Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Importin 7 enhances protective autophagy induced by nuclear translocation of homeobox A10 in oral squamous cell carcinoma

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Article Info

Article history:

Received: May 12, 2023
Accepted: February 15, 2024
Published: March 31, 2024

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Abstract

Oral squamous cell carcinoma (OSCC) is a common malignant tumor. Importin7 (IPO7) is responsible for nucleoplasmic transport of RNAs and proteins, and it has been confirmed to be involved in the development of human cancers. This study aimed to explore the function and mechanism of IPO7 in OSCC. IPO7 expression in tissues and cells was determined by RT-qPCR. Cell proliferative, migratory, and invasive capabilities were detected through transwell assay and colony formation assay. Mice xenograft models were established for evaluating tumor growth. Autophagy was estimated by the LC3 levels in cells through western blot and immunofluorescence (IF). Western blot was utilized to detect the key proteins in PERK/EIF2AK3/ATF4 pathway for assessing the endoplasmic reticulum stress (ERS). The interaction of IPO7 and homeobox A10 (HOXA10) was tested by GST pull-down assay and Co-IP assay. ChIP assay and luciferase reporter assay were utilized to determine the combination of HOXA10 and EIF2AK3. We proved that IPO7 was upregulated in OSCC tissues and cells, and its depletion reduced cell proliferation, migration, invasion and tumor growth. Furthermore, LC3 expression in cells was found to be reduced by IPO7 knockdown. IPO7 promoted OSCC tumor metastasis by activating autophagy. Additionally, we discovered that IPO7 could regulate ERS by activating the PERK/ATF4 pathway. EIF2AK3 upregulation can promote cell autophagy. Furthermore, IPO7 was proven to promote nuclear translocation of HOXA10 in cells. EIF2AK3 promoter can bind to HOXA10. Rescue assay confirmed that HOXA10 upregulation can reverse the effect of IPO7 silencing on OSCC progression. IPO7 can enhance proliferation, migration, invasion, and autophagy by nuclear translocation of HOXA10 and the activation of EIF2AK3/ATF4 pathway in OSCC.

Keywords: Autophagy, IPO7, Oral squamous cell carcinoma, Tumor metastasis, Unfolded protein response.

1. Introduction

Oral squamous cell carcinoma (OSCC) is a common tumor in the oral cavity, characterized by aggressive growth of cancer cells and a tendency to lymph node metastasis [1]. The cancer cells can metastasize through the body to remote areas such as the lungs thereby inducing new tumorigenesis [2]. Smoking, alcohol consumption, and betel nut chewing are some of the currently known risk factors for OSCC [3]. The incidence of OSCC patients has been increasing year by year in recent decades, but the survival rate is only about 40% to 50% [4]. Current treatment methods for OSCC rely on surgical resection, supplemented by radiation and chemotherapy, but the prognosis for patients is not satisfactory [5]. Therefore, deciphering the pathogenesis and metastasis mechanism of OSCC can help develop more effective therapeutic targets for OSCC.

Importin is a relatively conserved class of proteins consisting of α and β subunits [6, 7]. They can transport transcription factors, splicing factors and other proteins from the cytoplasm to the nucleus via the nuclear pore complex [8]. Importin7 (IPO7) belongs to the importin-β family and it is responsible for nucleoplasmic transport of RNAs, proteins, and transcription factors [9]. IPO7 takes part in regulating the development of some human cancers and exerts carcinogenic effects. For example, FOXM1 can promote glioma progression by enhancing the GLI1 nuclear localization via upregulating IPO7 expression [10]. IPO7 overexpression facilitates lung tumorigenesis in mice [11]. IPO7 overexpression remarkably enhanced pancreatic cancer cell proliferation, migration and invasion through modulating the ERBB pathway [12]. IPO7 is correlated with CD8 T cell infiltration and IPO7 overexpression accelerates the development of cervical cancer [13]. Bioinformatics shows that IPO7 expresses at a high level in OSCC, while the function of IPO7 remains unclear.

Autophagy is a complex and catabolic process that obviates protein agglomeration and organelle injury by lysosomal degradation pathway [14]. In recent years, numerous researches have demonstrated autophagy plays a double-edged role in tumor development [15]. Evidence has suggested autophagy can inhibit the growth of primary tumors. However, autophagy has also been shown to be required for tumor maintenance and progression [16, 17]. The protective autophagy role of cells is a survival mechanism. NUPR1 facilitates OSCC cell metastasis via the

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Doi: http://dx.doi.org/10.14715/cmb/2024.70.3.36

activation of TFE3-dependent autophagy [18]. Autophagy inhibition can enhance the anti-tumor function of metformin in OSCC [19]. However, whether IPO7 is involved in the progression of OSCC by regulating autophagy needs to be further investigated.

