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Original Article

Prognostic value of SATB2 expression in patients with esophageal squamous cell carcinoma

Guo-Jun Geng[1,2*], Ning Li[2*], Yan-Jun Mi[2*], Xiu-Yi Yu[2], Xian-Yang Luo[3], Jing Gao[3], Qi-Cong Luo[2], Jing-Dun Xie[4], Xian-En Fa[1], Jie Jiang[2]

[1]Department of Thoracic Surgery, The Second Affiliated Hospital, Zhengzhou University, Zhengzhou, China; [2]Department of Thoracic Surgery, The First Affiliated Hospital of Xiamen University, Xiamen, China; [3]Department of Head and Neck Surgery, The First Affiliated Hospital of Xiamen University, Xiamen, China; [4]Department of Anesthesiology, State Key Laboratory of Oncology in South China, Sun Yat-Sen University Cancer Center, Guangzhou, China. [*]Equal contributors.

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Abstract: SATB2, a member of the family of special AT-rich binding proteins, has been shown to affect numerous tumorigenesis. However, the role of SATB2 in esophageal squamous cell carcinoma (ESCC) remains unclear. In this study, the SATB2 expression was examined at mRNA and protein levels by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR), Western blotting, and immunohistochemistry in ESCC tissues and adjacent non-cancerous tissues. Statistical analyses were applied to test the associations between SATB2 expression, clinicopathologic factors, and prognosis. Western blotting and qRT-PCR showed that the expression levels of SATB2 mRNA and protein were both significantly lower in SATB2 tissues than those in non-cancerous tissues. Immunohistochemistry analysis showed that SATB2 expression was significantly correlated with clinical stage and Histological differentiation. The results of Kaplan-Meier analysis indicated that a low expression level of SATB2 resulted in a significantly poor prognosis of ESCC patients. Importantly, multivariate analysis showed that low SATB2 expression was an independent prognostic factor for ESCC patients. In sum, our data suggest that SATB2 plays an important role in ESCC progression, and that decreased expression of SATB2 in tumor tissues could be used as a potential prognostic marker for patients with ESCC.

Keywords: SATB2, esophageal cancer, prognosis, immunohistochemistry, biomarker, real-time PCR

Introduction

Esophageal cancer is the 6th leading cause of human cancer death worldwide [1]. Esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) are the 2 major histological types of esophageal cancer. In eastern Asia, ESCC accounts for more than 90% of esophageal cancer cases. Indeed, most patients suffering from ESCC are diagnosed at advanced stages of the disease, the 5-year survival rate is poor (9%-40%) and currently represents the fourth poorest among all forms of cancer [2]. ESCC is believed to develop owing to various risk factors including the accumulation of genetic mutations, tobacco and alcohol consumption, hot tea drinking, low intake of fresh fruit and vegetables, obesity and diet [1]. Until now, there are few specific biomarkers available for diagnostic use and in the development of targeted therapies against ESCC. Hence, Searching biomarkers for early detection, prognostic stratification, and novel therapeutic interventions are imperiously needed for effective treatment of ESCC.

Human special AT-rich sequence-binding protein-2 (SATB2) is a member of the satb family of transcription factors, which is involved in regulating gene expression by orchestrating chromatin structure and remodeling [3]. SATB2 acts as a molecular node in a transcriptional network regulating craniofacial development and cortical neurons differentiation. Knockout mouse models have showed roles for SATB2 in craniofacial morphogenesis and osteoblast differentiation in part via SATB2-mediated repression of Hoxa2 and cooperation with ATF4 and

Table 1. Correlation between patient's clinicopathologic features and the expression of SATB2 protein

	n	SATB2		*P*
		Low expressions	High expressions	
Age				
> 60	103	51	52	
< 60	100	52	48	0.723
Sex				
Male	135	70	65	
Female	68	33	35	0.655
Tumor size				
Diameter > 4	107	65	42	
Diameter < 4	96	48	48	0.124
Histological differentiation				
G1	75	30	45	
G2	64	33	31	
G3	64	40	24	0.030
Clinical stage				
I	7	3	4	
II	115	51	64	0.023*
III	67	37	30	
IV	14	12	2	
pT classification				
T1	6	3	3	
T2	36	17	19	
T3	143	73	70	0.650
T4	18	10	8	
pN classification				
No	109	52	57	0.352
Yes	94	51	43	

*stage I + II versus III + IV.

