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SOX4 is associated with poor prognosis in cholangiocarcinoma

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ABSTRACT

Overexpressions of EGFR and HER2 are thought to be prognostic factors of cholangiocarcinoma (CCA). The SOX4 transcription factor is involved in the development and cell fate decision. Although up-regulation of SOX4 has been described in multiple human malignancies, the prognostic value of SOX4 and its relationship to EGFR/HER2 in CCA remain unclear. In the current study, we showed that SOX4 and EGFR were overexpressed in 17 (29.3%), and 13 (22.4%) of the 58 intrahepatic cholangiocarcinomas (IHCCs), as well as 28 (29.8%), and 33 (35.1%) of the 94 extrahepatic cholangiocarcinomas (EHCCs), respectively. Overexpression of HER2 was exclusively identified in EHCCs, with the rate being 4.4% (4/90). In all, amplification of EGFR was identified in 1.8% (1/52) of IHCC cases, and in 2% (3/82) of EHCC cases. By contrast, HER2 amplification was present only in 3.5% (3/94) of the EHCC cases. Notably, Kaplan–Meier survival analysis suggested that SOX4 expression is a significant prognostic factor for poor prognosis in IHCC patients. Importantly, our findings suggested significant association of SOX4 and EGFR expression both in IHCC ($P < 0.001$) and EHCC ($P = 0.014$). SOX4 may modulate expression of EGFR, and SOX4+/EGFR+ defines a subset of CCA patients with poor prognosis. Finally, *in vitro* data indicated that SOX4 inhibits cellular migratory capacity and promotes epithelial–mesenchymal transition (EMT) process of CCA cells. Collectively, our results define an important role for SOX4 in CCA by orchestrating EMT and modulation on EGFR expression. SOX4 expression may serve as a prognostic marker for patients with IHCC.

1. Introduction

Cholangiocarcinoma (CCA) is a highly aggressive neoplasm with an extremely poor prognosis, which is featured of silent clinical course, early regional invasiveness and distant metastasis [1,2]. The morbidity and mortality of CCA have been steadily increasing worldwide in recent years [2]. Anatomically, CCA is classified into intrahepatic cholangiocarcinomas (IHCCs) and extrahepatic cholangiocarcinomas (EHCCs), the latter being further divided into perihilar CCAs and distal CCAs [3]. So far, complete resection is the only curative therapeutic option for patients with CCA but can be applied only in a limited number of patients with localized or locally advanced disease [1]. Therefore, identification of new biomarkers for early detection and/or development of novel therapeutic regiments are urgently needed.

Epidermal growth factor receptor (EGFR) and HER2 are members of the family of tyrosine kinase growth factor receptors (TKGFRs). They share approximately 50% overall homology and play a significant role in cellular growth and proliferation signaling [4]. In CCA, overexpressions of EGFR and HER2 are thought to be prognostic factors and it has been proposed that EGFR could be potential promising target for treatment of CCA [5,6]. To date, although a series of studies have revealed amplification, copy number alterations, and mutations of this gene, the exact molecular mechanisms underlying EGFR overexpression in CCA remains imperfectly understood [5,7,8].

The SOX4 (sex-determining region Y-box 4) gene, a transcription factor and member of the SOX family, has been shown to play important roles in development and cell fate decision [9]. It is overexpressed in a wide variety of malignancies, including

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leukemia [10], and cancers of the breast [11], liver [12], lung [13] and prostate [14]. In these tumors, deregulated expression of SOX4 has been associated with increased cancer cell proliferation and cell survival and tumor progression through the induction of an epithelial mesenchymal transition (EMT) and metastasis [15]. Previously, we have reported that SOX4 is an independent poor prognostic factor in prostate cancer (PCa) and contributes to PCa metastasis by initiating a transcriptional program that enables EMT phenotype [16]. However, in a limited subset of malignancies, SOX4 has also been reported to act as a tumor suppressor and its expression correlates with prolonged patient survival and slower disease progression, including bladder cancer [17], melanoma [18] and gallbladder cancer [19]. However, the clinicopathological significance of SOX4 in CCA remains largely unknown.

In the current study, we systematically characterized the expression and prognostic significance of SOX4/EGFR/HER2 in a cohort of Chinese CCA patients. For the first time, we demonstrated that SOX4 expression is an independent prognostic factor in CCA patients and may promotes EMT process in CCA cells. SOX4 overexpression is significantly associated with EGFR expression and ERG+/SOX4+ defines a subset of CCA patients with poor prognosis.

