Coexpression of *ANXA2*, *SOD2* and *HOXA13* predicts poor prognosis of esophageal squamous cell carcinoma

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Abstract. Esophageal squamous cell carcinoma (ESCC) is the main type of esophageal cancer, and is the sixth leading cause of cancer-related mortality among all types of cancers. Previously, we found that the homeobox A13 gene (*HOXA13*) plays a crucial role in the carcinogenesis of ESCC and both Annexin A2 (*ANXA2*) and superoxide dismutase 2 (*SOD2*) were its potential targets. Samples from 258 patients from two independent cohorts were collected. RT-qPCR and immunohistochemistry (IHC) were used to detect the expression levels of *HOXA13*, *ANXA2* and *SOD2*. Kaplan-Meier survival curve analysis and Cox proportional hazards regression model were employed to determine their prognostic significance. Results showed that ESCC tissues had higher *ANXA2* and *SOD2* mRNA and protein levels than the non-cancerous tissues. *ANXA2* and *SOD2* were found to be positively correlated with *HOXA13* expression not only at the mRNA level but also at the protein level. In both the study cohort and the validation cohort, the median overall survival time of patients with high expression of HOXA13, ANXA2 and SOD2 was shorter than the survival time of the patients with low expression. The Cox proportional hazards model revealed that both TNM stage and coexpression of HOXA13/ANXA2/SOD2 are independent predictors of overall survival of ESCC patients. In conclusion, the present study demonstrated that *ANXA2* and *SOD2* are potential target genes of *HOXA13* and their coexpression predicts the poor prognosis of ESCC patients.

Introduction

Esophageal cancer is the sixth leading cause of cancer-related mortality worldwide, and esophageal squamous cell carcinoma (ESCC) is the main histologic type of esophageal cancer (1). The incidence of ESCC varies widely by nearly 16-fold throughout the world, with the highest rates in Eastern Asia and Southern and Eastern Africa (2). Currently, surgical resection is the main curative therapeutic option. Although recent advances in diagnosis and treatment have improved patient prognosis, the 5-year survival rate is still low at ~30% (3).

Homeobox (*HOX*) genes encode a group of transcription factors, which can directly drive the transcription of target genes (4). However, further investigation of the detailed molecular mechanisms is needed. *HOX* genes play crucial roles in embryogenesis and tumorigenesis. There are three aberrations noted in *HOX* expression in solid tumors: re-expression, new expression, downregulation or deficiency (4). Takahashi *et al* comprehensively evaluated the expression of all *HOX* genes in 48 primary ESCC tissues and 7 normal esophageal tissues by RT-qPCR. Their data suggested that disordered expression of *HOX* genes was significantly associated with the tumorigenesis and development of ESCC (5). In our previous study, homeobox A13 gene (*HOXA13*) was found to be overexpressed in ESCC tissues when compared to that in normal tissues (3). Colony formation and nude mouse tumorigenicity assays revealed that *HOXA13* promoted tumorigenesis *in vitro* and *in vivo*. Moreover, the prognosis of HOXA13-positive patients was significantly worse than that of HOXA13-negative patients (6).

Considering that HOXA13 acts as a transcription factor, to identify its potential targets, protein expression changes after HOXA13 knockdown were detected by 2-dimensional electrophoresis (7). Among the proteins downregulated after *HOXA13* knockdown, Annexin A2 (ANXA2) and superoxide dismutase 2 (SOD2) were selected for further study. Consistent expression of *HOXA13*, *ANXA2* and *SOD2* was validated by western blotting. CHIP-DSL also revealed that *SOD2* and *ANXA2* were both potential targets of HOXA13. However, little is known concerning the clinical significance of *HOXA13*/*ANXA2* or *HOXA13*/*SOD2* coexpression in ESCC.

ANXA2, a calcium-dependent phospholipid binding protein, is implicated in apoptosis, calcium signaling, tumor invasion, metastasis and angiogenesis. Overexpression of *ANXA2*, as well as its prognostic value, has been described in colorectal (8), gastric (9) and pancreatic cancer (10,11), hepatocellular carcinoma (12), and lung (13) and breast cancer (14). However, little is known concerning its expression in ESCC.

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Superoxide dismutases (SODs) are a family of antioxidant enzymes that neutralize the reactive free superoxide radical (O_2^-) in mitochondrial reactive oxygen species (ROS) (15,16). *SOD2* (also named MnSOD) is highly expressed in cervical carcinoma (17), and in breast (18), gastric and colorectal cancer (19). Studies conducted on SOD2 in cancer focused mainly on its tumor-suppressor role, with a smaller but mounting number of studies suggesting that *SOD2* acts as an oncogene (15). It was reported that *SOD2* overexpression inhibited POX-induced apoptosis by avoiding oxidative damage to mitochondria (20-22). However, little is known concerning its role in ESCC carcinogenesis.