In this study, we aimed to explore the specific function and regulatory mechanism of IPO7 in OSCC, which may provide novel therapeutic targets for OSCC.

2. Materials and methods

2.1. Clinical tissue samples

The clinical samples were obtained from OSCC patients who received the surgical resection in The People's Hospital of Cangnan. The adjacent non-tumor tissues were gathered for control. Specimens were maintained in liquid nitrogen immediately after excision. The procedure was approved by the Ethics Committee The Centers for Disease Control and Prevention. All patients signed the written consent.

2.2. Cell culture

Three OSCC cell lines SCC25, SCC-9, and SCC-15 obtained from ATCC (Manassas, VA, USA) were incubated in DMEM/F12 with 10% FBS. HOK cells obtained from ScienCell (Santiago, CA, USA) were incubated in OKM medium (Invitrogen) with 10% FBS. Cells were cultured at 37 ℃ with 5% CO_2.

2.3. Cell transfection

Short hairpin RNA for IPO7 (sh-IPO7) and control negative control (sh-NC) were obtained from Genepharma (Shanghai, China). The full length of IPO7, EIF2AK3, or HOXA10 was cloned into the pcDNA3.1 vectors for gene overexpression. Lipofectamine 3000 (Invitrogen) was utilized for cell transfection for 48 h in accordance with user guides.

2.4. RT-qPCR

The total RNA was isolated from cells by TRIzol (Invitrogen). Then it was reverse transcribed for cDNA synthesis by Prime Script™ RT reagent kit (Takara Biotechnology, China). qPCR was performed with SYBR mix (Takara) on Step-One Plus System (Applied Biosystems, Foster City, CA, USA). Gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. GAPDH served as the reference gene.

2.5. Western blot

The proteins extracted from cells were lysed in the RIPA buffer. Then, the proteins were isolated on 10% SDS-PAGE and transferred to PVDF membranes. PVDF membranes were cultured with the primary antibodies (Abcam, USA) at 4℃ for one night, followed by incubating with secondary antibodies for further 2 h. The protein bands were determined by ECL luminescent liquid and analyzed by ImageJ (v1.8.0).

2.6. Colony formation assay

Cells were seeded in 6-well plates and cultured in culture medium for 14 days. Afterwards, cells were fixed with methanol and dyed with crystal violet. The quantity of colonies was measured by the microscope (Olympus, Japan).

2.7. Transwell assay

Cells in 200 μL serum-free medium were put in the upper chamber (8.0 μM pores, Corning, USA) without or with Matrigel. The complete culture medium was put in the lower chamber. Cells were cultured for 24 h. After that, the migrated cells were fixed by 4% polyformaldehyde and dyed by crystal violet. The microscope was utilized for observing the stained cells.

2.8. Immunofluorescence (IF)

Cells were subjected to fixation with 4% paraformaldehyde and permeation with 0.5% Triton X-100. Next, they were incubated with primary antibody anti-LC3B (Abcam, USA) for one night at 4 °C and then with a secondary antibody for 1 h at room temperature. Nuclei were dyed by DAPI solution. The microscope (Carl Zeiss, Oberkochen, Germany) was applied for observation. To detect the autophagic flux, cells were transiently transfected with mRFP-GFP-LC3B adenovirus (Hanbio Co. Ltd.) for 48 h. Then the quantity of LC3B puncta was detected through the confocal microscopy (Olympus).

2.9. Co-IP assay

Cells were lysed in lysis buffer and centrifugated at 16,000 g for 10 min at 4 ℃. Then, 900 μl aliquots were cultured with sepharose conjugated with IgG (GE Healthcare) or anti-HOXA10 or anti-IPO7 for 4 h at 4°C. The beads were rinsed with lysis buffer and eluted with SDS buffer through boiling for 10 min, followed by the western blot analysis.

2.10. Glutathione-S-Transferase (GST) pull-down assay

Fusion protein GST-IPO7 was constructed and the recombinant plasmids were transformed BL21 cells and induced by 1 mM IPTG. The soluble lysates were prepared utilizing protease and phosphatase inhibitors. The fusion proteins were subjected to purification by glutathione sepharose. The total protein was cultured with GST-IPO7 or GST at 4 °C, followed by incubating with glutathione-sepharose beads for 2 h. After washing with binding buffer, beads were boiled in SDS buffer for 10 min, followed by the western blot analysis.