2. Materials and methods

2.1. Patients

A total of 152 (male 83; female 69) patients with CCA were investigated in the present study. The patients had undergone surgery and been histologically diagnosed as having adenocarcinoma between 2005 and 2009, treated at the Qilu Hospital of Shandong University (Jinan, China), the Central Hospital of Jinan (Jinan, China) and General hospital of Liaocheng (Liaocheng, China). Patients who had other malignancies or had died within 1 month after surgery were excluded. Clinical and pathological data were obtained from the medical records and review of the slides. Follow-up data were available for 121 patients, ranging from 4 to 86 months (mean 27 months). The clinical and pathological characteristics of CCA cases in our cohort were summarized in Table 1. Informed written consents were obtained from the CCA patients. This study and the consent procedures were approved by the Institutional Review Board at the school of medicine of Shandong University.

2.2. Tissue microarray (TMA) construction

Tissue samples were fixed in buffered 4% formalin, embedded in paraffin, and used for tissue microarray (TMA) construction as previously described. Briefly, archived hematoxylin and eosin-stained slides were examined; representative tumor foci were selected and circled on the slide by a pathologist (B.H.). Corresponding paraffin blocks were then precisely aligned with the marked slides. The area of interest in the donor block was cored twice with a needle 1.0 mm in diameter and transferred to a recipient paraffin block. Detailed clinical and pathological profile was maintained in a secure relational database with TMA data.

Table 1
Summary of CCA patients' demographics.

Parameters	Categories	IHCC (%)	EHCC (%)
Age (year)	<60	33 (56.9)	59 (62.8)
	⩾60	25 (43.1)	35 (37.2)
Gender	Male	23 (39.7)	60 (63.8)
	Female	35 (60.3)	34 (36.2)
Tumor size (cm)*	<5	18 (31.0)	59 (62.8)
	⩾5	40 (69.0)	35 (37.2)
Histological differentiation	Well and moderately	37 (63.8)	75 (79.8)
	Poorly	21 (36.2)	19 (20.2)
T stage	I + II	41 (70.7)	43 (45.7)
	III + IV	17 (29.3)	51 (54.3)
N stage	Negative	45 (77.6)	64 (68.1)
	Positive	13 (22.4)	30 (31.9)
UICC stage	I + II	34 (58.6)	54 (57.4)
	III + IV	24 (41.4)	40 (42.8)

* In EHCC tumor size were categorized by <3 and ⩾3 cm, respectively.

2.3. Immunohistochemistry (IHC)

IHC was performed as previously described [16]. Immunohistochemical staining was done using the standardized labeled streptavidin biotin (LSAB) kit (DakoCytomation, Carpinteria, CA, USA) according to the manufacturer's instructions. Antigen retrieval was performed by microwave pretreatment in 0.01 M citrate buffer (pH 6.0) for 15 min. The primary antibodies used were anti-SOX4 (1:100 dilution, Abcam, Cambridge, MA, USA), anti-EGFR (1:500 dilution, DAKO, Carpinteria, CA, USA), and anti-HER2 (1:500 dilution, DAKO, Carpinteria, CA, USA). The slides were blindly evaluated by two independent observers (W.W. and B.H.) and the previously described scoring system was utilized to validate SOX4 expression. That is, the scores of 2 parameters were multiplied by the staining intensity (range, 0–3) and the percentage of positive cells (range, 0–4 [0, (0–10%), 1 (11–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%)]). Slides with scores of 8 or higher were classified as overexpression and slides with scores lower than 8 as non-overexpression. For EGFR and HER2, only the membrane immunostaining was scored following a four-step scale (scores 0, 1+, 2+, 3+). For HER2, we followed the consensus panel recommendations on HER2 scoring for breast cancer. Slides with a score of 2+ and 3+ were classified as positive or expressed, in contrast to slices with a score of 0 or 1+, which were defined as negative.

2.4. Fluorescence in situ hybridization (FISH)

Gene amplifications of *EGFR* and *HER2* were evaluated by FISH as previously described [16] on 4 μm TMA sections using the GLP *EGFR*/CSP 7 probe and GLP *HER2*/CSP17, respectively (GP Medical Technologies, Beijing, China). FISH was performed according to the manufacturer's protocol. The slides were examined using an ImagingZ1 microscope (Carl Zeiss, Oberkochen, Germany) and the hybridization signals of a minimum of 50 cancer cells with non-overlapping nuclei were manually counted. A previously documented method with minor modification was utilized to validate genetic aberrations of EGFR and HER2 [20,21].

2.5. Cell culture

The CCA cell line RBE was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured following the manufacturer's instructions.

