Received: 4 January 2023 | Revised: 20 April 2023 | Accepted: 25 April 2023

DOI: 10.1111/cas.15842

ORIGINAL ARTICLE

Cancer Science WILEY

Homeobox A7 promotes esophageal squamous cell carcinoma progression through C-C motif chemokine ligand 2-mediated tumor-associated macrophage recruitment

Anqi Feng[1] | Lingnan He[1] | Jiakai Jiang[2] | Yuan Chu[1] | Zehua Zhang[1] | Kang Fang[1] | Zeyu Wang[1] | Zhaoxing Li[1] | Mingchuang Sun[1] | Ziying Zhao[1] | Jianing Shi[1] | Li Zhang[3] | Tao Chen[1] | Meidong Xu[1]

[1]Endoscopy Center, Shanghai East Hospital, Tongji University School of Medicine, Shanghai, China

[2]Changzhou Third People's Hospital, Changzhou Medical Center, Nanjing Medical University, Nanjing, China

[3]Department of Pathology, Shanghai East Hospital, School of Medicine, Tongji University, Shanghai, China

Correspondence
Tao Chen and Meidong Xu, 150 Jimo Road, Pudong New Area, Shanghai 200120, China.
Email: chentao@tongji.edu.cn and 1800512@tongji.edu.cn

Funding information
Changzhou Medical Center, Nanjing Medical University, Grant/Award Number: CMCB202218; Top-level Clinical Discipline Project of Shanghai Pudong, Grant/Award Number: PWYgf2021-02; National Natural Science Foundation of China, Grant/Award Number: Nos.82203341; Shanghai Committee of Science and Technology, Grant/Award Number: 20XD1402900, 21XD1423100, 21JC1405200 and 18140900100

Abstract

Homeobox A7 (HOXA7) plays essential roles in multiple malignancies and was reported to be overexpressed in esophageal squamous cell carcinoma (ESCC). However, its functions in the ESCC tumor microenvironment remain to be explored. In this study, we showed that HOXA7 was overexpressed in ESCC among HOXA family members and correlated with tumor-associated macrophage (TAM) infiltration both in The Cancer Genome Atlas database and ESCC clinical samples. Moreover, transactivation of C-C motif chemokine ligand 2 (CCL2) by HOXA7 was identified (real-time quantitative PCR [RT-qPCR], western blot analysis, ELISA, and ChIP-qPCR), which was detected to drive chemotaxis and M2 polarization of macrophages both in vitro (Transwell assay) and in vivo (xenograft tumors models). In addition, CCL2 triggers macrophage expression of epidermal growth factor (EGF) (RT-qPCR and ELISA), which promotes tumor proliferation and metastasis by activating its receptor EGFR. In addition, EGF-induced ESCC cell proliferation and migration can be abrogated by HOXA7 knockdown (CCK-8 proliferation assay, EdU fluorescence, and Transwell assay). These results indicate a novel mechanistic role of HOXA7 in the cross-talk between ESCC and TAMs, which could be an underlying therapeutic target for ESCC.

KEYWORDS
C-C motif chemokine ligand 2 (CCL2), epidermal growth factor receptor (EGFR), esophageal squamous cell carcinoma (ESCC), homeobox A7 (HOXA7), tumor immunology, tumor-associated macrophage (TAM)

Abbreviations: Arg1, arginase 1; CCL, C-C motif chemokine ligand; CM, conditioned medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ESCA, esophageal carcinoma; ESCC, esophageal squamous cell carcinoma; HOXA7, homeobox A7; IF, immunofluorescence; IHC, immunohistochemistry; KEGG, Kyoto Encyclopedia of Genes and Genomes; M-CM, conditioned medium of M0 macrophages; NC, negative control; OE, overexpression; RT-qPCR, real-time quantitative PCR; shNC, short hairpin RNA-negative control; shRNA, short hairpin RNA; TAM, tumor-associated macrophage; TCGA, The Cancer Genome Atlas; TGF-β, transforming growth factor-β; TME, tumor microenvironment.

Anqi Feng, Lingnan He, and Jiakai Jiang contributed equally to this work.

1 | INTRODUCTION

Esophageal cancer is the sixth leading cause of cancer-related death worldwide,[1] and ESCC is the predominant histological subtype of esophageal cancer, which accounts for 90% of all cases globally and is highly prevalent in East Asia, East Africa, and South America.[2] Despite the progress made in endoscopy, surgery, chemotherapy, and radiotherapy, the mortality outcomes for ESCC remains high, even across high-income countries, with 5-year mortality rates well less than 20%.[2] Thus, it is urgent to elucidate the molecular mechanism that drives ESCC progression.

Generally, esophageal mucosa with chronic inflammatory irritation is prone to develop into ESCC,[3] and inflammatory irritation is closely related to immune cell infiltration, such as macrophages, neutrophils, and lymphocytes. Among these different cell types, TAMs have drawn great attention due to their strong correlation with poor prognosis of ESCC.[4] It is well known that macrophages polarize into antitumorigenic M1 or protumorigenic M2 phenotype under microenvironmental stimulations, and TAMs are prone to develop into M2 phenotype in TME.[5] Therefore, targeting TAMs in TME can be a promising method to cope with ESCC.

Homeobox genes were first identified as HOX clusters in mammals by Hart et al. in 1985.[6] Numerous studies have reported that HOX genes act as transcription factors, and these transcription products play key roles in the development of cancers.[7] HOXA family genes were identified as transcription factors that are involved in the development of various cancers, and have been reported to play important roles in TME.[8,9] Previous research has shown that HOXA7 is a member of the HOXA transcription factor family and was reported to play important roles in a number of malignancies including liver cancer,[10] colorectal cancer,[11] breast cancer,[12] and myeloid leukemia.[13] In addition, HOXA7 was significantly overexpressed in ESCC.[14] However, the role of HOXA7 in ESCC has not yet been explored, and how HOXA7 impacts on TME remains functionally uncharacterized.

In this study, we identified that HOXA7 is overexpressed in ESCC tumor tissues, and HOXA7 overexpression in ESCC cells stimulated TAM infiltration and polarization by transcriptionally promoting CCL2 secretion. Meanwhile, HOXA7 accelerated ESCC tumorigenesis by upregulating EGFR. We found there existed a positive feedback loop between ESCC cells and TAMs, and targeting this reciprocal cross-talk could be a promising strategy for conquering ESCC.

2 | MATERIALS AND METHODS

2.1 | Data collection

The Cancer Genome Atlas is a large-scale cancer genomics program, and it has molecularly characterized 33 primary cancer types including ESCC. The expression of the HOXA family in ESCC and HOXA7 in pan-cancer was investigated from the TCGA database.

2.2 | Functional enrichment analysis

Differentially expressed genes of HOXA family members in ESCC were screened out ($p < 0.05$), and a coexpression network was developed to investigate the genes that correlate with HOXA family members in ESCC. Gene Ontology and KEGG signaling pathway enrichment analyses was carried out and plotted according to the DAVID website (https://david.ncifcrf.gov).[15]

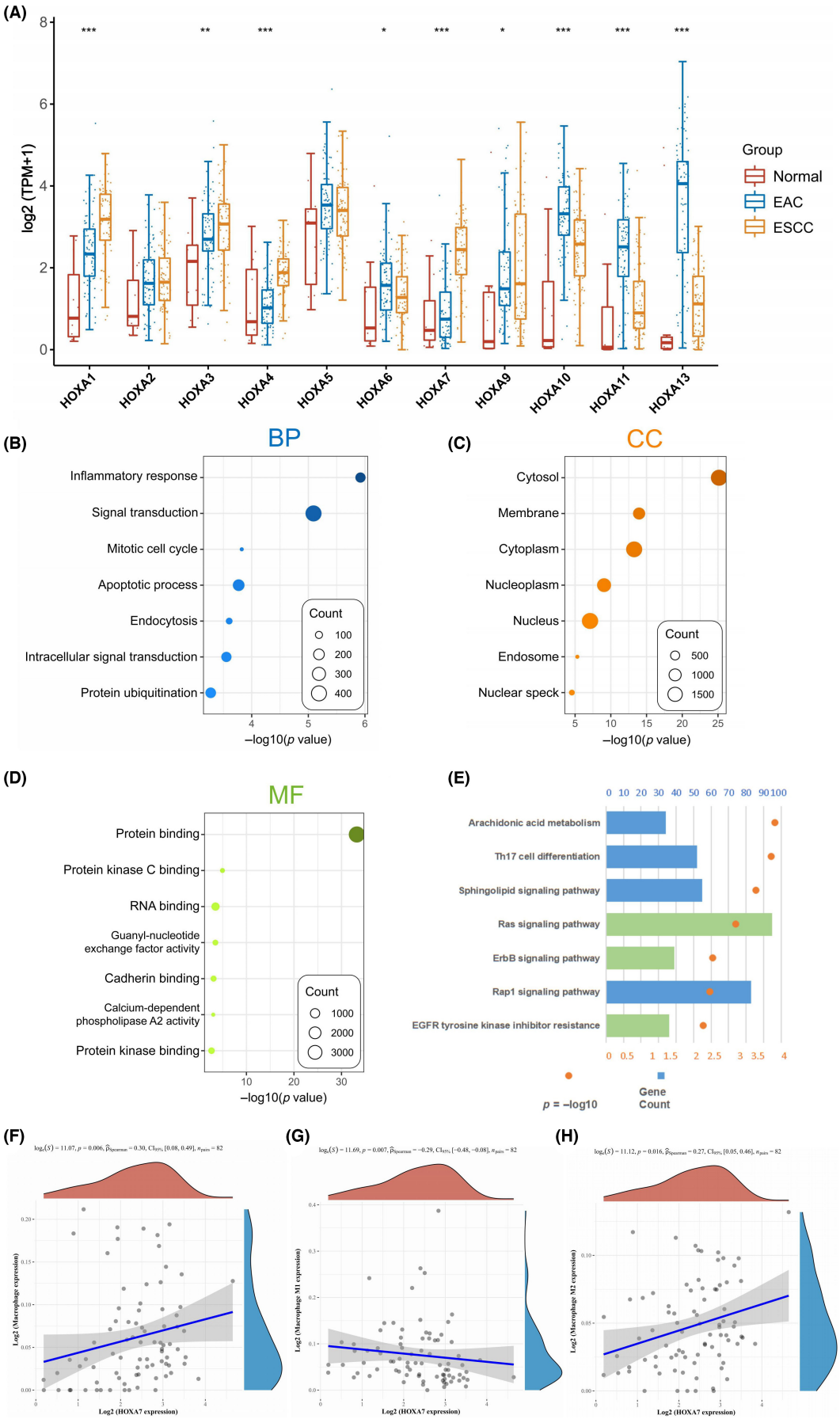
2.3 | Immune infiltration analysis

Tumor Immune Estimation Resource (TIMER; cistrome.shinyapps.io/timer)[16] and Quantiseq[17] analysis was applied to investigate molecular characterization of immune infiltration signatures according to HOXA7 expression.