In the present study, *HOXA13*, *ANXA2* and *SOD2* expression was evaluated in normal esophageal mucosa and ESCC tissues, and the correlation between *HOXA13* and *ANXA2* and *SOD2* expression was examined at both the transcriptional and translational levels. In addition, the association of *HOXA13*, *ANXA2* and *SOD2* coexpression and prognosis was investigated in ESCC patients.

Materials and methods

Patients and demographic data. The present study cohort consisted of 121 patients from a prospective database of esophageal cancer patients, and all of them underwent surgery at the Department of Thoracic Surgery I, Peking University School of Oncology from July 2000 to November 2009. Our validation cohort consisted of 137 ESCC patients in addition to the above-mentioned database, which were treated between February 1996 and June 2003 at the Department of Thoracic Surgery.

All of the patients underwent radical esophagectomy, and none of them received adjuvant chemotherapy or radiotherapy prior to surgery. Resected samples were immediately sent for histological examination with hematoxylin and eosin staining. Tumor-node-metastasis (TNM) stage was evaluated according to the criteria of the UICC, 1987. To clarify the survival conditions, life-long follow-up was available by regular review (examination records) after surgery or by direct telephone interview until recurrence, metastasis or death caused by tumor. The demographic information (gender and age) and tumor characteristics (histology, differentiation and TNM stage) were acquired from medical and pathological records. A total of 18 patients with fresh frozen cancerous and non-cancerous samples with complete clinical data were collected from Anyang Cancer Hospital, Henan. Tissues were collected immediately after surgical removal and snap-frozen in liquid nitrogen until further use. All participants provided informed consent, and the study was approved by the Ethics and Academic Committees of Peking University School of Oncology.

Immunohistochemistry (IHC). Formalin-fixed and paraffin-embedded 4-μm tissue sections were routinely immunostained. After deparaffinization in xylene and rehydration in a graded ethanol series, 3% hydrogen peroxide solution was put on the slide for 10 min, and antigen retrieval was carried out in citrate solution (pH 6.0) by microwave. The sections were blocked with goat serum for 15 min and then incubated with mouse monoclonal antibody to ANXA2 and SOD2 (Abcam) at 4˚C overnight. The mouse monoclonal antibody against human ANXA2 and SOD2 was used at 2 μg/ml (1:500) and 0.5 mg/ml (1:400), and the secondary antibody was goat anti-mouse biotin-conjugated IgG. Diaminobenzidine (DAB) chromogenic reaction was used for detection. Two experienced pathologists independently examined the immunohistochemical signals. The scores were evaluated according to the number of stained cells and staining intensity. The percentage of ANXA2 or SOD2-positive tumor cells was evaluated on a scale of 0-3 (0, no staining; 1+, ≤10%; 2+, 11-30%; 3+, 31-50%; 4+, >50%). Thus, the expression level of ANXA2 and SOD2 were divided into two groups in terms of the score: negative (0, 1+, 2+) and positive (3+, 4+). For the evaluation and scoring of *HOXA13*, the same criteria were used as described in our previous study (6).

Cell culture and RNA isolation. Human esophageal cancer EC109, EC9706, KYSE150, KYSE410 and KYSE510 cells were obtained from ATCC (Manassas, VA, USA). These cell lines were cultured in 1640 medium (HyClone, Logan, UT, USA) supplemented with heat-inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA) and 100 U/ml penicillin sodium in a humidified atmosphere with 5% CO_2 at 37˚C.

Total RNAs of fresh frozen tumor specimens and sorted cells (EC109, EC9706, KYSE150, KYSE410 and KYSE510) were extracted by TRIzol (Invitrogen, Carlsbad, CA, USA). RNAs were reverse transcripted to single strand cDNAs by two-step RT-PCR (Fermentas Life Sciences).

Real-time RT-qPCR. Quantitative real-time PCR was performed using SYBR-Green Real-Time PCR Master Mix (Applied Biosystems) to detect mRNA expression levels of the target genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenic control. Special primers were designed using Oligo Primer Analysis Software (version 5.0). The sequence of the primers used are as follows: for *HOA13,* forward 5'-AGCGCGTGCCTTATACCAAG-3' and reverse 5'-GCCGCTCAGAGAGATTCGT-3'; for *ANXA2*, forward 5'-CTCTACACCCCCAAGTGCAT-3' and reverse 5'-TCAGTGCTGATGCAAGTTCC-3'; for *SOD2*, forward 5'-AAGGGAGATGTTACAGCCCAGATA-3' and reverse 5'-TCCAGAAAATGCTATGATTGATATGAC-3'; for GAPDH, forward 5'-GACCCCTTCATTGACCTCAAC-3' and reverse 5'-CTTCTCCATGGTGGTGAAGA-3'. All assays were carried out in triplicate under the 7500 Real-Time PCR System (Applied Biosystems) and repeated three times according to the manufacturer's protocol. Evaluation of relative expression was calculated by comparative Ct (threshold cycle) method. $2^{-\Delta Ct}$ referred to the fold of the mRNA expression of the target gene compared to GAPDH expression in the same sample.

