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The oncogenic role of SOX8 in endometrial carcinoma

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ABSTRACT

Endometrial carcinoma (EC) remains one of the most prevalent forms of cancer to impact the female reproductive system, yet the mechanisms governing its development and progression are incompletely understood. We, therefore, sought to assess the relevance of SOX8 to EC progression and patient prognosis.

Array comparative genomic hybridization (aCGH) was performed using samples from 50 patients with EC. Samples were separated based upon whether patients were positive for lymph node metastasis (LN+ and LN−, respectively). Based on our initial results, the SOX8 gene was selected for further analysis. Immunohistochemical staining of 630 endometrial tissue samples was conducted to understand how SOX8 expression relates to specific EC clinicopathological characteristics. In addition, we explored the impact of SOX8 expression on the growth, invasion, and migration of EC cells through knockdown and overexpression experiments.

In our initial aCGH analysis, SOX family proteins and the Wnt and Notch signaling pathways were significantly associated with EC LN metastasis. SOX8 expression was markedly increased in EC tumor samples relative to normal endometrial tissue ($P=$ .003), and higher SOX8 expression was linked to a high tumor histological grade ($P=$ .032), LN metastasis ($P=$ .027), and shorter patient overall survival ($P=$ .031). When SOX8 was knocked down, this further impaired the proliferative, invasive, and migratory activity of EC cells, whereas overexpressing this gene had the opposite effect.

SOX8 may function in an oncogenic manner to drive EC development and progression, and higher SOX8 expression is associated with a poor EC patient prognosis.

Introduction

Endometrial carcinoma (EC) remains one of the most prevalent forms of gynecological cancer in the world, with 65,620 new diagnoses and 12,590 EC-related deaths predicted to occur in the United States alone in 2020,[1,2] and with rising rates of diagnosis in China.[3] In patients with early-stage EC, the 5-y survival rate is as high as 90%.[4] However, the course of EC is highly variable, and a subset of early-stage patients are at risk for tumor recurrence or metastasis, both of which are associated with an unfavorable prognosis.[5] The factors that govern whether EC patients ultimately suffer from a progressive disease, however, are not well understood. The mechanistic basis for EC pathogenesis, therefore, needs to be fully explored in an effort to elucidate how this disease progresses to a metastatic state in a subset of patients as a means of designing appropriate treatments for at-risk individuals.

Sex-determining region Y box (SOX) genes are conserved genes that encode transcription factors which regulate key developmental processes such as embryogenesis, organ development, sex differentiation, and neurogenesis.[6,7] These SOX genes contain conserved high-mobility group (HMG) box regions that have been used to classify SOX family proteins into defined subgroups.[8] Several studies to date have highlighted the pivotal roles played by certain SOX proteins in the context of tumor cell proliferation, migration, and invasion.[9,10] Consistent with these roles, some SOX proteins have been shown to be key markers of cancer patient prognosis in addition to being potentially viable therapeutic targets.[9] There is some evidence suggesting that SOX genes can govern EC pathogenesis,[11] with SOX3, SOX4, and SOX11 overexpression being associated with the enhanced EC cell proliferative migratory, and invasive activity that was suppressed upon the knockdown of these genes.[12–14] In contrast, SOX7, SOX15, and SOX17 were found to have the opposite effect, impairing EC cell proliferation.[15–17]

SOX8 is a member of the E subgroup of SOX proteins and is expressed at the highest levels in embryonic and adult brain tissues.[18] Elevated SOX8 expression has been associated with greater tumor aggression and poorer survival in patients with non-small lung cancer, triple-negative breast cancer, chemoresistant tongue squamous cell carcinoma, and colorectal cancer.[19–23] Whether SOX8 similarly controls EC tumor aggression and progression, however, remains to be determined. Herein, we, therefore, used an immunohistochemistry (IHC) staining

approach to analyze SOX8 expression in normal endometrium (NE), hyperplastic endometrium (HE), atypical hyperplastic endometrium (AHE), and EC tissues. We further examined the relationship between SOX8 expression and EC patient clinicopathological features and utilized *in vitro* approaches to directly examine the role of SOX8 expression in EC cells.

Results

Identification of CNVs in specific genes and pathways associated with EC lymph node metastasis

We began by utilizing gDNA extracted for 50 EC patient tumor samples in order to conduct a whole-genome aCGH analysis. The most frequently altered genomic regions in these samples were identified in an effort to identify specific cytoband variations that were significantly associated with LN metastasis in these EC patients (Figure 1 and Supplementary Table 1). We were able to determine that copy-number variations (CNVs) in certain genes in the SOX, Wnt, and Notch families were significantly more amplified in LN+ samples relative to LN− samples. Specifically, the levels of Wnt1, Wnt10a, Wnt10b, SOX4, SOX8, NCAM1, NOTCH2, NOTCH3, and NRXN2 CNVs were significantly higher in LN+ patient samples relative to those from LN− patientsLN patients. This suggests that these genes may be key regulators of EC metastasis. We have previously highlighted important roles for Wnt10a and Wnt10b in EC, and in the present study, we, therefore, elected to examine the role of SOX8 in this cancer type.