2.4 | Cell lines and incubation

The human esophageal epithelial cell line Het-1A and human monocyte cell line THP-1 were purchased from ATCC. THP-1 cells were differentiated into M0 macrophages with PMA (100 nM; Sigma-Aldrich) for 24 h. The human ESCC cell lines KYSE150, TE-1, KYSE30, ECA109, and KYSE410 were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences. All human cell lines were identified by short tandem repeat analysis, and the results of mycoplasma testing were negative. All human cell lines were cultured with 3 mL RPMI-1640 medium containing 10% FBS (Gibco) with 100 U/mL penicillin and streptomycin (Gibco), and incubated at 37°C and 5% CO_2 in a humidified incubator of 5% CO_2. The harvested media were centrifuged for 3 min at 1200 *g*, and the supernatant (CM) was collected and deposited at −80°C until use.

FIGURE 1 Overexpressed homeobox A7 (HOXA7) was associated with tumor-associated macrophage infiltration in esophageal squamous cell carcinoma (ESCC). (A) Expression of HOXA family genes in The Cancer Genome Atlas (TCGA)-ESCC database. (B) Biology process (BP) enrichment of overexpressed HOXA genes. (C) Cellular component (CC) enrichment of overexpressed HOXA genes. (D) Molecular functions (MF) enrichment of overexpressed HOXA genes. (E) Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of overexpressed HOXA genes. (F) Correlation of HOXA7 expression with macrophage infiltration based on TIMER scoring system. (G, H) HOXA7 expression and correlation with M1 and M2 macrophage infiltration based on Quantiseq scoring algorithm. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. EGFR, epidermal growth factor receptor.


(A)
log2 (TPM+1)
Group
Normal
EAC
ESCC
HOXA1
HOXA2
HOXA3
HOXA4
HOXA5
HOXA6
HOXA7
HOXA9
HOXA10
HOXA11
HOXA13
(B)
BP
Inflammatory response
Signal transduction
Mitotic cell cycle
Apoptotic process
Endocytosis
Intracellular signal transduction
Protein ubiquitination
Count
–log10(p value)
(C)
CC
Cytosol
Membrane
Cytoplasm
Nucleoplasm
Nucleus
Endosome
Nuclear speck
Count
–log10(p value)
(D)
MF
Protein binding
Protein kinase C binding
RNA binding
Guanyl-nucleotide exchange factor activity
Cadherin binding
Calcium-dependent phospholipase A2 activity
Protein kinase binding
Count
–log10(p value)
(E)
Arachidonic acid metabolism
Th17 cell differentiation
Sphingolipid signaling pathway
Ras signaling pathway
ErbB signaling pathway
Rap1 signaling pathway
EGFR tyrosine kinase inhibitor resistance
p = –log10
Gene Count
(F)
Log2 (Macrophage expression)
Log2 (HOXA7 expression)
(G)
Log2 (Macrophage M1 expression)
Log2 (HOXA7 expression)
(H)
Log2 (Macrophage M2 expression)
Log2 (HOXA7 expression)

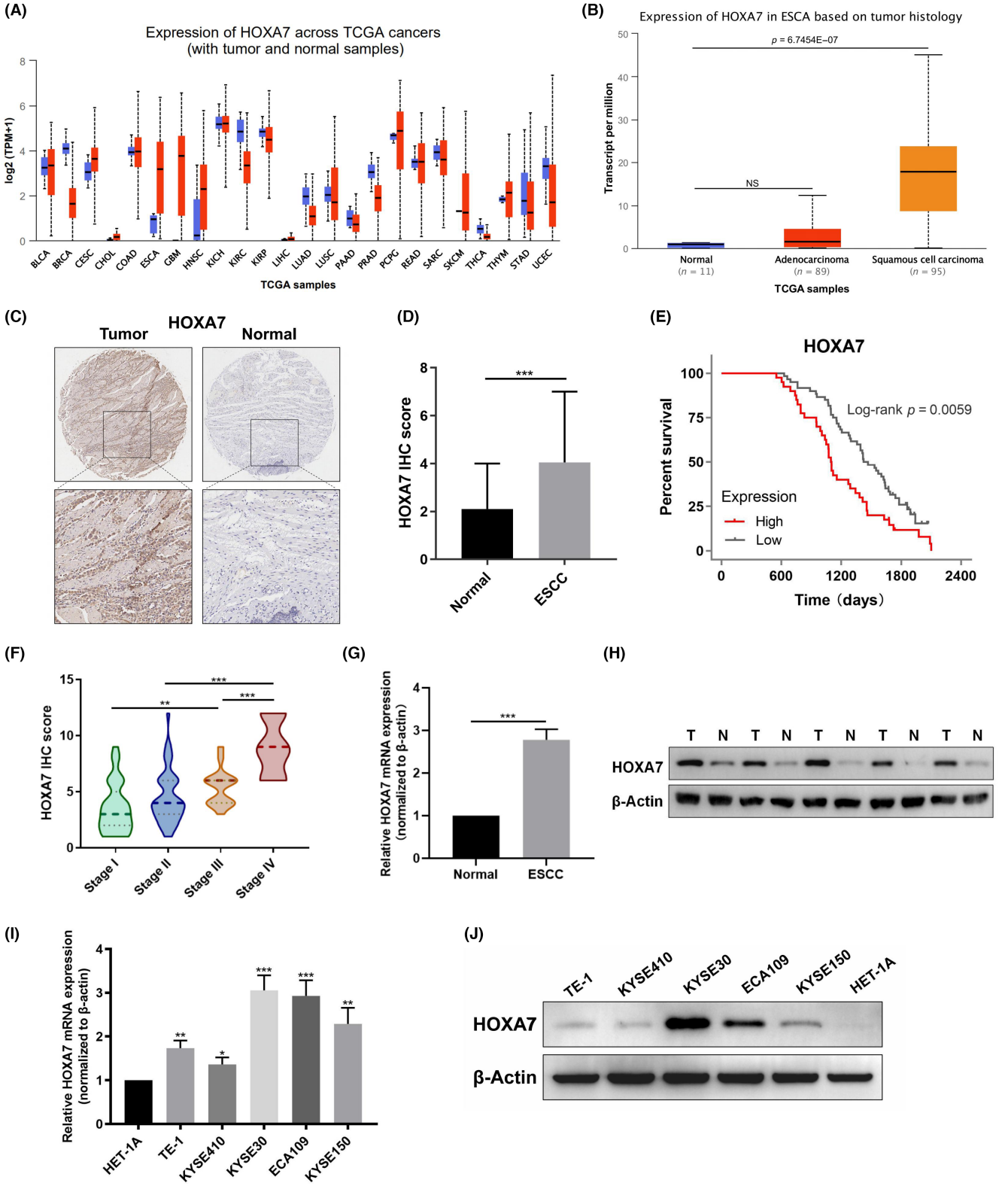


FIGURE 2 Homeobox A7 (HOXA7) expression correlates with poor prognosis. (A) Pan-cancer expression profiles of HOXA7 through The Cancer Genome Atlas (TCGA) database. (B) HOXA7 expression in TCGA-esophageal carcinoma (ESCA) based on histology type. (C) Representative immunohistochemical staining for HOXA7 in esophageal squamous cell carcinoma (ESCC) and adjacent nontumor tissues. (D) Quantification of (C). (E) Overall survival analysis plotted using Kaplan–Meier Plotter for patients with ESCC. (F) Analysis of correlation between the progression of ESCC and the expression of HOXA7. (G) HOXA7 expression level in ESCC tissues and adjacent nontumor tissues was detected by western blot. (H) HOXA7 expression level in ESCC tissues (T) and adjacent nontumor tissues (N) detected by real-time quantitative PCR. (I, J) HOXA7 gene and protein expression levels in ESCC cell lines and a normal esophageal epithelium cell line. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. ESCA, esophageal carcinoma; NS, not significant.

