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Citation for final published version:

Liu, Hong, Zhang, Zhancheng, Zhou, Shenli, Liu, Xianfang, Li, Guodong, Song, Bing and Xu, Wei 2022. Claudin-1/4 as directly target gene of HIF-1α can feedback regulating HIF-1α by PI3K-AKT-mTOR and impact the proliferation of esophageal squamous cell though Rho GTPase and p-JNK pathway. Cancer Gene Therapy 29 , pp. 665-682. 10.1038/s41417-021-00328-2

Publishers page: https://doi.org/10.1038/s41417-021-00328-2

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Claudin-1/4 as directly target gene of HIF-1α can feedback regulating HIF-1α by PI3K-AKT-mTOR and impact the proliferation of esophageal squamous cell though Rho GTPase and p-JNK pathway

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Abstract:

Immunohistochemical microarray comprising 80 patients with esophageal squamous cell carcinoma (ESCC) and discovered that the expression of CLDN1 and CLDN4 were significantly higher in cancer tissues compared to para-cancerous tissues. Furthermore, CLDN4 significantly affected the overall survival of cancer patients. When two ESCC cell lines (TE1, KYSE410) were exposed to hypoxia (0.1% O2), CLDN1/4 was shown to influence the occurrence and development of esophageal cancer. Compared with the control culture group, the cancer cells cultured under hypoxic conditions exhibited obvious changes in CLDN1 and CLDN4 expression at both the mRNA and protein levels. Through genetic intervention and Chip, we found that HIF-1α could directly regulate the expression of CLDN1 and CLDN4 in cancer cells. Hypoxia can affect the proliferation and apoptosis of cancer cells by regulating the PI3K-Akt-mTOR pathway. Molecular analysis further revealed that CLDN1 and CLDN4 can participate in the regulation process and had a feedback regulatory effect on HIF-1α expression in cancer cells. In vitro cellular experiments and vivo experiments in nude mice further revealed that changes in CLDN4 expression in cancer cells could affect the proliferation of cancer cells via regulation of Rho GTP and p-JNK pathway. Whether CLDN4 can be target for the treatment of ESCC needs further research.

Introduction

Globally, esophageal cancer is the eighth most common malignant cancer and the sixth leading cause of cancer-related death, with approximately 460,000 new diagnoses and more than 380,000 deaths annually [1, 2]. Esophageal adenocarcinoma and esophageal squamous cell carcinoma (ESCC) are the most prevalent types, with the latter accounting for approximately 90% of esophageal cancers each year [3].

Epidemiological studies report that tobacco use, heavy alcohol consumption, and ingestion of hot substances are all regarded as risk factors for ESCC [4–7]. However, the specific mechanism of ESCC is not clear. Reflux esophagitis is considered to be one of the major causes of Barrett's esophagus (BE), and several articles have reported that reflux esophagitis and BE can also promote the occurrence of ESCC and pharyngo-laryngeal cancer [8, 9]. In reflux esophagitis, CLDN1 and CLDN4 are abnormally expressed compared to that in healthy esophageal epithelial cells [10–12]. Hypoxia can induce expression changes in HIF-1α and CLDN1, and Ch-IP experiment showed a direct positive correlation between the two protein in eosinophilic esophagitis (EoE) [13].

Many papers have stated that abnormal expression of claudin (CLDN) proteins can affect the progression of esophageal cancer [14, 15]. Hypoxia also can affect Claudins protein expression in breast cancer, colon cancer, etc. [16, 17] and has been shown to play an important role in the occurrence, development, and resistance of esophageal cancer [18–22]. However, so far, we have not found any

reports on the possible correlation and mechanism between hypoxia and the expression of CLDN1/4 proteins in ESCC.

In this study, we analyzed the expression of CLDN1/4 in clinical samples from 80 esophageal cancer patients and 2 ESCC cancer cells. Culturing cancer cells under hypoxia conditions we found that CLDN1/CLDN4 are directly target genes of HIF-1α. CLDN1/CLDN4 both can feedback regulating HIF-1α by PI3K-AKT-mTOR pathway. CLDN4 also can further impact the proliferation of esophageal squamous cell though Rho GTP ase and p-JNK pathway which along with PI3K-AKT-mTOR pathway.

Combined with the literature report, we found that the correlation between HIF1α and CLDN1/4 protein exists in both normal esophageal epithelial cells and ESCC. This interaction may play an important role in the carcinogenesis of esophageal mucosal epithelial cells.

Results

CLDN1/CLDN4 expression levels are significantly different in cancer tissues vs. corresponding adjacent normal tissues

We compared CLDN1/CLDN4 expression with a tissue-microarray derived from both cancer tissues and adjacent normal tissues from 80 esophageal cancer patients (Table 1). The results showed that the expression of CLDN1/CLDN4 genes in the cancer tissues was significantly different from that in the paired adjacent normal tissues ($p < 0.05$). The expression of CLDN1 in 58.75% of the cancer tissues and CLDN4 in 35% of cancer tissues were significantly higher than that in the corresponding adjacent tissues (Fig. 1A–F). Moreover, there was a negative correlation between the upregulation of CLDN4 in cancer tissues and the overall survival time of patients (HR = 3.15; Log rank $p = 0.021$) (Fig. 1H). However, there was no significant correlation between the increase in CLDN1 in cancer tissues and the overall survival time of patients ($p = 0.551$) (Fig. 1G).

	n (%)	
Sex		
Male	59	(75.0)
Female	21	(25.0)
Age (y)	Median, 65.13 y	
Clinical stage		
I	4	(5.0)
II	40	(50.0)
III	36	(45.0)
T classification		
1	4	(5.0)
2	14	(17.5)
3	60	(75)
4	2	(2.5)
N classification		
0	39	(48.75)
1	26	(32.5)
2	9	(11.25)
3	5	(6.25)
4	1	(1.25)
Distant metastasis		
Yes	0	(0)
No	80	(100)
Expression of claudin 1		
Low	47	(58.75)
High	33	(41.25)
Expression of claudin 4		
Low	28	(35.0)
High	52	(65.0)

Table 1 Clincopathologic characteristics and expression of claudins in ECSS patients.

The proteins expression of CLDN1 and CLDN4 in the cancer tissues were significantly higher than that in the paired adjacent normal tissues (Fig. 1A–F). These results were consistent with that of TCGA database online (Supplementary Fig. 1A, B). However, only CLDN4 was negative correlation between the expression and the overall survival time of patients (Fig. 1H). The higher CLDN4 expressed in cancer tissues, the shorter the overall survival time of patients.

Hypoxia culture condition can significantly change the expression of CLDN1/CLDN4 in esophageal cancer cells

Real-time polymerase chain reaction (PCR) detected the mRNA expression of claudin-1 and claudin-4 in two cancer cells. In TE1 cells, there was significant high level expression of the claudin-4 mRNA, while the expression of claudin-1 was very low (Fig. 2A). In poorly differentiated KYSE410, the mRNA expression of claudin-1 were significantly higher than claudin-4 (Fig. 2A).

The result of Western Blot revealed that the protein expression of CLDN1/4 were consistent with mRNA expression, in two cancer cells. In TE1 cells, the CDLN4 protein expression was significant high, while the protein expression of CLDN1 was too low to detect (Fig. 2B). In KYSE410 cells, the CDLN1 protein expression was significantly higher than CLDN4 (Fig. 2B).

From the experiment results above, we found that the expression of CLDN1/4 in ESCC with degrees of differentiation were not the same (Fig. 2A, B).

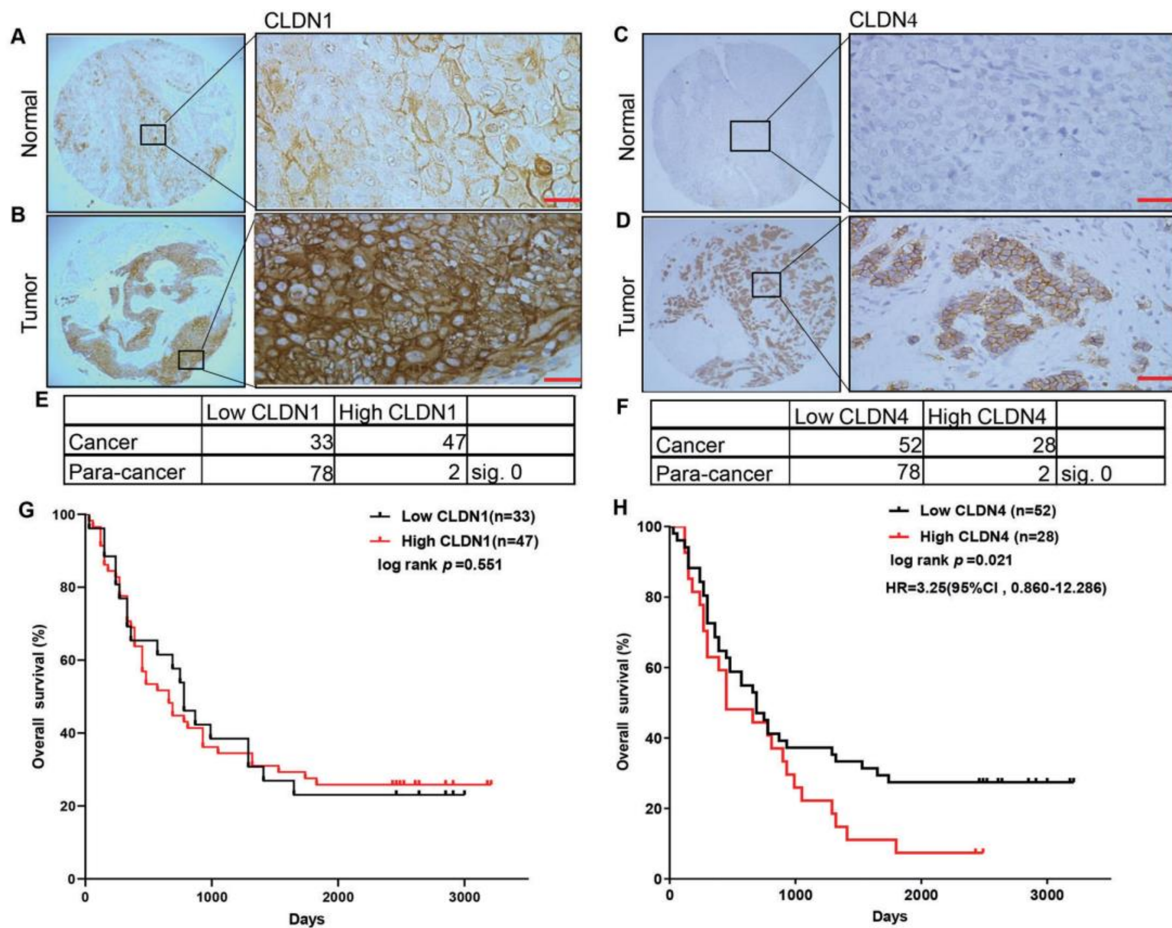
Hypoxia has been shown to play an important role in the occurrence, development, and resistance of esophageal cancer [18–22]. According to the data analysis of TCGA online database, we also found that the expression of HIF-1α in esophageal carcinoma was significantly higher than that in para-cancers [Supplementary Fig. 1C]. A primary transcriptional response to hypoxia is mediated by the hypoxia inducible factor (HIF), which is known as a pivotal regulator under hypoxia stress [23]. The HIF protein complex is a heterodimer consisting of an oxygen sensitive (alpha, A) and an oxygen stable (beta, B) subunit [24]. In mammals three different HIFA isoforms are present, among which HIF1A is expressed ubiquitously while HIF2A and HIF3A expression vary depending on the type of tissue cells [25]. Therefore, HIF-1α was regarded as biomarker of the hypoxia microenvironment [26]. The protein expression of CLDN1 and HIF-1α [Supplementary Fig. 1D] and CLDN4 and HIF-1α [Supplementary Fig. 1E] have significant correlation in ESCC, according to the data analysis of TCGA online database.