Statistical analysis. Analysis was performed using SPSS 17.0 software. The χ^2 test or Fisher's exact test was used to compare the relationship between *HOXA13*, ANXA2, SOD2 expression and clinicopathological characteristics of the ESCC patients. *HOXA13*, *ANXA2* and *SOD2* mRNA expression levels in cancerous and non-cancerous tissues are presented as the means ± SD and were compared by paired t-test. Wilcoxon test was employed to compare the protein levels of *HOXA13*,

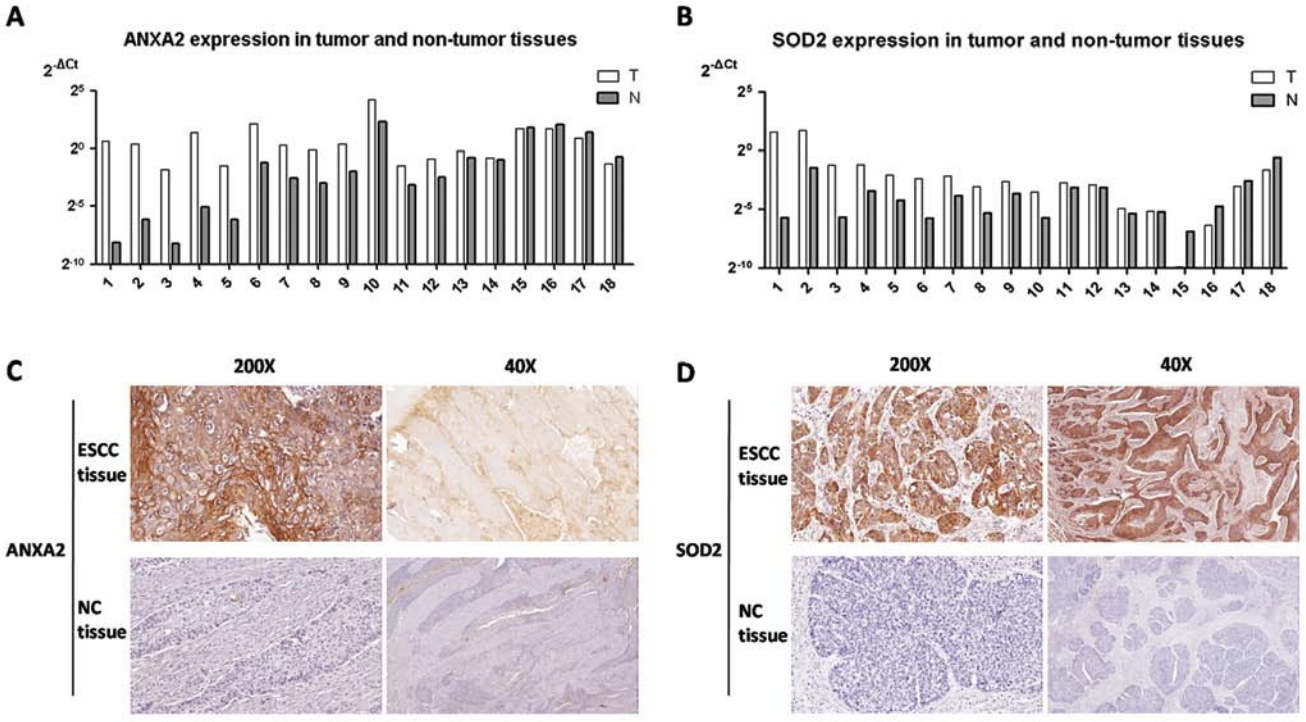

Figure 1. ESCC tissues exhibit higher ANXA2 and SOD2 expression when compared with non-cancerous tissues at both the mRNA and protein levels. Comparison of mRNA expression of (A) ANXA2 and (B) SOD2 in cancerous tissues (T) and non-cancerous tissues (N). Representative immunohistochemical staining of (C) ANXA2 and (D) SOD2 in ESCC and non-cancerous (NC) tissues. ESCC, esophageal squamous cell carcinoma; ANXA2, Annexin A2, SOD2, superoxide dismutase 2.

ANXA2 and *SOD2* between cancerous and non-cancerous tissues. Pearson's correlation coefficient analysis was applied to analyze the correlation of *HOXA13*, *ANXA2* and *SOD2* mRNA expression. Spearman's correlation coefficient analysis was applied to analyze the correlation of the protein levels.