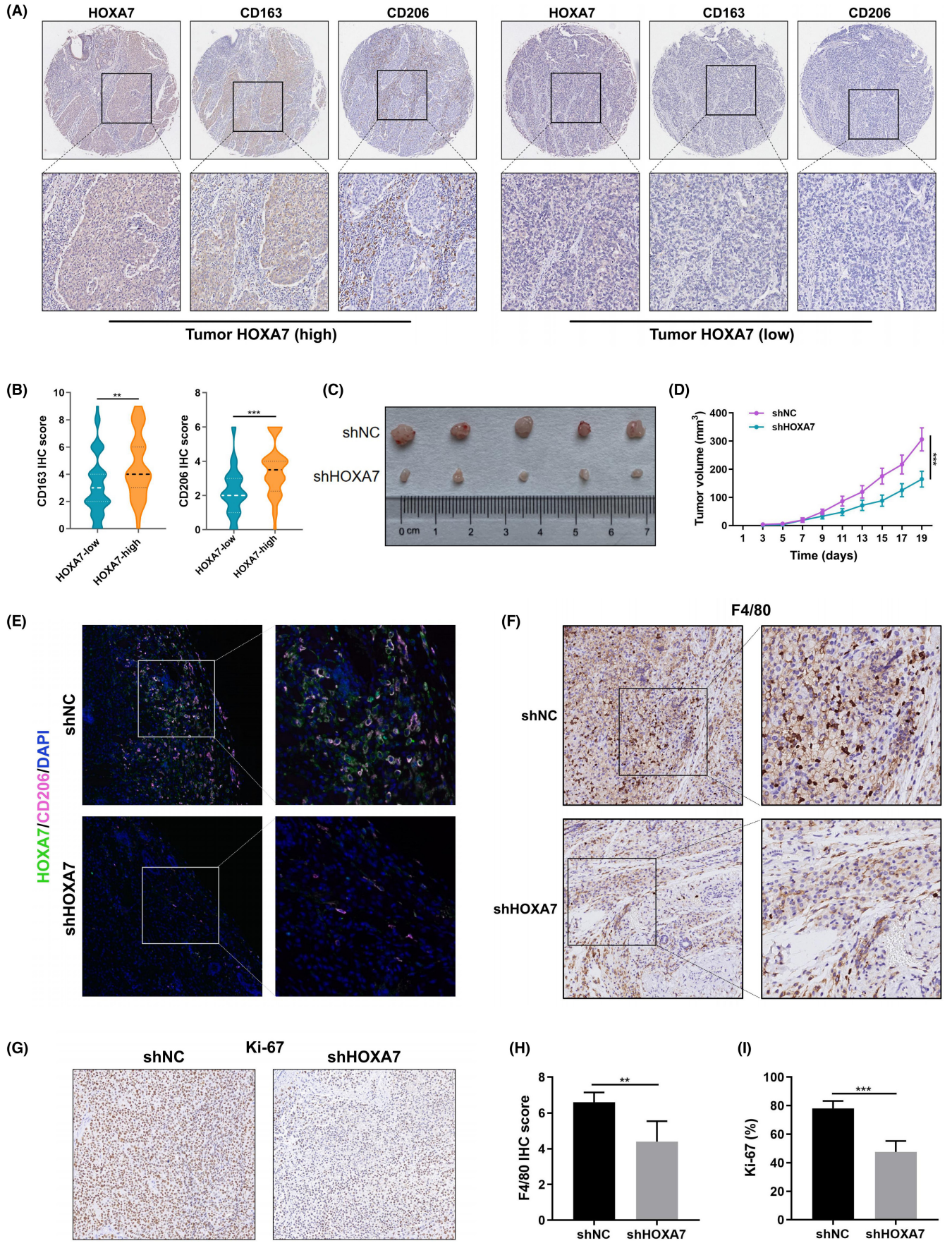


FIGURE 3 Legend on next page

FIGURE 3 Homeobox A7 (HOXA7) expression is correlated with macrophage infiltration in esophageal squamous cell carcinoma (ESCC) tissues and xenograft tumors. (A) Representative HOXA7, CD163, and CD206 immunohistochemistry (IHC) images in HOXA7 high and low groups in ESCC. (B) IHC staining scores of CD163 and CD206 in the HOXA7-high group ($n=41$) compared to the HOXA7-low group ($n=59$). (C) Image of subcutaneous tumors formed by ECA109/short hairpin RNA-negative control (shNC) and ECA109/shHOXA7 cells. (D) Growth curve of tumors described in (C). (E) Representative images of immunofluorescent staining with HOXA7 (green) and CD206 (red) in the shNC and shHOXA7 subcutaneous tumor tissues. (F, G) F4/80 and Ki-67 immunohistochemical staining of tumors described in (C). (H) Quantification of F4/80 staining in (E). (I) Quantification of Ki-67 staining in (F). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Medium without cells was cultivated under the same experimental conditions was prepared as a control.

2.5 | Clinical specimens

This study was approved by the institutional review board of Shanghai East Hospital, Tongji University. The human clinical sample was obtained from the Endoscopy Center of Shanghai East Hospital. All samples were obtained with the patients' informed consent, and the samples were processed with IHC staining for RNA extraction and western blotting.

2.6 | Animal studies

This in vivo study was approved by the animal care and use committee of Tongji University. BALB/c nude mice were purchased from Changzhou Cavens Laboratory Animal Co., Ltd and housed under standard pathogen-free conditions.

For in vivo tumor growth assay, 10 female BALB/c nude mice (6 weeks old) were used for animal studies. The animals were randomly divided into two groups (control and treated groups, five mice per group). A total number of 3×10^6 shHOXA7/shNC tumor cells were injected into the right flank of nude mice. After 4 weeks, mice were killed under anesthesia, and the samples were processed histologically.

For in vivo targeted inhibition experiments, 20 mice were randomly divided into five treatment groups (four per group): Control group (Ctrl), HOXA7 overexpression group (HOXA7-OE), Combination treatment group of HOXA7 overexpression and bindarit (CCL2 inhibitor) group (HOXA7 + bindarit), and Combination treatment group of HOXA7 overexpression and cetuximab (EGFR inhibitor) group (HOXA7 + cetuximab), and HOXA7-OE + bindarit + cetuximab (HOXA7 + B + C). A total number of 3 × 106 Ctrl/HOXA7-OE tumor cells were injected into the right flank of nude mice. When the average tumor size of the control group reached approximately 50 mm^3, the SPF (Specific Pathogen Free) grade 4-week-old female BALB/cA-nu mice were injected intraperitoneally with PBS (control), bindarit (100 mg/kg) or cetuximab (50 mg/kg) three times per week. Three weeks later, the mice were killed under anesthesia, and the tumor weight and volume were measured and calculated.

2.7 | Immunohistochemistry and IF

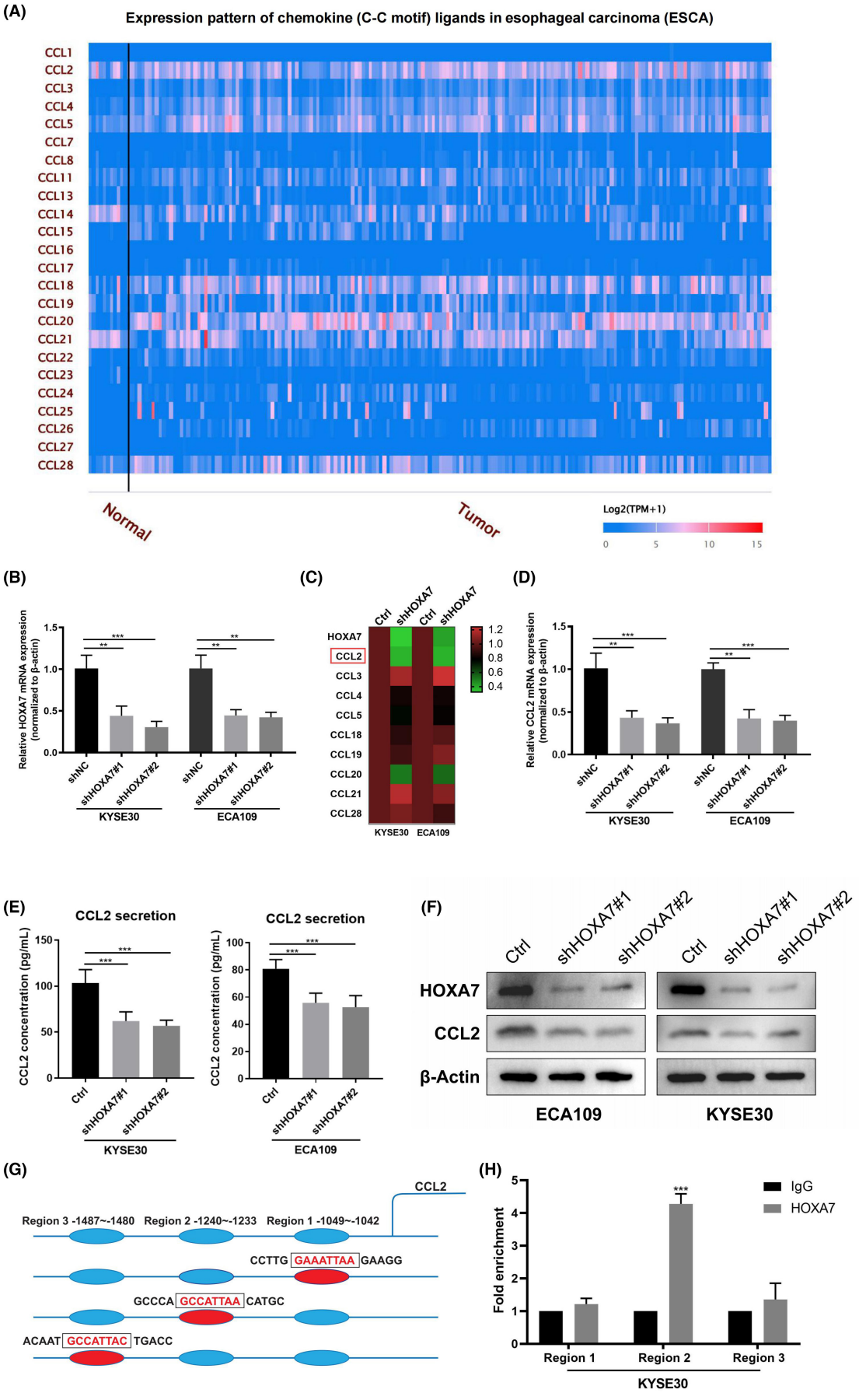
All paraffin-embedded material was sectioned at 4 μm. After dewaxing and hydration, the sections were incubated overnight with Abs against human HOXA7 (67112-1-Ig; Proteintech), human CD68 (sc-17,832; Santa Cruz), human CD163 (16646-1-AP; Proteintech), human CD206 (ab252921; Abcam), mouse F4/80 (ab6640; Abcam), mouse Ki-67 (ab1667; Abcam), and mouse CD206 (ab300621; Abcam). Primary Ab was detected with HRP-conjugated secondary Abs incubated for 8 min. Sections were washed in distilled water, counterstained with hematoxylin, dehydrated, and mounted. The whole tissue section was scored with staining intensity and percentage and the scoring scale was graded as follows: 0, no staining; 1, light brown staining; 2, brown staining; and 3, dark brown staining. The percentage of positive cells was divided into four levels: 1, <5%; 2, 5%–30%; 3, 31%–60%; and 4, 61%–100%. The IHC staining score was calculated as follows: intensity score × percentage score.

For IF, 5% BSA was used as the blocking buffer before incubation with primary Abs. We used DAPI (Invitrogen) to counterstain the tissues before mounting with fluorescent mounting medium (Dako).

2.8 | Virus construction and infection

The shRNA sequences targeting HOXA7 (shHOXA7#1 and shHOXA7#2) and lentivirus for HOXA7 overexpression was designed and constructed by Hanbio Technology Co. Ltd. Lentivirus was added to the culture medium of ESCC cells using polybrene (Hanbio Technology Co. Ltd) and puromycin (Hanbio Technology Co. Ltd) for screening out stable clones following the manufacturer's recommendations. The shHOXA7 sequences were as follows: CGGGC TTATACAATGTCAACA (shHOXA7#1) and CTTAAGAGACTCAC TGGTTT (shHOXA7#2).

FIGURE 4 Homeobox A7 (HOXA7) regulates the expression of chemokine (C-C motif) ligand 2 (CCL2). (A) Expression patterns of CCLs in esophageal carcinoma (ESCA) based on The Cancer Genome Atlas. (B) Real-time quantitative PCR (RT-qPCR) and western blot analysis of HOXA7 expression in esophageal squamous cell carcinoma cells after shRNA knockdown. (C) Expression patterns of screened CCLs after HOXA7 knockdown. (D) CCL2 expression detected by RT-qPCR after HOXA7 knockdown. (E) CCL2 secretion quantification detected by ELISA after HOXA7 knockdown. (F) HOXA7 and CCL2 expression in protein levels were measured by western blot after HOXA7 knockdown. (G) Schematic of the CCL2 promoter indicating the JASPAR-predicted binding site of HOXA7 (regions 1, 2, and 3). (H) ChIP-qPCR of the CCL2 promoter at the indicated regions in KYSE30 cells. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. shNC, short hairpin RNA-negative control.


