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ORIGINAL ARTICLE

Downregulation of HOXA13 sensitizes human esophageal squamous cell carcinoma to chemotherapy

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Keywords

Cisplatin; epithelial-mesenchymal transition; esophageal neoplasm; HOXA13.

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Abstract

Background: Chemoresistance often develops in esophageal squamous cell carcinoma (ESCC), leading to poor prognosis. *HOX* genes play a crucial role in embryonic development and cell differentiation. Studies have recently linked *HOX* with chemoresistance, thus we explored whether *HOXA13* is involved in ESCC chemoresistance.

Methods: One hundred thirty-one ESCC patients who received neoadjuvant chemotherapy were enrolled. *HOXA13* expression was examined by immunohistochemistry. RNA interference was used to knock down the *HOXA13* expression in KYSE70 and transfected HOXA13 plasmid to overexpress *HOXA13* in KYSE510 cells. We examined half-maximal inhibitory concentration of cisplatin, apoptosis, and epithelial-to-mesenchymal transition (EMT) in ESCC cell lines with different *HOXA13* expression levels by cell counting kit-8, flow cytometry, and transwell analysis.

Results: The median survival of patients with high *HOXA13* expression was significantly shorter than those with low expression ($P = 0.027$). *HOXA13* was associated with worse tumor regression grade ($P = 0.009$). Low *HOXA13* expressed cells decreased the half-maximal inhibitory concentration of cisplatin ($P < 0.05$), increased cisplatin-induced apoptosis ($P < 0.05$), and decreased EMT ($P < 0.05$) compared with high *HOXA13* expressed cells. In low *HOXA13* expressed cells, cleaved caspase-3 and cleaved PARP expression induced by cisplatin increased, while expression of E-cadherin and Snail protein, markers of EMT, was upregulated and downregulated, respectively. EMT decreased in low *HOXA13* expressed cells.

Conclusion: High *HOXA13* expression was associated with inferior tumor regression grade and poor overall survival in ESCC patients treated with neoadjuvant chemotherapy. *HOXA13* increased cisplatin-resistance and promoted EMT in ESCC cells.

Introduction

Esophageal carcinoma (EC) is the most common malignant tumor reported worldwide.[1] There are two major types of EC: esophageal adenocarcinoma and esophageal squamous cell carcinoma (ESCC). ESCC mainly occurs in eastern Asia, particularly in China.[2] Although the diagnostic and therapeutic landscape of ESCC has changed dramatically in the past decade, long-term survival of ESCC remains poor.[3] The cure rate for resectable ESCC has improved as a result of multimodality treatment approaches. The role of systemic therapy has also expanded to earlier stages of the disease. Currently, preoperative induction therapy, including platinum-based neoadjuvant chemotherapy and chemoradiation, is commonly

used for locally advanced cases.[4] However, because of chemoresistance, the efficacy of chemotherapy remains unsatisfactory; thus there is an urgent need to discover chemoresistance biomarkers to identify ESCC patients who could potentially respond to chemotherapy.[5]

The highly conserved *HOX* gene family, which was originally discovered in drosophila, is critical to embryonic development and encodes transcription factors that regulate cell proliferation and differentiation.[6] Alterations in the expression patterns of *HOX* genes cause dysregulation of HOX protein function leading to abnormal proliferation and differentiation.[7] Several studies have explored the expression of *HOX* genes, particularly *HOXA13*, which contribute to tumorigenesis in a variety of tumors.[8–11] Our previous studies determined that *HOXA13* is overexpressed in ESCC but not in normal tissues, indicating that *HOXA13* may play a crucial role in the tumorigenesis and development of ESCC.[12] In addition, we observed that the median survival duration of patients with high *HOXA13* expression was shorter than in patients with low expression.[13] Moreover, in vivo and in vitro experiments revealed that *HOXA13* knockdown suppressed ESCC cell proliferation.[13]

Recently, several studies have focused on the relationship between *HOX* genes and chemoresistance. In the carboplatin-resistant DMS53 SCLC cell line, some *HOX* members, including *HOXA7*, *HOXA9*, *HOXA13*, *HOXB2*, *HOXB5*, *HOXB7*, *HOXB8*, *HOXB9*, and *HOXC9*, were significantly overexpressed.[14] Furthermore, activation of *HOXA9*, *HOXA10*, *HOXB13*, *HOXC4*, *HOXC10*, *HOXC11*, *HOXC13*, and *HOXD1* was associated with drug resistance in temozolomide-resistant cell lines.[15] On the other hand, studies have found that the *HOX* gene is associated with epithelial-to-mesenchymal transition (EMT). *HOXA10* controls migration and EMT in oral squamous cell carcinoma.[16] *HOXB5* promotes EMT in breast cancer cells and non-small cell lung cancer.[17,18] In addition, several markers of EMT are associated with chemoresistance, such as Snail and E-cadherin.[19–22] However, the relationship between *HOXA13* dysregulation and chemoresistance or EMT in ESCC is not well understood. We investigated the relationship between *HOXA13* expression and the clinicopathological characteristics of ESCC patients who received neoadjuvant chemotherapy to evaluate whether *HOXA13* could serve as a predictor of chemotherapeutic response. We further explored the role of *HOXA13* in cisplatin-chemoresistance and EMT in ESCC cells.