2.6. In vitro overexpression of SOX4

Human SOX4 cDNA was subcloned into the pcDNA3.1 eukaryotic expression vectors. SOX4 and empty control plasmids were independently transfected into RBE cells using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

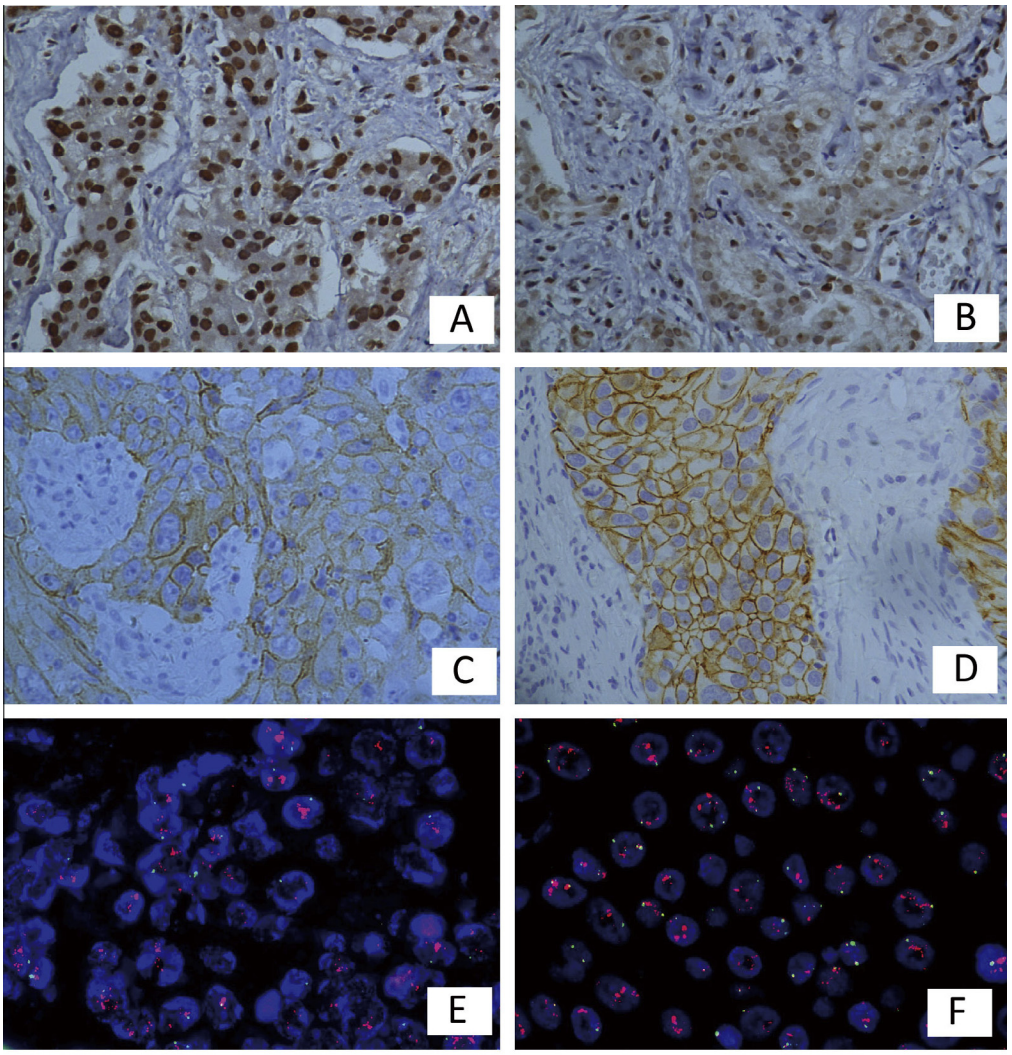
2.7. siRNA mediated SOX4 knockdown

Three SOX4 specific siRNAs were designed and synthesized by GenePharma (Shanghai, China), and the most effective single siRNA (sense strand: 5′-GGACAGACGAAGAGUUUAA-TT-3′ and anti-sense

strand: 5′-UUAAACUCUUCGUCUGUCC-TT-3′) was used for further experiments, Non-specific negative control siRNAs were also designed and synthesized (sense strand: 5′-UUCUCCGAACGUGUCACG-3′ and anti-sense strand: 5′-ACGUGACACGUUCGGAGAATT-3′).

2.8. Western blot analysis

Western blotting was performed as previously described [16]. The membrane was incubated with primary antibodies for SOX4 (Abcam, Cambridge, Cambridgeshire, UK), β-catenin (Cell Signaling



SOX4 IHC	EGFR IHC: 0 – 1+	EGFR IHC: 2+ – 3+
Moderate and strong	33	7
Negative and weak	5	11

IHCC (P<0.001)

SOX4 IHC	EGFR IHC: 0 – 1+	EGFR IHC: 2+ – 3+
Moderate and strong	48	18
Negative and weak	13	15

EHCC (P=0.014)

Fig. 1. Expressions and genetic aberrations of SOX4, EGFR and HER2 in CCA cases by IHC and FISH. (A) Strong nuclear staining for SOX4, ×400; (B) weak nuclear staining for SOX4, ×400; (C) strong membrane staining for EGFR, ×400; (D) strong membrane staining for HER2, ×400; (E) representative FISH image of EGFR with amplification. The ratio of red to green signals was more than 2. (F) Representative FISH image of HER2 with amplification. The ratio of red to green signals was more than 2. The bottom panel are contingency tables for SOX4 expression and EGFR expression status by IHC in patients with IHCC and EHCC, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Technology, MA, USA), Vimentin (Cell Signaling Technology, MA, USA) and GAPDH (Santa Cruz Biotechnology, CA, USA), respectively. The signals were detected with an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK).