The overall survival measured from the day of surgery was estimated by Kaplan-Meier curves, and the differences were analyzed by log-rank test. Cox proportional hazards model was used for multivariate survival analysis. The variables analyzed in the model included age, gender, tumor location, histology, tumor cell differentiation, TNM stage, *HOXA13*, *ANXA2* and *SOD2* expression. Hazard ratios and 95% confidence intervals were calculated. $p<0.05$ was considered to indicate a statistically significant result.

Results

ANXA2 and SOD2 are overexpressed in ESCC tissues. It has been demonstrated that *ANXA2* and *SOD2* are associated with gastric and colorectal cancer. To investigate whether they are also associated with ESCC, the mRNA levels of *ANXA2* and *SOD2* in ESCC and matched non-cancerous specimens were analyzed by RT-qPCR. When compared with normal esophageal tissues, *ANXA2* and *SOD2* showed higher expression in the ESCC tissues (Fig. 1A and B; Mann-Whitney test, *ANXA2* p=0.012, *SOD2* p=0.016). Furthermore, IHC was employed to assess the protein expression of ANXA2 and SOD2 in 18 ESCC and matched non-cancerous specimens. Positive expression of ANXA2 was observed in 20% of the ESCC tissues and in 0% of the non-cancerous tissues (Fig. 1C; Chi-square test, p=0.023). SOD2 overexpression was detected in 90% of the ESCC tissues and in 25% of the paired cancer margin tissues (Fig. 1D; Chi-square test, p=0.001). In conclusion, expression levels of *ANXA2* and *SOD2* were significantly higher in the ESCC tissues than levels in the non-cancerous specimens.

Expression levels of ANXA2 and SOD2 are positively correlated with HOXA13. To investigate the correlation between HOXA13 and its potential target genes, the levels of *HOXA13*, *ANXA2* and *SOD2* in 5 ESCC cell lines (EC109, EC9706, KYSE150, KYSE410 and KYSE510) were evaluated by RT-qPCR assay. Both *ANXA2* and *SOD2* showed a significant positive correlation with *HOXA13* in the 5 ESCC cell lines (data not shown; Pearson's correlation, *ANXA2* p=0.005, *SOD2* $p<0.001$), particularly *SOD2*, with a high R^2 score of 0.99.

In the 23 pairs of ESCC tissues, a strong positive correlation was observed between the mRNA expression of *ANXA2* and *HOXA13* (Pearson's correlation r=0.878, $p<0.001$). The same correlation was also found between *SOD2* and *HOXA13* (Pearson's correlation r=0.503, p=0.014). To further verify these potential positive correlations, IHC was applied in the study and validation cohorts. In the study cohort, a significant positive correlation was noted between ANXA2 and HOXA13 (Spearman correlation $r_s=0.200$, p=0.028) and the correlation between SOD2 and HOXA13 also approached significance (Spearman correlation $r_s=0.151$, p=0.098). Similar results were observed in the validation cohort. A significant positive correlation was noted between SOD2 and HOXA13 (Spearman correlation $r_s=0.148$, p=0.084) and the correlation

Table I. Association between HOXA13, ANXA2 and SOD2 expression and clinical characteristics of the patients with ESCC in the study cohort (n=121).

	HOXA13			ANXA2			SOD2		
Clinicopathological factors	High (n=21)	Low (n=100)	P-value	High (n=22)	Low (n=99)	P-value	High (n=54)	Low (n=67)	P-value
Age (years)			0.219			0.228			0.545
≤50	4	8		4	8		4	8	
>50	17	92		18	91		50	59	
Gender			0.254			0.780			0.827
Male	18	76		18	76		41	53	
Female	3	24		4	23		13	14	
Tumor location			0.950			0.090			0.117
Upper	4	48		1	21		14	8	
Middle	11	49		15	45		26	34	
Lower	6	33		6	33		14	25	
Tumor cell differentiation			0.793			0.533			0.363
Well	7	35		10	32		15	27	
Moderate	6	35		6	35		20	21	
Poor	8	30		6	32		19	19	
Tumor invasion (T)			0.075			0.004			0.698
T_1	3	25		1	27		18	10	
T_2	4	30		5	29		19	15	
T_3	9	39		10	38		24	24	
T_4	5	6		6	5		6	5	
Lymph node metastasis (N)			0.606			0.316			0.120
N_0	13	70		13	70		33	50	
N_1, N_2, N_3	8	30		9	29		21	17	
TNM stage			0.028			0.003			0.039
I, Tis	2	32		1	33		9	25	
IIa, IIb	8	42		9	41		25	25	
IIIa, IIIb, IIIc	11	26		12	25		20	17	

HOXA13, homeobox A13 gene; ANXA2, Annexin A2; SOD2, superoxide dismutase 2; ESCC, esophageal squamous cell carcinoma; TNM, tumor-node-metastasis.

between ANXA2 and HOXA13 also approached significance (Spearman correlation r_s=0.198, p=0.021).

Collectively, expression of *ANXA2* and *SOD2* was positively correlated with *HOXA13* in the ESCC cell lines and tissues.