We detected the expression of HIF-1α and CLDN1/4 change from mRNA and protein level when put the two cancer cells in hypoxia culture condition.

We placed cancer cells in a hypoxic incubator for 48 h, and measured the changes of HIF-1a, CLDN1, and CLDN4 in mRNA and protein levels every 8 h. In TE1 cells, the mRNA change of the above three genes increased first and then decreased. After 8–16 h of hypoxic treatment, the expression of these three genes increased significantly at the mRNA level, and decreased gradually in 16–48 h (Fig. 2C). Western Blot results were consistent with the changes in mRNA levels (Fig. 2D). In KYSE410 cells, the mRNA of HIF-1α and CLDN1 decreased gradually after 8 h hypoxia treatment, and the decrease was most obvious at 48 h. CLDN4 mRNA was significantly upregulated from 8 h (Fig. 2E). Western Blot results were consistent with the changes of mRNA (Fig. 2F).

Overall, in the two cancer cells, 0.1% of hypoxic culture condition could significantly affect the HIF-1α, CLDN1 and CDLN4 expression both at mRNA and protein level, but the changes were not the same. In TE1 cell, hypoxic culture condition could promote the HIF-1α, CLDN1 and CDLN4 expression both at mRNA and protein level (Fig. 2C, D).

In KYSE410 cells, hypoxic culture inhibited the expression of HIF-1α and CLDN1 both in mRNA and protein levels, while promoted CLDN4 (Fig. 2E, F).



	Low CLDN1	High CLDN1	
Cancer	33	47	
Para-cancer	78	2	sig. 0

	Low CLDN4	High CLDN4	
Cancer	52	28	
Para-cancer	78	2	sig. 0

Fig. 1 CLDN1/4 expression analysis in ESCC patients. A, B CLDN1 protein expression in para-cancerous and carcinoma tissues, respectively. C, D CLDN4 protein expression in para-cancerous and carcinoma tissues, respectively. E CLDN1expression was significantly elevated in carcinoma tissues than in para-cancerous tissues ($p = 0$). F CLDN4 expression was significantly elevated in carcinoma tissues than in para-cancerous tissues ($p = 0$). G Survival analysis comparing the overall survival status of cancer patients and CLDN1 expression levels (high vs. low) in cancer tissues. CLDN1 expression and overall survival of patients with cancer were not correlated ($p = 0.551$). H Survival analysis comparing the overall survival status of cancer patients and CLDN4 expression levels (high vs. low) in cancer tissues. CLDN4 expression significantly correlated with the overall survival of patients, with a negative correlation coefficient r at −0.222 (Pearson's chi-squared/Fisher's exact test, $p = 0.021$). HR = 3.25, 95% CI (0.860–12.286).

CLDN1/CLDN4 were directly target genes of HIF-1α in esophageal cancer cells

By transfecting siRNA targeting HIF-1α (si-HIF-1α) into two cancer cell lines to downregulate the expression of HIF-1α, we further investigated the mechanism by which CLDN1/4 protein changes were induced under hypoxia conditions.

Knockdown HIF-1α in 2 cancer cells could lead to the decrease of claudin-1 at mRNA level (Fig. 2G, H). Proteins changes of CLDN1 in KYSE410 cells were consistent with mRNA (Fig. 2J). Knockdown HIF-1α also could lead to the down-regulation of claudin-4 at mRNA level in KYSE410, while promoted it in TE1 cells (Fig. 2G, H). The protein changes of CLDN4 in TE1 was consistent with mRNA (Fig. 2I).

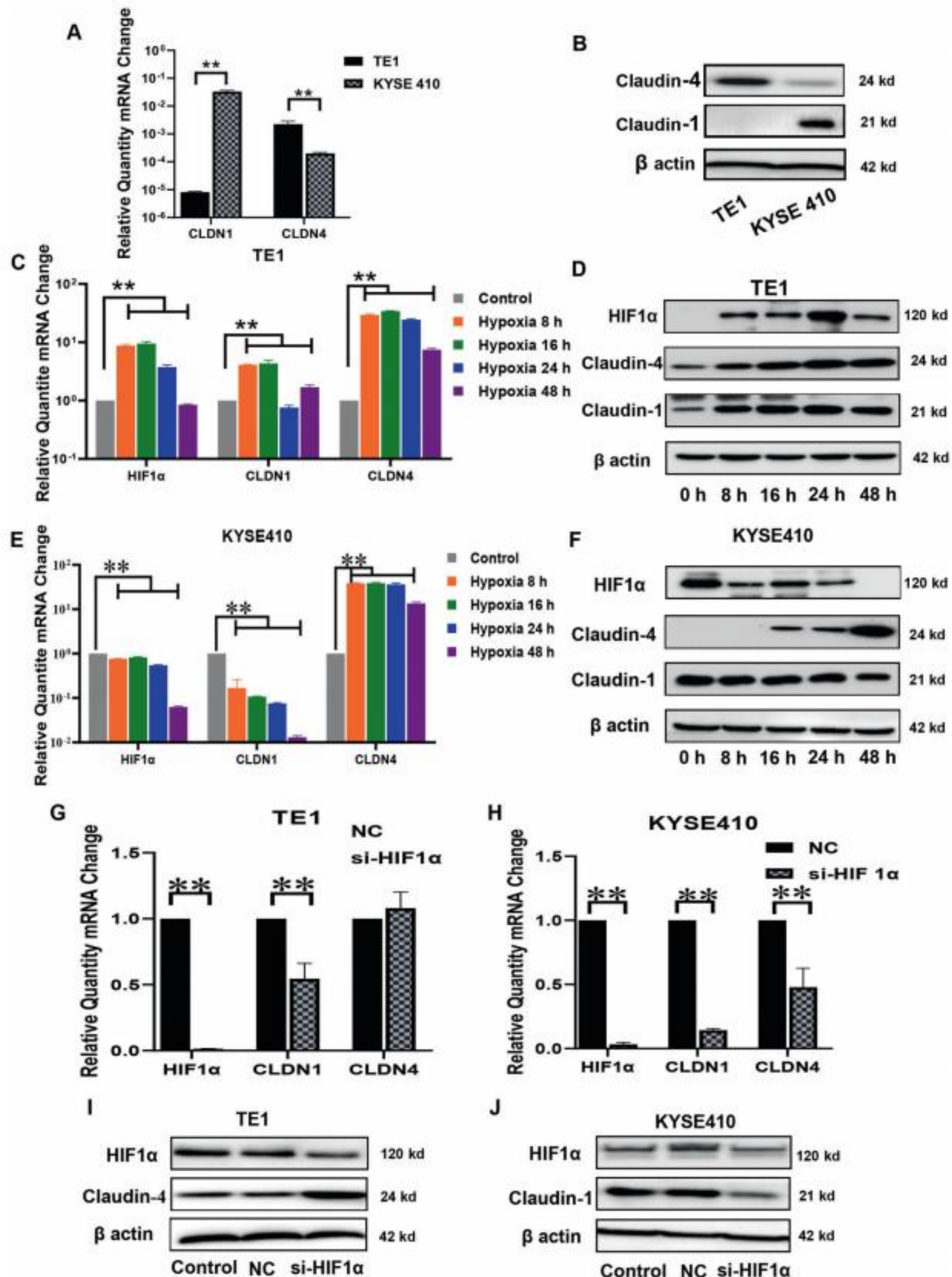


Fig. 2 Two cancer cell lines were treated with 0.1% O_2. A, B CLDN1 and CLDN4 mRNA and associated protein expression of two ESCC cell lines. RT-PCR (A) and Western-blot (B) affirmed that TE1 cells expressed both CLDN1 and CLDN4.KYSE410 cells showed the highest CLDN1 and lowest CLDN4 expression. C–F Two kinds of cancer cells after different periods of hypoxic culture, claudin-1/4 changes in mRNA and protein levels. Hypoxic culture can promote the expression of HIF-1α, claudin-1, and claudin-4 in mRNA levels in TE1. Western bolt also detected these changes in protein levels in TE1 cell. Hypoxic culture inhibited the expression of HIF-1α and CLDN1 both in mRNA and protein levels, while promoted CLDN4 in KYSE410 cells. G–J siRNA was transfected to inhibit the expression of HIF-1α in two cancer cells. Knock-down HIF-1α can down-regulate the

expression of claudin-1 and claudin-4 in KYSE410, while decrease claudin-1 in TE1, at the mRNA level. I, J The proteins change of knock down HIF-1α in two cancer cells. Knockdown HIF-1α in TE1 slightly increase claudin-4 expression in m RNA level, while it can promote CLDN4 expression significantly in protein level. Data are presented as the mean ± SD of the three independent experiments (* $p < 0.05$, ** $p < 0.01$).