Methods

Patients

Clinicopathological data of 131 ESCC patients including age, gender, clinical stage, pathological primary tumor and lymph node stage, and tumor regression grade were retrieved from our prospective EC database, established in January 2000 at the Department of Thoracic Surgery I, Peking University Cancer Hospital (Beijing, China). The clinical features of the tumor samples were defined according to the seventh edition of the Union for International Cancer Control (UICC) Tumor Node Metastasis (TNM) classification. Follow-up visits took place every three months for up to two years, every six months up to five years, then once per year up to 10 years after surgery. Follow-up was performed by a single surgical team at the outpatient clinic of Peking University Cancer Hospital, and consisted of standardized patient history, physical examination, chest contrast computed tomography (CT) scan, abdominal and supraclavicular regional ultrasonography, and a combination of cranial magnetic resonance imaging and whole-body bone scintigraphy or positron emission tomography (PET)-CT. Results were documented in a standardized form. The ethics committee of the Peking University Cancer Hospital approved the study.

Neoadjuvant chemotherapy

A platinum-based doublet was administered every three weeks, including: cisplatin 75 mg/m^2 intravenous infusion over two hours on day 1 followed by paclitaxel 175 mg/m^2 on day 1 (more than 95%); and cisplatin 75 mg/m^2 intravenous infusion over two hours on day 1, followed by 5-FU 1000 mg/m^2 intravenous daily as continuous infusion over 24 hours, days 1–4 (< 5%). After each cycle, a restaging evaluation was performed, and the single surgical oncology team determined the number of cycles in terms of response and resectability.

Tumor regression grade (TRG)

The degree of histomorphological regression was determined by the ratio of residual tumor cells in possible areas of recurrence, especially resection edges. The degree of regression, defined as the tumor regression grade (TRG), was classified into four categories according to method used by Chirieac *et al.*: TRG1: complete response without any residual tumor cells; TRG2: 1–10% residual tumor cells; TRG3: 11–50% residual tumor cells; and TRG4 >50% vital residual tumor cells.[23] Two experienced pathologists who were blinded to all clinical and genetic data obtained the grading results.

Immunohistochemistry

Patient specimens were retrieved from the Department of Pathology, Peking University Cancer Hospital. After routine deparaffinization and rehydration, the tissue sections

were incubated with 3% hydrogen peroxide and heated in ethylene-diamine-tetraacetic acid (EDTA, pH 9.0) for antigen retrieval. Following blocking with goat serum, the sections were incubated overnight at 4°C with a primary antibody against *HOXA13* (1:500; Abcam, Cambridge, MA, USA) to allow antigen-antibody binding. Goat anti-rabbit biotin-conjugated immunoglobulin G secondary antibody was then used. The degrees of antigen-antibody binding were determined using a streptavidin/peroxidase amplification kit (SPN-9001; ZSGB-BIO, Beijing, China). Peroxidase activity was assessed with diaminobenzidine. All sections were counterstained with hematoxylin to stain the nuclei. Two independent pathologists graded immunohistochemistry images. Each score was calculated as the product of the staining intensity. The relative intensities of staining were categorized as: 0: negative; 1: weak; 2: moderate; and 3: strong (Fig 1a). Scores 0–1 were considered indicators of lower expression, whereas scores 2–3 indicated higher expression.

Cell culture and treatments

Human ESCC cell lines KYSE70, KYSE150, KYSE180, KYSE450, and KYSE510 were purchased from the Cancer Hospital of the Chinese Academy of Medical Sciences (Beijing, China). The identities of the cell lines were confirmed by standard short tandem repeat analysis, and results were matched with data from the American Tissue Culture Collection and the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. All cells were cultured for < 1 year before use and were cultured in RPMI-1640 medium (HyClone; GE Healthcare, Logan, UT, USA) with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin solution at 37°C in a humidified atmosphere containing 5% CO_2. Cisplatin was purchased from Sigma (C2210000, Darmstadt, Germany).

Western blotting

Cells were harvested and lysed in cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and proteinase inhibitors). Equal amounts of total protein were boiled, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Non-specific protein interactions were blocked by incubation with 5% non-fat milk in tris-buffered saline plus tween 20 at room temperature for one hour. Subsequently, the membrane was incubated overnight with antibodies against *HOXA13* (1 μg/ml), Snail (1 μg/ml), and E-cadherin (1:2000; Abcam, Cambridge, MA, USA); glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000; ZSGB-BIO, China); and caspase 3 (1:1000) and cleaved-PARP (1:1000; Cell Signaling Technology, Boston, MA, USA).

Real-time PCR

Total cellular RNA was extracted with Trizol reagent (Tiangen, Beijing, China) according to the manufacturer's instructions. Complimentary (c)DNA was synthesized using QuantiTect SYBR Green PCR Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Messenger RNA (mRNA) expression of GAPDH was used as an internal control. Finally, real-time (RT) PCR was performed to analyze the mRNA levels of *HOXA13* with HOXA13 and GAPDH specific primers. The primers used were as follows: HOXA13 sense primer, 5′ CTGGCATT TTCCTCTCCCGAA 3′; HOXA13 antisense primer, 5′ ATTACCATCTAACGCAGTGTCC 3′; GAPDH sense primer, TCATTGACCTCAACTACATGG; and GAPDH antisense primer, TCGCTCCTGGAAGATGGTG. The cycle threshold difference between the internal control and *HOXA13* was presented as $-\Delta CT$. $2^{-\Delta CT}$ is an exponential value of $-\Delta CT$, which indicates the expression of HOXA13 relative to that of the internal control, GAPDH.