2.11. Luciferase reporter assay

EIF2AK3 promoter was generated in pGL3 luciferase vector. Cells were subjected to co-transfection with the control pRL-Renilla (Promega, Madison, WI) and the indicated plasmids utilizing Lipofectamine 3000. Luciferase activity was determined by Dual Luciferase Assay System (Promega).

2.12. Chromatin immunoprecipitation (ChIP) assay

EZ-ChIP kit (Millipore) was applied for this assay in accordance with user guides. Cells were fixed with 1% formaldehyde and cell lysates were sonicated into DNA fragments in the range of 200-1000 bp. Then, the chromatin was immunoprecipitated with anti-HOXA10 or normal IgG (Abcam) at 4℃ overnight. After cross-linking, elution and extraction, the purified chromatin DNA was analyzed through RT-qPCR.

2.13. Mice xenograft models

BALB/c nude mice (4–6 weeks old, female) were pur-

chased from Gempharmatech Co., Ltd (Nanjing, China). All animal experiments were approved by the Ethics Committee of The People's Hospital of Cangnan. 5×10^6 SCC-25 cells transfected with sh-NC or sh-IPO7 were suspended in PBS and were injected subcutaneously to the left back of each mouse. Tumor volumes were detected every five days. After 30 days, mice were euthanized, and the tumor tissues were weighed and maintained at -80°C.

2.14. Statistical analyses

Data were displayed as the means ± SD from three individual repeats. Statistical analysis was performed utilizing GraphPad Prism software (version 7.0, USA). Student's t-test was applied for comparison between two groups. The comparison among multiple groups was analyzed by the one-way ANOVA followed by Tukey's *post hoc* analysis. $P<0.05$ was considered to indicate statistical significance.

3. Results

3.1. IPO7 facilitates OSCC cell proliferation, migration, invasion and tumor growth

Through UALCAN database (https://ualcan.path.uab.edu/index.html), we found that IPO7 expressed at a high level in the HNSS tissues (Fig. 1A). Then, GSE74530 dataset illustrated that IPO7 was highly expressed in the tumor tissues of OSCC patients in comparison of the adjacent tissues (Fig. 1B). Furthermore, through the performance of western blot assay, we proved that IPO7 level in OSCC tumor tissues was higher than in normal tissues (Fig. 1C). Similarly, in OSCC cell lines, we observed a notable upregulation of IPO7 expression (Fig. 1D). Kaplan-Meier analysis further manifested that patients with high IPO7 expression possessed the lower survival rate than patients with low IPO7 expression (Fig. 1E). Thus, IPO7 upregulation was identified in OSCC.

The biological functions of IPO7 in OSCC was investigated. IPO7 was silenced in SCC-25 and SCC-9 cells by the sh-IPO7 transfection (Fig. 1F). Then, it was manifested by Transwell assay that cell migration was inhibited after IPO7 silencing (Fig. 1G). Transwell assay further verified that IPO7 depletion notably weakened the cell migratory and invasive capabilities (Fig. 1H). Moreover, cell proliferation was assessed by colony formation assay and we discovered that the quantity of colonies was declined by IPO7 inhibition (Fig. 1I). Then we performed the *in vivo* assay by establishing the mice model. We observed that compared with the control mice, the tumor size, volume, and weight were all reduced in mice injected with sh-IPO7-transfected OSCC cells (Fig. 1J-L). Thus, we confirmed that IPO7 facilitated OSCC cell proliferation, migration, invasion and tumor growth.

3.2. IPO7 promotes OSCC tumor metastasis by activating autophagy

Using the Linkedomics database (http://www.linkedomics.org/login.php), we analyzed the genes associated with IPO7 expression in HNSCC. As shown in 2A, we obtained 310 positively correlated genes and 222 negatively correlated genes. Next, we performed KEGG analysis on these co-expressed genes of IPO7 and found that they were related to biological processes such as autophagy (Fig. 2B). Therefore, we speculate that IPO7 may mediate cell autophagy process in OSCC. Through western blot, we found that compared to the control group, LC3-II level was markedly reduced in the transfection group, and the ratio of LC3-II/LC3-I was notably reduced, indicating IPO7 knockdown inhibited autophagy (Fig. 2C). Subsequently IF results further manifested that, in comparison of control cells, LC3 illustrated a weaker fluorescence intensity in sh-IPO7-transfected cells (Fig. 2D). To further verify, we transfected the cells with mRFP-GFP-LC3 adenovirus to monitor autophagic flux. The results demonstrated that autophagic flux was inhibited in IPO7-knockdown cells because of the declined quantity of red and yellow dots (Fig. 2E). Therefore, we believed that IPO7 facilitated cell autophagy in OSCC.

