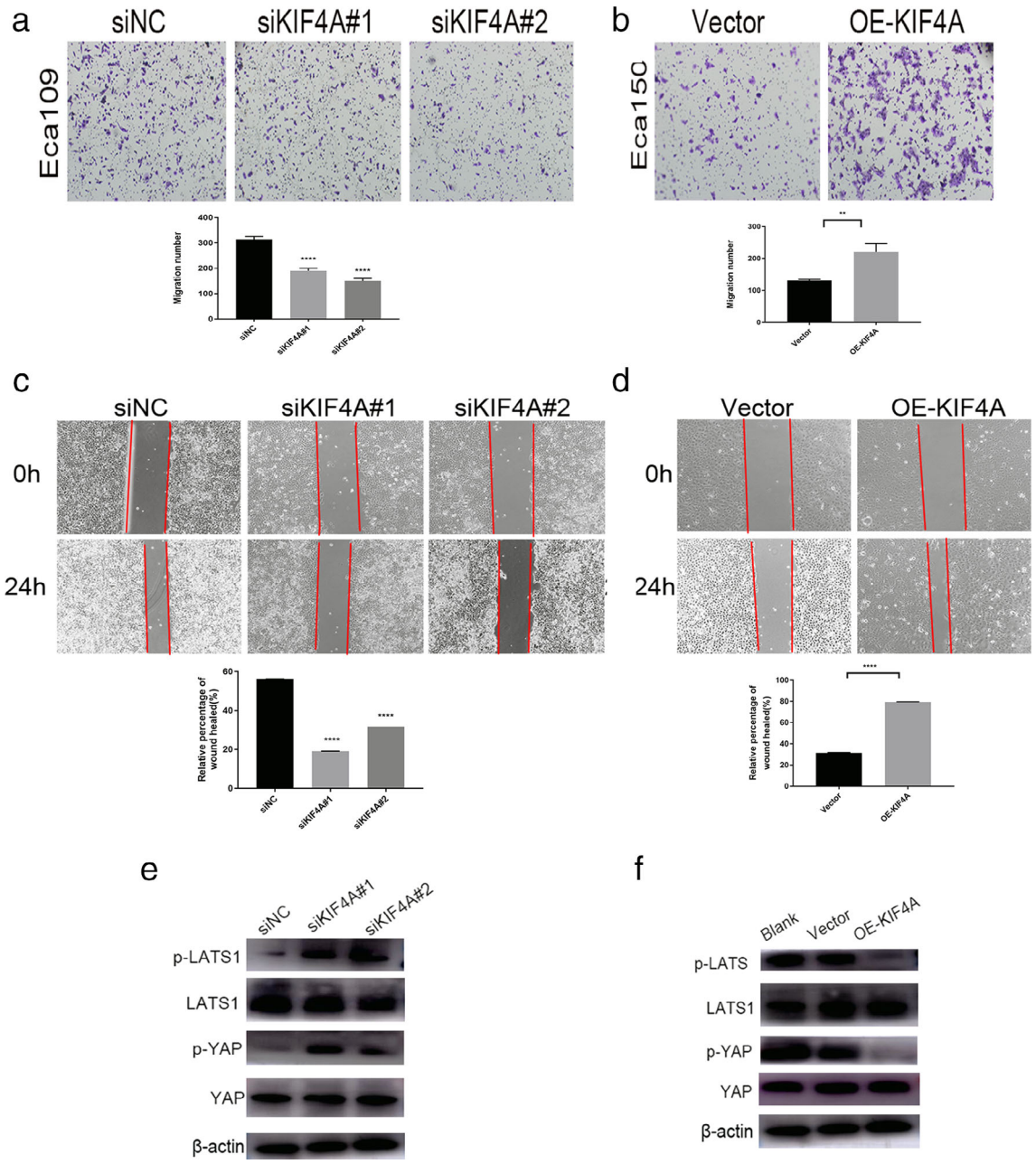

Figure 5 KIF4A regulates esophageal squamous cell carcinoma (ESCC) cell migration in vitro (**a** and **b**). The effects of KIF4A on the migration potential of ESCC cells were measured by transwell assay. (**c** and **d**) The effects of KIF4A on the migratory capability of ESCC cells was measured by wound healing assay. (**e** and **f**) The effect of KIF4A on the Hippo pathway was detected by western blot analysis (**$P < 0.01$, ****$P < 0.0001$).