Overexpression of ANXA2 or SOD2 indicates poor prognosis of ESCC patients, respectively. Our previous study revealed that high expression of HOXA13 indicates poor survival; thus, the potential target genes *ANXA2* and *SOD2* may also have prognostic value in ESCC. To study the correlation of *ANXA2* and *SOD2* with *HOXA13* and their roles in ESCC, we analyzed the expression of *HOXA13*, *ANXA2* and *SOD2* in both the study and validation cohorts. In the study cohort, HOXA13, ANXA2 and SOD2 expression was significantly correlated with TNM stage (Table I). In the univariate analysis, tumor invasion (T), lymph node metastasis (N), TNM stage, and expression of HOXA13, ANXA2 and SOD2 were statistically associated with poor prognosis, respectively (Table II). The validation cohort also supported a similar conclusion (data not shown).

In the study cohort, Kaplan-Meier curve analysis indicated that the median survival time was 22 months for the ANXA2-positive patients, which was significantly shorter than the 64 months for ANXA2-negative patients (Fig. 2A, log-rank p=0.026). As for SOD2, the median survival time was 31 and 84 months for SOD2-positive and SOD2-negative patients, respectively (Fig. 2B, log-rank p=0.039). In conclusion, overexpression of ANXA2 or SOD2 indicated poor prognosis of ESCC patients, respectively. This was similar in the validation cohort. Kaplan-Meier curve analysis indicated that the median survival time was 15 months for ANXA2-positive

Table II. Clinicopathological features, tumor markers and patient survival in the study cohort (n=121, univariate analysis).

Variables	Hazard ratio (95% CI)	P-value
Age, years	1.009 (0.982-1.037)	0.514
Gender	1.229 (0.682-2.217)	0.492
Tumor location (upper/middle vs. lower)		0.561
Middle vs. upper	0.895 (0.307-2.610)	0.337
Lower vs. upper	0.825 (0.474-1.437)	0.497
Tumor cell differentiation (poor/moderate vs. well)		0.534
Moderate vs. poor	1.809 (0.675-4.848)	0.239
Well vs. poor	1.207 (0.519-2.808)	0.662
Tumor invasion (T)		0.021
T_1 vs. T_4	0.338 (0.142-0.806)	0.014
T_2 vs. T_4	0.417 (0.184-0.946)	0.036
T_3 vs. T_4	0.745 (0.354-1.566)	0.437
Lymph node metastasis (N)	0.447 (0.274-0.728)	0.001
TNM stage (I/IIa/IIb vs. III)		0.003
I vs. III	0.194 (0.025-1.537)	0.121
IIa vs. III	0.265 (0.107-0.659)	0.004
IIb vs. III	0.494 (0.210-1.159)	0.105
HOXA13 expression (yes vs. no)	2.020 (1.294-3.145)	0.002
ANXA2 expression (yes vs. no)	2.074 (1.344-3.202)	0.001
SOD2 expression (yes vs. no)	1.764 (1.181-2.634)	0.006

CI, confidence interval; TNM, tumor-node-metastasis; HOXA13, homeobox A13 gene; ANXA2, Annexin A2; SOD2, superoxide dismutase 2; ESCC, esophageal squamous cell carcinoma.