We further detected whether IPO7 promoted OSCC

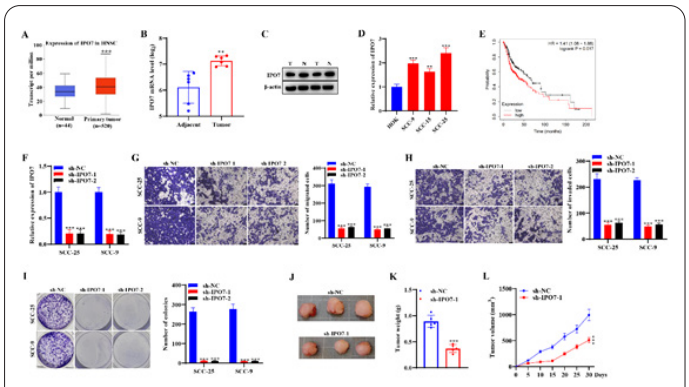

Fig. 1. IPO7 facilitates OSCC cell proliferation, migration, invasion and tumor growth. (A) UALCAN analysis of IPO7 expression in HNSS tissues and normal tissues. (B) GSE74530 analysis of IPO7 expression in OSCC tissues and adjacent tissues. (C) Western blot outcomes of IPO7 protein levels in OSCC tissues and normal tissues. (D) IPO7 expression in OSCC cells was tested through RT-qPCR. (E) Kaplan-Meier analysis of the survival rate of OSCC patients with high or low IPO7. (F) RT-qPCR outcomes of the transfection efficiency of sh-IPO7 in SCC-25 and SCC-9 cells. (G-H) transwell assay was implemented for testing cell migratory and invasive capabilities when IPO7 was silenced. (I) Cell proliferative capability was assessed by colony formation assay after inhibiting IPO7. (J-L) Tumor size, volume and weight in mice were measured. *P<0.05; #P<0.05.

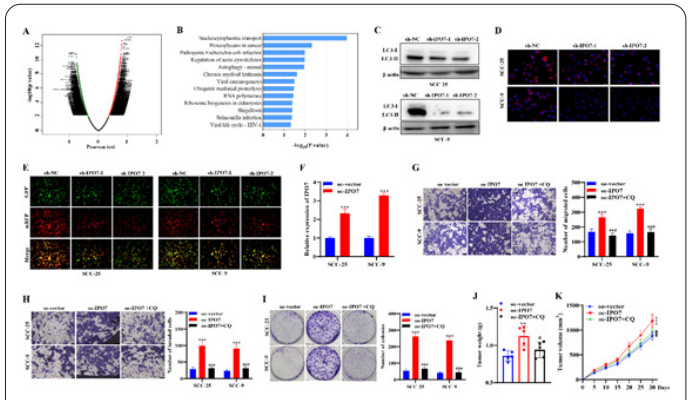

Fig. 2. IPO7 facilitates OSCC tumor metastasis by activating autophagy. (A) Linkedomics analysis of genes related to IPO7 expression in HNSCC. (B) KEGG analysis of co-expressed genes of IPO7. (C) Western blot outcomes of LC3 protein levels in cells. (D) IF was utilized to determine the LC3 expression in cells. (E) The autophagic flux was assessed by the transfection of LC3-GFP or mRFP adenovirus. (F) The overexpression efficiency of IPO7 was determined by RT-qPCR. (G-H) transwell assay was utilized for cell migration and invasion when IPO7 was overexpressed or CQ was added. (I) Cell proliferative capability was assessed by colony formation assay. (J-K) Tumor volume and weight in mice were measured. *P<0.05; #P<0.05.

growth by inducing autophagy. The cells were transfected with pcDNA3.1-IPO7 to construct the IPO7-overexpressed cell line (Fig. 2F). CQ was utilized to treat cells for suppressing autophagy. Functional assays were then conducted to test the impacts of IPO7 upregulation and CQ treatment on cell behaviors. We discovered that the migration and invasion elevated by IPO7 upregulation were reduced by CQ treatment (Fig. 2G-H). Additionally, colony formation assay results illustrated that cell proliferative capability promoted by IPO7 overexpression was attenuated by CQ (Fig. 2I). Further, we found that CQ treatment diminished the increase of tumor volume and weight in vivo induced by IPO7 overexpression (Fig. 2J-K). Overall, IPO7 upregulation promotes OSCC cell malignant phenotypes and tumor metastasis by activating autophagy.