2.9. Statistical analysis

The software used for statistical analyses was Statistical Package for Social Sciences, version 19.0 (SPSS). Two-sided Student's *t* test and Mann–Whitney test were used for statistical comparisons; correlations between SOX4 overexpression with clinicopathological parameters were evaluated by Spearman's test. The Kaplan–Meier method and Cox regression hazard tests were applied for the analysis of follow-up data, and hazard ratio (HR) with 95% confidence intervals (CI) were calculated. A *P*-value of <0.05 was considered significant.

3. Results

3.1. SOX4, EGFR and HER2 expression in cholangiocarcinomas and association with clinicopathological variables

Representative images of positive IHC staining for SOX4, EGFR, and HER2 were shown in Fig. 1(A–D). The relationship between SOX4 expression level and clinicopathologic variables in IHCC and EHCC cases were summarized in Tables 2 and 3. Overall, SOX4 overexpression was identified in 17 (29.3%) of the 58 IHCCs, and 28 (29.8%) of the 94 EHCCs, respectively. Interestingly, SOX4 expression was significantly associated with older age in IHCC ($P = 0.009$). The incidence of SOX4 overexpression was more frequently observed in elderly patients ($\geqslant$60 years) than younger ones. Similarly, increased SOX4 expression was marginally associated with poorly histological differentiation ($P = 0.061$), gender ($P = 0.078$) and higher UICC stage ($P = 0.089$), but not with tumor size ($P = 0.758$), T stage ($P = 0.753$) or lymph node metastasis ($P = 0.494$). In EHCC, there was no significant association between SOX4 expression and any clinicopathological factors.

Overexpression of EGFR was demonstrated in 13 (22.4%) of the 58 IHCC, and 33 (35.1%) of the 94 EHCC cases. Increased expression of EGFR was significantly correlated with older age ($P = 0.045$) in EHCC, and poorly histological differentiation both in IHCC ($P = 0.031$) and EHCC cases ($P = 0.012$). No other clinicopathological parameters were associated with EGFR expression in our cohort. In all, amplification of EGFR was identified in 1.8% (1/52) of IHCC cases, and in 2% (3/82) in patients with EHCC.

HER2 overexpression was exclusively identified in patients with EHCC, among which the rate was 4.4% (4/90). Similarly, HER2 amplification was present only in EHCC cases, and the positive rate

Table 2
Association of expressions of SOX4 and EGFR with clinicopathological parameters in IHCC.

Parameters		SOX4		*P*	EGFR		*P*
		Not overexpressed (%)	Over-expressed (%)		Not overexpressed (%)	Over-expressed (%)	
Age (year)	<60	28 (84.8)	5 (15.2)		27 (81.8)	6 (18.2)	
	⩾60	13 (52.0)	12 (48.0)	0.009	18 (72.0)	7 (28.0)	0.375
Gender	Male	13 (56.5)	10 (43.5)		17 (73.9)	6 (26.1)	
	Female	28 (80.0)	7 (20.0)	0.078	28 (80.0)	7 (20.0)	0.587
Tumor size (cm)	<5	12 (66.7)	6 (33.3)		13 (72.2)	5 (27.8)	
	⩾5	29 (72.5)	11 (27.5)	0.758	32 (80.0)	8 (20.0)	0.511
Histological differentiation	Well and moderately	29 (78.4)	8 (21.6)		32 (86.5)	5 (13.5)	
	Poorly	12 (57.1)	9 (42.9)	0.087	13 (61.9)	8 (38.1)	0.031
T stage	I + II	28 (68.3)	13 (31.7)		31 (75.6)	10 (24.4)	
	III + IV	13 (76.5)	4 (23.5)	0.753	14 (82.4)	3 (17.6)	0.917
Lymph node metastasis	Negative	33 (73.3)	12 (26.7)		35 (77.8)	10 (22.2)	
	Positive	8 (61.5)	5 (38.5)	0.494	10 (76.9)	3 (23.1)	0.759
UICC stage	I + II	21 (61.8)	13 (38.2)		27 (79.4)	7 (20.6)	
	III + IV	20 (83.3)	4 (26.7)	0.089	18 (75.0)	6 (25.0)	0.801

Table 3
Association of expressions of SOX4 and EGFR with clinicopathological parameters in EHCC.