We continuously examined whether CLDN1/4 were directly target genes of HIF-1α in cancer cells. By using chromatin immunoprecipitation (Ch-IP), we confirmed HIF-1α's capacity to bind to the CLDN1 and CLDN4 gene promoters (Fig. 3C–H). We identified two hypoxiaresponsive elements (HREs) 226 and 589 bp upstream of the CLDN1 transcription start site (Fig. 3C). There were also two HREs 353 and 583 bp upstream of the CLDN4 transcription start site (Fig. 3F). The results of Ch-IP were examined by real-time PCR.

Through the above studies, we found that hypoxic culture condition could increase the protein expression of HIF-1α in the cytoplasm which can enter the nucleus and act on the transcription factor regulation site of CLDN1 and CLDN4. HIF-1α was directly regulating the expression of CLDN1/4 protein from the transcription level (Fig. 3C–H).

Hypoxia promoted the apoptosis of esophageal cancer cells via PI3K-AKT-mTOR signal pathway

Apoptosis analysis by flow cytometry revealed that the apoptosis of two types of cancer cells increased significantly with the prolonged hypoxia culture time (Fig. 4A, B).

Mitochondria played a central role in apoptotic signaling pathways. Upon exposure to apoptotic stimuli, mitochondria released cytochrome c to the cytoplasm and activated caspase cascade leading to cell death [27]. Caspases were an evolutionary conserved family of cysteine proteases that are centrally involved in cell death and inflammation responses [28]. Caspases 9 played a key role in apoptosis [29], which function was exemplified by the activation of cleave-caspase-3 [30].

In this experiment, we first detected the proteins expression of Caspase 9, Caspase 3 and cytochrome C in cancer cells treated with hypoxia. In the 2 cancer cells, the expression of Caspase 9, Caspase 3 and their activator proteins Cleave Caspase 9 and Cleave Caspase 3 decreased gradually with the prolongation of hypoxic treatment. The expression of cytochrome C protein was also significantly reduced (Fig. 4C).

The above experimental results showed that the increase of apoptosis of TE1 and KYSE410 cells in the hypoxic culture condition was not by regulating the mitochondrial apoptosis pathway. PI3k-AKT-mTOR signal pathway can affect the proliferation and apoptosis of esophageal cancer cells [31, 32]. We further detected the expression of AKT, p-AKT and mTOR to study the mechanism of apoptosis induced by 0.1% hypoxic culture condition in esophageal carcinoma cells.

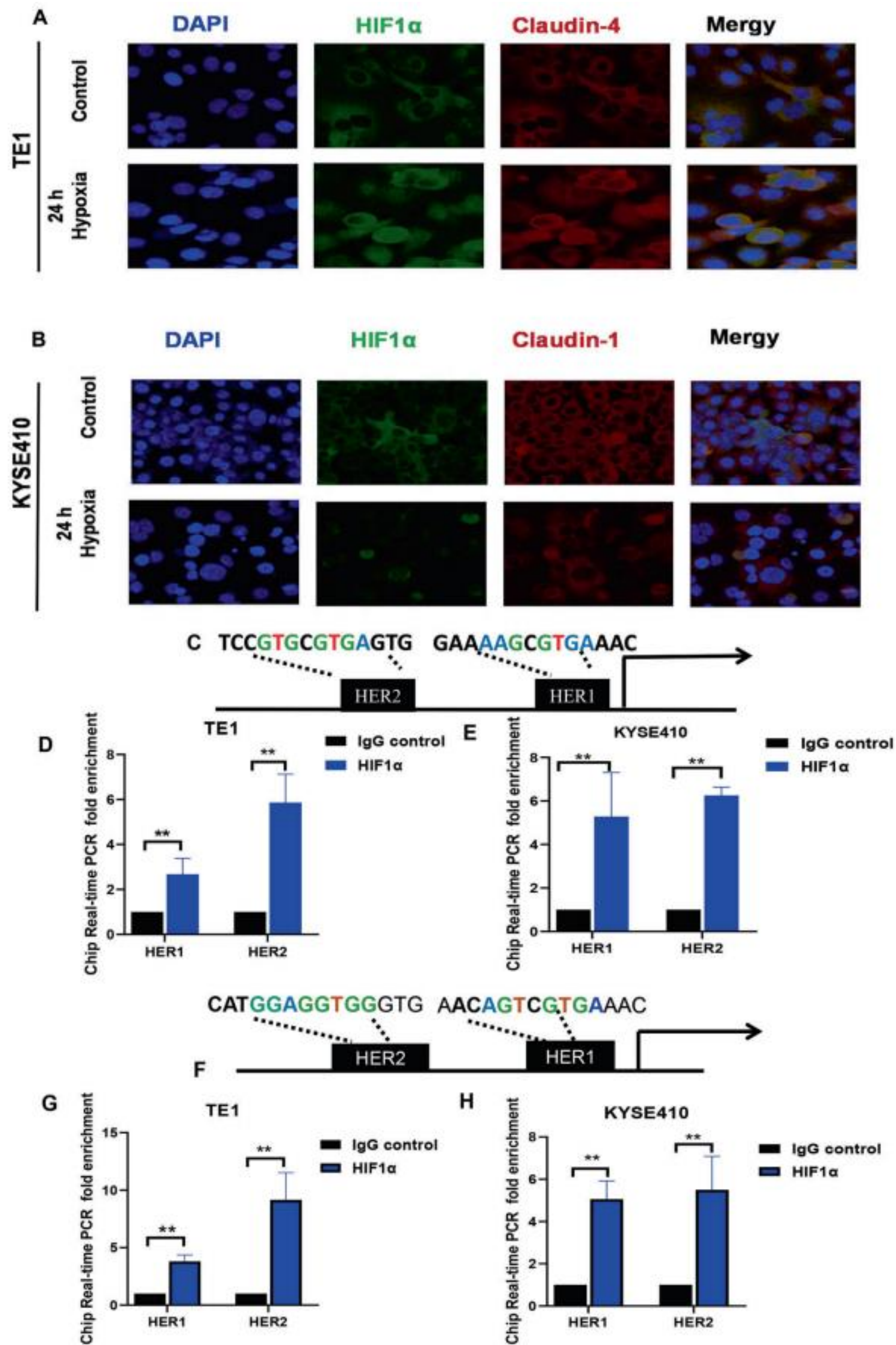


Fig. 3 HIF-1α can directly regulate the CLDN1/4 protein expression in ESCC cell lines. A, B Immunofluorescence results of two cells cultured in hypoxia for 24 h. Hypoxic culture can affect the expression of HIF-1α and CLDNs protein in esophageal cancer cell lines. Compared with the control group, hypoxia for 24 h could increase the expression of HIF-1α and CLDN4 in TE1 cells (A), and downregulate the expression of HIF-1α and CLDN1 in KYSE410 cells (B). C–E Proximal human claudin-1 promoter sequence identified 2 potential hypoxia-responsive elements (HREs). ChIP using a HIF-1α antibody on two cells, followed by PCR using primers spanning HRE2 and HRE1 sites, was normalized to PCRs performed on ChIP product generated using an isotype IgG control antibody. F–H Proximal human claudin-4 promoter sequence identified two potential hypoxia-responsive elements (HREs). ChIP using a HIF-1α antibody on two cells, followed by PCR using primers spanning HRE2 and HRE1 sites, was normalized to PCRs performed on ChIP product generated

using an isotype IgG control antibody. Data are presented as the mean ± SD of the three independent experiments (* $p < 0.05$, ** $p < 0.01$).

The expression of proteins including AKT, p-AKT and mTOR were changed significantly in two cancer cells in the hypoxic culture condition. Hypoxia could promote the phosphorylation of AKT at Thr308 sites and inhibit the expression of m-TOR (Fig. 4C).

Overall, 0.1% hypoxia culture condition promoted the apoptosis of esophageal cancer cells via PI3K-AKT-mTOR signal pathway not though caspases-related mitochondria apoptosis.

CLDN1/4 modulation could affect the proteins expression of PI3K-AKT-mTOR signal pathway in cancer cells

Through lentivirus and plasmid transfection of cancer cells, stable cell lines were constructed that interfered with CLDN1/4 protein expression in two cancer cells. Protein detection showed that interfering with the expression of CLDN1/4 in cancer cells could significantly affect the expression of AKT, p-AKT (Thr308), and m-TOR in cancer cells.

CLDN1 over-expression in TE1 could promote the protein of AKT phosphorylation and inhibit the expression of m-TOR (Fig. 5A). CLDN1 downregulation in KYSE410 decreased the expression of m-TOR, while had not significant impact to the expression of p-AKT.