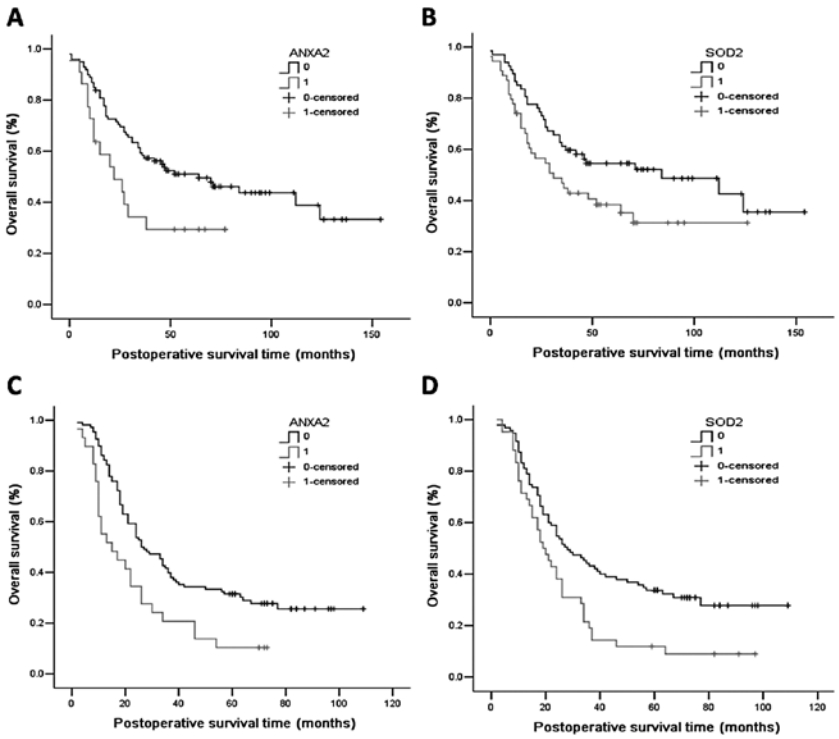

Figure 2. Kaplan-Meier survival curves for ESCC patients bearing tumors with or without ANXA2 and SOD2 expression. The overall survival of patients bearing tumors with ANXA2 expression was significantly shorter than that of patients bearing tumors without ANXA2 expression in both the (A) study and (C) validation cohort. The overall survival of patients with SOD2 expression was significantly shorter than that of patients bearing tumors without SOD2 expression in both the (B) study and (D) validation cohort. ESCC, esophageal squamous cell carcinoma; ANXA2, Annexin A2, SOD2, superoxide dismutase 2. 0, negative expression; 1, positive expression.