3.3. IPO7 promotes autophagy by enhancing ERS through PERK/ATF4 pathway

Endoplasmic reticulum (ER) stress (ERS) signal transduction is closely associated with autophagy in tumor cells [20]. Therefore, we further tested whether IPO7 has an impact on ERS. Western blot was utilized to detect ERS sensing proteins and key downstream proteins. As a result, only EIF2AK3/ATF4 pathway activity was markedly affected by IPO7 knockdown in SCC-25 and SCC-9 cells (Fig. 3A). Therefore, we further tested the level changes of key proteins on EIF2AK3/ATF4 pathway. The result manifested that p-EIF2α and CHOP levels decreased markedly after knockdown of IPO7, indicating that IPO7 depletion may inhibit p-EIF2α to regulate ERS (Fig. 3B). The analysis outcomes from LinkedOmics database illustrated a positive correlation between p-EIF2α and IPO7 protein expression (Fig. 3C). Next, we utilized western blot to determine the expression of kinases that mediate EIF2α phosphorylation. In addition to EIF2AK3, the kinases that regulate p-EIF2α also include HRI, PKR, GCN2, and GADD34. We found no significant alterations in HRI, PKR, GCN2, and GADD34 levels after interference with IPO7, confirming that IPO7 regulates p-EIF2α through EIF2AK3 (Fig. 3D). Moreover, through the GEPIA database (http://gepia2.cancer-pku.cn/#index), we found a positive correlation between IPO7 and EIF2AK3 mRNA expression (Fig. 3E). These results indicated that IPO7 regulated ERS by the PERK/ATF4 pathway.

EIF2AK3 was overexpressed in SCC-25 and SCC-9 cells by transfecting with the pcDNA3.1-EIF2AK3 vectors (Fig. 3F). Then RT-qPCR and western blot manifested that EIF2AK3 mRNA and protein levels were reduced in the sh-IPO7 group, while restoring in the sh-IPO7+oe-EIF2AK3 group (Fig. 3G). In addition, we observed that p-EIF2α, ATF4 and CHOP levels decreased by IPO7 depletion were reversed by EIF2AK3 overexpression, suggesting IPO7 activated the EIF2AK3/ATF4 pathway through upregulating EIF2AK3 (Fig. 3H). Then, we detected the impact of IPO7 depletion and EIF2AK3 overexpression on cell autophagy. We observed that the LC3-II/LC3-I ratio reduced by IPO7 downregulation was increased by EIF2AK3 upregulation (Fig. 3I). LC3 fluorescence intensity was weakened in the sh-IPO7 group, while recovering in the sh-IPO7+oe-EIF2AK3 group (Fig. 3J). The mRFP-GFP-LC3B adenovirus assay further displayed that EIF2AK3 upregulation abolished the inhibitory function of IPO7 depletion on autophagic flux (Fig. 3K). Thus, we confirmed that IPO7 promoted autophagy by enhancing ERS.

3.4. IPO7 promotes nuclear translocation of HOXA10, and HOXA10 transcriptionally activates EIF2AK3

We further explored the specific regulatory mechanism between IPO7 and EIF2AK3. Through luciferase reporter assay, we found that IPO7 silencing markedly inhibited the transcription activity of EIF2AK3 (Fig. 4A). However, methylation-specific PCR results manifested that IPO7 depletion had no significant impact on the methylation level of the EIF2AK3 promoter (Fig. 4B). Therefore, we speculate that IPO7 may regulate EIF2AK3 through transcription factors. Co-IP and GST pull-down assays verified the binding of transcription factor homeobox A10 (HOXA10) and IPO7 in cells (Fig. 4C-D). Western blot indicated that there was no notable change in the total amount of HOXA10 protein in the IPO7-silenced cells, but the level of HOXA10 protein in the nucleus was markedly reduced (Fig. 4E-F). Subsequently, the cells processed the exonuclear transport inhibitor LMB and then performed the IF assay to evaluate the subcellular localization of HOXA10. The outcomes demonstrated that after IPO7 knockdown, the activated HOXA10 in the nucleus decreased and HOXA10 in the cytoplasm increased (Fig. 4G). Therefore, we believe that IPO7 can promote the nuclear translocation of HOXA10 in OSCC cells.