CLDN4 overexpression in KYSE410 could promote the protein of AKT phosphorylation while inhibiting the expression of m-TOR (Fig. 5A). In TE1 cells, overexpression CLDN4 triggered the reduced expression of p-AKT and increased the expression of m-TOR, whereas CLDN4 downregulation in TE1 cells promoted the protein expression of p-AKT and down-regulated the expression of m-TOR (Fig. 5A).

We also detected the protein expression of Caspase 9, Caspase 3 and cytochrome C after regulating CLDN1/4 protein expression in TE1 and KYSE410 cells. CLDN1 over-expression in TE1 could inhibit the protein of cytochrome C and slightly downregulated Caspase 3, while Caspase 9, Cleave Caspase 9 and Cleave Caspase 3 had not significantly change. CLDN1 downregulated in KYSE410 could promote the protein expression of cytochrome C and slightly up-regulated Caspase 9 and Cleave Caspase 9, while Caspase 3, Cleave Caspase 3 had not change (Fig. 5A).

CLDN4 overexpression in KYSE410 could promote the protein expression of cytochrome C and slightly upregulated Caspase 9, Cleave Caspase 9, while Caspase3 and Cleave Caspase 3 had not change. CLDN4 overexpression in TE1 had not affect to the expression of above proteins. CLDN4 downregulated in TE1 inhibited the protein expression of cytochrome C and slightly upregulated Caspase 9 and Cleave Caspase 9, while Caspase 3, Cleave Caspase 3 had not change (Fig. 5A).

The results of above showed that CLDN1/4 modulation could affected the apoptosis of cancer cells though PI3K-AKT-mTOR signal pathway.

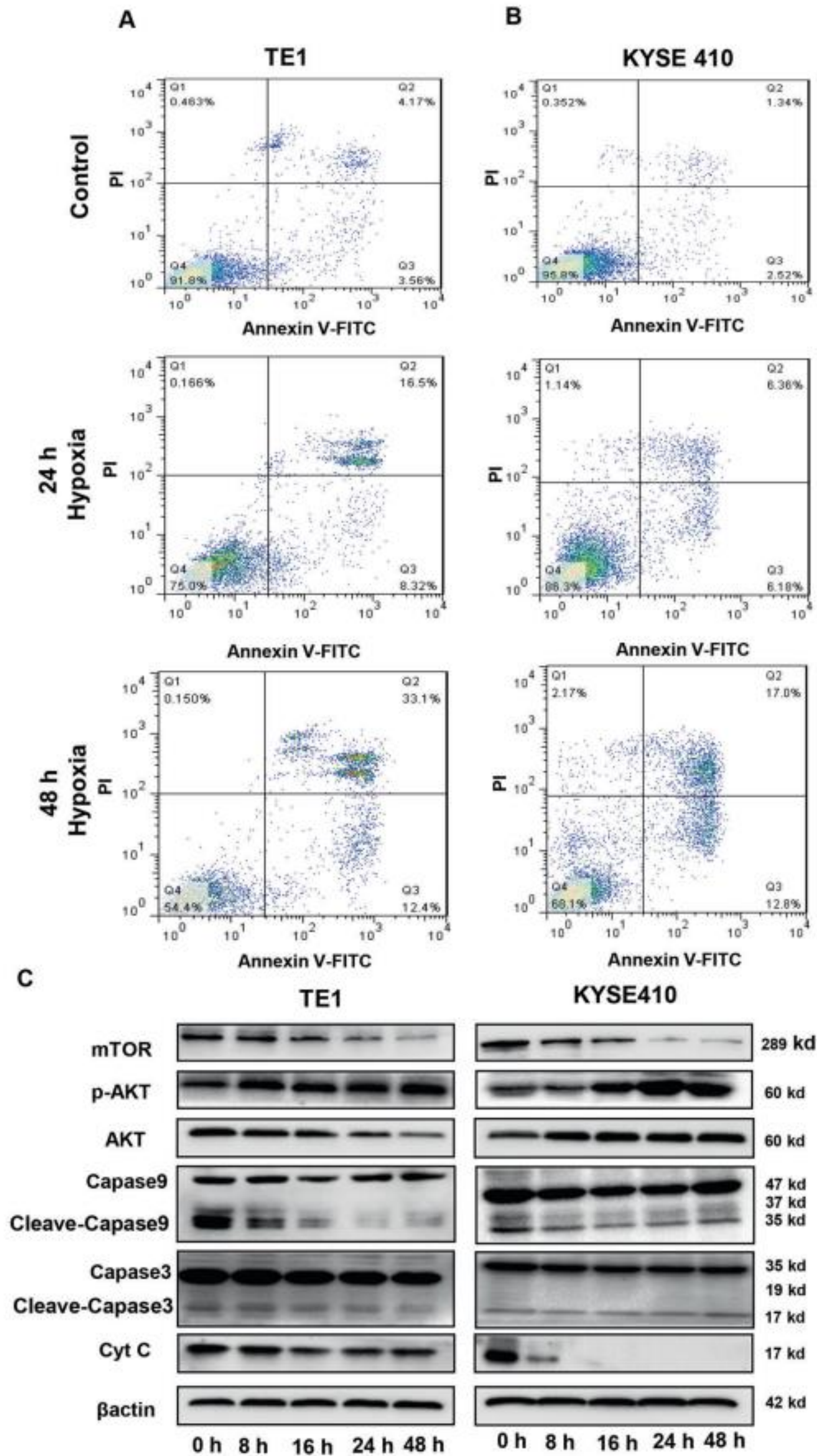


Fig. 4 Effect of hypoxic culture on cell apoptosis of cancer cells. A, B The results of the apoptosis analysis by flow cytometry assays of two cancer cells in a hypoxic environment. The hypoxic environment can significantly promote the apoptosis of cancer cells. C Hypoxic culture promoted phosphorylation of AKT protein at Thr308 sites in cancer cells and downregulated the expression of mTOR protein. In the two cancer cells, the expression of Caspase 9, Caspase 3, and their activator proteins Cleave Caspase 9 and Cleave Caspase 3 decreased gradually with the prolongation of hypoxic treatment. The expression of cytochrome C protein was also significantly reduced. All independent experiments were repeated three times and had statistic difference, $p < 0.05$.

CLDN1/4 could feedback on the expression of HIF-1α in cancer cells through PI3K-AKT-mTOR signal pathway

PI3K-AKT-mTOR pathway can regulate the expression of HIF-1α in oxygen-independent condition in cancer [33]. As the results above that CLDN1/4 could affect the proteins expression of PI3K-AKT-mTOR signal pathway in two cancer cells. We further detected the m RNA and protein changes of HIF-1α in the stable cell lines which we constructed above.

Downregulating CLDN1 or CLDN4 in 2 cancer cells could result in decreased expression of HIF-1α. The results of Real-time PCR and Western blot showed that the expression of HIF-1α decreased significantly at both the mRNA and protein levels in TE1 CLDN4− and KYSE410 CLDN1− stable cancer cells compare to the control (Fig. 5D, E, G).

CLDN1 overexpression in TE1 had reduced the expression of HIF-1α in protein level (Fig. 5G). CLDN4 overexpression in TE1 upregulated the expression of HIF-1α both at the mRNA and protein levels (Fig. 5C, G). However, overexpression CLDN4 in KYSE410 could inhibit the expression of HIF-1α compare to the control (Fig. 5F, G).

CLDN1/4 could feedback regulate on the expression of HIF-1α in cancer cells through PI3K-AKT-mTOR signal pathway in oxygen-independent condition.

Hypoxia inhibits the proliferation and viability of esophageal cancer cells

We used Edu to detect the changes in the cell activity of esophageal cancer cells in a hypoxia environment.

The experimental results showed that a hypoxia environment could significantly inhibit the activity of cancer cells, and the effect on the highly differentiated esophageal cancer cell line TE1 was significantly greater than that on the less differentiated cell lines KYSE410 (Fig. 6A–C, E). The results of the cell proliferation assay (MTT) were consistent with those of the Edu assay (Fig. 6D, F).