Flow cytometry

After 8 μg/ml cisplatin was added in cells for eight hours, cells were washed three times in phosphate buffered saline and incubated in 1 ml trypsin (without EDTA) at 37°C for approximately 10 minutes. The cells were then re-suspended in binding buffer at a concentration of 1×10^6 cells/ml, with 5 μl of Annexin V-FITC antibody and 5 μl of propidium iodide (Dojindo Molecular Technologies Inc., Kumamoto, Japan). The samples were incubated for 15 minutes at room temperature in the dark and analyzed by FACScan cytometry (BD Biosciences, Franklin Lakes, NJ, USA) within an hour.

Cell proliferation assay

Cells were seeded in 96-well plates at a density of 5000 cells per well and incubated overnight at 37°C. The cells were then treated with gradient dilutions of cisplatin (0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 μg/ml) for 48 hours. Following cisplatin treatment, 10 μl of Cell Counting Kit-8 reagent (Dojindo Molecular Technologies Inc., Japan) was added to each well and the samples were incubated for two hours at 37°C. Finally, the absorbance of each well was measured at 450 nm using an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

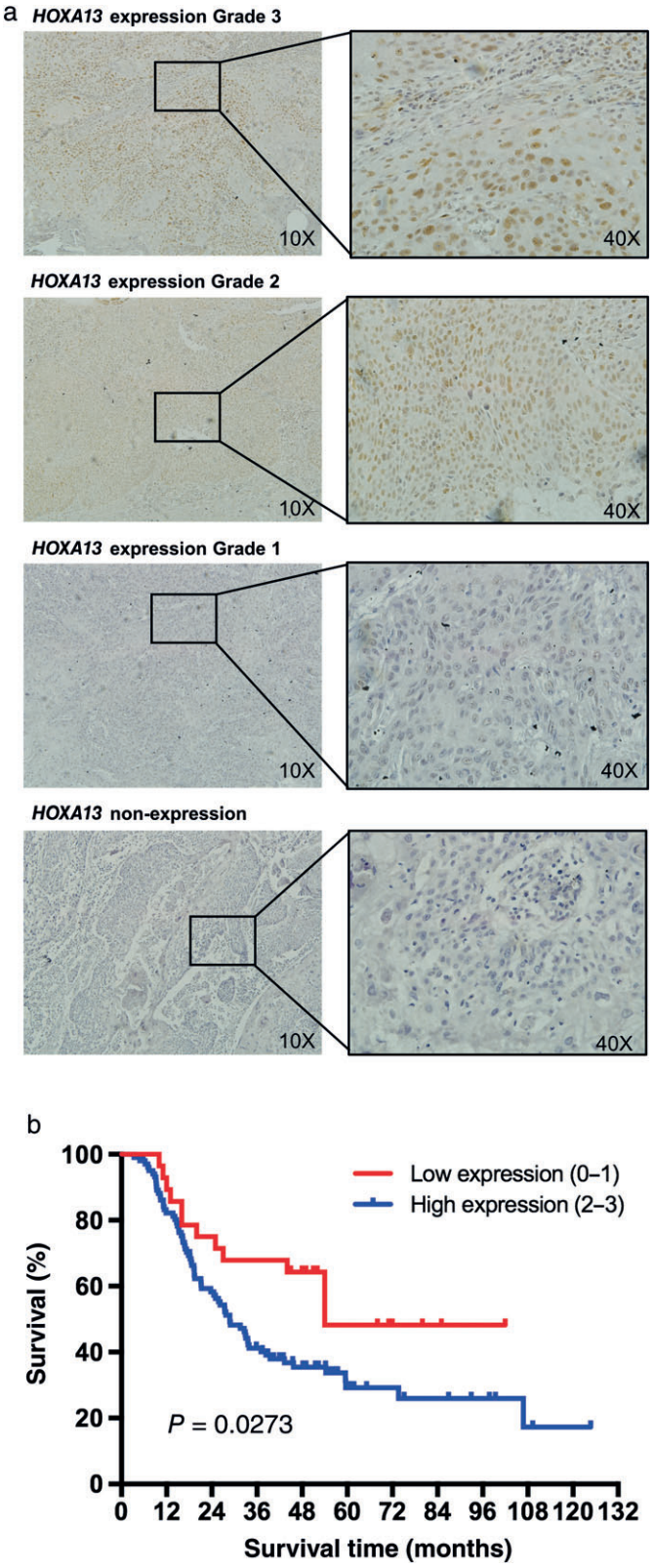

Figure 1 *HOXA13* expression was associated with prognosis in esophageal squamous cell carcinoma (ESCC) patients who received neoadjuvant chemotherapy. (**a**) The different staining intensities of *HOXA13* in ESCC samples. (**b**) Kaplan–Meier survival curves for low or high *HOXA13* expression. Survival of patients with high *HOXA13* expression was significantly shorter than patients with low *HOXA13* expression ($P = 0.027$).

Knockdown of *HOXA13* in KYSE70

HOXA13 small interfering RNA (siRNA[h]) sc-45 666 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were seeded in a six-well plate (2×10^5 cells per well) and incubated at 37°C for approximately 24 hours. The subsequent steps were followed according to the siRNA transfection protocol (Santa Cruz Biotechnology, USA). RT-PCR and Western blotting (WB) were used to determine *HOXA13* expression in KYSE70 and KYSE70-siHOXA13.

Plasmid construction of *HOXA13* and cell transfection in KYSE510

HOXA13 was cloned from the cDNA of 293T cells using specific primers: sense primer 5′-CTAGTGGAGGATACC CATACGACGTCCCAGACTACGCTTAAGA TATCA-3′ and antisense primer 5′-CTAGTGATATCTTAAGCGTAG TCTGGGA CGTCGTATGGGTATCCTCCA-3′ (GenBank accession number NM_000522.4). The full-length human *HOXA13* cDNA was inserted into pcDNA3.1 vector (Invitrogen, Shanghai, China) using the EcoRV site. The primers were synthesized by Invitrogen (China). Cells were seeded on six-well plates (2×10^5 cells per well) and transiently transfected with the control pcDNA3.1 vector using Lipofectamine 3000 (Invitrogen, China). RT-PCR and WB were used to determine *HOXA13* expression in KYSE510 and KYSE510-HOXA13.