Elevated SOX8 expression is linked with EC progression and development

We next measured SOX8 expression levels in TMAs prepared using NE, HE, AHE, and EC tissue samples via IHC, the representative image is shown in Figure 2a. We found that EC tumor samples exhibited significantly higher SOX8 expression relative to NE tissues ($\chi 2 = 11.685$, $P= .003$, Figure 2b). High SOX8 expression was almost exclusively detected in EC and AHE samples (7.9% and 12%, respectively), while negative SOX8 expression was most frequently observed in NE and HE samples (74.1% and 69.8%, respectively) (Table 2, Figure 2b). In order to further explore how SOX8 expression levels relate to EC patient clinicopathological parameters, we next conducted a correlation analysis of the 277 EC patients included within this TMA sample cohort. Elevated SOX8 expression was found to be significantly associated with higher tumor histological grade ($\chi 2 = 6.907$, $P= .032$) and with LN metastasis (Fisher, $P= .027$) (Figure 2c and d, Table 3). There were also trends toward higher SOX8 expression in samples exhibiting deep myometrial invasion, advanced FIGO stage, and 2 type samples ($P> .05$).

3. Elevated SOX8 expression correlated with poor prognosis

We next examined the relationship between SOX8 expression and the overall survival (OS) of EC patients from the date of surgery to death or last follow-up. A Kaplan–Meier curve analysis revealed that high SOX8 expression was linked to shorter OS relative to low or no SOX8 expression in these EC patients ($\chi 2 = 6.930$, $P= .031$) (Figure 2e).

4. SOX8 promotes the proliferative, migratory, and invasive activity of EC cells

In order to confirm the functional role of SOX8 in the context of EC, we next assessed the expression of this protein in five common EC lines via Western blotting. This analysis revealed that SOX8 levels were higher in HEC-1B cells and lower in
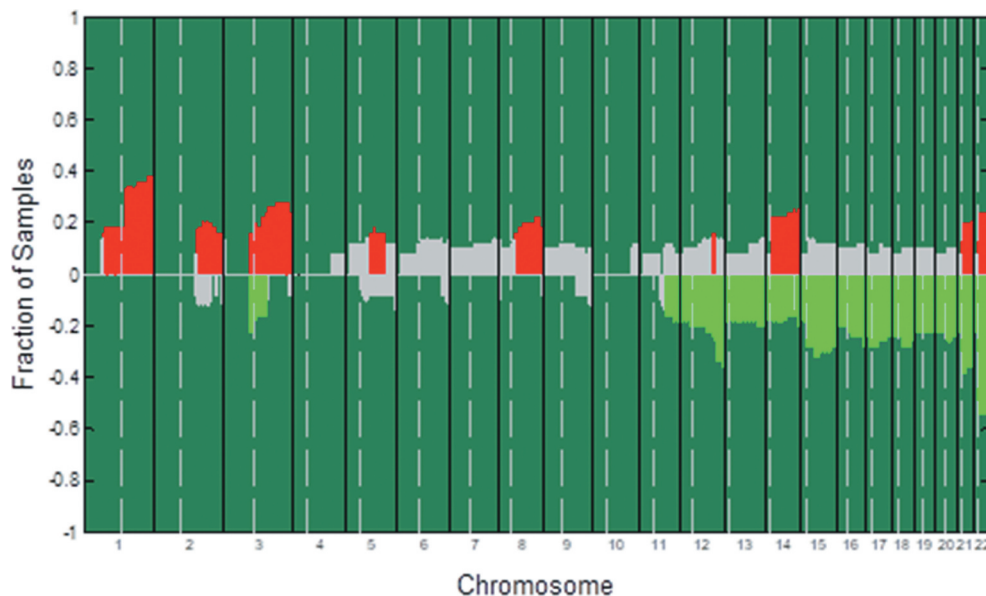