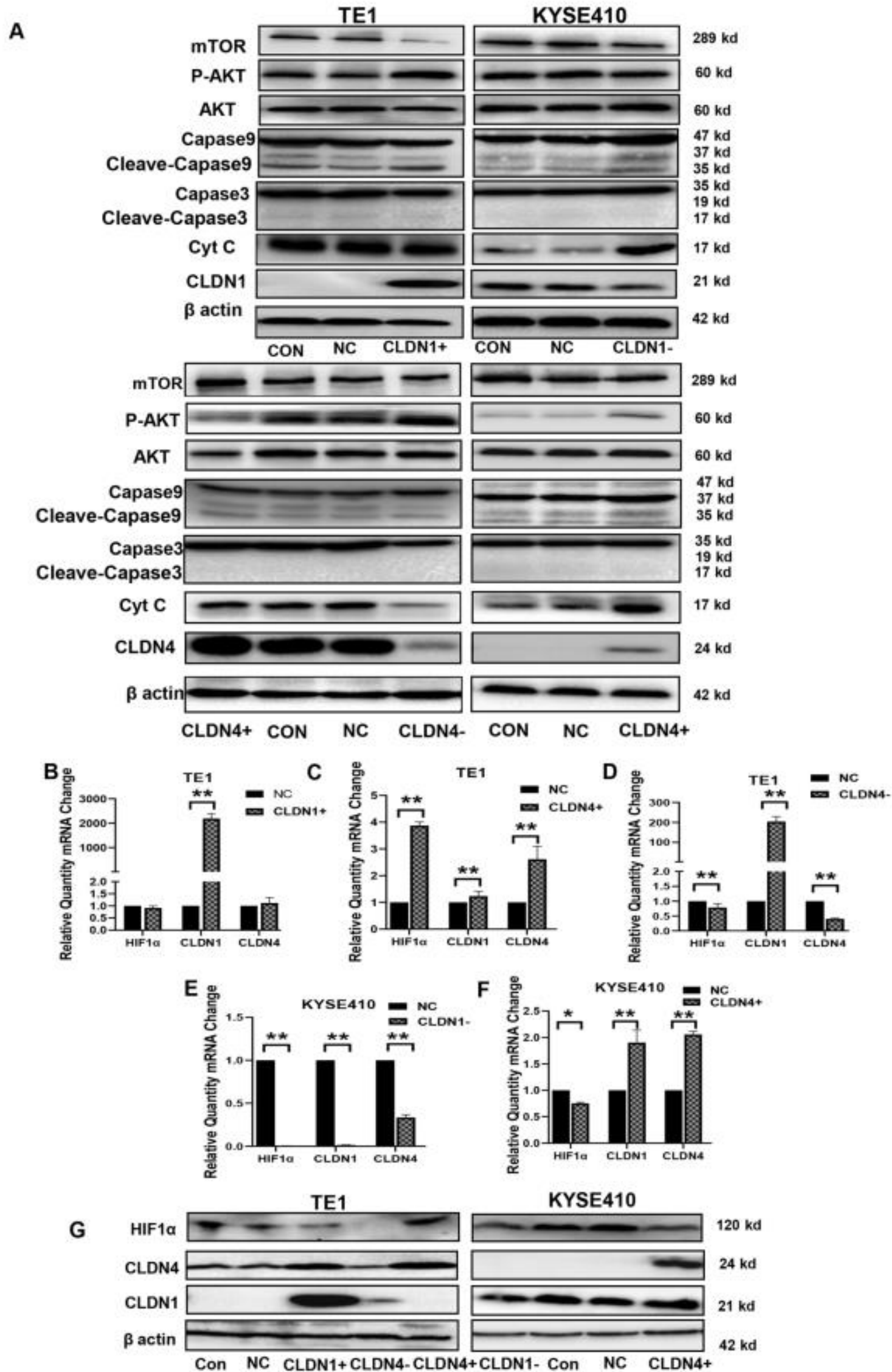


Fig. 5 Interfering with the expression of CLDN1/4 in cancer cells can affect the expression of p-AKT and mTOR proteins and feedback regulate HIF-1α in normoxia condition. A Using plasmids and viruses to overexpress and inhibit the claudin 1/4 genes in cancer cells, we observed their effect on the expression of p-AKT

and mTOR of cancer cells. A In TE1, CLDN1 overexpression could promote the expression of p-AKT and down-regulate the expression of mTOR. In TE1 cells, CLDN4 downregulation could promote the expression of p-AKT and mTOR, while CLDN4 overexpression can inhibit the phosphorylation of AKT protein and down-regulate m-TOR. In KYSE410, CLDN1 inhibition could downregulate the expression of mTOR protein and overexpressd-CLDN4 could promote the phosphorylation of AKT and downregulate the expression of mTOR protein. CLDN1 overexpression in TE1 could inhibit the protein of cytochrome C and slightly downregulated Caspase 3, while Caspase 9, Cleave Caspase 9 and Cleave Caspase 3 had not significantly change. CLDN1 downregulated in KYSE410 could promote the protein expression of cytochrome C and slightly upregulated Caspase 9 and Cleave Caspase 9, while Caspase 3, Cleave Caspase 3 had not change. CLDN4 overexpression in KYSE410 could promote the protein expression of cytochrome C and slightly up-regulated Caspase 9, Cleave Caspase 9, while Caspase3 and Cleave Caspase 3 had not change. CLDN4 overexpression in TE1 had not affect to the expression of above proteins. CLDN4 downregulated in TE1 inhibited the protein expression of cytochrome C and slightly up-regulated Caspase 9 and Cleave Caspase 9, while Caspase 3, Cleave Caspase 3 had not change. B–G CLDN1 and CLDN4 affect the expression of each other and feedback regulate HIF-1α, in normoxia condition. B, G Overexpressed CLDN1, in TE1 cells, had no effect on claudin-4 and HIF-1α at mRNA level, but we detected that upregulate CLDN1 could increased the expression of CLDN4 and decreased the expression of HIF-1α at protein level. C, G Overexpressed CLDN4 in TE1 cells, which can up-regulate HIF-1α and CLDN1 expression at both mRNA and protein levels. D, G Knockdown CLDN4 in TE1 cells promoted the expression of CLDN1 both at mRNA and protein levels, while down-regulated the expression of HIF-1α. E, G Knockdown CLDN1 in KYSE410 cells can down-regulate the expression of HIF-1α at mRNA and protein levels. F, G Overexpressed CLDN4 in KYSE410 cells could down-regulated the expression of HIF-1α while up-regulated CLDN1 in both mRNA and protein levels. Data are presented as the mean ± SD of the three independent experiments (*$p < 0.05$, **$p < 0.01$).

CLDN4 modulation affects the proliferation of cancer cells via Rho GTPases and p-JNK signal pathway both in MTT and in vivo assays

CLDN1 had not significant affect to the proliferation of cancer cells

Compared with the control, the results of MTT assay (Fig. 7C) and xenograft tumor transplantation experiments in nude mice (Fig. 8C, D) showed that CLDN1 knockdown in KYSE410 had not significant change the cell proliferation. The protein expression of RhoA, Cdc42, Rac1/2/3, and p-JNK (Fig. 7E) had no changes in KYSE410 CLDN1- cell compare to the control. Although Rac-1/2/3 was slightly upregulated in CLDN1 over-expression TE1 cells (Fig. 7E), no changes in the proliferation were detected in the in vitro and in vivo assays (Fig. 7A, E and Fig. 8A, B).

CLDN4 can affect the proliferation of cells by influencing the expression of Rho GTPase and p-JNK in cancer cells

CLDN4 overexpression in TE1 cells increased the protein expression of RhoA, Rac1/2/3, Cdc42, and p-JNK and promoted cancer cell proliferation in MTT and vivo assays (Fig. 7B, F and Fig. 8A, B), whereas CLDN4 downregulation in TE1 cells inhibited the protein expression of RhoA, Rac1/ 2/3, Cdc42, and p-JNK and reduced cancer cell proliferation (Fig. 7B, F and Fig. 8A, B). In KYSE410 cells, CLDN4 overexpression obviously increased the expression of RhoA, Cdc42, and p-JNK and accelerated the proliferation of cancer cells (Fig. 7F). CLDN4 overexpression in KYSE410 cells promoted the proliferation of cancer cells in the MTT and in vivo assays (Fig. 7D, F and Fig. 8C, D).

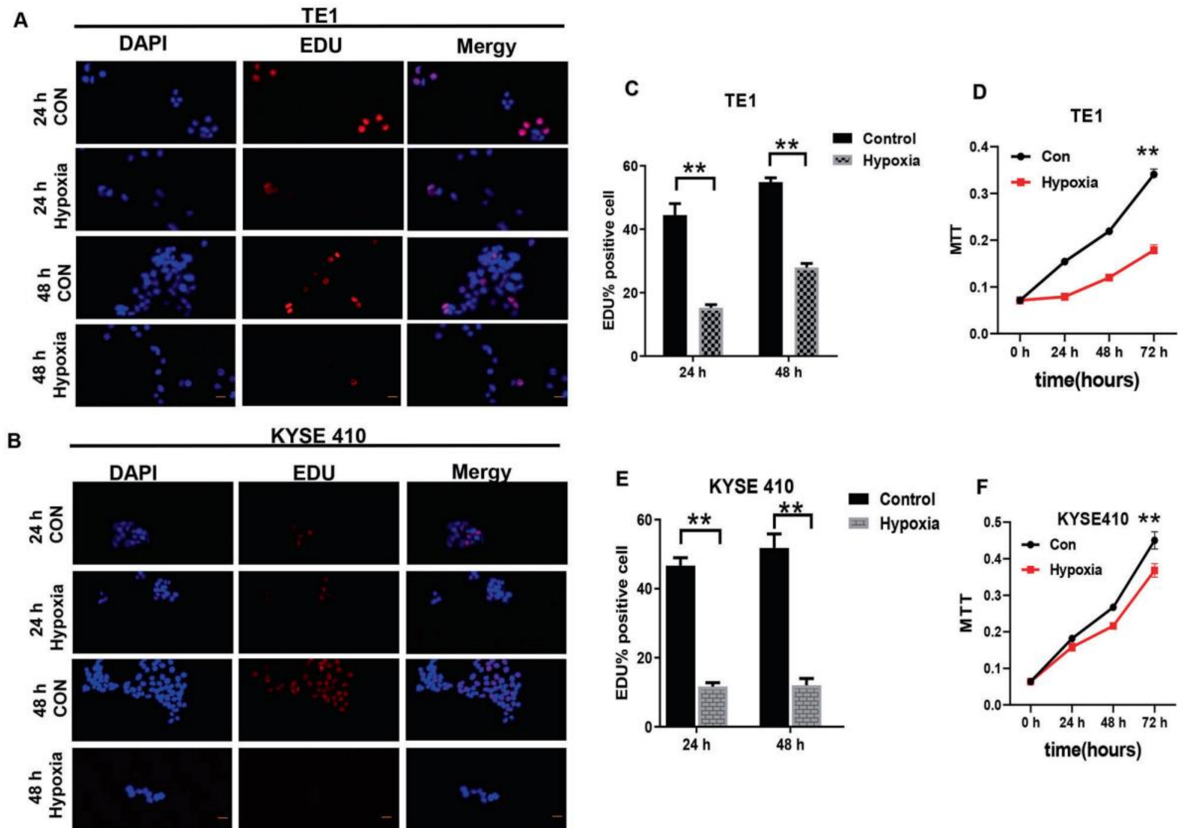


Fig. 6 Effect of hypoxic culture on cell proliferation and cell viability of cancer cells. A, C, D The results of the Edu and MTT assays of TE1 in a hypoxic environment. B, E, F The results of Edu and MTT assays of KYSE410 cells in a hypoxic environment. Hypoxic environment can significantly inhibit the proliferation and cell activity of cancer cells. Data are presented as the mean ± SD of the three independent experiments (*$p < 0.05$; **$p < 0.01$).