We then conducted assays to confirm whether HOXA10 can regulate EIF2AK3 transcription in OSCC cells. HOXA10 expression in cells was notably upregulated after transfection with the pcDNA3.1-HOXA10 vector (Fig. 4H). Further, we observed that HOXA10 upregulation increased the mRNA and protein levels of EIF2AK3 (Fig. 4I-J). The luciferase report assay illustrated that HOXA10

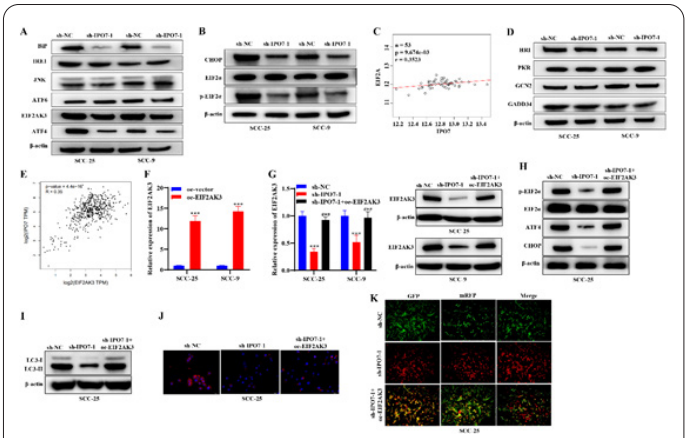

Fig. 3. IPO7 promotes autophagy by enhancing ERS through PERK/ATF4 pathway. (A) Western blot outcomes of BiP, IRE1, JNK, ATF6, EIF2AK3 and ATF4 protein expression levels. (B) Western blot outcomes of CHOP, EIF2α and p-EIF2α protein levels. (C) LinkedOmics database was applied to predict the correlation of IPO7 and p-EIF2α. (D) Western blot outcomes of HRI, PKR, GCN2, and GADD34 protein levels. (E) The GEPIA database was applied to predict the correlation of IPO7 and EIF2AK3. (F) The overexpression efficiency of EIF2AK3 was tested via RT-qPCR. (G) EIF2AK3 expression was tested by RT-qPCR and western blot in cells when IPO7 was silenced and EIF2AK3 was upregulated. (H) Western blot outcomes of EIF2α, p-EIF2α, ATF4 and CHOP protein levels in cells. (I) Western blot outcomes of LC3 protein levels. (J) The IF was utilized to determine the LC3 expression. (K) The autophagic flux was assessed by the transfection of LC3-GFP or mRFP adenovirus

upregulation enhanced the activity of the EIF2AK3 promoter (Fig. 4K). ChIP further verified that HOXA10 can bind to EIF2AK3 promoter, and HOXA10 overexpression further enhanced the binding between them (Fig. 4L). Next, we found that HOXA10 upregulation can reverse the expression level of EIF2AK3 inhibited by IPO7 knockdown (Fig. 4M-N). The positive correlation between HOXA10 and EIF2AK3 expression was further verified through the GEPIA database (Fig. 4O). Collectively, these results demonstrated that HOXA10 could transcriptionally activate EIF2AK3 in OSCC cells.

3.5. IPO7 promotes OSCC progression by upregulating HOXA10

Analysis of the UALCAN database and GSE74530 dataset showed that HOXA10 was notably overexpressed in OSCC (Fig. 5A-B). Moreover, through the survival curve of Kaplan-Meier analysis, we discovered that compared with patients with low HOXA10, patients with high HOXA10 had a poor prognosis (Fig. 5C). Additionally, through functional assays, we found the inhibitory function of IPO7 silencing on cell migratory, invasive, and proliferative capabilities were reversed by HOXA10 overexpression (Fig. 5D-E). Thus, IPO7 promoted the growth of OSCC via overexpressing HOXA10.

4. Discussion

Studies have shown that OSCC patients with advanced stage have a postoperative survival time of less than 30 months [21]. Therefore, the search for tumor biomarkers for molecular diagnosis and targeted therapy is of great importance for curing OSCC. IPO7 belongs to the nucleoplasmic transporter protein family which primarily mediates the nuclear import of proteins [9]. Importantly, IPO7 is upregulated in a variety of cancers, including cervical cancer and pancreatic cancer, with oncogenic functions [12, 13]. Herein, we discovered that IPO7 expression was upregulated in OSCC tissues and cells. Functional assays manifested that IPO7 knockdown effectively inhibited OSCC cell proliferative, migratory, and invasive capabilities, and enhanced tumor growth. In contrast, IPO7 overexpression facilitated cell malignant phenotypes and enhanced the lung metastasis of the tumor in mice. Therefore, we suggested that IPO7 could be involved in regulating OSCC development by acting as an oncogene.