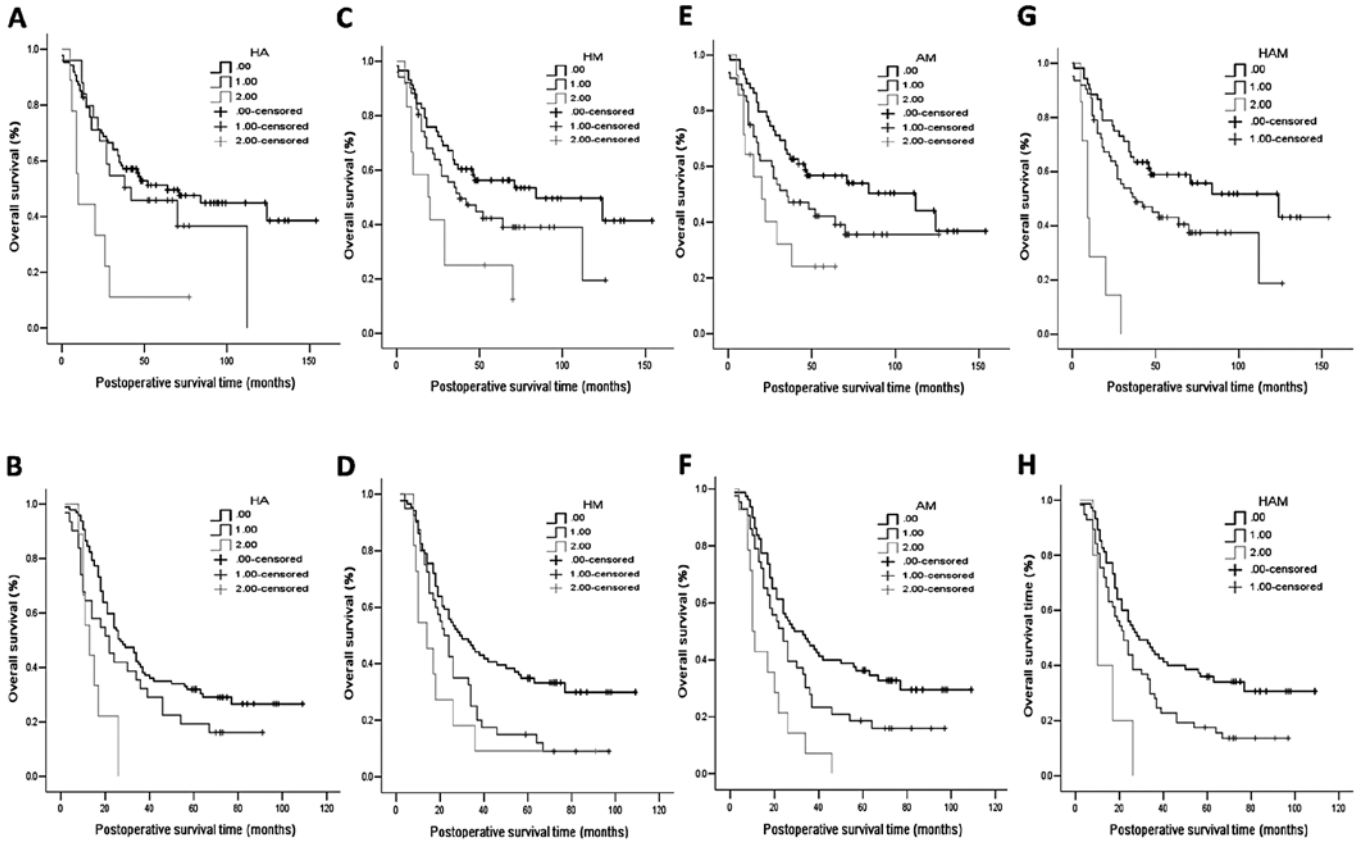

Figure 3. Kaplan-Meier survival curves for ESCC patients subgrouped according to the combined expression of HOXA13, ANXA2 and SOD2. The survival of patients with HOXA13^{+}/ANXA2^{+} expression was significantly shorter than that of the patients with HOXA13^{-}/ANXA2^{-} expression in both the (A) study and (B) validation cohort. The survival of patients with HOXA13^{+}/SOD2^{+} expression was significantly shorter than that of patients with HOXA13^{-}/SOD2^{-} expression in both the (C) study and (D) validation cohort. The survival of patients with ANXA2^{+}/SOD2^{+} expression was significantly shorter than that of patients with ANXA2^{-}/SOD2^{-} expression in both the (E) study and (F) validation cohort. The survival of patients with HOXA13^{+}, ANXA^{+} and SOD2^{+} (triple-positive) expression was significantly shorter than that of the triple-negative patients in both the study cohort (G) and (H) validation cohort. ESCC, esophageal squamous cell carcinoma; ANXA2, Annexin A2, SOD2, superoxide dismutase 2. HA, HOXA13+ANXA2 coexpression; HM, HOXA13+SOD2 coexpression; AM, ANXA2+SOD2 coexpression; HAM, HOXA13+ANXA2+SOD2 coexpression. 0, no markers positive; 1, some markers positive; 2, all markers positive.

patients, which was significantly lower than the 26 months for ANXA2-negative patients (Fig. 2C, log-rank p=0.003). As for SOD2, the median survival time was 19 and 28 months for SOD2-positive and SOD2-negative patients, respectively (Fig. 2D, log-rank p=0.003).

Combined expression of HOXA13, ANXA2 and SOD2 has increased prognostic value in ESCC. The coexpression of HOXA13 and its potential targets ANXA2 and SOD2 were analyzed. On the basis of HOXA13 and ANXA2 expression, in the study cohort, all of the patients were categorized into three groups: double-positive (HOXA13^{+}/ANXA2^{+}), single-positive (HOXA13^{+}/ANXA2^{-} and HOXA13^{-}/ANXA2^{+}) and double-negative (HOXA13^{-}/ANXA2^{-}). The median survival time of double-positive patients was 10 months, significantly lower than the 64 months for the double-negative patients and 42 months for the single-positive patients (Fig. 3A, log-rank p=0.002). A similar conclusion was found for the validation cohort. The median survival time of the double-positive patients was 13 months, significantly less than the 27 months for the double-negative patients and 22 months for the single-positive patients (Fig. 3B, log-rank p=0.001). For coexpression of HOXA13/SOD2, in the study cohort, the median survival time was 19, 38 and 84 months for the double-positive, single-positive and double-negative patients, respectively (Fig. 3C, log-rank p=0.010). In the validation cohort, the median survival time was 14, 22 and 29 months for the double-positive, single-positive and double-negative patients, respectively (Fig. 3D, log-rank p=0.004). For ANXA2 and SOD2, coexpression of ANXA2 and SOD2 was also predictive of a poor prognosis in the study cohort (Fig. 3E, log-rank p=0.018) and the validation cohort (Fig. 3F, p=0.001).

Moreover, when combining the expression of HOXA13, ANXA2 and SOD2, a better prognostic model was obtained in the two cohorts. We found that coexpression of HOXA13, ANXA2 and SOD2 was significantly associated with overall survival in the study cohort (Fig. 3G, log-rank p<0.001) as well as in the validation cohort (Fig. 3H, log-rank p=0.001). In the study cohort, TNM stage (p=0.006) and HOXA13/ANXA2/SOD2 (p=0.002) coexpression are both independent poor predictors of overall survival time in the multivariate analysis (Table III). In the validation cohort, consistent with the above, multivariate analysis showed that TNM stage (p=0.002) and HOXA13/ANXA2/SOD2 (p=0.017) coexpression are both independent predictors of poor overall survival (Table IV).

Table III. Independent predictors of the overall survival time in the study cohort (multivariate analysis, n=121).

Variables	Hazard ratio (95% CI)	P-value
TNM stage (I/IIa/IIb vs. III)		0.006
I vs. III	0.404 (0.208-0.784)	0.007
IIa vs. III	0.358 (0.152-0.840)	0.018
IIb vs. III	0.382 (0.206-0.711)	0.002
HOXA13/ANXA2/SOD2		0.002
None positive vs. all positive	0.171 (0.068-0.433)	<0.001
Partly positive vs. all positive	0.294 (0.124-0.699)	0.294

CI, confidence interval; TNM, tumor-node-metastasis; HOXA13, homeobox A13 gene; ANXA2, Annexin A2; SOD2, superoxide dismutase 2; ESCC, esophageal squamous cell carcinoma.

Table IV. Independent predictors of the overall survival time in the validation cohort (multivariate analysis, n=137).