CLDN1 and CLDN4 affect the expression of each other in cancer cells

By detecting the m RNA and protein changes of CLDN1/ CLDN4 in the stable cell lines which we constructed above, we further discovered that CLDN1 and CLDN4 affect the expression of each other in cancer cells.

The result of Western blot showed that overexpression of CLDN1 significantly increased the protein expression of CLDN4 in TE1 cells (Fig. 5G). Furthermore, knockdown of CLDN1 in KYSE410 cells triggered a clear reduction in claudin-4 expression at the mRNA level (Fig. 5E).

Overexpression of CLDN4 in TE1 and KYSE410 cells both can upregulate the expression of claudin-1 at both the mRNA and protein levels (Fig. 5D, F, G). Knockdown CLDN4 in TE1 cells also increased the mRNA and protein expression of CLDN1 (Fig. 5C, G).

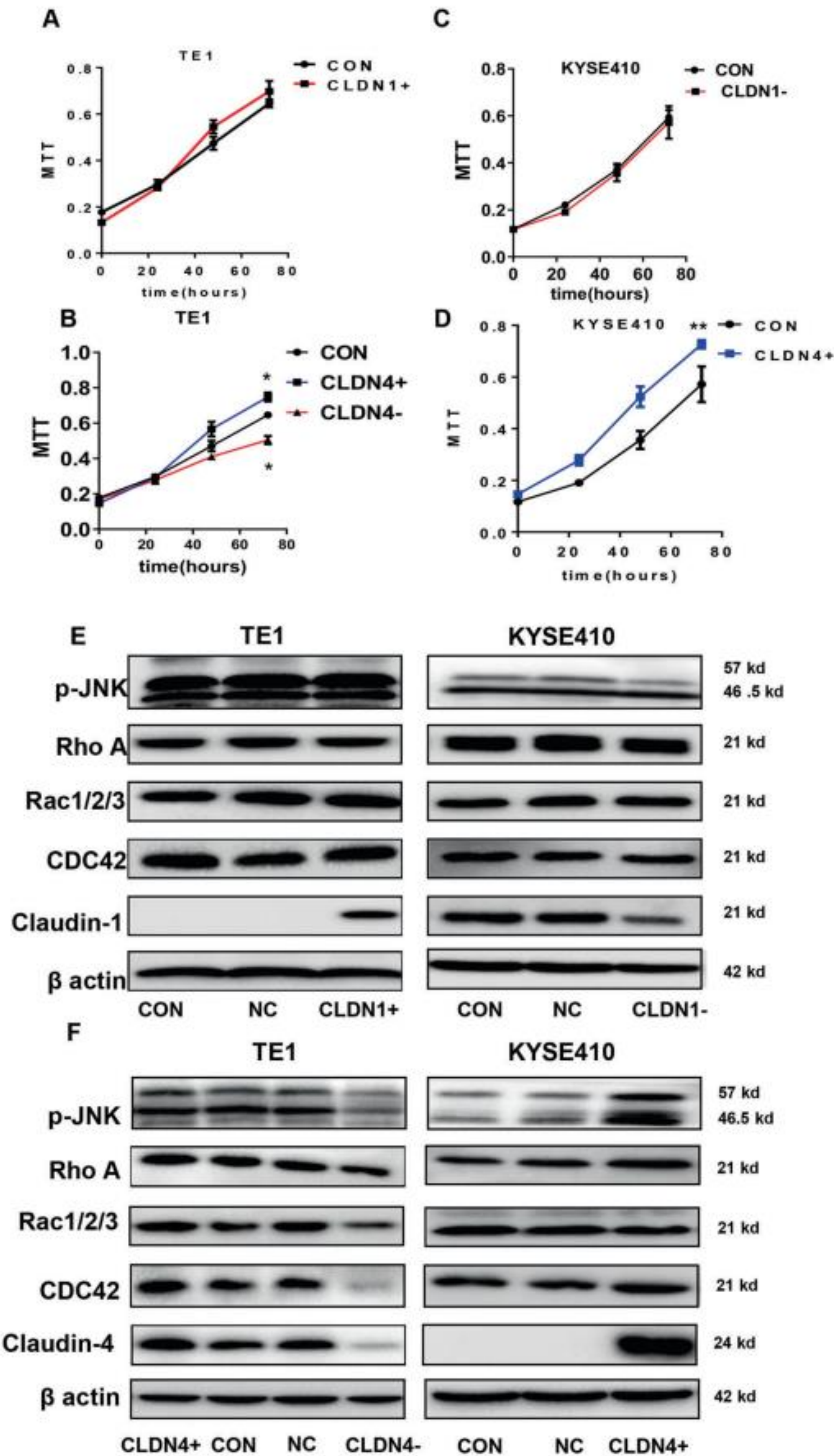


Fig. 7 CLDN1 and CLDN4 affect the proliferation of cancer cells though Rho GTPase and p-JNK pathway. A–D Change the expression of CLDN1/4 protein in cancer cells and observe their effect on the proliferation of cancer cells by the MTT assay. E, F In esophageal squamous cell carcinoma, Western blot analysis was used to investigate the effect of CLDN1/4 protein expression on RhoA, Rac1/2/3, Cdc42, and p-JNK protein expression. CLDN4 can influence the proliferation of cancer cells by affecting the expression of RhoA, Rac1/2/3, Cdc42, and p-JNK in TE1. In KYSE410, CLDN4 overexpressioncan promote the proliferation of cancer cells by affecting the expression of RhoA, CDC42, and p-JNK. All data are presented as the mean ± SD of the three independent experiments (*p < 0.05, **p < 0.01).

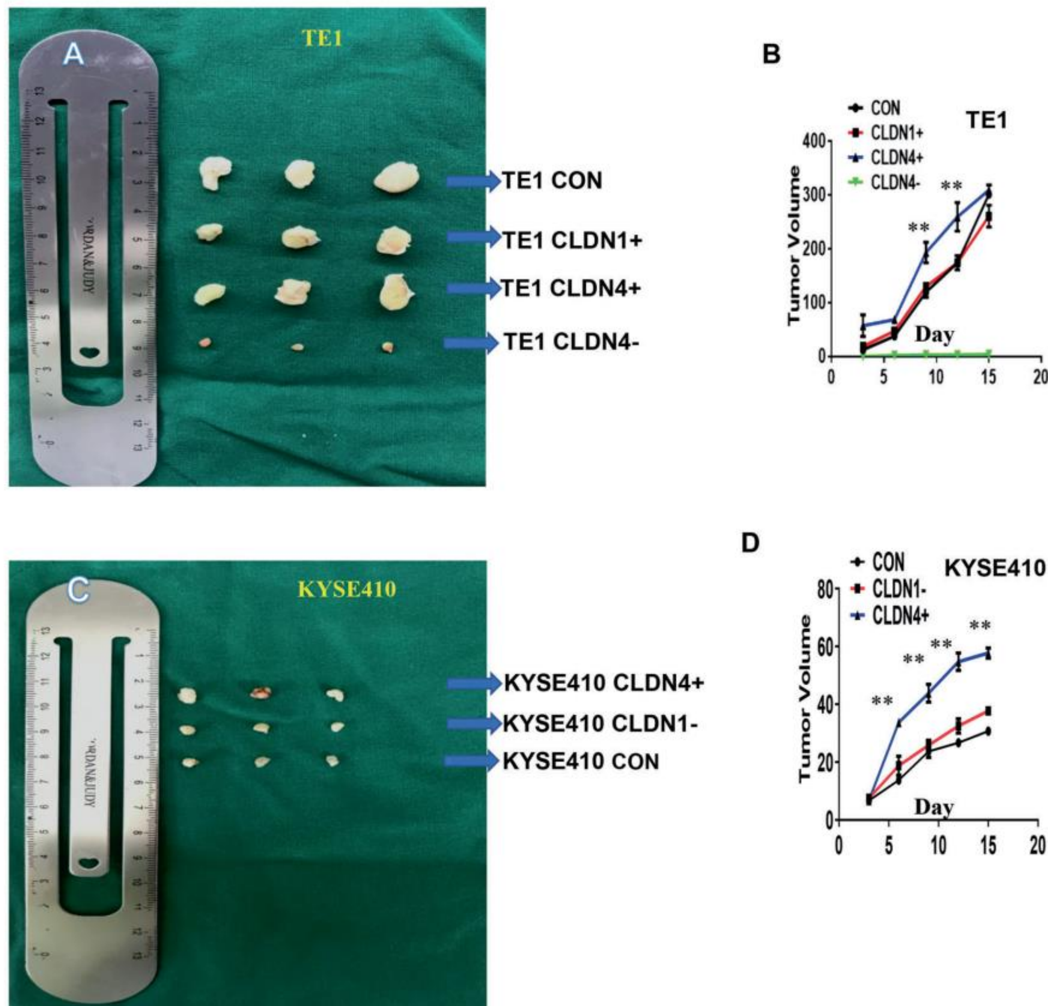


Fig. 8 CLDN1 and CLDN4 can affect the proliferation of cancer cells in vivo experiment. Nude mice of control and subject which were subcutaneously injected with cancer cells. A, B Vivo experiment of TE1, the resulting xenografts were removed to measure the size of the tumors. C, D Vivo experiment of KYSE410, the resulting xenografts were removed to measure the size of the tumors. Data are presented as the mean ± SD of the experiment (*p < 0.05; **p < 0.01).