(A)
Expression pattern of chemokine (C-C motif) ligands in esophageal carcinoma (ESCA)
CCL1
CCL2
CCL3
CCL4
CCL5
CCL7
CCL8
CCL11
CCL13
CCL14
CCL15
CCL16
CCL17
CCL18
CCL19
CCL20
CCL21
CCL22
CCL23
CCL24
CCL25
CCL26
CCL27
CCL28
Normal
Tumor
Log2(TPM+1)
0
5
10
15
(B)
Relative HOXA7 mRNA expression (normalized to β-actin)
shNC
shHOXA7#1
shHOXA7#2
KYSE30
ECA109
(C)
Ctrl
shHOXA7
HOXA7
CCL2
CCL3
CCL4
CCL5
CCL18
CCL19
CCL20
CCL21
CCL28
KYSE30
ECA109
(D)
Relative CCL2 mRNA expression (normalized to β-actin)
(E)
CCL2 secretion
CCL2 concentration (pg/mL)
Ctrl
shHOXA7#1
shHOXA7#2
KYSE30
ECA109
(F)
HOXA7
CCL2
β-Actin
(G)
CCL2
Region 3 -1487~-1480
Region 2 -1240~-1233
Region 1 -1049~-1042
CCTTG GAAATTAA GAAGG
GCCCA GCCATTAA CATGC
ACAAT GCCATTAC TGACC
(H)
Fold enrichment
IgG
HOXA7
Region 1
Region 2
Region 3
KYSE30

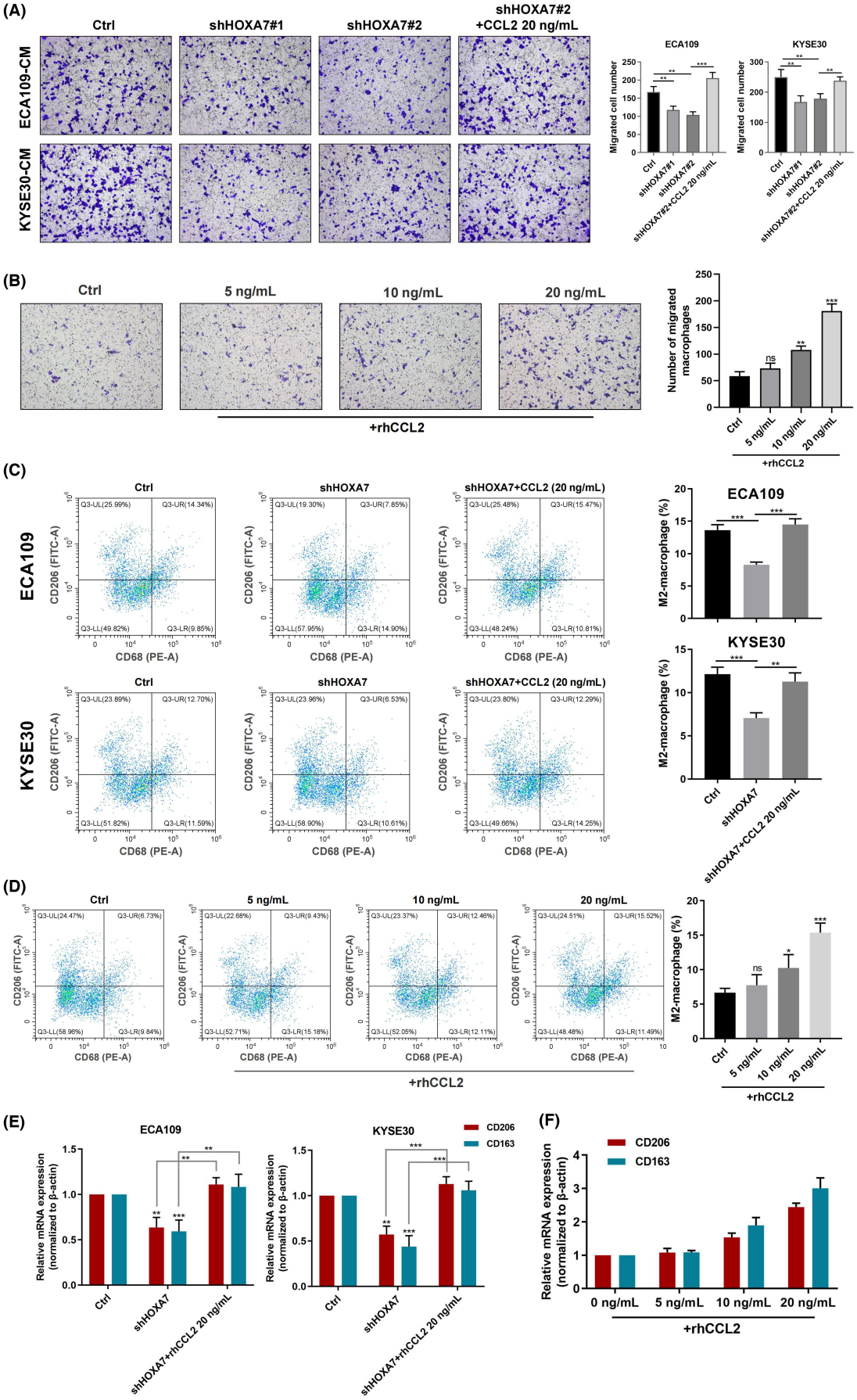

(A)
Ctrl
shHOXA7#1
shHOXA7#2
shHOXA7#2 +CCL2 20 ng/mL
ECA109-CM
KYSE30-CM
ECA109
KYSE30
Migrated cell number
(B)
Ctrl
5 ng/mL
10 ng/mL
20 ng/mL
+rhCCL2
Number of migrated macrophages
ns
(C)
Ctrl
shHOXA7
shHOXA7+CCL2 (20 ng/mL)
ECA109
KYSE30
CD206 (FITC-A)
CD68 (PE-A)
M2-macrophage (%)
(D)
5 ng/mL
10 ng/mL
20 ng/mL
+rhCCL2
(E)
ECA109
KYSE30
CD206
CD163
Relative mRNA expression (normalized to β-actin)
shHOXA7+rhCCL2 20 ng/mL
(F)
CD206
CD163
Relative mRNA expression (normalized to β-actin)
0 ng/mL
5 ng/mL
10 ng/mL
20 ng/mL
+rhCCL2

FIGURE 5 Homeobox A7 (HOXA7) induces tumor-associated macrophage infiltration and M2 polarization by promoting chemokine (C-C motif) ligand 2 (CCL2) secretion. (A) Transwell assay of PMA-induced M0 macrophages stimulated by conditioned medium (CM) from ECA109 and KYSE30 cells transfected with short hairpin RNA-negative control(shNC)/shHOXA7 or shHOXA7 together with CCL2 recombinant (20 ng/mL). (B) Transwell assay of M0 macrophages stimulated by different concentrations of CCL2 recombinant. (C-D) Flow cytometric analysis for CD68 and CD206 expression in macrophages stimulated by CM from shNC, shHOXA7 tumor cells, or CCL2 recombinant. (E-F) Relative expression of M2 macrophage markers CD206 and CD163 in M0 macrophages stimulated by tumor cells transfected with shNC and shHOXA7 or with rhCCL2 detected using real-time quantitative PCR. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. ns, not significant.

2.9 | RNA extraction and RT-qPCR

Total RNAs were extracted from cells by using TRIzol (Sigma-Aldrich) solution, according to the instructions of the manufacturer, quantified by using Nanodrop 2000 (Thermofisher), and stored at −80°C. RNA (1000 ng) was reverse transcribed into cDNA using a reverse transcription system (Takara). Real-time qPCR was carried out to quantify the transcripts using SYBR Green PCR Master Mix (Takara). The relative abundance of RNA was normalized to GAPDH. We used $2-(\Delta\Delta Ct)$ to calculate the relative abundance of mRNA. The relevant primers are summarized in Table S1.

2.10 | Western blot analysis

Cells were lysed in cold RIPA buffer containing protease inhibitors. Equal amounts of total protein were separated by SDS-PAGE (Shanghai Epizyme Biomedical Technology Co., Ltd), transferred to PVDF membranes (Millipore), and blocked in 5% milk. The primary Abs against HOXA7 (67112-1-Ig; Proteintech), CCL2 (ab214819; Abcam), and EGFR (ab52894; Abcam) were diluted according to the instructions. Membranes were incubated overnight with primary Abs at 4°C, followed by secondary Abs for 1 h at room temperature. Blots were developed using Millipore Immobilon Western Chemiluminescent HRP Substrate. β-Actin and GAPDH were used as loading controls.

2.11 | Enzyme-linked immunosorbent assay

Esophageal squamous cell carcinoma cells were seeded in 6-well plates at a density of 1×10^6 cells per well and incubated for 48 h. The supernatant was collected to detect the secretion of CCL2 and EGF. The CCL2 ELISA kit (RK00381) and EGF ELISA kit (RK00024HS) were purchased from Abclonal; ELISA was carried out as per the manufacturer's instructions.

2.12 | Flow cytometry

Cells were collected, washed, and incubated for 30 min at 4°C with florescence-conjugated Abs against CD68 (PE-65187; Proteintech) and CD206 (FITC-65155; Proteintech). To facilitate intracellular staining, cells were fixed and permeabilized with a fixation/permeabilization solution kit (BD Cytofix/Cytoperm) and 1% paraformaldehyde. The results were analyzed using the CytExpert 2.4 software program.

2.13 | In vitro proliferation assay

Cells were seeded in 96-well plates at 1000 cells/well. Cell proliferation was detected using a CCK-8 cell proliferation assay kit at 450 nm following the manufacturer's instructions.

2.14 | Chromatin immunoprecipitation qPCR

For ChIP assays, a ChIP kit (Active Motif) protocol was carried out. Esophageal squamous cell carcinoma cells were used for each immunoprecipitation. The protein and DNA were cross-linked with 1% formaldehyde, cleaved with SDS buffer, and cut by ultrasound. One HOXA7 Ab (sc-81,290; Santa Cruz) was used for immunoprecipitation. Next, ChIP RT-qPCR was carried out to validate the pulled samples. The ChIP-qPCR primer sequences are listed in Table S2.

2.15 | In vitro migration assay

Cells (5×10^4) were suspended in serum-free medium and added to the upper chamber of the 24-well insert (Corning). Complete medium or conditioned medium (500 μL) was added into the lower chamber as the chemical attractant. After incubation for 24 h at 37°C in an incubator, unmigrated cells on the top membrane were removed with cotton swabs. Cells that migrated were fixed in 4% paraformaldehyde for 20 min and stained with 1% crystal violet for 10 min. Cells were counted in five different fields using an inverted light microscope.

2.16 | Statistical analysis

All experiments were repeated at least three times, and the results are expressed as means ± SD. Student's *t*-test or the one-way ANOVA was used to analyze the data, and the χ^2-test was used to analyze differences in other variables, as appropriate. All statistical analyses were undertaken using GraphPad Prism software. A *p* value <0.05 was considered statistically significant for all datasets ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

3 | RESULTS

3.1 | Overexpressed HOXA7 significantly associated with TAM infiltration in ESCC

Bioinformatic analysis of HOXA family gene expression by histology type was carried out using TCGA[18] in ESCA, which includes ESCC and esophageal adenocarcinoma. As shown in Figure 1A, HOXA7 was the most significantly upregulated gene among the 11 HOXA family genes compared with normal esophagus tissue ($p = 1.1E\text{-}16$).