Autophagy is closely related to tumor development, and it is a major pathway for organisms to remove damaged, senescent, degenerative proteins and organelles [14]. LC3 protein is a key marker for autophagy, and LC-II to LC3-I rate can reflect the autophagy status [22]. Bioinformatics tools illustrated that the co-expressed genes of IPO7 were closely related to biological processes such as cellular autophagy. Therefore, we evaluated the impact of IPO7 on autophagy in OSCC cells. IPO7 silencing inhibited the LC-II/LC3-I ratio, reduced the formation of autophagosomes, and suppressed autophagic flux. Further, rescue assays proved that treatment with autophagy inhibitor CQ notably abolished the promoting function of IPO7 overexpression on the malignant phenotype of OSCC cells as well as tumor metastasis, which reinforced that IPO7 promoted OSCC development by promoting autophagy.

ERS caused by the anomalous accumulation of unfolded or misfolded proteins at the ER has been considered

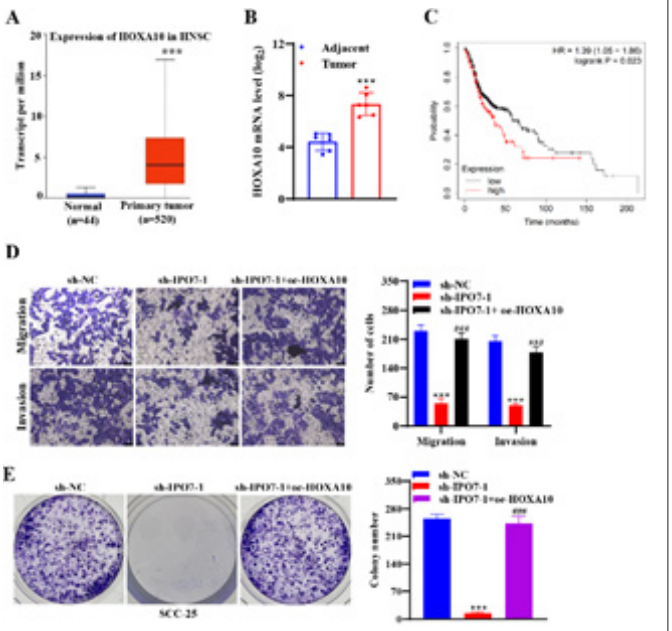

Fig. 2. IPO7 promotes OSCC progression by upregulating HOXA10. (A) UALCAN analysis of HOXA10 expression in HNSS tissues and normal tissues. (B) GSE74530 analysis of HOXA10 expression in OSCC tissues and adjacent tissues. (C) Kaplan-Meier analysis of the survival rate of OSCC patients with high or low HOXA10. (D) Transwell assay was implemented to test cell migration and invasion when IPO7 was silenced and HOXA10 was overexpressed. (E) Cell proliferative capability was assessed by colony formation assay when IPO7 was silenced and HOXA10 was overexpressed. *P<0.05; #P<0.05.

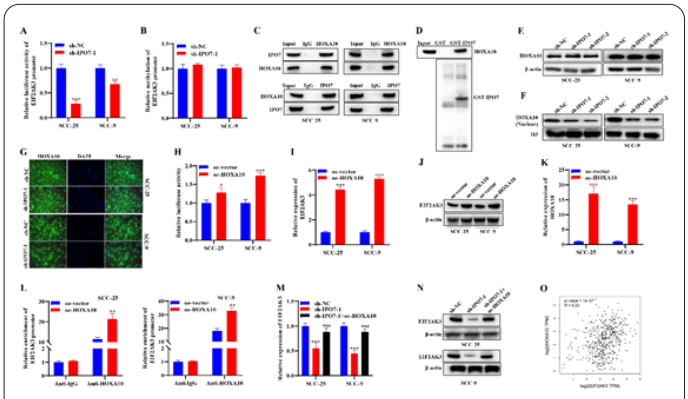

Fig. 4. IPO7 accelerates nuclear translocation of HOXA10, and HOXA10 transcriptionally activates EIF2AK3. (A) Luciferase reporter assay was applied to determine the transcriptional activity of EIF2AK3 in cells transfected with sh-NC or sh-IPO7. (B) Methylation-specific PCR assay was used to detect EIF2AK3 methylation levels in different transfection groups. (C-D) Co-IP and GST pull-down assays were applied to verify the combination of IPO7 and HOXA10. (E) Western blot outcomes of the total protein content of HOXA10 in cells when IPO7 was silenced. (F) Western blot was utilized to assess the nuclear HOXA10 level in different cells. (G) IF assay was utilized to determine the subcellular location of HOXA10 in LMB-treated cells. (H) The overexpression efficiency of HOXA10 was tested via RT-qPCR. (I-J) EIF2AK3 expression was tested by RT-qPCR and western blot in cells when HOXA10 was overexpressed. (K-L) Luciferase reporter assay and ChIP assay were utilized to determine the combination of HOXA10 and EIF2AK3 promoter. (M-N) RT-qPCR and western blot outcomes of EIF2AK3 expression in cells when IPO7 was silenced and HOXA10 was overexpressed. (O) GEPIA database was utilized to predict the correlation of HOXA10 and EIF2AK3. *P<0.05; #P<0.05.