Runx2 to promote osteoblast [4]. SATB2 also regulates neuronal specification and skeletal development [5]. In head and neck squamous cell carcinoma (HNSCC), SATB2 augments ΔNp63α activity to promote chemoresistance and SATB2 expression was associated with more advanced stage HNSCC [6]. SATB2 has also been reported to be upregulated in cancer-associated fibroblasts mediating migration of endometrial cancer cells, and higher SATB2 mRNA levels were detected in breast cancer tissue compared with normal matched tissue [7]. In contrast, SATB2 might involve in the development and progression of LSCC as a tumor suppressor [8]. The data of these studies were controversial, suggesting that further studies are required to evaluate the relationship between SATB2 expression and cancer progression. To date, there is no report on its role in tumorigenesis and progression of esophageal carcinoma. In this study, we thus investigated the expression of SATB2 and its clinical significance in human esophageal carcinoma.

Materials and methods

Patients and tumor specimens

The study was approved by the Cancer Center of Sun Yat-Sen University, Guangdong, China. Informed consent was obtained from patients prior to surgery. 10 pairs of fresh ESCC tissue specimens and corresponding nontumorous specimens were obtained from patients with ESCC who underwent surgical esophageal tissue resection at the Cancer Center of Sun Yat-Sen University. All excised samples were obtained within 1 h after operation and immediately frozen in liquid nitrogen and stored at -70°C until use. In addition, a total of 203 paraffin-embedded ESCC samples which were histologically and clinically diagnosed between 2002 and 2007 at the Cancer Center of Sun Yat-Sen University were also included in this study. Clinical and pathological data of the 203 patients with ESCC were collected, such as age, tumor size, stage, differentiation grade, lymph node metastases, treatment and recurrence. The characteristics of these 203 patients are listed in **Table 1**. None of the patients underwent radiotherapy or chemotherapy before surgery. The All patients' disease stages were classified according to the pathological TNM classification. Clinical follow-up information was obtained by telephone or from the outpatients' records.

Real-time PCR

Total RNA from human tissues was extracted using TRIzol solution (Invitrogen; Carlsbad, CA) according to the manufacturer's protocol; RNase-free DNase I was used to remove the DNA contamination. M-MLV reverse transcriptase (Fermentas; American) was used according to the manufacturer's recommendations to treat 2 μg of the total RNA for synthesizing the first-strand cDNA. The cDNA was subjected to real-time quantitative PCR for the evaluation of the relative mRNA levels of SATB2 and GAPDH (as an internal control) with the corresponding

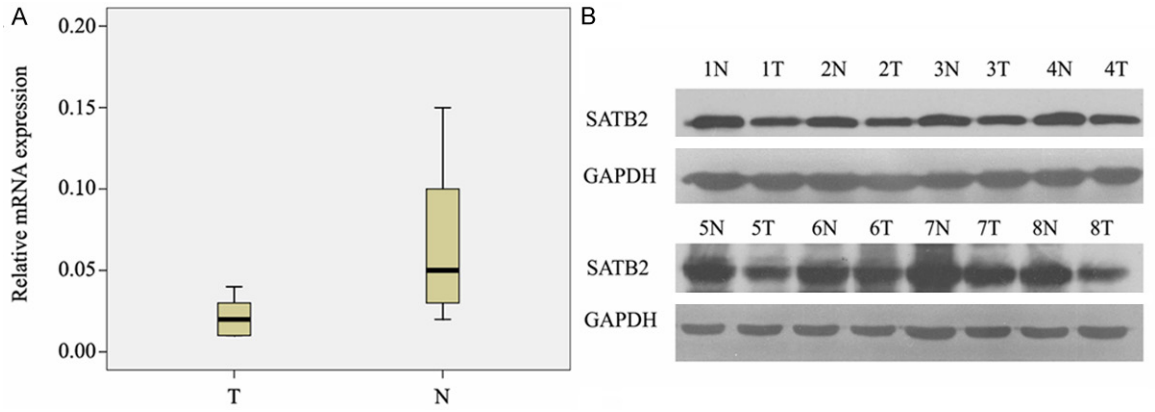

Figure 1. Expression levels of SATB2 in esophageal squamous cell carcinoma tissues by real-time PCR (A) and Western blots (B). (A) The relative expression of SATB2 mRNA in ESCC tumor tissues samples was lower than that in the paired adjacent normal (N) tissue samples (n = 10, $P < 0.01$). The bottom and the top of the box represent whisker represent the 25th and the 75th percentile, respectively, and the band near the middle of the box is the 50th percentile (the median). The ends of the whiskers represent the 2.5th percentile and the 97.5 percentile. (B) Western blots results in 8 pairs of esophageal tissues. (N means normal and T means tumor).