To determine the functions of dysregulated HOXA genes, a co-expression network of mRNA with dysregulated ($p < 0.05$) HOXA family genes was constructed, and functional annotation assay was performed on the coexpressed genes (Figure 1B–E). Biological

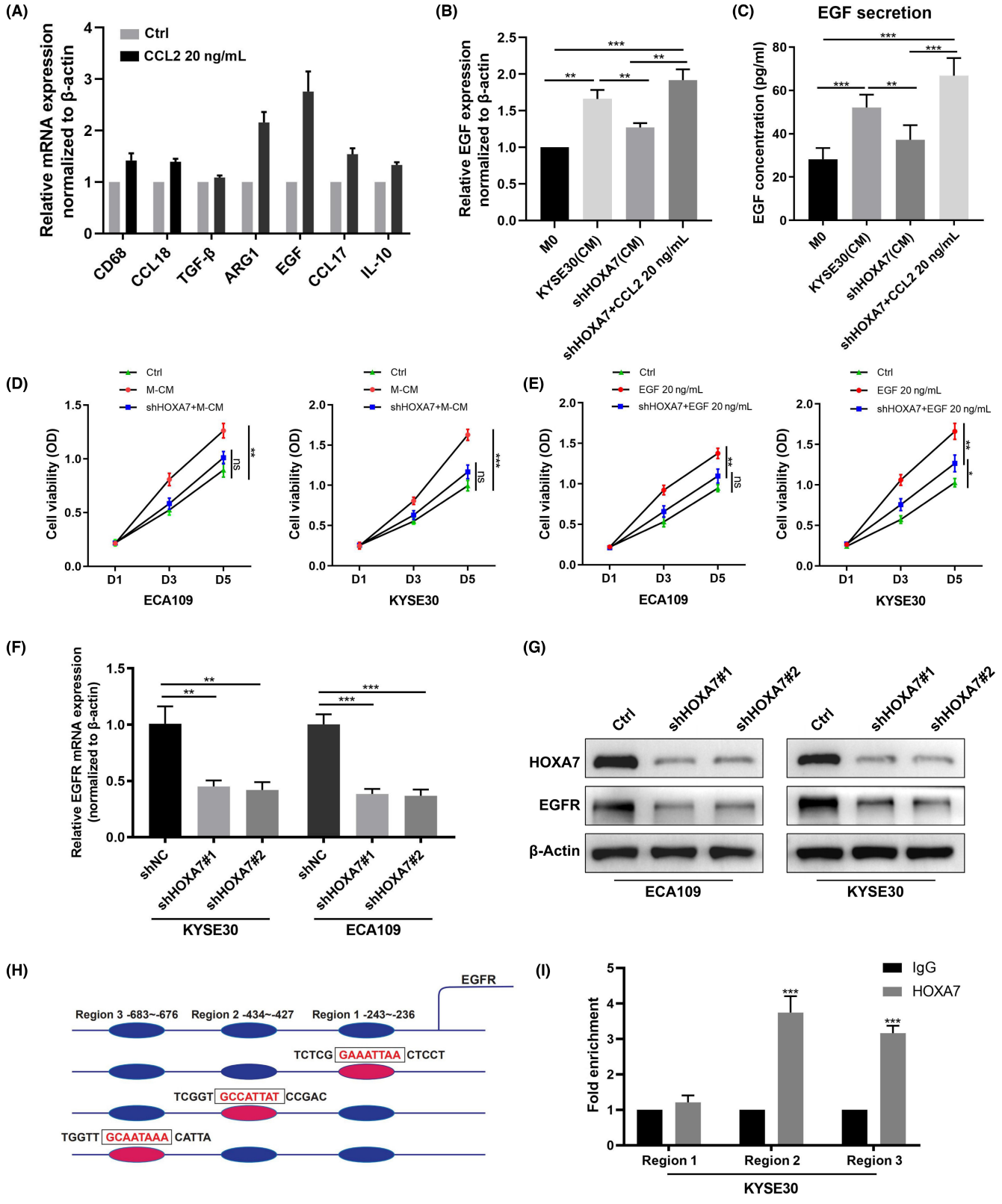


FIGURE 6 Homeobox A7 (HOXA7) modulates expression of epidermal growth factor receptor (EGFR). (A) Relative expression of M2 macrophage markers in M0 macrophages and M0 macrophages stimulated by chemokine (C-C motif) ligand 2 (CCL2) recombinant (20 ng/mL) detected using real-time quantitative PCR (qPCR). (B) Relative epidermal growth factor (EGF) expression in macrophages stimulated by shNC, shHOXA7, and rhCCL2. (C) Relative EGF concentration in macrophage medium stimulated by KYSE30 cells with addition of short hairpin RNA-negative control (shNC), shHOXA7, or rhCCL2. (D, E) CCK-8 proliferation assay of shNC or shHOXA7 cell lines stimulated by CCL2-treated conditioned medium of M0 macrophages (M-CM) or EGF recombinant (20 ng/mL). (F) Relative EGFR mRNA expression in esophageal squamous cell carcinoma cell lines after HOXA7 knockdown. (G) Western blot analysis indicating EGFR protein expression after HOXA7 knockdown. (H) Schematic of the EGFR promoter indicating the JASPAR-predicted binding site of HOXA7 (regions 1, 2, and 3). (I) ChIP-qPCR of the EGFR promoter at the indicated regions in KYSE30 cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ARG1, arginase 1; Ctrl, control; IL-10, interleukin-10; OD, optical density; TGF-β, transforming growth factor-β.

process analysis revealed significant enrichment in inflammatory response, and KEGG pathway enrichment analysis showed Ras signaling pathway, ErbB signaling pathway, and EGFR tyrosine kinase inhibitor resistance were remarkably correlated. These results indicated that dysregulated HOXA family genes are possibly playing crucial roles in the cross-talk of ESCC with TME.

To further elucidate the specific roles of HOXA genes in ESCC, the most significantly dysregulated HOXA7 was chosen to undertake the immune infiltration analysis. According to TIMER scoring system, HOXA7 expression was significantly associated with macrophage infiltration in ESCC (Figure 1F). Moreover, based on the Quantiseq scoring algorithm, we observed that HOXA7 was negatively correlated with tumor-suppressive M1 macrophages (Figure 1G) and was positively related to carcinogenic M2 macrophage infiltration (Figure 1H).

3.2 | Overexpression of HOXA7 in ESCC correlates with poor prognosis

Previous studies have reported that HOXA7 was overexpressed in numerous human malignancies, including glioma,[19] leukemia,[13] ovarian cancer,[20] and hepatocellular cancer.[10] According to gene expression profiles through TCGA, we found that HOXA7 was overexpressed in breast carcinoma, head and neck squamous cell carcinoma, and thymoma (Figure 2A). HOXA7 expression profiles based on histology type was screened out and the expression of HOXA7 was significantly overexpressed in ESCA, and even higher in ESCC (Figure 2B).

To further determine the overexpression of HOXA7 in ESCC, a TMA consisting of 100 paired ESCC tissue samples was analyzed by IHC. Notably, HOXA7 was clearly upregulated in ESCC tissues and IHC results showed that the average expression score of HOXA7 was significantly higher in ESCC tissues than in adjacent nontumor tissues (Figure 2C,D). Based on HOXA7 expression, patients were divided into HOXA7-high ($n = 41$) and HOXA7-low groups ($n = 59$; cut-off value = 6), and incomplete clinical and pathological data were excluded from the analysis. The relationship between clinicopathologic features and HOXA7 expression is shown in Table S3. Kaplan–Meier survival analysis showed that HOXA7-high patients had poorer clinical outcomes than HOXA7-low patients (log-rank $p = 0.0059$; Figure 2E). The correlation analysis between the progression (stage I–IV) of ESCC and the expression of HOXA7 was carried out, and results showed that HOXA7 expression elevated in consistent with ESCC tumor stages (Figure 2F). Then HOXA7 expression was examined and we consistently detected significantly higher expression levels of HOXA7 ESCC tissue samples than in paired normal ones (Figure 2G,H). In addition, the expression of HOXA7 in ESCC cell lines and normal esophagus epithelial cell line Het-1A was evaluated using RT-qPCR and western blotting. The results showed elevated expression of HOXA7 in ESCC cell lines (Figure 2I,J).

3.3 | HOXA7 overexpression correlated with macrophage infiltration in ESCC tissues and xenograft tumors

To analyze the correlation between HOXA7 and TAMs, IHC staining of HOXA7 and pan-macrophage markers CD163 and CD206 was carried out. Our results indicated that more $CD163^{+}$ and $CD206^{+}$ M2 macrophages infiltrated the tumor stroma in tumor tissues where HOXA7 was highly expressed, which indicated that HOXA7 might be capable of regulating the infiltration of M2 macrophages (Figure 3A,B).

To further investigate whether HOXA7 and macrophage infiltration is positively correlated in vivo, a nude mouse xenograft tumor model was established to investigate the effect of HOXA7 knockdown. The results showed that the tumors generated by shHOXA7 cells exhibited smaller volume and size (Figure 3C,D). Moreover, the tumor tissues were collected and analyzed with IHC and IF. In the shHOXA7 group, M2-TAM marker CD206 (Figure 3E) and murine oriented pan-macrophage marker F4/80 (Figure 3F,H) were remarkably decreased. Immunohistochemical staining of Ki-67 showed reduced numbers of proliferating cells in shHOXA7 tumors compared to shNC tumors (Figure 3G,I).

3.4 | HOXA7 regulates expression of CCL2

The C-C motifs are macrophage chemotactic factors that are crucial messengers mediating the cross-talk between tumor cells and TAMs, and play pivotal roles in the recruitment, polarization, and exertion of TAMs.[21] In order to explore whether HOXA7 can regulate macrophage chemotactic factors, expression levels of 24 chemokine CCLs in ESCA were analyzed according to the TCGA database (Figure 4A). Based the expression patterns of 24 CCLs in TCGA, nine CCL genes that turned out to be highly expressed in ESCA (*CCL2*, *3*,