Cell invasion

We suspended 1×10^5 KYSE70, KYSE70-siHOXA13, KYSE510, and KYSE510-HOXA13 in 200 μl of serum-free medium. The cells were seeded into the upper chambers with Matrigel (BD Biosciences, USA) in a 24-well transwell chamber (8 μM, Corning, Tewksbury, MA, USA); 700 μl of complete medium with 10% fetal bovine serum was added to the bottom wells. The cells were cultured for 16 hours, and those that invaded through the membrane were stained with 0.1% crystal violet and imaged under a light microscope (Olympus, Tokyo, Japan).

Statistical analysis

Associations between *HOXA13* expression and clinicopathological characteristics were determined using chi-squared or Fisher's exact tests. Overall survival after surgery was presented in the form of Kaplan–Meier curves, and significance was assessed by log rank test. All in vitro experiments were performed at least three times. When the data from different groups were compared, normal analysis and homogeneity of variance were checked first, followed by unpaired two-tailed *t*-testing. Data are presented as mean ± standard error of the mean. Differences of $P < 0.05$ were considered statistically significant. All data were analyzed using SPSS version 24.0 (IBM Corp., Armonk, NY, USA) or GraphPad Prism version 6.01 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Clinicopathological characteristics of enrolled esophageal squamous cell carcinoma (ESCC) patients

From January 2000 to December 2012, 131 ESCC patients (108 men, 23 women) underwent esophagectomy following neoadjuvant chemotherapy and were enrolled in the study. The latest date of follow-up was 1 June 2017. The median patient age was 59 years. Thirty-four cases were at clinical stage II and 97 at stage III. Neoadjuvant chemotherapy was administered at a median of two cycles (range 1–4). A total of 131 formalin-fixed paraffin-embedded blocks of resected specimens were collected.

HOXA13 expression levels were associated with prognosis of ESCC patients who received neoadjuvant chemotherapy

HOXA13 expression in ESCC tissue samples was determined by immunohistochemistry. The positive staining rate of nuclear *HOXA13* expression was 93.8% (123/131). We further explored whether *HOXA13* expression was associated with ESCC progression by classifying the patients into groups of high and low expression. The different staining intensities of *HOXA13* in ESCC samples are shown in Figure 1a. There were no significant differences between the two groups in terms of age, gender, clinical stage, or pathological N stage (Table 1). Notably, *HOXA13* expression was associated with pathological T stage ($P = 0.010$) (Table 1) and survival ($P = 0.027$) (Fig1b) of ESCC patients. The five-year survival rates of patients in the low and high expression groups were 48.2% and 28.9%, respectively.

HOXA13 expression was associated with TRG in ESCC patients

Our results demonstrated that in 131 cases of resected specimens, *HOXA13* expression was strongly associated with TRG ($P = 0.009$) (Table 1). The patients in the low *HOXA13* expression group achieved better TRG (TRG1) than those in the high *HOXA13* expression group ($P = 0.001$) (Tabl2).

Table 1 Association between *HOXA13* expression of and clinicopathological characteristics in 131 ESCC patients who received neoadjuvant chemotherapy

	HOXA13 expression intensity		
Clinicopathologic data	Low (0–1)	High (2–3)	*P*
Age (years)			0.749
< 59	14/28 (50.0%)	48/103 (46.6%)	
≥ 59	14/28 (50.0%)	55/103 (53.4%)	
Gender			0.283
Male	25/28 (89.3%)	83/103 (80.6%)	
Female	3/28 (10.7%)	20/103 (19.4%)	
Clinical stage †			0.722
II	8/28 (28.6%)	26/103 (25.2%)	
III	20/28 (71.4%)	77/103 (74.8%)	
Pathological T stage †			0.010*
T0/1	11/28 (39.3%)	14/103 (13.6%)	
T2	4/28 (14.3%)	23/103 (22.3%)	
T3	13/28 (46.4%)	58/103 (56.3%)	
T4	0/28 (0%)	8/103 (7.8%)	
Pathological N stage †			0.380
N0	16/28 (57.1%)	48/103 (46.6%)	
N1	5/28 (17.9%)	31/103 (30.1%)	
N2	3/28 (10.7%)	16/103 (15.5%)	
N3	4/28 (14.3%)	8/103 (7.8%)	
TRG †			0.009**
1	4/28 (14.3%)	1/103 (1.0%)	
2	5/28 (17.9%)	14/103 (13.6%)	
3	5/28 (17.9%)	28/103 (27.2%)	
4	14/28 (50.0%)	60/103 (58.3%)	

*$P < 0.05$; **$P < 0.01$. †Tumor stage was defined according to the seventh edition of the Union for International Cancer Control Tumor Node Metastasis classification. ESCC, esophageal squamous cell carcinoma; TRG, tumor regression grade.