Parameters		SOX4		*P*	EGFR		*P*
		Not over-expressed (%)	Over-expressed (%)		Not over-expressed (%)	Over-expressed (%)	
Age (year)	<60	42 (71.2)	17 (28.8)		43 (72.9)	16 (27.1)	
	⩾60	24 (68.6)	11 (31.4)	0.789	18 (51.4)	17 (48.7)	0.045
Gender	Male	44 (73.3)	16 (26.7)		39 (65.0)	21 (35.0)	
	Female	22 (64.7)	12 (35.3)	0.379	22 (64.7)	12 (35.3)	1.000
Tumor size (cm)	<3	40 (67.8)	19 (32.2)		39 (66.1)	20 (33.9)	
	⩾3	26 (74.3)	9 (25.7)	0.424	22 (62.9)	13 (37.1)	0.824
Histological differentiation	Well and moderate	52 (69.3)	23 (30.7)		53 (70.6)	22 (29.4)	
	Poor	14 (73.7)	5 (26.3)	0.711	8 (42.1)	11 (57.9)	0.019
T stage	I + II	29 (67.4)	14 (32.6)		27 (62.8)	16 (37.2)	
	III + IV	37 (72.5)	14 (27.5)	0.590	34 (66.7)	17 (33.1)	0.829
Lymph node metastasis	Negative	45 (70.3)	19 (29.7)		45 (70.3)	19 (29.7)	
	Positive	21 (70.0)	9 (30.0)	0.975	16 (53.3)	14 (46.7)	0.164
UICC stage	I + II	36 (66.7)	18 (33.3)		35 (64.8)	19 (35.2)	
	III + IV	30 (75.0)	10 (25.0)	0.382	26 (65.0)	14 (35.0)	1.000

was 3.5% (3/94). A significant association was identified between HER2 amplification and HER2 overexpression ($P < 0.01$). Representative FISH images of EGFR and HER2 were shown in Fig. 1(E and F).

3.2. Prognostic value of SOX4 expression in CCA

To investigate the possible associations between the protein levels of SOX4 and overall survival, we compared overall survival rates between patients with or without SOX4 overexpression in univariate and multivariate models. In IHCC, the group of the patients who were with SOX4 overexpression had a much greater rate of mortality than patients who are not ($P = 0.038$). On the basis of the Kaplan–Meier survival estimates, SOX4 overexpression was significantly linked to cancer-related mortality in our cohort (Fig. 2). By contrast, no statistical significance was identified between SOX4 overexpression and overall survival in EHCC.

In univariate Cox regression analysis, SOX4 overexpression was a prognostic factor for cancer mortality (hazard ratio = 2.35, 95% CI = 1.104–5.786, $P = 0.029$) (Table 4) in IHCC. Additionally, lymph node metastasis and EGFR overexpression were also significantly related to overall survival. In a multivariate analysis, lymph node metastasis remained its predictive value, whereas SOX4 and EGFR expression lost (Table 4).

In EHCC, SOX4 expression failed to be related to overall survival of CCA patients. 3 factors including EGFR overexpression were identified as prognostic factors by univariate analysis. In multivariate analysis, as shown in Table 5, only UICC stage was an independent prognostic factor (HR (95% CI): 3.110 (1.602–5.123), $P < 0.01$). Collectively, these data suggested that SOX4 was an unfavorable prognostic indicator in Chinese patients with IHCC.

3.3. SOX4 expression is associated with EGFR overexpression in CCA

Genetic aberrations including gene amplification and gene mutation contribute to EGFR overexpression in multiple cancers. In an attempt of characterizing novel mechanisms for EGFR overexpression in CCA, we successfully analyzed a total of 135 CCA cases for both SOX4 and EGFR expression by IHC. Notably, SOX4 overexpression was significantly associated with overexpression of EGFR both in IHCC ($P < 0.001$) and EHCC ($P = 0.034$) (Fig. 1).

3.4. SOX4+ /EGFR+ defines a subset of CCA patients with poor prognosis

In the current study, we next determined whether combining markers further improved prognostic value. The prognostic effects of EGFR aberration and SOX4 overexpression were directly compared in combination. The Kaplan–Meier analyses were therefore conducted using the group with no SOX4 overexpression and no EGFR overexpression as the reference. As shown in Fig. 3, the largest group, which comprised those who had both EGFR and SOX4 overexpression, had a greater survival when compared with the three other groups. Notably, the subset of patients with EGFR and SOX4 overexpression had the worst cancer-related survival.