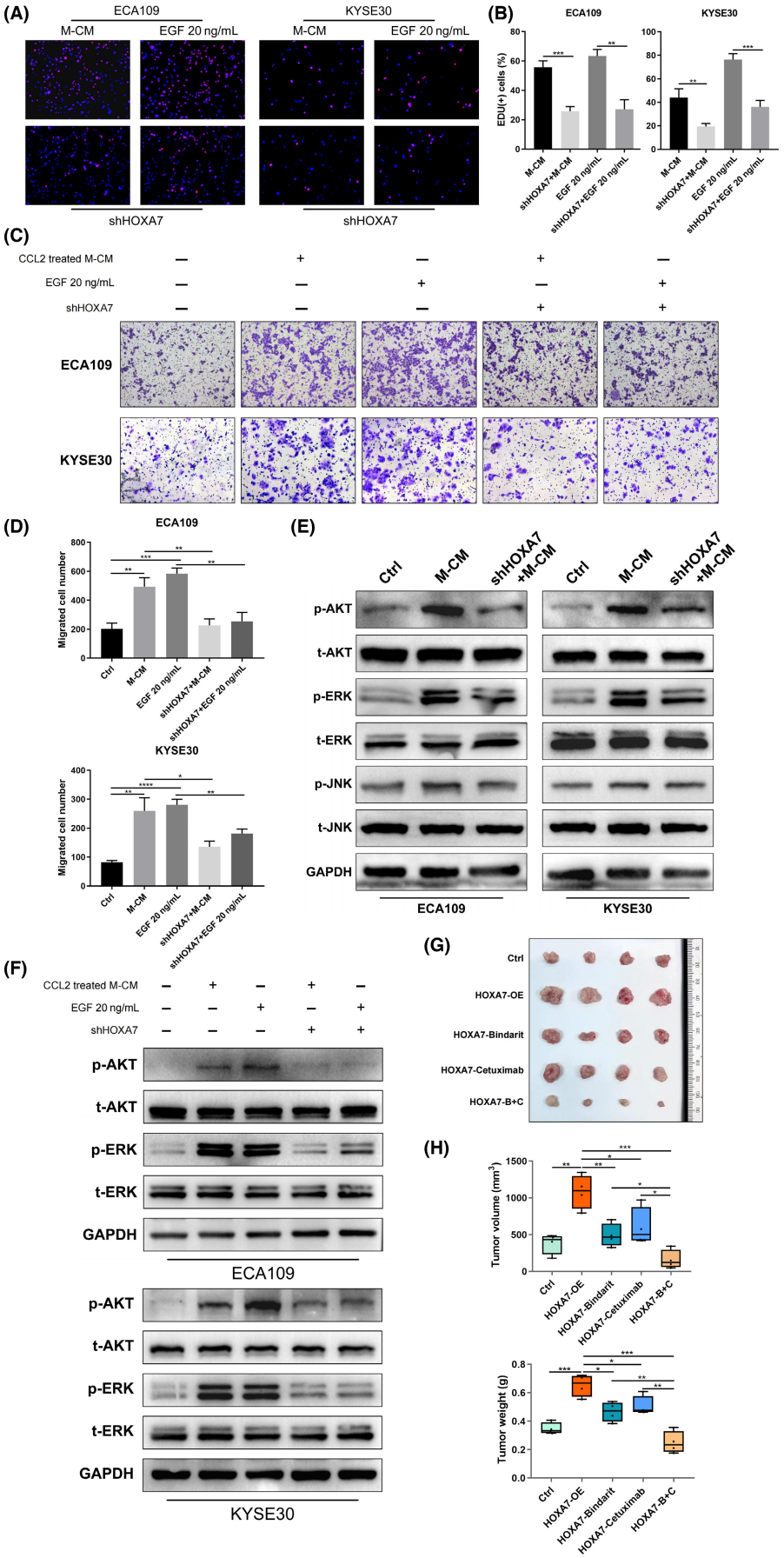

(A)
ECA109
KYSE30
M-CM
EGF 20 ng/mL
shHOXA7
(B)
ECA109
KYSE30
EDU(+) cells (%)
M-CM
shHOXA7+M-CM
EGF 20 ng/mL
shHOXA7+EGF 20 ng/mL
(C)
CCL2 treated M-CM
EGF 20 ng/mL
shHOXA7
ECA109
KYSE30
(D)
ECA109
KYSE30
Migrated cell number
Ctrl
M-CM
EGF 20 ng/mL
shHOXA7+M-CM
shHOXA7+EGF 20 ng/mL
(E)
Ctrl
M-CM
shHOXA7 +M-CM
p-AKT
t-AKT
p-ERK
t-ERK
p-JNK
t-JNK
GAPDH
ECA109
KYSE30
(F)
CCL2 treated M-CM
EGF 20 ng/mL
shHOXA7
p-AKT
t-AKT
p-ERK
t-ERK
GAPDH
ECA109
KYSE30
(G)
Ctrl
HOXA7-OE
HOXA7-Bindarit
HOXA7-Cetuximab
HOXA7-B+C
(H)
Tumor volume (mm3)
Tumor weight (g)
Ctrl
HOXA7-OE
HOXA7-Bindarit
HOXA7-Cetuximab
HOXA7-B+C

FIGURE 7 Homeobox A7 (HOXA7) modulates epidermal growth factor receptor (EGFR) to facilitate the chemokine (C-C motif) ligand 2 (CCL2)/epidermal growth factor (EGF)/EGFR axis. (A) Representative EdU immunofluorescence staining images of short hairpin RNA-negative control (shNC) or shHOXA7 tumor cells stimulated by CCL2 treated conditioned medium of M0 macrophages (M-CM) or recombinant EGF (20 ng/mL). (B) Quantification of (A). (C) Transwell migration assay on shNC or shHOXA7 tumor cells in response to CCL2 treated M-CM or recombinant EGF (20 ng/mL). (D) Quantification of (C). (E) Western blotting indicating the protein levels of AKT, p-AKT, ERK1/2, p-ERK1/2, JNK, and p-JNK expressions in shNC or shHOXA7 tumor cells in response to CCL2 treated M-CM. (F) Protein levels of AKT, p-AKT, ERK1/2, p-ERK1/2, JNK, and p-JNK expressions in response to CCL2 treated M-CM or EGF in shNC or shHOXA7 cells were measured by western blot. (G) Image of subcutaneous tumors formed by ECA109/control (Ctrl) and ECA109/HOXA7-overexpression (OE) cells together with treatment with bindarit (B; CCL2 inhibitor) and cetuximab (C; EGFR inhibitor). (H) Statistical analysis of tumor volume (mm^3) and weight (g). $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

4, *5*, *18*, *19*, *20*, *21*, and *28*) were taken into consideration. The knockdown efficiency of shHOXA7#1 and shHOXA7#2 was confirmed by RT-qPCR (Figure 4B). Our results showed that depletion of HOXA7 consistently resulted in reduction in CCL2 mRNA levels in ESCC cell lines, which was the most significant among the nine selected CCLs (Figure 4C,D). This phenomenon was further confirmed by ELISA and western blot (Figure 4E,F). To determine whether the direct regulation of CCL2 by HOXA7 is a possible regulation pathway, we assessed the binding of HOXA7 to the CCL2 promoter, which was predicted to contain three putative binding sites at region 1 (−239 to−232), region 2 (−1240 to−1233), and region 3 (−1487 to−1480) in the JASPAR database (Figure 4G). Analysis with ChIP-qPCR showed that HOXA7 binds directly to region 2 (Figure 4H). Collectively, our data reveal that HOXA7 functions as a key transcriptional regulator of CCL2.

3.5 | HOXA7 induces TAM infiltration and M2 polarization by promoting CCL2 secretion

We established a coculture system with ESCC tumor cells and M0 macrophages. To verify the hypothesis that HOXA7 is responsible for TAM infiltration, Transwell migration assays of tumor-stimulated M0 macrophages were carried out. The results showed that the number of migrated macrophages remarkably decreased after 48 h of incubation of shHOXA7 tumor CM compared with the control group, whereas migration of macrophages could be recovered after pretreatment of recombinant rhCCL2 protein (Figure 5A). We subsequently used recombinant rhCCL2 protein to determine its function in inducing M0 macrophage migration and found that CCL2 could promote macrophage infiltration in a dose-dependent way (Figure 5B).

Similarly, M0 macrophages were incubated with CM collected from different ESCC cell lines, and the expression of TAM polarization markers was analyzed by flow cytometry. Results showed that the percentage of M2 macrophages ($CD206^+/CD68^+$) was decreased when M0 macrophages were cultured with shHOXA7 tumor CM. Moreover, the ability to induce M2 polarization was restored when M0 macrophages were treated with shHOXA7-CM together with addition of rhCCL2 (Figure 5C).

C-C motif chemokine ligand 2 has previously been reported to be capable of inducing macrophage recruitment and polarization,[22] and we showed that HOXA7 directly regulates the expression of CCL2. Additionally, HOXA7 exerts the function to induce M0 macrophage polarization toward M2 type. Hence, we hypothesized that HOXA7 induces macrophage recruitment and M2-like polarization by promoting CCL2 secretion. Furthermore, M0 macrophages were treated with recombinant CCL2 in different concentrations. The results of flow cytometry confirmed that CCL2 induced M2-like macrophage polarization in a dose-dependent manner (Figure 5D).

In addition, M2 macrophage markers CD206 and CD163 (another M2 macrophage-specific marker) were analyzed by RT-qPCR. The results showed that shHOXA7 tumor cells induced evidently lower expression levels of CD163 and CD206 (Figure 5E). In contrast, elevated levels of CD206 and CD163 were observed when macrophages were treated with rhCCL2 (Figure 5E,F).

3.6 | Macrophage secretion of EGF induced by CCL2 promotes ESCC tumor growth

M2 macrophages secrete tumor-promoting factors including Arg1, TGF-β, vascular endothelial growth factor, and EGF, which are stimulative in ESCC progression.[23] Real-time qPCR was undertaken in M0, Ctrl, and shHOXA7 tumor cells and CCL2-induced M0 macrophages to detect the expression of molecules that were secreted by macrophages. The results of RT-qPCR and ELISA showed that the expression and secretion of EGF was most significantly upregulated by CCL2 (Figure 6A), suggesting that EGF is a key macrophage-secreted factor that regulates tumor cell proliferation. This result coordinates with previous study which demonstrated that CCL2 not only drives the chemotaxis of macrophages but also stimulates macrophage expression of EGF.[24] RT-qPCR and ELISA showed shHOXA7 conditioned medium from tumor cells significantly reduced EGF secretion but can be restored after addition of CCL2 (Figure 6B,C).

To explore the tumor-promoting effects of TAMs, M-CM stimulated by CCL2 was collected to stimulate ESCC tumor cells. The CCK-8 cell proliferation assay was carried out on macrophage stimulated cells and nontreated ones. The results showed that M-CM significantly contributed to tumor cell proliferation. Nevertheless, we discovered that the growth-enhancing function of M-CM could be evidently abrogated by shHOXA7 (Figure 6D), and similarly, shHOXA7 attenuated the growth-stimulative functions of EGF on ESCC cells (Figure 6E).

3.7 | HOXA7 modulates EGFR to facilitate the CCL2/EGF/EGFR axis through MAPK/ERK1/2 and PI3K/AKT pathways

Previous studies have shown that HOXA7 increases cell proliferation by upregulating EGFR expression in human granulosa cells.[25] Given the research above, we suppose that HOXA7 is a key transcription factor to facilitate TAM and tumor cell interaction through regulating the CCL2/EGF/EGFR axis. To verify the function of shHOXA7 in abrogating macrophage stimulation by modulating EGFR, the expression level of EGFR in ESCC cells in shHOXA7 and shNC cells was detected at both mRNA and protein levels. Knockdown of HOXA7 in ESCC cells significantly reduced EGFR expression (Figure 6F,G). The ChIP-qPCR analysis was undertaken to identify the direct combination of HOXA7 on the EGFR promoting region and three putative binding sites at region 1 (−243 to −236), region 2 (−434 to −427), and region 3 (−683 to −676) were predicted by the JASPAR database (Figure 6H). The ChIP-qPCR analysis showed that HOXA7 binds directly to regions 2 and 3 (Figure 6I). This result validated that EGFR was upregulated by HOXA7 to facilitate the CCL2/EGF/EGFR axis in order to promote tumor–macrophage interaction and tumor growth in ESCC.