Table 2 Association between *HOXA13* expression and TRG/pathological stage in 131 ESCC patients who received neoadjuvant chemotherapy

	HOXA13 expression intensity		
Item	Low (0–1)	High (2–3)	*P*
TRG			0.001***
1	4/28 (14.3%)	1/103 (1.0%)	
2/3/4	24/28 (85.7%)	102/103 (99.0%)	
pStage†			0.006**
I/PCR	9/28 (32.1%)	9/103 (8.7%)	
II	9/28 (32.1%)	44/103 (42.7%)	
III	10/28 (35.7%)	50/103 (48.5%)	

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. †Pathological tumor stage was defined according to the seventh edition of the Union for International Cancer Control Tumor Node Metastasis classification. ESCC, esophageal squamous cell carcinoma; TRG, tumor regression grade.

Low expression of *HOXA13* sensitized KYSE70 and KYSE510 to cisplatin-induced growth inhibition and apoptosis

The baseline expression levels of *HOXA13* were determined by RT-PCR and WB in several human ESCC cell lines, including KYSE70, KYSE150, KYSE180, KYSE450, and KYSE510 (Fig 2a,b). KYSE70 and KYSE510 were selected for further analysis because *HOXA13* expression was highest in KYSE70 and lowest in KYSE510 of the five ESCC cell lines. We knocked down the *HOXA13* expression by RNA interference to establish KYSE70-siHOXA13 and overexpressed *HOXA13* by transfecting HOXA13 plasmid into KYSE510. The knockdown and overexpression efficiency was evaluated by RT-PCR and WB (Fig 2c,d).

Treatment with cisplatin for 48 hours decreased the half-maximal inhibitory concentration (IC_{50}) of KYSE70-siHOXA13 and KYSE510-HOXA13 compared to wild-type cells (Fig 3a,b). We then tested whether *HOXA13* was involved in cisplatin-induced apoptosis in KYSE70 and KYSE510. Indeed, knockdown of *HOXA13* sensitized KYSE70 to cisplatin-induced apoptosis, and flow cytometry analysis demonstrated that cisplatin-induced apoptosis was significantly increased in KYSE70-siHOXA13 (Fig 3c). Overexpression of *HOXA13* inhibited cisplatin-induced apoptosis in KYSE510 (Fig 3d). Apoptosis was further confirmed with two apoptosis markers, namely, cleaved-PARP and cleaved-caspase 3. After cisplatin treatment, the levels of these markers were increased in KYSE70-siHOXA13 and decreased in KYSE510-HOXA13(Fig 3e,f).

Low *HOXA13* expression inhibited KYSE70 and KYSE510 epithelial-to-mesenchymal transition

Many studies have indicated that Snail[20,24] and E-cadherin,[25,26] markers of EMT, induce chemoresistance.[19] Thus, we examined the expression of Snail and E-cadherin in KYSE70, KYSE70-siHOXA13, KYSE510, and KYSE510-HOXA13. Snail expression was decreased and E-cadherin increased in KYSE70-siHOXA13 (Fig 4a). Similarly, Snail expression was increased and E-cadherin decreased in KYSE510-HOXA13 (Fig 4b). Interestingly, we found that low *HOXA13* expression decreased the ability of cell invasion (Fig 4c,d). These results suggested that low *HOXA13* expression might inhibit EMT by decreasing Snail and increasing E-cadherin levels in ESCC cells.

Discussion

Platinum-based doublet regimens are the most common chemotherapy regimens applied for the treatment of solid tumors, including ESCC. Multimodality treatment has been developed to improve the prognosis of patients with locally advanced EC.[27,28] However, previous studies of ESCC have demonstrated that the efficacy associated with neoadjuvant therapy is limited to patients who respond to chemotherapy, whereas the prognosis of non-responders is poorer