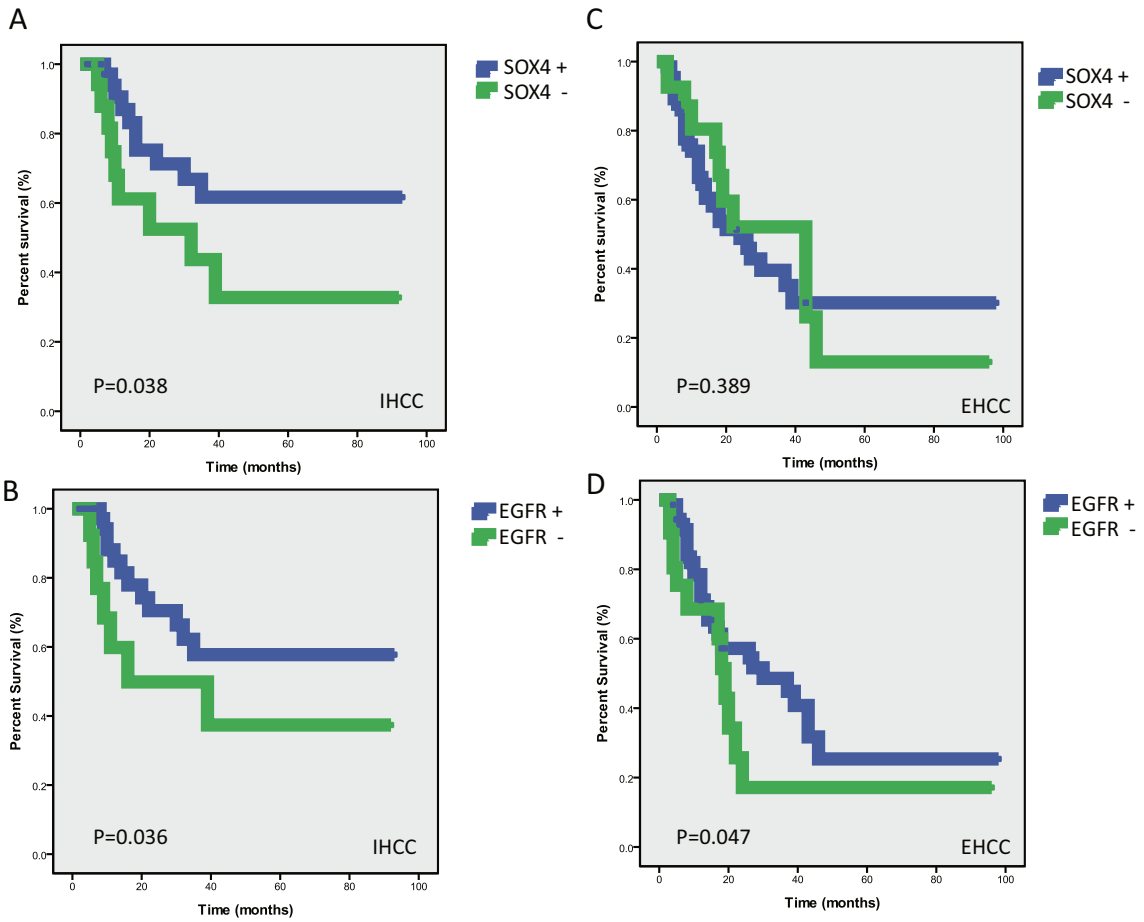


Fig. 2. Kaplan–Meier survival analysis of SOX4 and EGFR expression in Chinese CCA patients. (A) and (C) showed survival curves stratified by SOX4 expression in IHCC and EHCC patients, respectively; (B) and (D) showed survival curves stratified by EGFR expression in IHCC and EHCC patients, respectively. Cancer-related death was used as the end point.

Table 4
Univariate and multivariate analysis for overall survival in IHCC.

Parameters	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (year)						
<60	1	(Reference)		1	(Reference)	
⩾60	1.499	0.607–3.703	0.381	—	—	—
Gender						
Male	1	(Reference)		1	(Reference)	
Female	0.843	0.342–2.079	0.711	—	—	—
Tumor size (cm)						
<5	1	(Reference)		1	(Reference)	
⩾5	0.786	0.298–2.026	0.627	—	—	—
Histological differentiation						
Well and moderate	1	(Reference)		1	(Reference)	
Poor	0.823	0.311–2.178	0.695	—	—	—
T stage						
I + II	1	(Reference)		1	(Reference)	
III + IV	1.967	0.783–4.937	0.150	—	—	—
Lymph node metastasis						
Negative	1	(Reference)		1	(Reference)	
Positive	2.599	1.253–5.644	0.011	1.963	1.090–5.454	0.039
UICC stage						
I + II	1	(Reference)		1	(Reference)	
III + IV	1.056	0.424–2.630	0.907	—	—	—
SOX4 expression						
Not overexpressed	1	(Reference)		1	(Reference)	
Overexpressed	2.35	1.104–5.786	0.029	Non significance		
EGFR expression						
Not overexpressed	1	(Reference)		1	(Reference)	
Overexpressed	2.087	1.021–5.305	0.022	Non significance		

Table 5
Univariate and multivariate analysis for overall survival in EHCC.