Figure 1. An aCGH-based analysis of DNA copy-number alteration frequencies in 50 endometrial carcinoma samples. Gains or losses for each measured sequence are shown on the y-axis, with sequences being aligned along the x-axis in chromosomal order. The significance threshold is represented by a dashed line. Significant DNA copy-number gain frequencies are marked by red lines, while significant DNA copy-number losses are marked by green lines. Non-significant changes are marked in gray. Vertical bars demarcate different chromosomes, with dashed vertical bars marking the separation between short and long arms of individual chromosomes.
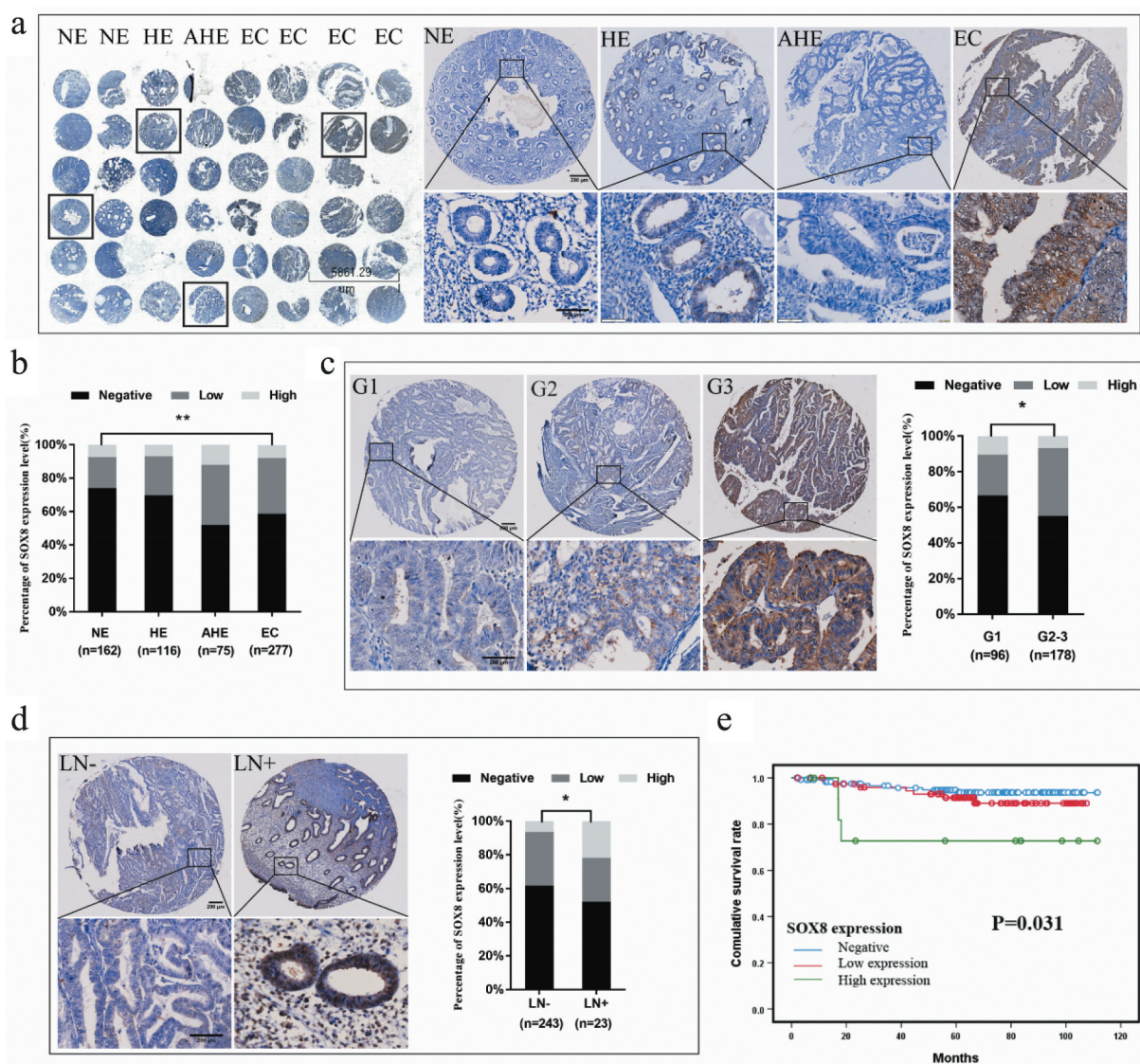
Figure 2. SOX8 expression levels in human EC tissue samples correlate with aggressive clinicopathological findings. (a) An IHC approach was used to measure SOX8 levels in TMAs. The resultant data revealed that SOX8 levels were higher in EC and AHE samples relative to HE and NE samples. (b) The expression of SOX8 in EC samples was significantly higher than that in NE samples. (c) SOX8 overexpression was linked to high EC histological grade. *Left*: Representative images of EC tissue SOX8 staining (G1 – G3). *Right*: Elevated SOX8 expression was confirmed to be significantly associated with high EC histological grade in these patient samples. (d) SOX8 overexpression was associated with EC lymph node metastasis. *Left*: Representative SOX8 staining in LN− and LN+ EC samples. *Right*: Elevated SOX8 expression was confirmed to be significantly associated with EC lymph node metastasis in these patient samples. (e) The relationship between SOX8 expression and post-operative EC patient OS was assessed using a Kaplan-Meier curve. (**$p < .01$, *$p < .05$).

Table 1. Gene alterations in endometrial carcinoma.

			LN[a] negative	LN positive
Gene name	Chromosome	Band label	Amplification (%)	Amplification (%)
Wnt1	12	q13.11	–	33.33
Wnt6	2	q35	40.63	50
Wnt10A	2	q35	–	44.44
Wnt10B	12	q13.11	–	50
SOX4	6	p22.3	31.25	38.89
SOX8	16	p13.3	–	33.33
SYP	X	p11.23-p11.22	4	2
NCAM1	11	q23.1	–	38.89
NOTCH2	1	p13-p11	–	33.33
NOTCH3	19	p13.2-p13.1	–	33.33
NOTCH4	6	p21.33	10.5	8
NRXN2	11	q12.3	–	27.8

[a]LN indicates lymph node metastasis.