Discussion

CLDN4 can be used as an indicator of long-term survival of esophageal cancer

The results of the immunohistochemical microarray showed that CLDN1 and CLDN4 expression was significantly higher in cancer tissue than in para-cancerous tissue. However, only the expression of CLDN4 was negatively correlated with the overall survival time of patients. The higher the expression of CLDN4, the shorter the overall survival time of cancer patients. Rho GTPase may affect the development of esophageal cancer [34, 35]. Treatment with shRNA targeting RhoA suppresses the growth of ESCC cells in vitro and in vivo [36]. miR-107 functions as a tumor suppressor in human ESCC and targets Cdc42 [37]. Suppression of p-JNK could lead to apoptosis and reduce proliferation in ESCC cells [38, 39].

The results of the MTT assay in cells and in vivo nude mouse experiments showed that upregulation of CLDN4 expression in TE1 cells and KYSE410 cells can promote their proliferation via Rho GTPase and p-JNK pathway. Inhibiting the expression of CLDN4 in TE1 could decrease the proliferation ability of cancer cells via Rho GTPase and p-JNK pathway.

At the same time, CLDN4 could also affect the apoptosis of cancer cells by regulating the phosphorylation of AKT protein. Downregulating the expression of CLDN4 in TE1 could promote the phosphorylation of AKT protein and increase the apoptosis of cancer cells. Overexpression CLDN4 in TE1 cells could lead to decrease of p-AKT and reduced the apoptosis of cancer cells. However, in KYSE410 cells, overexpression CLDN4 could lead the expression of p-AKT increased. We speculate that the different roles of CLDN4 in TE1 and KYSE 410 may be related to the different degrees of differentiation of cancer cells.

Transmembrane protein Claudins are critical components of the tight junctions essential for the tissue integrity, and represent a useful target for various therapeutic strategies, as demonstrated by the anti-claudin 18.2 antibody used as a new targeted therapy for advanced gastric cancer [40]. Therefore, how to utllise CLDN4 as a marker to predict the long-term survival of ESCC patients and target gene of therapy are the focus of our future research.

CLDN1/CLDN4 as direct target genes of HIF-1α may promote the carcinogenesis of epithelial cells in hypoxia condition

In reflux esophagitis, CLDN1 and CLDN4 are abnormally expressed compared to that in healthy esophageal epithelial cells [10, 12, 41]. Hypoxia can induce expression changes in HIF-1α and CLDN1, and a chip experiment showed a direct positive correlation between the two proteins in EoE [13]. Several articles have reported that reflux esophagitis characteristics of reflux esophagitis and EoE, we proposed the following hypothesis based on the results of this study.

Acid reflux, chronic esophageal inflammation with tissue remodeling and structural changes in the esophagus can cause chronic inflammation of the esophageal mucosa. Edema of the mucosal tissue and venous congestion lead to a hypoxic micro environment around the epithelial cells of the esophagus. Abnormal expression of HIF-1α and CLDN1/4 can further influence epithelial cell proliferation and apoptosis ability through Rho GTPases and p-JNK pathway and affect the phosphorylation of AKT protein.

If mucosal cells with accelerated proliferation fail to undergo apoptosis normally, the mucosa epithelium will develop abnormal hyperplasia, which further subjects epithelial cells to ischemia and hypoxia and induces the carcinogenesis of epithelial cells.

Mutual regulation between CLDN1 and CLDN4 may affect the polarity of epithelial cells and promote the carcinogenesis of epithelial cells

The immunohistochemical results of the tissue microarray showed that both CLDN1 and CLDN4 were expressed at the protein level in normal esophageal mucosa at approximately the same level. In TE1 cells, which are a well-differentiated cancer cell line, Western blotting could detect CLDN1 and CLDN4 expression, but CLDN4 expression was significantly higher than that of CLDN1 (Fig. 2A, B). In poorly differentiated KYSE410, CLDN1 and CLDN4 expression was already significantly out of balance (Fig. 2A, B). In the esophagus, claudin-1 expression is decreased in gastroesophageal reflux disease [42] and EoE [43].

If there is also mutual regulation between CLDN1 and CLDN4 proteins in normal epithelial cells, it would be interesting to determine whether such regulation will aggravate changes in CLDN1 and CLDN4 protein expression induced by HIF-1α, make epithelial cells lose their normal polarity and induce cells canceration.

The feedback regulation of CLDN1/4 on the expression of HIF-1α in cancer cells through PI3K-AKT-mTOR may assist the carcinogenesis of epithelial cell and promote the development of ESCC

HIFs and pathways (PI3K/AKT and mTOR/p70S6K1) that lead to normoxic HIF activation are considered potential therapeutic targets in growth factors, deregulated oncogenes, and tumor suppressors [33].

CLDN1/4 could feedback on the expression of HIF-1α in cancer cells through PI3K-AKT-mTOR signal pathway. Knockdown CLDN1 in KYSE410 could decrease the expression of m-TOR and reduced the expression of HIF-1α. Knockdown CLDN4 in TE1 decreased the expression of m-TOR, while which reduced the expression of HIF-1α. Overexpression CLDN4 in TE1 could upregulate HIF-1α both at mRNA and protein levels by increase the expression of mTOR (Fig. 5A, C–E, G).

CLDN1 overexpression in TE1 promoted the phosphorylation of AKT protein and reduced the expression of m-TOR, which down-regulate the protein expression of HIF-1α (Fig. 5A, G). Overexpression of CLDN4 protein in highly differentiated TE1 cells can promote the expression of HIF-1 α in normoxic environment by upregulate the expression of mTOR.

The feedback regulation of CLDN1 and CLDN4 on HIF-1α can help maintain the balance of HIF-1α expression in cancer cells and promote the development of ESCC. This relationship of CLDN1/CLDN4 with HIF-1α may also help epithelial cells to tolerate hypoxic conditions. In future experiments, by observing the biological behavior changes in the cancer cells at different oxygen concentrations and different durations under hypoxic conditions, we will clarify the effect of CLDN1/4 protein on cancer cell hypoxia tolerance to determine whether CLDN1/4 can be targeted to inhibit the occurrence and development of esophageal cancer.

Materials and methods

Antibodies and reagents

Antibodies (Abs) against HIF-1α (ab16066), CLDN1 (ab15098), and CLDN4 (ab210796) were purchased from Abcam PLC. Abs against p-AKT (2965), AKT (2920S), mTOR (2972), Rac-1/2/3 (2465S), RhoA (2117S), Cdc42 (2466S), and p-SAPK/JNK (9251S) were purchased from Cell Signaling Technology. Abs against β-actin (TA09) were purchased from Origene, ZSGB Bio, China. Ch-IP performed as the manufacturer's instructions (PIERCR ChIP Kit, 26156, Thermo fish). Tissue chips for immunohistochemistry were purchased from Shanghai Outdo Biotech Co, Ltd. (number: heso-squ180sur-04). Small interfering RNAs (siRNAs), plasmids, and lentiviruses related to HIF-1α, claudin-1 and claudin-4 were constructed and synthesized by Gene-Pharma, Suzhou, Jiangsu. The human esophageal cancer cell lines TE1, KYSE410 were purchased in 2016 from the China Tumor Cell Institute, Beijing, China. Hypoxia condition were generated using a hypoxia workstation (Ruskin Technologies, UK).

Immunohistochemistry and clinical data collection

The immunohistochemical microarray (number: hesosqu180sur-04) contained 180 tissue samples from 100 patients; upon exclusion of those who were lost to follow-up and those who had not been clinically staged, 95 patients remained. Among them, 80 cases had both cancer and paracancerous tissue samples, and 15 cases had only cancer tissue samples. Patients underwent surgery between January 2006 and October 2008, and the follow-up time was through September 2014 (5.8–7.8 years). Adjacent tissues were selected and processed onto chips for immunohistochemical staining for CLDN1 and CLDN4. Using two pre-experimental tissue microarrays, primary antibodies against CLDN1/ CLDN4 were added, and the negative controls were handled in the same way except that phosphate-buffered saline (PBS) was applied instead of primary antibodies. Experiments were performed in accordance with the instructions of the Streptomycin Peroxide Kits (Sigma-Aldrich, USA). Primary antibodies against CLDN1 and CLDN4 were used at concentrations of 1:200 and 1:200, respectively. Immunostaining was observed and imaged under a light microscope, with brown membranous and/or cytoplasmic staining classified as positive expression. The number of positive cells and the total cell number from a minimum of five randomly selected visual fields were counted in each specimen.

Cell culture and stable cell line construction

TE1 is a well-differentiated esophageal squamous cancer cell line, whereas KYSE410 is poorly differentiated ESCC cell. Cancer cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ ml penicillin and 100 μg/ml streptomycin (Life Technologies, Carlsbad, CA, USA) at 37 °C and 5% CO2 in an incubator. To mimic severe hypoxia conditions, cells were cultured at 37 °C in 5% CO2, 94.9% N2, and 0.1% O2 in a hypoxia incubator (Ruskin Technologies, UK).

Table 2 LV sequences.

Gene	Sense1	Sense2
CLDN1	5′-GCAAAGTCTTTGACTCCTTGC-3’	5′-GGGTGCGATATTTCTTCTTGC-3′
CLDN4	5′-GCTGAACAATGGCCTCCAT-3′	5′-GCGAAGGTGCTGTAAACAGGT-3′

Table 3 siRNA sequences.

Gene	Sense	Anti-sense
si-HIF1α	5′-GGCCGCUCAAUUUAUGAAUTT-3′	5′-AUUCAUAAAUUGAGCGGCCTT-3′
si-con	5′-UUCUCCGAACGUGUCACGUTT-3′	5′-ACGUGACACGUUCGGAGAATT-3′

Table 4 Primers used for PCR.