a potential driver of human cancers [23]. ER is a common organelle and the vital position for the synthesis and modification of proteins, lipids and carbohydrates [24]. In order to maintain ER homeostasis, unfolded protein response (UPR) has evolved into signal transduction pathways to reduce the load of unfolded or misfolded proteins [24, 25]. EIF2AK3 encodes the protein kinase RNA-like ER kinase (PERK) which is the key regulator of UPR in response to ERS [26]. In tumors, assorted cellular stress conditions can trigger ERS, further activating UPR, leading to the EIF2α phosphorylation activated PERK and the subsequent transient attenuation of protein synthesis [27]. This can prevent newly synthesized proteins from fluxing into ER, thereby limiting the misfolding load of proteins [27]. ATF4 is a stress-inducible transcription factor which can activate the expression of genes involved in autophagy, protein synthesis, and redox homeostasis [28]. ATF4 can promote the expression of the transcription factor CHOP and increase the nascent protein loading in ER, thereby promoting ERS and cell death [27]. There is substantial evidence that PERK/EIF2α/ATF4 signaling have been shown to be crucial in regulating autophagy induced by ERS [20]. It is reported that Nox4 facilitates autophagy and osteoclastogenesis by the activation of the PERK/EIF2α/ATF4 signaling [29]. Atiprimod-induced ERS-mediated autophagy through regulating the PERK/EIF2α/ATF4/CHOP axis in breast cancer [30]. In this study, we found that IPO7 depletion markedly suppressed the EIF2AK3, ATF4, CHOP, and p-EIF2α levels. Thus, we confirmed that IPO7 could activate the PERK/ATF4 pathway to regulate ERS. Bioinformatics prediction tools predicted that IPO7 was positively correlated with EIF2AK3 expression. We further found that EIF2AK3 overexpression could reverse the inhibitory effects of IPO7 knockdown on autophagy. Therefore, we confirmed that IPO7 promoted autophagy in OSCC cells by enhancing ERS via the PERK/ATF4 pathway.

HOXA10 is a highly conserved transcription factor that plays key roles in embryogenesis and it belongs to Homeobox (HOX) gene family [31]. HOX genes exert the crucial function in cell proliferation, apoptosis, and migration [32]. HOXA10 overexpression is identified in various human cancers and has been proven to take part in tumorigenesis. For example, HOXA10 accelerates pancreatic cancer development by the activation of NF-κB pathway [33]. HOXA10 transcriptionally activates BCL2 expression to facilitate cell growth in gastric cancer [34]. Importantly, HOXA10 is reported to upregulate in OSCC and promote cell proliferation, migration and invasion [35]. Furthermore, IPO7 can transport transcription factors into the nucleus to regulate protein activity and cellular behaviors [10, 13]. Xue et al. have confirmed that IPO7 can promote glioblastoma cell proliferative and migratory capabilities via increasing the nuclear import of GLI1 [10]. Similarly, in this study, we found that IPO7 can bind and interact with HOXA10 in OSCC cells. Moreover, IPO7 knockdown notably reduced HOXA10 expression in the nucleus, indicating IPO7 can regulate the nuclear translocation of HOXA10. Next, we confirmed that HOXA10 can combine with the EIF2AK3 promoter and transcriptionally activate EIF2AK3 in OSCC. Subsequently, HOXA10 was proved to be highly expressed in OSCC, and its overexpression can reverse the inhibitory functions of IPO7 silencing on cell proliferation, migration, and invasion, further indicating HOXA10 acts as an oncogene in OSCC.

5. Conclusion

Taken together, this study proved that IPO7 enhanced tumor metastasis and protective autophagy by promoting nuclear translocation of HOXA10 and activating EIF2AK3/ATF4 pathway in OSCC. These findings may provide novel therapeutic targets for OSCC.

Conflict of interests

The authors declare no competing interests.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

We have received approval from the Ethics Committee of The People's Hospital of Cangnan.

Informed consent

We have received informed consent from the Ethics Committee of The People's Hospital of Cangnan.

Availability of data and material

The data that support the findings of this work are available from the corresponding author upon reasonable request.

Authors' contributions

PJ contributed to the study conception and design. The experimental operation, data collection and analysis were performed by CS. The first draft of the manuscript was written by CS and all authors commented on previous versions of the manuscript.

Funding

Not applicable.

Acknowledgements

Not applicable.

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