Ishikawa cells relative to other tested cell lines (Figure 3a), leading us to select these cells for downstream experimentation. We then generated versions of these cells in which SOX8 had been knocked down (HEC-1B-si-SOX8#1, HEC-1B-si-SOX8#2) or overexpressed (Ishikawa-LV-SOX8), with these changes in SOX8 expression relative to cells transfected with negative control (NC) constructs being confirmed by Western blotting (Figure 3b-c) and RT-PCR (Figure 3d-e). Using a CCK8 assay, we found that HEC-1B cells in which SOX8 had been knocked down were significantly less proliferative relative to control and NC cells (*P*< .05, Figure 3f), whereas SOX8 overexpression had the opposite effect on Ishikawa cell proliferation (*P*< .01, Figure 3g). This was in line with the results of a colony-formation assay, wherein SOX8 knockdown decreased colony numbers relative to control and NC groups

Table 2. SOX8 expression in NE, HE, AHE, and EC tissues.

Group	Total	SOX8 expression[n(%)]			χ2 trend	P
		Negative	Low	High		
NE [a]	162	120(74.1)	30(18.5)	12(7.4)	18.298	**0.006***
HE	116	81(69.8)	27(23.3)	8(6.9)		
AHE	75	39(52.0)	27(36.0)	9(12.0)		
EC	277	163(58.8)	92(33.2)	22(7.9)		

1. The data is presented as number of cases (percentage of cases).
2. Abbreviations: NE, normal endometrium; HE, hyperplastic endometrium; AHE, atypical hyperplastic endometrium; EC, endometrial cancer.
3. [a]Compared with EC, χ2 = 11.658, P= 0.003.
4. * means P < 0.05.

(251.3 ± 23.25, 436.3 ± 9.615 vs 732 ± 14.57, vs 744.3 ± 32.2, P< .001) while SOX8 overexpression increased colony numbers relative to control and NC groups (681 ± 20 vs 427 ± 1, vs 403 ± 5, P< .01) (Figure 3h-i).

In a Transwell assay system, we further found that SOX8 knockdown in HEC-1B cells impaired their migration relative to control and NC treatment (17.4 ± 1.364, 22.8 ± 1.393 vs 70 ± 3.619, vs 73 ± 2.387, P< .0001), and findings in an invasion assay were comparable (7 ± 0.3162, 15.2 ± 0.860 vs 51.8 ± 1.685, vs 50.6 ± 2.4, P< .0001) (Figure 4a). In contrast, overexpressing SOX8 enhanced Ishikawa cell migration (81.4 ± 5.437 vs 26.2 ± 1.02, vs 31.8 ± 2.99, P< .0001), and invasion (66.2 ± 2.709 vs 21 ± 0.8367, vs 19.4 ± 2.694, P< .0001) relative to control and NC groups (Figure 4b).

Discussion

EC is currently among a limited number of cancers for which mortality rates are rising.[24] Most patients with EC are diagnosed while the disease is still in its early stages due to obvious symptoms such as postmenopausal vaginal bleeding. While these women typically have favorable disease outcomes, some women are not diagnosed until the disease is in an advanced state, while others present with low-grade, early-stage, well-differentiated endometrioid tumors that recur unexpectedly and that have a poor prognosis. EC LN metastasis is correlated with poorer patient outcomes. However, there is a clear need for the identification of LN metastasis-related biomarkers that can be used to evaluate EC patients in an effort to improve treatment efficacy. In the present study, we compared genomic alterations in LN+ and LN− EC tumor samples via an aCGH approach, revealing SOX8 to be amplified with higher frequency in LN+ samples relative to LN− samples (CNV levels of 33.33% and 0%, respectively), suggesting a role for SOX8 in EC LN metastasis. Recent work has highlighted a role for SOX family proteins in tumor development and progression.[9] This, coupled with our aCGH analysis results, suggested that SOX8 may play a yet-to-be-characterized role as a risk factor for EC progression. We, therefore, elected to study the prognostic and functional relevance of SOX8 in EC as a means of understanding how this protein impacts EC development.

This is the first study we are aware of to have demonstrated a difference in SOX8 levels between LN+ and LN− EC patient samples. We further confirmed that SOX8 expression was significantly higher in EC tumor tissue samples relative to NE samples, and we found that elevated SOX8 expression correlated with LN metastasis, high histologic grade, and poor prognosis. When we extended this work in vitro, we confirmed that SOX8 is able to directly drive the migratory and proliferative activity of EC cell lines. Together, these results thus suggest that SOX8 overexpression may be indicative of a higher risk of EC progression or development.

SOX8 dysregulation has previously been documented in multiple human tumor types. For example, SOX8 upregulation has been observed in hepatocellular carcinoma and colorectal cancer (CRC) tumor samples relative to levels in paracancerous tissues.[19,21] Chemoresistant tongue squamous cell carcinoma patients were found to express significantly higher levels of SOX8, suggesting that this gene may also influence therapeutic resistance.[23] In this analysis, we found that SOX8 expression

Table 3. Correlation between SOX8 expression and clinicopathological parameters in EC cases.