ESCC cells, the phosphorylation levels of LATS1 and YAP in the cells increased significantly, whereas the total LATS1 and YAP protein contents remained unchanged. After overexpression of KIF4A in ESCC cells, the phosphorylation levels of LATS1 and YAP in the OE-KIF4A group decreased significantly, whereas the total LATS1 and YAP protein contents remained unchanged. The results showed that KIF4A maintained cell survival by affecting the Hippo pathway (Fig 5f).

Discussion

Esophageal squamous cell carcinoma is a malignant tumor of the digestive tract[16] which occurs in the esophageal epithelium. Because ESCC does not become specifically manifested in the early stage, most patients in the sample were in the middle and late stages when they visited the doctor.[17] Therefore, early diagnosis and assessment of

disease progression trend are effective ways to improve the prognosis of ESCC patients. In recent years, with the development of molecular bioinformatics, there has been a gradual increase in the use of the expression of related genes in the diagnosis and the prognostic assessment of malignant tumors because this method provides a certain point of reference for the prevention and treatment of diseases.[18,19]

In this study, we found a total of 53 DEGs by analyzing three ESCC microarray expression spectrum data sets in the GEO database. The GO functional enrichment analysis of DEGs showed that DEGs mainly enriched and participated in monooxygenase activity, actin-binding, cell cortex, midbody, collagen catabolic process, extracellular matrix organization, etc. KEGG pathway analysis revealed that DEGs were involved in transcriptional misregulation in cancer, drug metabolism—cytochrome P450, protein digestion and absorption, etc. The differentially expressed genes were analyzed by PPI and the most significant gene modules were selected. Survival and prognosis analysis of the six most significant core genes using the TCGA database found that KIF4A was significantly correlated with the prognosis of esophageal cancer. Based on the above analysis, KIF4A was selected as the key ESCC gene in this study. Western blotting and RT-qPCR were used to detect the collected clinical ESCC and adjacent tissues, and it was found that the expression level of KIF4A in ESCC tissues was significantly higher than that in corresponding adjacent tissues. IHC staining appeared brown. Results revealed that KIF4A was mainly positively expressed in the cytoplasm and the expression level of KIF4A in patients with advanced ESCC was significantly greater. In a series of cell function experiments, KIF4A overexpression was found to increase the migratory capability and decrease the apoptotic ability of ESCC cells, suggesting that KIF4A may be an oncogene of ESCC.

A Hippo signaling pathway is an evolutionarily conserved signaling pathway involved in cell tissue growth and development.[20] The Hippo pathway responds to a variety of external stimuli, including cell density and growth factors.[21] At the core of the Hippo signaling pathway are YAP and TAZ proteins that are important transcription-assisted activators that regulate cell growth and the transcription process of related genes. Previous studies suggested that YAP was highly expressed in ESCC and had adverse effects on the prognosis of ESCC.[13] The transcriptional activation of downstream genes by YAP is regulated by kinase complexes, including LATS1, LATS2, etc. LATS kinase mediates YAP phosphorylation which results in YAP retention in the cytoplasm and inhibits its role in transcriptional activation.[21,22] Our experimental results suggest that KIF4A may inhibit YAP phosphorylation by inhibiting the upstream kinase activity of YAP, resulting in increased YAP expression level in the nucleus, and, thereby, promoting the proliferation and migration of ESCC cells.

This study has several limitations. We were limited by the sample size of TCGA and clinical specimens, which may have resulted in a slight bias. Further research will help to further validate our findings and better understand the mechanisms that promote migration and proliferation.

Acknowledgments

This study was supported by Special Fund for Taishan Scholar Project (grant number.ts20190973). We thank Laboratory of Basic Medical Sciences and all the members in the lab and our team who provided us with a favorable environment to finish the study and offered us the help whenever required. This study was approved by the Ethics committee of Qilu Hospital, Cheeloo College of Medicine, Shandong University, and patients' informed consent was acquired before the study. All procedures were consistent with the declaration of Helsinki.

Disclosure

The authors declare that there are no conflicts of interest.