primer pairs (SATB2 sense strand: 5'-GGAGAACGACAGCGAGGAA-3', SATB2 antisense strand: 5'-CCGATGTATTGCTTTGCCTAGT-3', GAPDH sense strand: 5'-CTCCTCCTGTT-CGACAGTC-AGC-3', GAPDH antisense strand: 5'-CCCAATACGACCAAATC-CGTT-3'). Gene-specific amplification was performed using an Applied Biosystems (ABI 7000) real-time PCR machine with a 20 μl PCR reaction mixture containing 1 μl of cDNA (synthesized as described above), 10 μl SYBR Green master mix (Invitrogen; Carlsbad, CA), and 40 nM of each pair of oligonucleotide primers. The amplification conditions were 94°C (14 min) and 40 cycles of 94°C (15 sec), 55°C (40 sec), and 72°C (20 sec). Relative expression levels of the target genes were normalized to the geometric mean of the internal control gene, GAPDH. The data was analyzed using the comparative threshold cycle ($2^{-\Delta CT}$) method. To ensure reproducibility of results, all reactions were run in triplicate in three independent experiments.

Immunohistochemical analysis

Immunohistochemistry was performed as described previously [9]. In brief, paraffin-embedded specimens were cut into 4 mm sections and baked at 65°C for 1 hour. The sections were deparaffined with xylenes and rehydrated, submerged into 0.01 M citrate buffer (pH 6.0) antigen retrieval buffer, and microwaved for antigenic retrieval. They were treated with 0.3% H_2O_2 for 15 min to block the endogenous peroxidase at RT, and then were treated with normal goat serum for 30 min to reduce the nonspecific binding. Consequently, the sections were incubated with rabbit polyclonal anti-SATB2 antibody (1:200) overnight at 4°C. After being washed, the sections were treated with MaxVision^{TM} HRP-Polymer anti-rabbit HIC Kit (Maixin Bio; Fujian, China) at 37°C for 30 min. The tissue sections were immersed in 3-amino-9-ethyl carbazole, counterstained with Mayer's hematoxylin, dehydrated, and finally mounted in Crystal Mount.

The immunohistochemically stained tissue sections were scored independently by two pathologists blinded to the clinical parameters. The final score for SATB2 was the average of the scores obtained by the two observers. The intensity and extent of the staining were used as criteria of evaluation. The staining intensity was scored as 0 (no staining), 1 (light yellow), 2 (yellow brown), or 3 (brown). Extent of staining was scored as 0 (0%), 1 (< 5%), 2 (5 to 25%), 3 (26 to 50%), 4 (51 to 75%), or 5 (76 to 100%), according to the percentages of the positive staining areas in relation to the whole carcinoma area or entire section for the normal samples. Staining index was calculated as the multiplication of staining intensity score and the proportion of SATB2-positive tumor cells. We evaluated SATB2 expression in benign esophageal tissue and malignant lesions on the basis of the staining index values, with scores of 0, 1, 2, 3, 4, 5, 6, 8, 9, 10, 12, and 15. An optimal