Variables	Total	SOX8 expression[n(%)]			χ2 trend	P
		negative	low	high		
Histological grade (n = 274)[a]						
G1	96	64(66.7)	22(22.9)	10(10.4)	6.907	**0.032***
G2-G3	178	98(55.1)	68(38.2)	12(6.7)		
Myometrial invasion						
<1/2	210	125(59.5)	68(32.4)	17(8.1)	0.275	0.827
≥1/2	67	38(56.7)	24(35.8)	5(7.5)		
FIGO stage						
I	225	137(60.9)	72(32.0)	16(7.1)	2.424	0.298
II-IV	52	26(50.0)	20(38.5)	6(11.5)		
Lymph node metastasis (n = 262)[b]						
No	241	151(62.7)	75(31.1)	15(6.2)	Fisher	**0.027*[c]**
Yes	21	12(57.1)	4(19.0)	5(23.8)		
Pathologic						
Endometrioid	249	149(59.8)	82(32.9)	18(7.2)	Fisher	0.374[c]
Non-endometrioid	28	14(50.0)	10(35.7)	4(14.3)		

1. The data is presented as number of cases (percentage of cases).
2. [a]The other 3 cases were adenosquamous carcinoma.
3. [b]The other 15 cases did not undergo lymph node dissection.
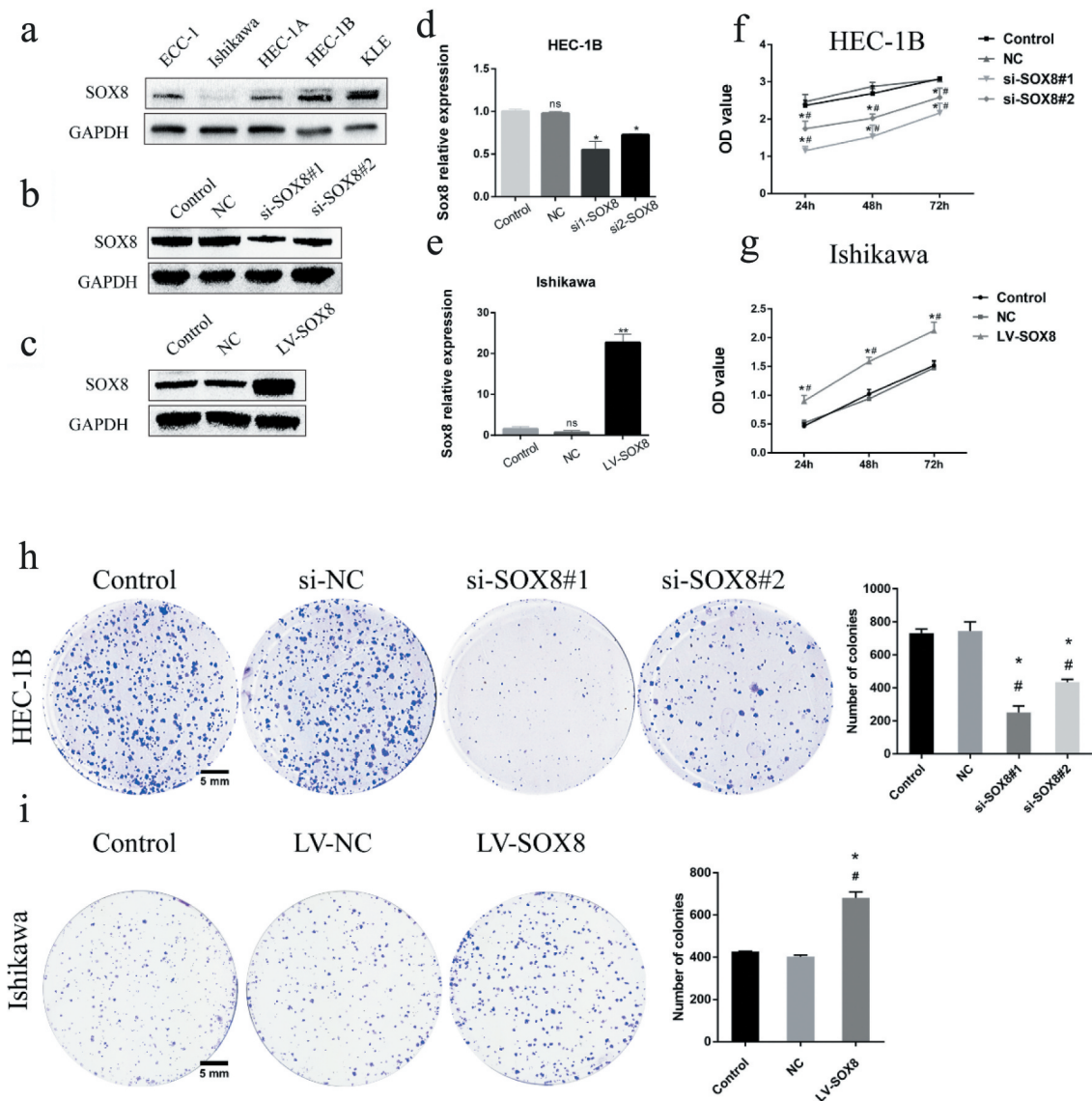4. [c]Fisher test was used.
5. * means P < 0.05.