References

1 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68** (6): 394–424.
2 Chen W, Zheng R, Baade P *et al.* Cancer statistics in China, 2015. *CA Cancer J Clin* 2016; **66** (2): 115–32.
3 Hao J, Lin D, Dinh H *et al.* Spatial intratumoral heterogeneity and temporal clonal evolution in esophageal squamous cell carcinoma. *Nat Genet* 2016; **48** (12): 1500–7.
4 Xu Y, Peng Y, Xu L *et al.* Autoantibodies: Potential clinical applications in early detection of esophageal squamous cell carcinoma and esophagogastric junction adenocarcinoma. *World J Gastroenterol* 2019; **25** (34): 5049–68.
5 Wang M, Smith J, Wei W. Tissue protein biomarker candidates to predict progression of esophageal squamous cell carcinoma and precancerous lesions. *Ann N Y Acad Sci* 2018; **1434** (1): 59–69.
6 Barisic M, Aguiar P, Geley S, Maiato H. Kinetochore motors drive congression of peripheral polar chromosomes by overcoming random arm-ejection forces. *Nat Cell Biol* 2014; **16** (12): 1249–56.
7 Chen J, Li M, Fang S *et al.* KIF4A: A potential biomarker for prediction and prognostic of prostate cancer. *Clin Invest Med* 2020; **43** (3): E49–59.
8 Stumpff J, Wagenbach M, Franck A, Asbury CL, Wordeman L. Kif18A and chromokinesins confine centromere movements

via microtubule growth suppression and spatial control of kinetochore tension. *Dev Cell* 2012; **22** (5): 1017–29.
9 Luan Y, Liu S, Sun Y *et al*. Whole genome expression microarray reveals novel roles for Kif4 in monocyte/macrophage cells. *Eur Rev Med Pharmacol Sci* 2019; **23** (16): 7016–23.
10 Heng B, Zhang X, Aubel D et al. An overview of signaling pathways regulating YAP/TAZ activity. *Cell Mol Life Sci* 2020. http://dx.doi.org/10.1007/s00018-020-03579-8.
11 Liu G, Lu Y, Li L *et al*. The kinesin motor protein KIF4A as a potential therapeutic target in renal cell carcinoma. *Invest New Drugs* 2020; **38** (6): 1730–42.
12 Ibar C, Irvine K. Integration of hippo-YAP signaling with metabolism. *Dev Cell* 2020; **54** (2): 256–67.
13 Zhou X, Li Y, Wang W *et al*. Regulation of hippo/YAP signaling and esophageal squamous carcinoma progression by an E3 ubiquitin ligase PARK2. *Theranostics* 2020; **10** (21): 9443–57.
14 Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K. KEGG: New perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res* 2017; **45**: D353–D61.
15 Chen L, Zhang Y, Wang S *et al*. Prediction and analysis of essential genes using the enrichments of gene ontology and KEGG pathways. *PLOS One* 2017; **12** (9): e0184129.
16 Shin D, Kim H, Cho J et al. Conditional survival of patients who underwent curative resection for esophageal squamous cell carcinoma. *Ann Surg* 2020. http://dx.doi.org/10.1097/sla.0000000000004473.
17 Li Y, Beck M, Päßler T *et al*. A FDG-PET radiomics signature detects esophageal squamous cell carcinoma patients who do not benefit from chemoradiation. *Sci Rep* 2020; **10** (1): 17671.
18 Leek J, Johnson W, Parker H *et al*. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics* 2012; **28** (6): 882–3.
19 Hu J, Li R, Miao H, Wen Z. Identification of key genes for esophageal squamous cell carcinoma via integrated bioinformatics analysis and experimental confirmation. *J Thorac Dis* 2020; **12** (6): 3188–99.
20 Jain P, Guerreiro P, Canato S *et al*. The spectraplakin dystonin antagonizes YAP activity and suppresses tumourigenesis. *Sci Rep* 2019; **9** (1): 19843.
21 Sarmasti Emami S, Zhang D, Yang X. Interaction of the hippo pathway and phosphatases in tumorigenesis. *Cancer* 2020; **12** (9): 2438. http://dx.doi.org/10.3390/cancers12092438.
22 Irie K, Nagai T, Mizuno K. Furry protein suppresses nuclear localization of yes-associated protein (YAP) by activating NDR kinase and binding to YAP. *J Biol Chem* 2020; **295** (10): 3017–28.