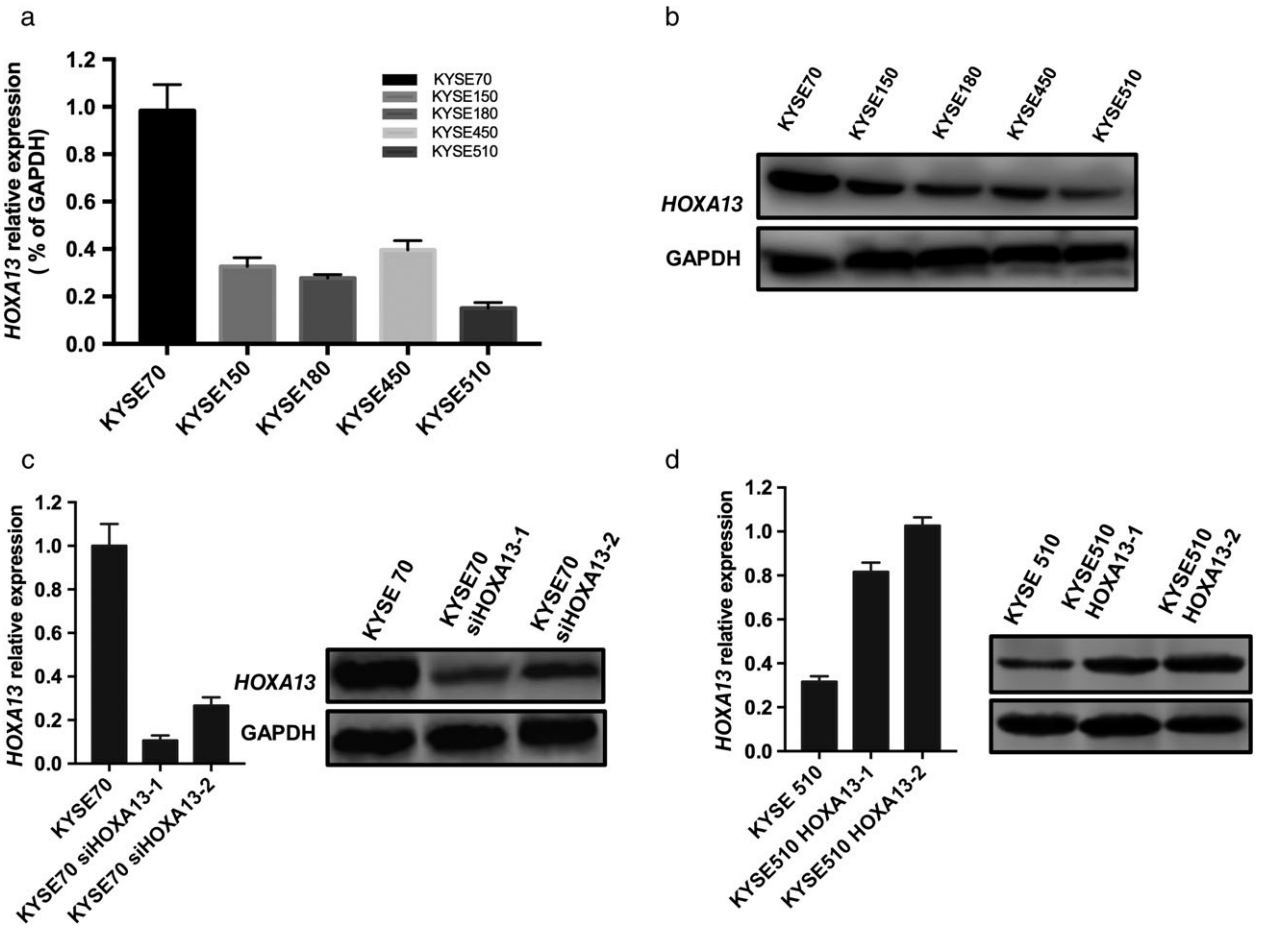

Figure 2 The basal level of *HOXA13* expression in esophageal squamous cell carcinoma (ESCC) cell lines, knockdown of *HOXA13* in KYSE70, and overexpression of *HOXA13* in KYSE510. (**a**) Relative messenger RNA (mRNA) levels of *HOXA13* in five ESCC cell lines (KESE70, KYSE150, KYSE180, KYSE450, and KYSE510) were examined by real-time (RT) PCR. (**b**) Protein levels of *HOXA13* in ESCC cell lines were analyzed by Western blot (WB). After (**c**) KYSE70 was transfected with HOXA13 small interfering RNA for 48 hours and (**d**) KYSE510 was transfected with HOXA13 plasmid for 48 hours, HOXA13 mRNA and protein levels were examined by RT-PCR and WB, respectively. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

than in patients who undergo surgery alone.[29] One of the reasons for this chemotherapeutic inefficacy is the existence of chemoresistance. One of the best strategies to deal with chemoresistance is to discover novel biomarkers to identify patients who respond well to the treatment. In this study, we explored the relationship between *HOXA13* expression and the prognosis of ESCC patients who underwent neoadjuvant chemotherapy. Higher *HOXA13* expression indicated poorer TRG and overall survival in ESCC patients. Therefore, our results reveal that *HOXA13* could serve as a potential biomarker for predicting the efficacy of chemotherapy in ESCC patients.

As key factors in regulating embryonic morphogenesis and differentiation, *HOX* genes are reported to be associated with carcinogenesis and chemoresistance. Knockdown of *HOXA5* expression by short hairpin RNA in acute myeloid leukemia cells inhibits cell proliferation and enhances cytarabine chemosensitivity.[30] Knockdown of *HOXA1* expression affects small cell lung cancer cell survival and sensitivity to chemotherapy.[31] However, it is unclear whether *HOXA13* is involved in chemoresistance in ESCC patients. To further explore the role of *HOXA13* in ESCC cisplatin-chemoresistance, we knocked down *HOXA13* expression by HOXA13 siRNA in KYSE70 and overexpressed *HOXA13* in KYSE510. The evaluation of cisplatin-induced inhibition and apoptosis in ESCC cells revealed that low *HOXA13* expression increased KYSE70 and KYSE510 sensitivity to cisplatin. Furthermore, we found that the depletion of *HOXA13* promoted cisplatin-induced apoptosis in KYSE70 and KYSE510. These finding suggest a close relationship between *HOXA13* and cisplatin-chemoresistance in ESCC.

According to previous research, EMT is a procedure in which cells lose their epithelial characteristics, accompanied by changes in the expression levels of some proteins, such as E-cadherin, which is downregulated during EMT.