Figure 3. SOX8 controls EC cell proliferation and growth. (a) Endogenous SOX8 levels in ECC-1, Ishikawa, HEC1A, HEC1B, and KLE cells were assessed by Western blotting. SOX8 knockdown in HEC-1B cells (b,d) and SOX8 overexpression in Ishikawa cells (c,e) was confirmed via Western blotting and qPCR. A CCK8 assay revealed that SOX8 knockdown significantly impaired cell proliferation (f), whereas SOX8 overexpression had the opposite effect (g). A colony-formation assay indicated that SOX8 knockdown impaired EC cell growth (h), while SOX8 overexpression significantly enhanced proliferation (i). Data are means ± SD from triplicate experiments. #p < .05 vs. control; *p < .05 vs. NC control.

was significantly upregulated in EC samples relative to NE controls, and we found that this increased expression was correlated with LN metastasis, in line with our aCGH results. This is also consistent with work demonstrating that SOX8 upregulation correlates with LN metastasis in non-small cell lung cancer (NSCLC), chemo-resistant tongue squamous cell carcinoma, and CRC, suggesting that SOX8 is a metastasis-related oncogene.[20,21,23] Our Kaplan-Meier survival analyses further revealed that high SOX8 expression was associated with poor EC patient prognosis, consistent with findings in studies of NSCLC, tongue squamous cell carcinoma, CRC, and triple-negative breast cancer.[22] SOX8 may thus be a key regulator of EC progression and a valuable prognostic biomarker in patients with this disease.

Using *in vitro* knockdown and overexpression techniques, we further sought to confirm the role of SOX8 in regulating EC progression and proliferation. The results of these experiments indicated that knocking down this transcription factor markedly impaired EC cell proliferative, migratory, and invasive activity, whereas its overexpression had the opposite effect. This is in line with findings in several other cancer types. For example, SOX8 knockdown inhibited chemoresistance, tumorsphere formation, and EMT via Wnt/β-catenin signaling in cisplatin-resistant tongue squamous cell carcinoma cells, whereas its overexpression in triple-negative breast cancer cells enhanced their migratory and proliferative activity. SOX8 overexpression in hepatocellular carcinoma cell lines also enhanced their proliferation via a mechanism associated with Wnt/β-catenin signaling. Our
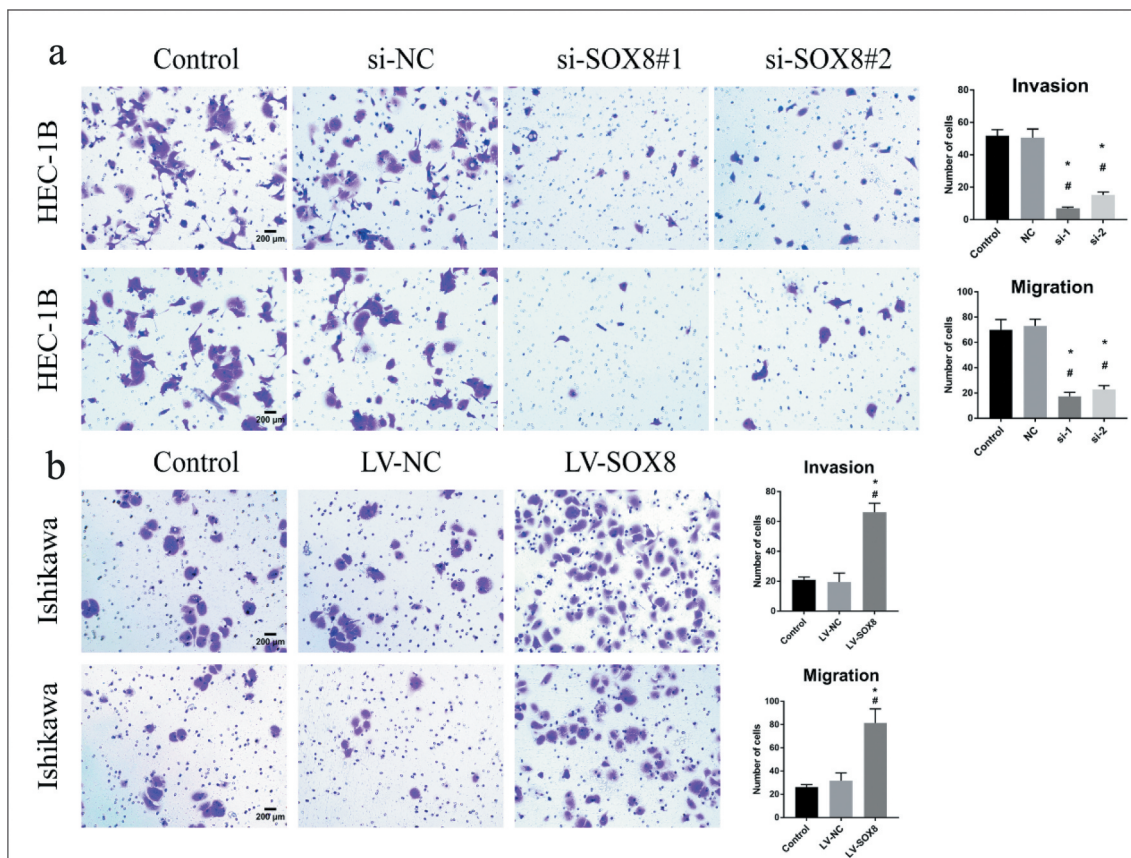
Figure 4. SOX8 drives EC cell migratory and invasive activity *in vitro*. (a) The invasive and migratory activity of HEC-1B cells was significantly decreased following SOX8 knockdown. (b) Overexpression of SOX8 significantly enhanced Ishikawa cell migratory and invasive activity (×100).