Gene	Sense	Antisense
CLDN1	5′-CCCTATGACCCCAGTCAATG-3′	5′-ACCTCCCAGAAGGCAGAGA-3′
CLDN4	5′-CATCGGCAGCAACATTGTCA-3′	5′-CGAGTCGTACACCTTGCACT-3′
HIF1α	5′-ACCTATGACCTGCTTGGTGC-3′	5′- GGCTGTGTCGACTGAGGAAA-3′
β-actin	5′-TGGCACCCAGCACAATGAA-3′	5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′

Stable cell lines were constructed with lentiviruses. The cells were seeded in a six-well plate at 30% overnight. The next morning, the tumor cells were transfected with lentiviruses. The titer of the lentiviruses we constructed is 109 . Approximately, 70% of the virus MOI successfully transduced TE1 and KYSE410 cells. Added 7 µl lentivirus to the RPMI 1640 medium with 10% FBS, without penicillin and streptomycin. The total volume of medium and lentiviruses should no more than 1 ml. After 24 h, replaced the medium of the transfected cells with normal medium. The efficiency of transfection of each virus and plasmid was mainly based on immunofluorescence, and the efficiency of inhibition and overexpression of related genes was mainly based on Real-time PCR and Western blot assays. Stable cell lines were selected by adding 8 µg/ml puromycin into the medium, to remove the uninfected tumor cells and change the cell culture medium daily. The sequences are shown in Table 2.

RNA interference by synthetic siRNA

Selective targeting of HIF-1α was performed using specific siRNAs synthesized commercially by Gene-Pharma (Suzhou, Jiangsu, China). As a control, siRNA sequence (si-NC) that does not target any gene in the human genome and has been vetted by microarray analysis was employed. The sequences are shown in Table 3. Transfection of the siRNA (final concentration 100 nM) was performed with Lipofectamine 2000.

RNA extraction and real-time PCR

First, real-time PCR was used to compare the expression of CLDN1 and CLDN4 mRNA levels in different esophageal cancer cell lines. The cancer cells were cultured in hypoxia environment (0.1% O2) for 8, 16, 24, and 48 h. The mRNA changes of HIF1α, CLDN1 and CLDN4 were analyzed by real-time PCR. Total RNA was extracted from the cells using Trizol (Invitrogen, USA) following the manufacturer’s instructions. Because HIF-1α was easy degradable in normal oxygen condition, therefore, when extracting RNA from cells which cultured in a hypoxic environment, it should to

minimize the operation time, finished all operations within 30 s, removed the culture medium, washed the cells with ice PBS, and added Trizol to the cells to lyse the cells. To synthesize cDNA, 1 μg of total RNA was subjected to reverse transcription using the Prime-Script RTTM Reagent Reverse Kit (TaKaRa), and 1 μg of cDNA was used for PCR. HIF-1α, CLDN1 and CLDN4 were amplified along with β-actin (as an internal control) with SYBR Premix Ex TaqTMII (TaKaRa, Japan) following the manufacturer's instructions. The PCR conditions were 95 °C 10 s (Hold), after that were 95 °C 5 s, 60 °C 30 s, 40 cycles to emplify. The primer sequences of HIF-1α, claudin-1, claudin-4, and β-actin are shown in Table 4. The amplification efficiency of all primers was close to 100% and the 2ΔΔ CT method was used to analyze the data of real-time PCR.

Cell viability assay

Cell viability was examined with the MTT assay according to the manufacturer's instructions. A total of 2 × 103 cells/ well were incubated for 24, 48 or 72 h in 96-well plates at a final volume of 100 μl/well. After the addition of MTT reagent (10 μg/well), the cells were further incubated for 4–6 h in a 37 °C incubator and then agitated for 10 min on a shaker. The cell number was determined based on the standardization from control cells used in the experiment. The absorbance of the samples at 490 nm (reference wavelength: 690 nm) against the background control was read using a 96-well plate reader.

Cell proliferation assay

Cells were seeded at 2 × 103 cells/well in poly-D-lysine-coated 96-well chamber slides. Four hour prior to the end of the culture, 40 μl of Edu was added from a 100 mM 1:10 DMSO: H2O stock. Cells were then fixed for 20 min in 4% PFA in PBS, and Edu was detected according to the manufacturer's protocol (KGA 337-100, Key-GEN Biotech, Nanjing, China). Cell proliferation was quantified as the percentage of Edu incorporating cells against the total number of cells determined by DAPI nuclear staining.

Flow cytometry

The cells were incubated in six-well plates overnight, before synchronized next morning. The cells were treated in 0.1% hypoxia culture condition with fresh culture medium containing 10% FBS and penicillin/streptomycin for 24 and 48 h. Cells were harvested, Annexin-V FITC /PI kit was used for apoptosis detection in accordance with the operation instructions. Ten thousand cells were collected from each sample, and the percentage of apoptotic cells was calculated. Mean values were obtained by repeated three times.

Chromatin immunoprecipitation

For Ch-IP, cells fixed in 1% formaldehyde for 10 min at 4 °C. Cells were sonicated, and the resulting sheared chromatin was incubated with 5 μg of control goat IgG or goat anti-human HIF-1α antibody and ChIP performed per the manufacturer's instructions (PIERCR ChIP Kit, 26156, Thermo fish). Input

DNA, beads, IgG, and DNA replicates enriched for HIF-1α binding were amplified by real-time reverse transcriptase PCR (RT-PCR) using CLDN1/CLDN4 promoters HRE-spanning primers (Table 5).

Table 5 Primers used for PCR of ch-IP.

Gene	Sense	Anti-sense
CLDN1 HER 1	5′-GTGTTAGGATTTACAAGTACAGTGC-3′	5′-CTCGTGGATCTTCTCGCTCC-3′
CLDN1 HER 2	5′-ATTGGGAGGGGAGCCAAGTA-3′	5′-ACTTGTCTCTGCAAGGAGCC-3′
CLDN4 HER 1	5′-GCCGCAAAGACTGCTGG-3′	5′-ATTGTCATTTCTTGTCCTTACCAGT-3′
CLDN4 HER 2	5′-AGGCTGGATCCCGGCAG-3′	5′-ACATGATGCACCTTCAGCCA-3′

Immunofluorescence

Cells were seeded at 2 × 103 cells/well in poly-D-lysinecoated 96-well chamber slides and incubated for 24 h at a final volume of 100 μl/well. Cells were then fixed for 20 min in 4% PFA in PBS, permeabilized in 0.1% Triton X-100 (Sigma) for 1 h and immunoblocked with 1% fish gelatin solution (G7765, Sigma) for 1 h. The cells were incubated with different primary antibodies: diluted in 1% bovine serum albumin (BSA)–PBS, respectively, at 4 °C overnight. After 30 min wash in PBS, the cells were incubated with FITC-conjugated donkey anti-rabbit, anti-mouse secondary antibodies (1:1000, Life Technologies, Carlsbad, CA), diluted in 1% BSA–PBS at 1:100 for 1 h at room temperature in the dark. After a final wash with PBS, specimens were mounted on slides with anti-fade mounting media and imaged using a Leica scanning confocal microscope (Leica, Germany).

Tumorigenicity assay in nude mice

A 5-week-old female nude mice were randomly divided into experimental group and control group according to the principle of equal probability. Cancer cells were implanted into 5-week-old female nude mice (BALB/c–nu) by s.c. injection (1.5 × 106 cells per dose) using 25-gauge needles. Mice were anesthetized by intraperitoneal injection of ketamine and xylazine. To mix: Add 0.13 ml of xylazine (100 mg/ml) + 0.87 ml of Ketamine (100 mg/ml) + 9 ml sterile water. Dose at 0.1 ml/10 g BW. Tumor cells were inoculated subcutaneously under the right axilla. Tumor size was measured and recorded every 3 days for a 15-day period. Measuring tool: vernier caliper. The maximum diameter and length of the tumor were L (mm) and W (mm). The volume of the tumor was L*W2 /2 (mm3). At the end of the experiment, the mice were put to death in a carbon dioxide euthanasia incubator to minimize the suffering of the experimental animals. The animals' welfare and the experimental procedures were approved by the Animal Ethics Committee of The Shandong Provincial ENT Hospital (No. XYK-LL20190203).

Western blot analysis

Cells were washed with ice-cold PBS, harvested and lysed in lysis buffer containing 0.1% PMSF (KAIJI, Nanjing, China). Because HIF-1α was easy degradable in normal oxygen condition, it is important to minimize the time required to extract the protein from cells cultured under hypoxia condition. The protein samples could be broken by ultrasound on ice to assist the release of HIF-1α

from the nucleus. The protein concentration of the supernatant was determined with a BCA protein assay kit according to the manufacturer's instructions. Added loading buffer to the protein sample, heated to 95°, and denaturate the protein. Proteins were separated by. sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Amersham). All primary antibody was diluted with 5% BSA at a dilution ratio were 1:1000. The dilution ratio of the corresponding secondary antibody is 1:5000.

Statistical analyses

Data were expressed as the mean ± s.d. of at least three independent experiments. The statistical differences between the two groups were analyzed using the Student's t test. The correlations between two variables were evaluated using the Pearson chi-squared and Fisher's exact tests. The prognostic value of CLDN1 and CLDN4 expression on overall survival was determined with Cox's proportional hazard regression model. Overall survival was calculated as time from surgery to time of death. Compare of CLDN1/4 expression in cancer para tissue and cancer with analyzed by nonparametric test. The relationship of expression changing of CLDN1/4 with overall survival status was analyzed by correlation test. The normal and homoschedastic have been detected in Student's t test and nonparametric test. Statistical analysis was performed using the Statistical Package of Social Sciences software, version 20.0 (SPSS, Chicago, IL, USA), a two-sided p value of 0.05 was considered as the significant statistical level.

Acknowledgements

We are grateful to Darren Edwards for comments on the paper. The present study was supported by grants from the National Natural Science Foundation of China (grant No. 81902763) and Major Science and Technology Plan Project of Hainan Province (grant No. ZDKJ202005).

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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