To further confirm that M-CM and EGF stimulate ESCC cell activation, EdU proliferation staining and Transwell migration assay were carried out. Compared with controls, shHOXA7 notably attenuated ESCC cell proliferation stimulated by M-CM and EGF (Figure 7A,B), suggesting that HOXA7 is a key regulator of TAM–ESCC cross-talk in the CCL2/EGF/EGFR axis. Moreover, after treating with CM from CCL2-stimulated macrophages and EGF, the migration rate of KYSE30 and ECA109 cells was remarkably elevated compared with the control group, while the migration rate of HOXA7 knockdown cells was significantly attenuated (Figure 7C,D).

Previous studies reported that EGF derived from M2 macrophages facilitates cancer cell proliferation through EGFR- (specific receptor of EGF) mediated MAPK/ERK and PI3K/Akt activation.[26,27] Western blot analysis showed that M-CM and EGF stimulated the phosphorylation of Akt and ERK1/2, while shHOXA7 abrogated the impact and significantly reduced phosphorylation levels of ERK1/2 and Akt (Figure 7E). However, the JNK signaling pathway did not exert significant changes in phosphorylation after M-CM stimulation or HOXA7 knockdown (Figure 7F), indicating that MAPK/ERK and PI3K/Akt are dominating pathways taking part in growth-enhancing functions stimulated by M-CM and EGF, and HOXA7 is the key regulator of the CCL2/EGF/EGFR axis in TAM–ESCC cross-talk.

To further investigate the cross-talk of ESCC cells and TAMs in vivo, HOXA7 was overexpressed and HOXA7 and EGFR mRNA expression in transplanted tumors was analyzed by RT-qPCR (Figure S1A). The biological functions of HOXA7-OE tumor cells were also assessed (Figure S1B–D). Bindarit (CCL2 inhibitor) and cetuximab (EGFR inhibitor) was applied for in vivo targeted inhibition. As shown in Figure 7G,H, tumor weight and volume were significantly increased in the HOXA7-OE group but could be reduced through treatment with bindarit or cetuximab. Combined treatment with bindarit and cetuximab showed a significantly synergistic effect on tumor weight and volume. Moreover, M2 macrophage infiltration was analyzed by CD206 immunofluorescence (Figure S1E).

4 | DISCUSSION

The family of homeodomain-containing transcription factors are known to be key regulators of embryonic development.[28] Moreover, the HOXA family has been reported to be associated with TME and the cross-talk with tumor cells.[9,29] However, the functions of HOXA genes in ESCC remains largely unknown. Here we report that *HOXA7* was the most significant dysregulated HOXA gene in ESCC and was correlated with TAM infiltration. In further experiments, we verified that HOXA7 is frequently upregulated in ESCC tissues compared with adjacent nontumor counterparts and provided the first evidence that HOXA7 expression is negatively correlated with prognosis of ESCC patients, as HOXA7 expression is significantly higher in the advanced ESCC than in the early stages. These results suggest that HOXA7 could possibly participate in ESCC carcinogenesis.

The results of IHC staining also verified our bioinformatic analysis as the M2-macrophage markers CD163 and CD206 were positively correlated with HOXA7 in expression levels. Tumor-associated macrophages are myeloid-derived immune suppressive cells, and contribute to tumor development and metastasis in various ways.[30] Tumor-associated macrophages exert immunosuppressive effects on tumor-infiltrating lymphocytes and secrete anti-inflammatory cytokines and chemokines including Arg1,[31] interleukin-10,[32] and TGF-β,[33] which strongly contribute to tumor proliferation and migration. HOXA7 has been reported to be responsible for upregulating chemokines to draw myeloid-derived immune suppressive cells into the TME.[29] Consistently, our results found that HOXA7 recruits TAMs through upregulation of CCL2, which has been reported to shape macrophage polarization under the influence of macrophage colony-stimulating factor[34] and promotes TAM recruitment through its receptor CCR2.[35] Our results showed that knockdown of HOXA7 significantly attenuated CCL2 secretion instead of other CCLs, thus inhibiting TAM infiltration and polarization. These findings underscore the importance of the HOXA7-CCL2 axis in interactions between ESCC cells and TAMs.

It has been reported that CCL2 derived from keratinocytes triggers infiltrated macrophage expression of EGF, which in turn activates basal epidermal keratinocyte proliferation.[24] Additionally, CCL2/EGF formed a positive feedback loop between head and neck squamous cell carcinoma and macrophages.[36] Nevertheless, it is still unclear whether ESCC cell-derived CCL2 can stimulate TAM secretion of EGF. In this study, we first showed CCL2 played facilitative roles in triggering TAM production of EGF, and in turn accelerated ESCC progression. However, surprisingly, we discovered that knockdown of HOXA7 also attenuated tumor cell proliferation activated by EGF. A previous study confirmed that HOXA7 upregulates EGFR in human granulosa cells,[25] suggesting

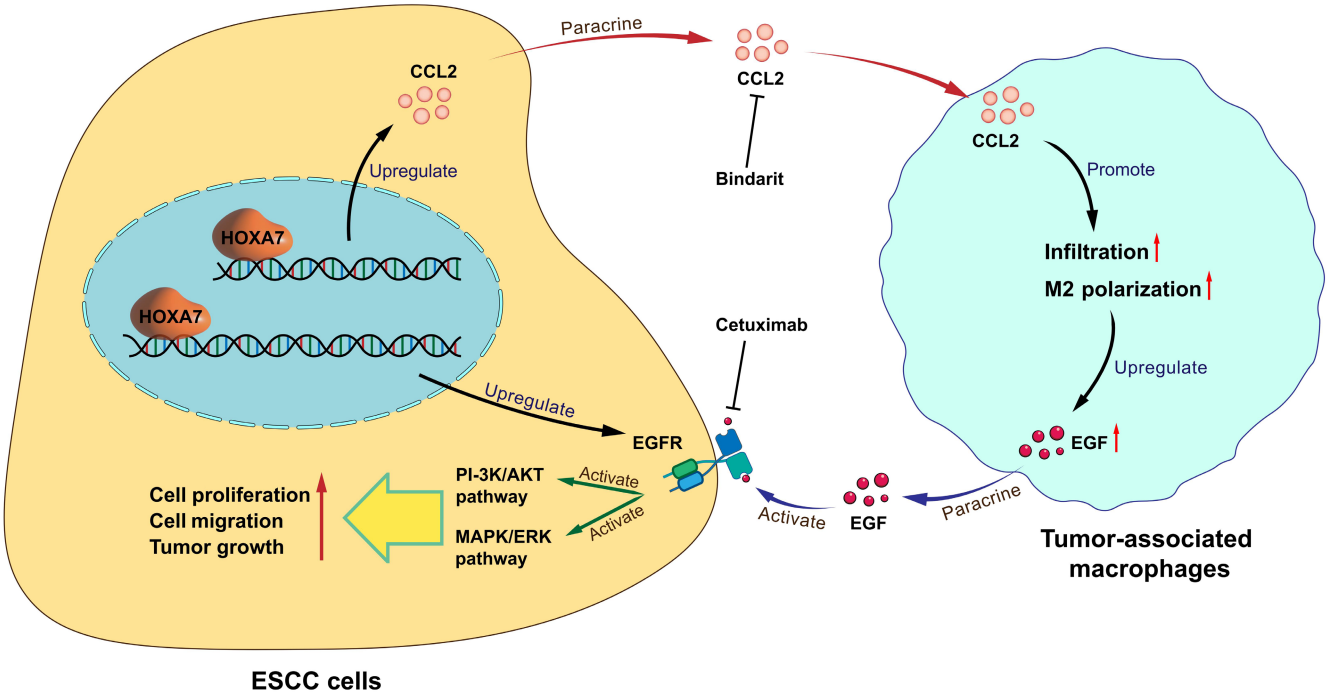


FIGURE 8 Schema summarizing the research results of intercellular signaling between esophageal squamous cell carcinoma (ESCC) cells and tumor-associated macrophages. CCL2, chemokine (C-C motif) ligand 2; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HOXA7, homeobox A7.

that EGFR is a downstream effector of HOXA7. In agreement with the aforementioned findings, we identified that HOXA7 directly transcriptionally controls EGFR in ESCC cells, thereby shaping a HOXA7/CCL2/EGF/EGFR feedback loop. Intracellular signaling plays predominant roles in regulating tumor malignant features.[37] In ESCC, EGF-induced activation of PI3K/AKT and ERK1/2 pathways was well elucidated.[38,39] Our results are consistent with the findings of previous studies, demonstrating that these intercellular signaling pathways were under the regulation of HOXA7 both in vitro and in vivo. Taken together, our data indicate that the HOXA7/CCL2/EGF/EGFR feedback loop mediates reciprocal interactions between ESCC cells and TAMs (Figure 8), providing a promising therapeutic target for ESCC patients.

Our research found that HOXA7 transcriptionally activates CCL2, which was described as a member of the CCL family, and was reported to induce TAM infiltration and polarization.[22] Meanwhile, CCL2 was also reported to exert essential functions on tumor-activated neutrophils[40] and regulatory T cells.[41] Thus, future investigations would be interesting to identify whether HOXA7/CCL2/EGF/EGFR causes other cancer features in ESCC, such as chemoresistance, autophagy, and immune escape, which are of great research value. As EGFR inhibitors have not been certified for the treatment of ESCC based on the results of several clinical trials, we hope our study could provide evidence for further clinical research.

AUTHOR CONTRIBUTIONS

M.-D.X. and T.C. conceived and designed the study. A.-Q.F., L.-N.H., and J.-K.J conducted most of the experiments. Y.C., Z.-H.Z, K.F, J.-N.S., Z.-X.L., and Z.-Y.Z. helped in some experiments. A.-Q.F. and L.-N.H. wrote the original draft of the manuscript. M.-D.X., T.C., and L.-N.H. contributed to draft revising and funding. L.Z., A.-Q.F., and L.-N.H. contributed to sample collection and data analysis. J.-K.J., Z.-Y.W., Z.-X.L., and M.-C.S. participated in methodology supervision and draft revising. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank Dr. Guohan Chen (Shanghai East Hospital, Tongji University School of Medicine, Shanghai, China) for assistance with sample collection.

FUNDING INFORMATION

This work was supported by Shanghai Committee of Science and Technology (20XD1402900, 21XD1423100, 21JC1405200, and 18140900100), the Top-level Clinical Discipline Project of Shanghai Pudong (PWYgf2021-2), National Natural Science Foundation of China (Nos.82203341), and Changzhou Medical Center, Nanjing Medical University (CMCB202218).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

ETHICS STATEMENTS

Approval of the research protocol by an institutional review board: All procedures followed were in accordance with the ethical standards of the institutional review board of Tongji University School of Medicine with the Declaration of Helsinki 1964 and later versions.