current study did not explore the mechanistic basis whereby SOX8 drives the progression and development of EC. However, our aCGH analysis revealed that in addition to SOX8, amplifications were also common in several Wnt and Notch signaling pathway proteins in LN+ EC patient samples. Wnt and Notch signaling are well known to be linked to EC activation and development,[25–29] and SOX genes have been shown to interact with the Wnt/β-catenin signaling pathway in many cancers.[10,30] We also found that SOX8 is predicted to interact with the Wnt/β-catenin signaling pathway via CTNNB1 (β-catenin) in the STRING database (https://string-db.org/). Future studies exploring whether SOX8 influences EC cell proliferation and metastasis via the Wnt/β-catenin signaling pathway are therefore warranted.

In conclusion, in the present study, we determined that EC samples exhibit significantly elevated SOX8 expression relative to NE tissues. We additionally provided novel evidence indicating that high expression of SOX8 is correlated with LN metastasis, a high histologic grade, and poor patient prognosis. Using cell lines, we further confirmed that SOX8 can directly promote EC cell proliferative, migratory, and invasive activity, confirming the oncogenic relevance of this protein. Together, these findings suggest that SOX8 may be a viable prognostic biomarker and therapeutic target in EC, although further analyses will be necessary in order to understand the molecular mechanisms whereby SOX8 influences the development and progression of this form of cancer.

Materials and methods

Patient sample collection

For this study, we analyzed previously collected samples from 50 EC patients that had been archived by Tianjin Medical University General Hospital (TMUGH, Tianjin, China) between 2000 and 2009. These samples were utilized for aCGH and were separated into two groups based upon whether they were positive for lymph node metastasis (LN+, 18 cases) or negative for lymph node metastasis (LN−, 32 cases). These tissues had been snap-frozen within 30 minutes of isolation and stored at −80°C. In addition, we utilized a tissue microarray that had been constructed from 630 endometrial tissue samples collected at TMUGH from 2008 to 2016. This latter cohort included samples from 277 EC patients, 75 patients with AHE, 116 patients with HE, and 162 patients with NE tissue collected during hysterectomy. Carcinosarcoma was not included in EC. Two pathologists had independently graded the histological typing of each sample based on the WHO classification criteria, and EC tumor staging was conducted using the 2014 FIGO staging system. Patients in this study that did not have EC had been admitted to the hospital for the treatment of uterine prolapse, cystocele, or urethrocele. No patients had undergone radio- or chemotherapy prior to tissue collection. The Ethics Committee of TMUGH approved these sample collection procedures.

Array comparative genomic hybridization (aCGH)

A DNA/RNA Prep Kit (Qiagen, CA, USA) was used to isolate gDNA from the 50 EC patient samples, after which labeled DNA was hybridized to a human whole-genome CGH microarray (4 × 44 k; Agilent Technologies, CA, USA). This array covered 43,000 coding and non-coding genomic sequences, with an average spatial resolution of 35kbp per oligonucleotide probe. The array contained a minimum of one target sequence per well-characterized gene and a minimum of two probes per cancer-related gene. The University of California Santa Cruz hg17 human genome (National Center for Biotechnology Information, NCBI Build 35) was used to guide probe design.

Immunohistochemistry

IHC staining of TMA samples was conducted as in prior studies,[31] using anti-SOX8 from Proteintech (20627-1-AP, China) and an appropriate secondary from ZSGH-Bio (PV9001, China). Hematoxylin and eosin (H&E) were used to counterstain slides based on standard protocols. Both staining frequency and intensity were then used to assess SOX8 staining levels, the scoring system was derived from the previous report.[21] Scores for the percentage of positively stained cells were as follows: 0 = 0–5%; 1 = 6%-25%; 2 = 26%-50%; 3 = 51–75%; 4, 76%-100%. Intensity scoring was conducted as follows: 0 = no staining; 1 = weak; 2 = moderate; 3 = strong. These two scores were then multiplied together to yield a final score, which was graded as follows: 0 [0] = negative expression; 1 [1–3] = low expression; 2 [4–12] = high expression. Two pathologists independently scored each sample.