Variables	Hazard ratio (95% CI)	P-value
TNM stage (I/IIa/IIb vs.III)		0.002
I vs. III	0.197 (0.048-0.813)	0.025
IIa vs. III	0.592 (0.390-0.899)	0.014
IIb vs. III	0.363 (0.164-0.806)	0.013
HOXA13/ANXA2/SOD2		0.017
None positive vs. all positive	0.332 (0.129-0.858)	0.023
Partly positive vs. all positive	0.535 (0.208-1.374)	0.194

CI, confidence interval; TNM, tumor-node-metastasis; HOXA13, homeobox A13 gene; ANXA2, Annexin A2; SOD2, superoxide dismutase 2; ESCC, esophageal squamous cell carcinoma.

Discussion

Homeobox (*HOX*) genes function as primary regulators in embryogenesis and tumorgenesis. Transcriptional factors encoded by *HOX*, which have been detected as deregulated in various types of tumors, regulate cell proliferation and differentiation (4). Previously, we performed the first comprehensive investigation on the 39 *HOX* genes in ESCC; 8 of the 39 *HOX* genes were detected in cancerous tissues rather than non-cancerous tissues. The upregulation of *HOXA13* was observed in ESCC cell lines and cancerous tissues. Colony formation and nude mouse tumorigenicity assays revealed that *HOXA13* promotes tumor cell proliferation *in vitro* and *in vivo*, and *HOXA13* expression is significantly associated with disease-free survival. Subsequently, a proteomics study and CHIP-DSL revealed that *ANXA2* and *SOD2* are potential targets of HOXA13.

In the present study, we revealed that both *ANXA2* and *SOD2* were overexpressed in ESCC tissues when compared to the levels in the normal esophageal tissues. Further analysis of *ANXA2* and *SOD2* expression combined with *HOXA13* expression in the same series of ESCC tissues indicated a significantly positive correlation between them at both the protein and mRNA levels. Collectively, *ANXA2* and *SOD2* may participate in ESCC tumorigenesis as well as *HOXA13*.

However, to date, the molecular pathway linking HOXA13 and its potential targets is not yet clear. As a transcriptional factor, the core binding motif of HOXA13 has been identified: a core sequence of TAA, and TAA-containing sequences were TAAA (50%), TAAC (30%) and TAAT (20%) (23), which were also found in the promotor region of both *ANXA2* and *SOD2* (data not shown).

ANXA2 is a member of the calcium and phospholipid-dependent proteins. Binding of t-PA and ANXA2 on the membrane of pancreatic cancer cells was found to activate tumor cell invasion (10). ANXA2 was found to facilitate cell cycle and proliferation in non-small cell lung cancer by inhibiting p53, while the silencing of ANXA2 increased p53 expression, which led to p53-dependent and -independent G2 arrest (13). The present study suggests that overexpression of ANXA2 is indicative of the poor prognosis of ESCC patients, which corroborates the role of oncogenic ANXA2 revealed by such mechanistic studies.

SOD2 is a member of the manganese superoxide dismutase family, which encodes a mitochondrial protein. Studies suggest that *SOD2* overexpression is associated with tumor invasion and metastasis. NF-κB was found to reduce tumor progression through binding to intronic enhancer element to activate the expression of *SOD2* (18). Our results also indicated that over-

expression of SOD2 is predictive of poor prognosis of ESCC patients.

Considering the oncogenic role of *ANXA2* and *SOD2*, and our previous result of their coexpression in ESCC, we speculate that *HOXA13* may act as an oncogene in ESCC by regulating *ANXA2* and *SOD2* expression, which still needs further investigation. Revealing the specific mechanism of the above association may further our understanding of ESCC carcinogenesis, with the potential to develop new drug targets of ESCC and possibly, to establish a more personalized prognosis for each patient.

Since *ANXA2* and *SOD2* were found to be involved in ESCC and were associated with *HOXA13*, elucidation of their clinical significance was of great concern. We revealed that not only *ANXA2* or *SOD2* expression alone but also their coexpression with *HOXA13* was significantly correlated with the overall survival of ESCC patients. Kaplan-Meier survival curve analysis showed that coexpression of HOXA13/ANXA2/SOD2 was indicative of a poor prognosis of ESCC patients, while Cox proportional hazards regression model indicated that coexpression of HOXA13/ANXA2/SOD2, as well as TNM stage, are both independent prognosis factors of ESCC. To strengthen our conclusion, all of the results were validated in two independent cohorts. Collectively, both ANXA2 and SOD2 had a significant prognostic value for ESCC patient, and their coexpression with HOXA13 may have added prognostic value, as a complement to the TNM staging system.

In conclusion, HOXA13 as well as its target genes ANXA2 and SOD2 are potential negative predictors of overall survival time of ESCC patients. Thus, combination of their expression profile and the TNM stage classification may provide a more accurate prediction of the postoperative outcome of ESCC patients.

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