Informed consent: All human samples were obtained with the patients' written informed consent.

Registry and registration no. of the study/trial: N/A.

Animal studies: All institutional and national guidelines for the care and use of laboratory animals were followed.

ORCID

Anqi Feng https://orcid.org/0000-0002-9306-0189
Lingnan He https://orcid.org/0000-0002-0760-9789

REFERENCES

1. Smyth EC, Lagergren J, Fitzgerald RC, et al. Oesophageal cancer. *Nat Rev Dis Primers*. 2017;27(3):17048. doi:10.1038/nrdp.2017.48
2. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209-249. doi:10.3322/caac.21660
3. Arnold M, Soerjomataram I, Ferlay J, Forman D. Global incidence of oesophageal cancer by histological subtype in 2012. *Gut*. 2015;64(3):381-387. doi:10.1136/gutjnl-2014-308124
4. Yagi T, Baba Y, Okadome K, et al. Tumour-associated macrophages are associated with poor prognosis and programmed death ligand 1 expression in oesophageal cancer. *Eur J Cancer*. 2019;111:38-49. doi:10.1016/j.ejca.2019.01.018
5. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol*. 2017;14(7):399-416. doi:10.1038/nrclinonc.2016.217
6. Hart CP, Awgulewitsch A, Fainsod A, McGinnis W, Ruddle FH. Homeo box gene complex on mouse chromosome 11: molecular cloning, expression in embryogenesis, and homology to a human homeo box locus. *Cell*. 1985;43(1):9-18. doi:10.1016/0092-8674(85)90007-8
7. Bhatlekar S, Fields JZ, Boman BM. HOX genes and their role in the development of human cancers. *J Mol Med (Berl)*. 2014;92(8):811-823. doi:10.1007/s00109-014-1181-y
8. Zhao F, Tian H, Liu X, et al. Homeobox A1 facilitates immune escape and alleviates oxidative stress in lung adenocarcinoma. *Oxid Med Cell Longev*. 2022;2022:4102666. doi:10.1155/2022/4102666
9. Zhong X, Prinz A, Steger J, et al. HoxA9 transforms murine myeloid cells by a feedback loop driving expression of key oncogenes and cell cycle control genes. *Blood Adv*. 2018;2(22):3137-3148. doi:10.1182/bloodadvances.2018025866
10. Tang B, Qi G, Sun X, et al. HOXA7 plays a critical role in metastasis of liver cancer associated with activation of snail. *Mol Cancer*. 2016;15(1):57. doi:10.1186/s12943-016-0540-4
11. Pao JB, Lu TL, Ting WC, Chen LM, Bao BY. Association of Genetic Variants of small non-coding RNAs with survival in colorectal cancer. *Int J Med Sci*. 2018;15(3):217-222. doi:10.7150/ijms.22402
12. Zhang Y, Cheng JC, Huang HF, Leung PC. Homeobox A7 stimulates breast cancer cell proliferation by up-regulating estrogen receptor-alpha. *Biochem Biophys Res Commun*. 2013;440(4):652-657. doi:10.1016/j.bbrc.2013.09.121
13. Eklund EA. The role of HOX genes in malignant myeloid disease. *Curr Opin Hematol*. 2007;14(2):85-89. doi:10.1097/MOH.0b013e32801684b6
14. Chen KN, Gu ZD, Ke Y, Li JY, Shi XT, Xu GW. Expression of 11 HOX genes is dysregulated in esophageal squamous cell carcinoma. *Clin Cancer Res*. 2005;11(3):1044-1049.
15. Dennis G Jr, Sherman BT, Hosack DA, et al. DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol*. 2003;4(5):P3.
16. Li T, Fan J, Wang B, et al. TIMER: a web server for comprehensive analysis of tumor-infiltrating immune cells. *Cancer Res*. 2017;77(21):e108-e110. doi:10.1158/0008-5472
17. Finotello F, Mayer C, Plattner C, et al. Molecular and pharmacological modulators of the tumor immune contexture revealed by deconvolution of RNA-seq data. *Genome Med*. 2019;11(1):34. doi:10.1186/s13073-019-0638-6
18. PCAWG Transcriptome Core Group, Calabrese C, Davidson NR, et al. PCAWG consortium. Genomic basis for RNA alterations in cancer. *Nature*. 2020;578(7793):129-136. doi:10.1038/s41586-020-1970-0
19. Xiulin J, Wang C, Guo J, Wang C, Pan C, Nie Z. Next-generation sequencing identifies HOXA6 as a novel oncogenic gene in low grade glioma. *Aging (Albany NY)*. 2022;14(6):2819-2854. doi:10.18632/aging.203977
20. Ota T, Choi KB, Gilks CB, Leung PC, Auersperg N. Cell type- and stage-specific changes in HOXA7 protein expression in human ovarian folliculogenesis: possible role of GDF-9. *Differentiation*. 2006;74(1):1-10. doi:10.1111/j.1432-0436.2006.00053.x
21. Qin R, Ren W, Ya G, et al. Role of chemokines in the crosstalk between tumor and tumor-associated macrophages. *Clin Exp Med*. 2022. doi:10.1007/s10238-022-00888-z. Online ahead of print.
22. Li B, Qi ZP, He DL, et al. NLRP7 deubiquitination by USP10 promotes tumor progression and tumor-associated macrophage polarization in colorectal cancer. *J Exp Clin Cancer Res*. 2021;40(1):126. doi:10.1186/s13046-021-01920-y
23. Li J, Shan F, Xiong G, et al. EGF-induced C/EBPβ participates in EMT by decreasing the expression of miR-203 in esophageal squamous cell carcinoma cells. *J Cell Sci*. 2014;127(Pt 17):3735-3744. doi:10.1242/jcs.148759
24. Villarreal-Ponce A, Tiruneh MW, Lee J, et al. Keratinocyte-macrophage crosstalk by the Nrf2/Ccl2/EGF signaling Axis orchestrates tissue repair. *Cell Rep*. 2020;33(8):108417. doi:10.1016/j.celrep.2020.108417
25. Zhang Y, Huang Q, Cheng JC, et al. Homeobox A7 increases cell proliferation by up-regulation of epidermal growth factor receptor expression in human granulosa cells. *Reprod Biol Endocrinol*. 2010;14(8):61. doi:10.1186/1477-7827-8-61
26. Zeng XY, Xie H, Yuan J, et al. M2-like tumor-associated macrophages-secreted EGF promotes epithelial ovarian cancer metastasis via activating EGFR-ERK signaling and suppressing lncRNA LIMT expression. *Cancer Biol Ther*. 2019;20(7):956-966. doi:10.1080/15384047.2018.1564567
27. Nabeshima A, Matsumoto Y, Fukushi J, et al. Tumour-associated macrophages correlate with poor prognosis in myxoid liposarcoma and promote cell motility and invasion via the HB-EGF-EGFR-PI3K/Akt pathways. *Br J Cancer*. 2015;112(3):547-555. doi:10.1038/bjc.2014.637
28. Gehring WJ, Hiromi Y. Homeotic genes and the homeobox. *Annu Rev Genet*. 1986;20:147-173. doi:10.1146/annurev.ge.20.120186.001051
29. Dang Y, Yu J, Zhao S, Cao X, Wang Q. HOXA7 promotes the metastasis of KRAS mutant colorectal cancer by regulating myeloid-derived suppressor cells. *Cancer Cell Int*. 2022;22(1):88. doi:10.1186/s12935-022-02519-9
30. Nakamura K, Smyth MJ. Myeloid immunosuppression and immune checkpoints in the tumor microenvironment. *Cell Mol Immunol*. 2020;17(1):1-12. doi:10.1038/s41423-019-0306-1
31. Arlauckas SP, Garren SB, Garris CS, et al. Arg1 expression defines immunosuppressive subsets of tumor-associated macrophages. *Theranostics*. 2018;8(21):5842-5854. doi:10.7150/thno.26888
32. Kim TH, Yang K, Kim M, Kim HS, Kang JL. Apoptosis inhibitor of macrophage (AIM) contributes to IL-10-induced anti-inflammatory response through inhibition of inflammasome activation. *Cell Death Dis*. 2021;12(1):19. doi:10.1038/s41419-020-03332-w
33. Batlle E, Massagué J. Transforming growth factor-β signaling in immunity and cancer. *Immunity*. 2019;50(4):924-940. doi:10.1016/j.immuni.2019.03.024

34. Sierra-Filardi E, Nieto C, Domínguez-Soto A, et al. CCL2 shapes macrophage polarization by GM-CSF and M-CSF: identification of CCL2/CCR2-dependent gene expression profile. *J Immunol*. 2014;192(8):3858-3867. doi:10.4049/jimmunol.1302821
35. Yang H, Zhang Q, Xu M, et al. CCL2-CCR2 axis recruits tumor associated macrophages to induce immune evasion through PD-1 signaling in esophageal carcinogenesis. *Mol Cancer*. 2020;19(1):41. doi:10.1186/s12943-020-01165-x
36. Gao L, Wang FQ, Li HM, et al. CCL2/EGF positive feedback loop between cancer cells and macrophages promotes cell migration and invasion in head and neck squamous cell carcinoma. *Oncotarget*. 2016;7(52):87037-87051. doi:10.18632/oncotarget.13523
37. Chang W, Luo Q, Wu X, et al. OTUB2 exerts tumor-suppressive roles via STAT1-mediated CALML3 activation and increased phosphatidylserine synthesis. *Cell Rep*. 2022;41(4):111561. doi:10.1016/j.celrep.2022.111561
38. Ye L, Zhang J, Zhang Y, Gu B, Zhu H, Mao X. Isoliquiritigenin suppressed esophageal squamous carcinoma growth by blocking EGFR activation and inducing cell cycle arrest. *Biomed Res Int*. 2020;2020:9259852. doi:10.1155/2020/9259852
39. Qi J, Li H, Liu N, et al. The implications and mechanisms of the extra-nuclear nucleolin in the esophageal squamous cell carcinomas. *Med Oncol*. 2015;32(3):45. doi:10.1007/s12032-015-0484-3
40. Cheng Y, Li H, Deng Y, et al. Cancer-associated fibroblasts induce PDL1+ neutrophils through the IL6-STAT3 pathway that foster immune suppression in hepatocellular carcinoma. *Cell Death Dis*. 2018;9(4):422. doi:10.1038/s41419-018-0458-4
41. Fei L, Ren X, Yu H, Zhan Y. Targeting the CCL2/CCR2 Axis in cancer immunotherapy: one stone, three birds? *Front Immunol*. 2021;12:771210. doi:10.3389/fimmu.2021.771210

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Feng A, He L, Jiang J, et al. Homeobox A7 promotes esophageal squamous cell carcinoma progression through C-C motif chemokine ligand 2-mediated tumor-associated macrophage recruitment. *Cancer Sci*. 2023;114:3270-3286. doi:10.1111/cas.15842