Cell culture

The Ishikawa and HEC-1B cell lines were obtained from the China Infrastructure of Cell Line Resource. The ECC-1, HEC-1A, and KLE EC cell lines were provided by the TMUGH obstetrics and gynecology lab. All cells were cultured using standard protocols, as detailed previously.[32] No mycoplasma contamination in all cells during cell culture.

RT-PCR

Total RNA was isolated from cells according to TRIzol protocol (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Total RNA was converted to cDNA using a Reverse Transcriptase Kit. Then, real-time PCR analyses were carried out in triplicate for each sample. The PCR primers were listed as follows: SOX8: forward: CCGTGTCGCAGGTGCTCA, reverse: CGCCGTGGCTGGTACTTGTAG; GAPDH: forward: TGACTTCAACAGCGACACCCA, reverse: CACCCTGTT GCTGTAGCCAAA.

Western blotting

Standard western blotting procedures were used in this study.[33] Using the following primary antibodies: anti-SOX8 (ab125858, 1:1000), anti-GAPDH (ZSGH-Bio, 1:5000). In addition, an appropriate secondary antibody was used (1:8000; ZSGH-Bio) , and Immobilon Western HRP (Millipore, USA) was used for protein visualization.

Modulation of SOX8 expression

The Ubi-SOX8-3 FLAG-CBh-gcGFP-IRES-puromycin lentiviral vectors were constructed by GeneChem (Shanghai, China). These viruses were used to transduce appropriate target cells, and stably transduced cells were selected using puromycin. Western blotting was used to confirm SOX8 overexpression. SOX8 knockdown was conducted by transducing cells with siRNA constructs specific for SOX8 from Integrated Biotech Solutions (Shanghai, China). Sequences for these constructs were as follows: 5′-AGAACAUCGACUUCAGCAACG-3′ and 5′-UCCACGAG UUCGACCAGUATT-3′. In addition, a negative control siRNA with the following sequence was used: 5'-UUCUCCGA ACGUGUCACGUdTdT-3′. Manufacturer's protocols were used to conduct SOX8 knockdown and overexpression experiments.

Cell proliferation assay

Proliferation was measured with a Cell Counting Kit-8 (CCK8) (Dojindo, Japan) assay. Briefly, 6×10^3 Ishikawa cells or 5×10^3 HEC-1B cells were added into each well of a 96-well plate in a 100 μL volume. At appropriate time points (24, 48, or 72 h), 10 μL of CCK-8 solution was added per well and absorbance at 450 nm was assessed after a 4 h incubation at 37°C using a microplate reader (EnSpire, China).

Colony-formation assay

A total of 1000 Ishikawa or HEC-1B cells were added per well of a 6-well plate, and colony formation was assessed via microscopy each day. Following a 10-d incubation, 4% paraformaldehyde was used to fix colonies for 20 minutes, after which they were stained with 2.5% crystal violet and counted.

Cell migration/invasion assays

A Transwell system (8.0 μm pore size; Corning, USA) with or without a Matrigel coating (Corning) was used based on provided directions, as in prior studies.[34] Briefly, appropriately transfected cells in serum-free media were added to the upper chamber of this system (8×10^4/100 μl for Ishikawa; 10×10^4/100 μl for HEC-1B), while a 500 μl volume of media containing 20% FBS was added to the lower well. After 24 or 48 h, cells on the upper surface were gently removed with a swab, with cells that had undergone migration or invasion were fixed with 4% paraformaldehyde and stained using 2.5% crystal violet. A ZEISS HB100 (×100) was then used to count cells in five random fields of view, with data being compiled from three experimental replicates.

Statistical analysis

SPSS v25.0 (IBM, USA) was used for statistical testing, while GraphPad Prism 7.0 (GraphPad, USA) was used to prepare figures. The relationships between SOX8 expression levels and clinicopathological variables were assessed via $\chi2$ or Fisher's exact tests, as appropriate, with the former being used when values

had an expected frequency of ≤5. Survival analyses were conducted using the Kaplan–Meier method, and the relationship between SOX8 expression and postoperative survival in EC patients was also assessed via univariate analyses. Data generated *in vitro* were compared using ANOVAs and Student's t-tests as appropriate. $P < .05$ was the significance threshold.

Acknowledgments

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Authorship

Yingmei Wang and Fengxia Xue: study design, supervision of the study, review, and editing the manuscript.

Wenyan Tian: collection of tissue specimens and scoring IHC results.

Zhanghuan Li: conducting the cell biological experiment, statistical integration, writing, review, and editing the manuscript.

Lu Bai: conductingthe aCGH experiment.

Lingli Chen: conducting the qPCR experiment.

Ye Yan: database maintenance and statistical analysis.

Huihui Li: conducting the IHC experiment and collection of patient information

Yanyan Han: scoring IHC results.

Fei Teng and Chao Gao: collection of tissue specimens and follow-up of the patient.

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