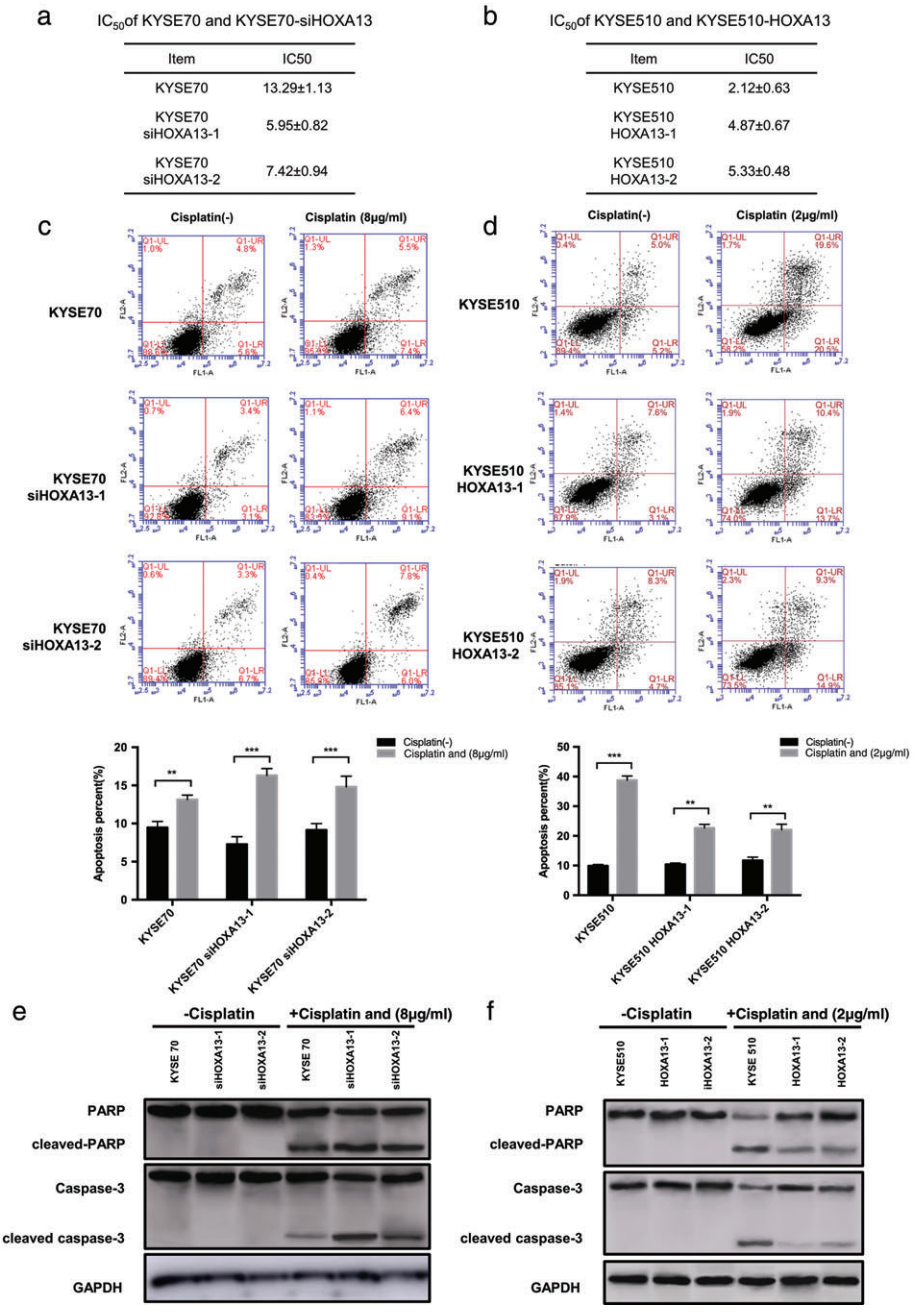

Figure 3 Low *HOXA13* expression sensitizes KYSE70 and KYSE510 to cisplatin-induced apoptosis. KYSE70, KYSE70-siHOXA13, KYSE510, and KYSE510-HOXA13 were seeded in 96-well plates and treated with gradient dilutions of cisplatin (0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 μg/ml) for 48 hours. Cell viability was analyzed by cell counting kit-8 and optical density 450 values were obtained. The half-maximal inhibitory concentration (IC_{50}) of cisplatin in (**a**) KYSE70-siHOXA13 was significantly lower than KYSE70 and in (**b**) KYSE510-HOXA13 was significantly higher than KYSE510. (**c**) KYSE70 and KYSE70-siHOXA13 were treated with 8 μg/ml cisplatin for eight hours. (**d**) KYSE510 and KYSE510-HOXA13 were treated with 2 μg/ml cisplatin for eight hours. The cells were then harvested and apoptosis was analyzed by flow cytometry. The proportion of apoptosis (presented by histogram) in KYSE70-siHOXA13 was higher than in the control. (**e**) KYSE70 and KYSE70-siHOXA13 were treated as in (**c**). (**f**) KYSE510 and KYSE510-HOXA13 were treated as in (**d**). The cells were then lysed and analyzed by Western blot with antibodies against PARP and caspase-3, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as a loading control. The data are presented as mean ± standard error of the mean. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; Student's *t*-test.

Recent studies have indicated that Snail and E-cadherin promote chemoresistance. In ovarian cancer cells, Snail mediates chemoresistance by antagonizing p53-mediated apoptosis.[20] In pancreatic cancer, Snail expression promotes chemoresistance.[24] Knockdown of Snail increases A549 cell sensitivity to cisplatin.[21] E-cadherin-dependent intercellular adhesion enhances chemoresistance in Lovo and MCF-7 cells.[22] Hepatocyte induced re-expression of E-cadherin in breast and prostate cancer cells increases chemoresistance.[32] In addition, some research suggests that the expression of *HOX* genes is closely related to EMT. For example, *HOXA13* exerts a beneficial effect in albumin-induced EMT via the glucocorticoid receptor pathway in human renal tubular epithelial cells,[33] and *HOXB13* overexpression is correlated with the aberrant expression of EMT markers in pancreatic carcinoma.[34] In the current study, Snail and E-cadherin, markers of EMT, were deregulated and upregulated in cells with low expression, respectively