Parameters	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (year)						
<60	1	(Reference)		1	(Reference)	
⩾60	0.917	0.501–1.679	0.779	—	—	—
Gender						
Male	1	(Reference)		1	(Reference)	
Female	0.583	0.302–1.125	0.108	—	—	—
Tumor size (cm)						
<3	1	(Reference)		1	(Reference)	
⩾3	1.528	0.837–2.790	0.167	—	—	—
Histological differentiation						
Well and moderate	1	(Reference)		1	(Reference)	
Poor	1.573	0.809–3.058	0.182	—	—	—
T stage						
I + II	1	(Reference)		1	(Reference)	
III + IV	1.84	1.004–3.373	0.049	Non significance		
Lymph node metastasis						
Negative	1	(Reference)		1	(Reference)	
Positive	2.36	1.283–4.343	0.006	Non significance		
UICC stage						
I + II	1	(Reference)		1	(Reference)	
III + IV	3.249	1.769–5.965	<0.001	3.11	1.602–5.123	<0.001
SOX4 expression						
Not overexpressed	1	(Reference)		1	(Reference)	
Overexpressed	0.854	0.430–1.695	0.652	—	—	—
EGFR expression						
Not overexpressed	1	(Reference)		1	(Reference)	
Overexpressed	1.846	0.948–3.594	0.071	—	—	—

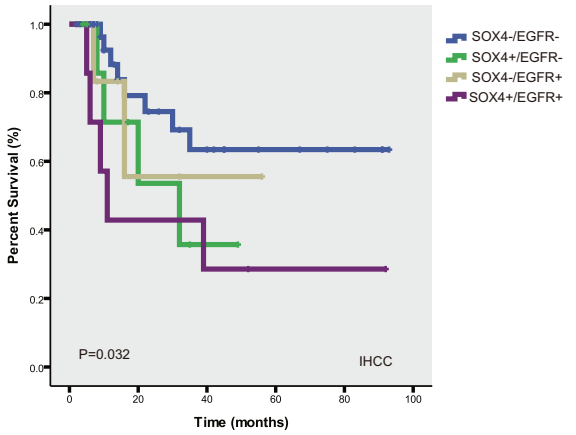


Fig. 3. Kaplan–Meier curves illustrating overall survival among IHCC patients stratified by SOX4 and EGFR expression in combination. The analysis with Kaplan–Meier method clearly showed that IHCC patients having tumors with both SOX4 and EGFR overexpression had the worst cancer-related survival compared with other groups.

3.5. Modulation of EGFR expression by SOX4 in CCA

As shown in Fig. 4, siRNA knockdown of SOX4 significantly decreased EGFR protein expression level in RBE cells. By contrast, transfection of SOX4 vector in RBE cells increased expression level of EGFR levels. Interestingly, protein level of phosphorylated EGFR was not altered in above treatment. These data suggested SOX4 modulates EGFR expression *in vitro* in CCA.

3.6. siRNA knockdown of SOX4 inhibits cellular migration in CCA cells

As shown in Fig. 4, wound healing assay indicated that siRNA-HER4-transfected RBE cells displayed a significant decrease in cell migration ability compared to control conditions.

3.7. In vitro effect of SOX4 on EMT

To explore whether SOX4 was a regulator of EMT in CCA, expression of epithelial markers (E-cadherin, and β-catenin) and mesenchymal markers (Vimentin, and N-cadherin) were analyzed after transiently siRNA knockdown of SOX4. As shown in Fig. 4, expressions of E-cadherin and β-catenin were significantly downregulated, while Fibronectin and N-cadherin were upregulated by Western blot analysis in RBE cells.

4. Discussion

In the current study, we systematically characterize SOX4 expression in a Chinese CCA cohort. As a developmental transcription factor, SOX4 gene regulates progenitor development and Wnt signaling [22]. SOX4 is overexpressed in multiple human malignancies and has been recognized as one of the 64 'cancer signature' genes, suggesting a fundamental role in tumor development and progression [15,22]. Nevertheless, how SOX4 contributes to cancer invasion and progression remain undefined. Recent studies have suggested SOX4 may promote metastasis in part by inhibiting terminal differentiation and promoting the EMT process in liver and breast cancer [11,12]. EMT is a developmental program and associated with cancer invasion and metastasis [22]. Most recently, we have demonstrated that SOX4 overexpression may contribute to EMT process in PCa metastasis [16]. In line with these findings, our data suggested that SOX4 overexpression could promote EMT and siRNA knockdown of SOX4 could disturb EMT phenotype in RBE cells. Taken together, these data supported the concept that SOX4 is a common regulator or EMT in cancer. Additionally, our results offered a novel mechanism of SOX4 associated with EMT that can be explored to identify new target drugs for CCA.