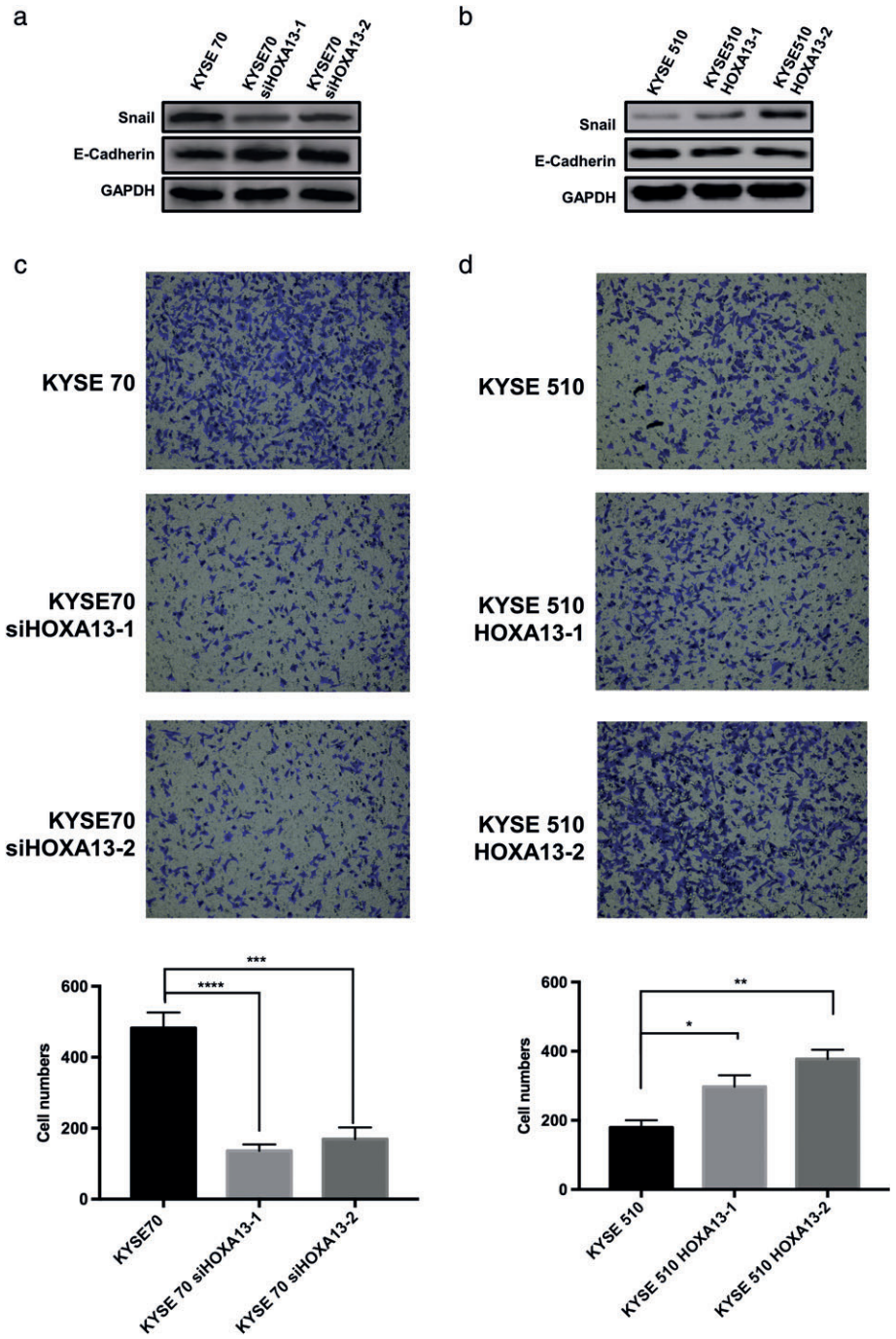

Figure 4 *HOXA13* enhances epithelial-to-mesenchymal transition (EMT) of KYSE70 and KYSE510 by regulating Snail and E-cadherin expression. The proportions of (**a**) KYSE70 and KYSE70-siHOXA13 and (**b**) KYSE510 and KYSE510-HOXA13 were collected and analyzed to determine Snail and E-cadherin expression by Western blotting. After (**c**) knockdown of *HOXA13* in KYSE70 and (**d**) overexpression of *HOXA13* in KYSE510, the ability of transfecting through the well was significantly increased. Transwell cell numbers are presented by histogram. The data are presented as mean ± standard error of the mean. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; Student's *t*-test.

(i.e. KYSE70 and KYSE510). Transwell analysis indicated that the ability of EMT was decreased in KYSE70 and KYSE510 with low *HOXA13* expression. *HOXA13* is associated not only with chemoresistance, but also EMT. We posit that *HOXA13* might regulate EMT to promote chemoresistance. Therefore, exploration of the relationship between EMT and chemoresistance in ESCC may be a direction for future research.

Our current study has some limitations. First, the chemotherapy cycles were not pre-specified or standardized to all treated subjects. In other words, the chemotherapy cycles and the timing of surgery are subject to biases because they were not pre-specified. Second, chemotherapy may change *HOXA13* protein expression levels, but the original *HOXA13* levels were not verified in the postoperative specimens of patients administered neoadjuvant

chemotherapy. Third, the acquisition of pretreatment samples from all patients was difficult and an analysis of matched biopsy tissues with surgical samples was not possible. Finally, we routinely use platinum-based doublet regimens for ESCC patients. In the current study, we explored *HOXA13* and cisplatin-resistance. The relationship between paclitaxel resistance and *HOXA13* should be explored in future studies.

In conclusion, high *HOXA13* expression was associated with inferior TRG and poor overall survival in ESCC patients treated with neoadjuvant chemotherapy. Moreover, high expression enhances cisplatin-chemoresistance and promotes EMT in ESCC cell lines.

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Disclosure

No authors report any conflict of interest.

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