Clinically, how to precisely stratify CCA patients according to their clinical prognosis in alignment with therapeutic options remains a great challenge [1]. To date, a series of reports have identified several biomarkers which appear to carry prognostic significance. Of these, p53 mutation, cyclins, CA19-9 and growth factors appeared to hold potential as predictors of outcome [4]. In the current study, we confirmed a strong link of EGFR to the biological aggressiveness of CCA and demonstrated that EGFR expression is an independent prognostic factor in Chinese CCA patients. More importantly, for the first time, we showed a negative correlation between SOX4 expression and overall survival in IHCC patients. To date, the prognostic role of SOX4 expression in cancer remains controversial. Several studies demonstrated that SOX4 plays an oncogenic role in cancer progression. Previously, we and others suggested that SOX4 expression correlates with more aggressive tumors and poor prognosis in Pca [14,16]. Andersen et al. reported high levels of SOX4 in microsatellite stable stage II cancers correlate with recurrence, linked to metastatic capacity of primary

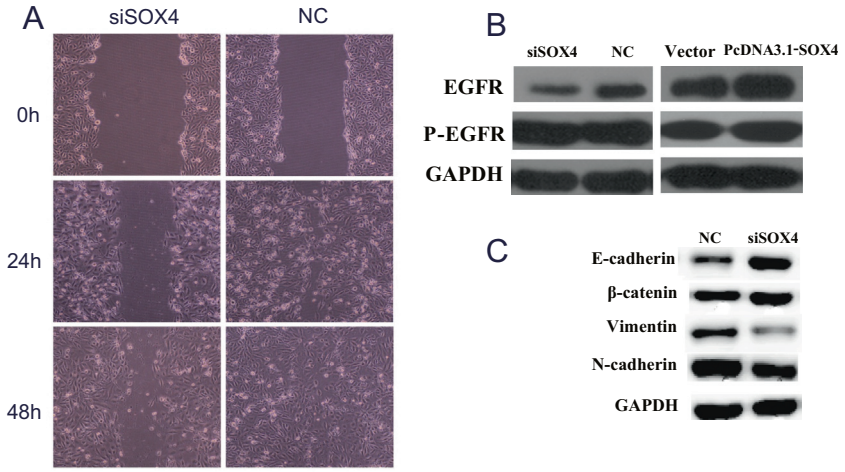


Fig. 4. The biological role of SOX4 and its regulation on EGFR in RBE cells. (A) Effects of siRNA SOX4 on RBE cell migration by wound healing assay. Original magnification, ×100; NC: negative control. (B) Modulation of EGFR expression by SOX4 in RBE cells. The protein levels of EGFR and phosphorylated EGFR were evaluated after siRNA knockdown of SOX4 and pcDNA3.1-SOX4 transfection in RBE cells. (C) The protein expression levels of the epithelial markers (E-cadherin, β-catenin) and mesenchymal markers (Vimentin, N-cadherin) were shown after siRNA SOX4 in RBE cells.

tumors [23]. By contrast, others reported a significant association of SOX4 expression with a favorable prognosis. Aaboe et al. found that SOX4 overexpression was correlated with increased survival of patients with bladder cancer [17]. Similarly, Wang et al. reported that the overexpression of SOX4 in gallbladder cancer was significantly associated with favorable clinicopathologic features and was an independent prognostic factor for better overall in patients [19]. These opposing roles suggested that the outcome of SOX4 activation depends on the cellular context and the tumor origin.

In the current study, for the first time, we demonstrated the positive correlation of SOX4 and EGFR overexpression both in IHCC and EHCC patients. EGFR gene amplification is a relatively rare event in CCA [24]. Indeed, amplification of EGFR was identified in 1.8% (1/52) of IHCC cases, and in 2% (3/82) in patients with EHCC in our cohort. Although several studies have demonstrated somatic mutations of EGFR in CCA [25], there is a subset of EGFR+ cases that were absent for gene amplification or mutation. Herein, we proposed a novel mechanism for EGFR overexpression in CCA, that is, SOX4 may induce EGFR expression as a transcription factor. Previously, a number of studies have investigated SOX4-mediated regulation of transcriptional targets in a variety of human cancers [15,22]. Analysis of potential SOX4 target genes in PCa, hepatocellular carcinoma and lung cancer revealed that SOX4 could be involved in the regulation of many key cellular processes, including apoptosis, cell cycle control, microRNA processing, differentiation and growth factor signaling [15].

In prostate cancer, it has been reported that EGFR is one of the direct targets of SOX4 [26]. Interestingly, our data revealed the modulation of EGFR expression by SOX4. More importantly, SOX4+/EGFR+ defines a subset of CCA patients with poor prognosis. Therefore, how SOX4 regulates EGFR expression in CCA merits further investigation.

HER2 is another important EGFR family growth factor receptor and known to regulate tumor progression in many different cancer types [27]. Of note, HER2 overexpression was exclusively identified in EHCC cases, but absent with clinical variables, out data suggested that HER2 overexpression might has limited significance in the development and progression of CCA.

In conclusion, this is the first analysis to show that the overexpression of SOX4 is an independent prognostic factor in Chinese CCA patients with IHCC. SOX4 overexpression in surgically excised CCA tissues might help predict overall survival of the patients. Notably, our findings suggested significant association of SOX4 and EGFR expression in a subset of CCA cases with poor prognosis. Finally, SOX4 may induce EMT process of CCA cells.

Conflict of interest

None.

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