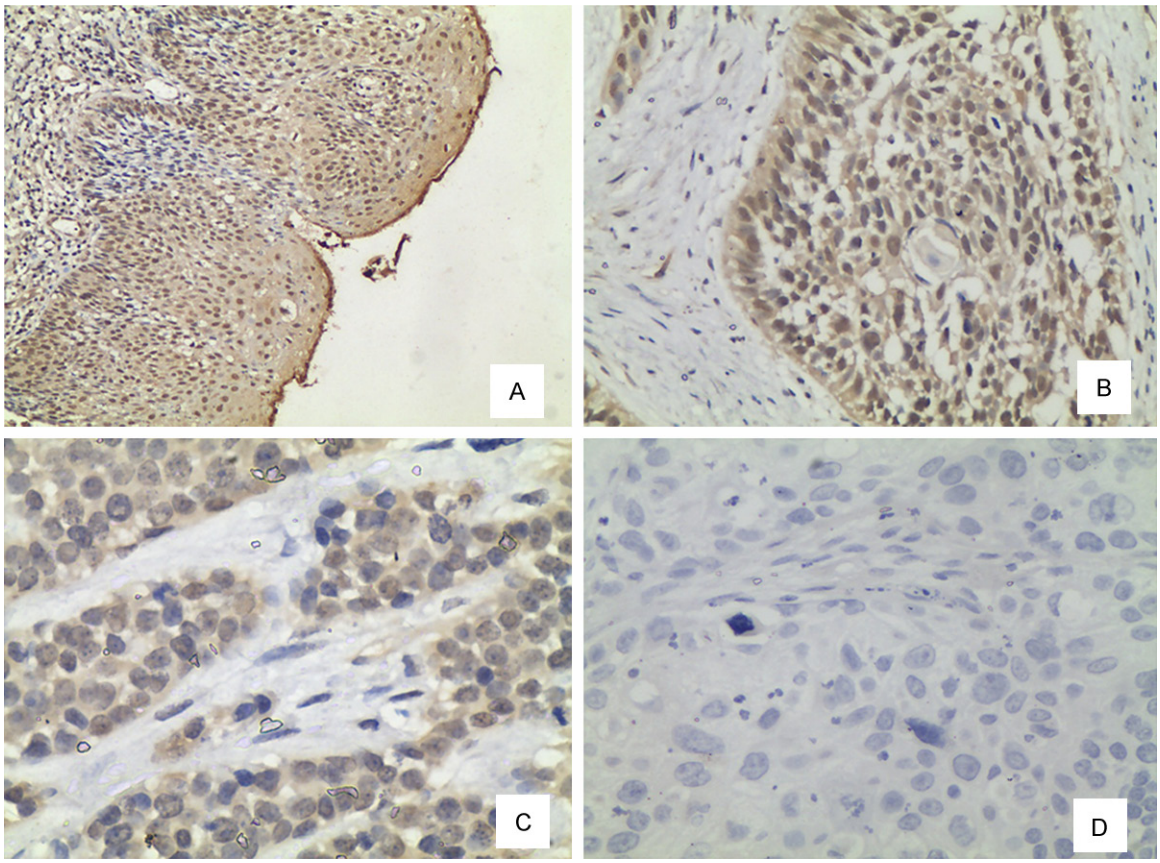

Figure 2. Analysis of SATB2 protein by immunohistochemistry in ESCC. SATB2 expression was mainly localized within nuclei of tumor cells, and its expression was observed in tumor cells. A. Staining of SATB2 staining in normal esophageal epithelial tissue (200 ×). B. Strong SATB2 staining in esophageal carcinoma tissues (400 ×). C. Weak SATB2 staining in esophageal carcinoma tissues (400 ×). D. Negative SATB2 staining in esophageal carcinoma tissues (400 ×).

cutoff value was identified: a staining index score of ≥ 5 was considered as high SATB2 expression, whereas a staining index score of ≤ 4 was considered as low SATB2 expression.

Western blotting analysis

Tissue samples were solubilized in SDS lysis buffer containing protease inhibitor. The protein concentration was quantified using the BCA protein assay kit (PIERCE, Rockford, IL). Equivalent amounts of protein were resolved by SDS-polyacrylamide gel and transferred to PVDF membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were blocked with 5% non-fat milk solution for 1 h, and incubated with a primary monoclonal antibody to SATB2 for 2 h at room temperature. After washing with TBST, the membranes were incubated with a secondary antibody. The membranes were washed and protein was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The membranes were then stripped and probed with an anti-GAPDH mouse monoclonal antibody to confirm equal loading of the samples.

Statistical analysis

All statistical analyses were carried out using the SPSS18.0 statistical software package. In the real-time RT-PCR and immunohistochemical assays, paired-sample t tests were used to analyze the significance of the differences in mRNA and protein expression between ESCCs and the adjacent normal tissues. The Chi-square and Fisher's exact tests were used to analyze the relationship between SATB2 expression and clinical-pathological characteristics. Survival curves were plotted by the Kaplan-Meier method and compared by the

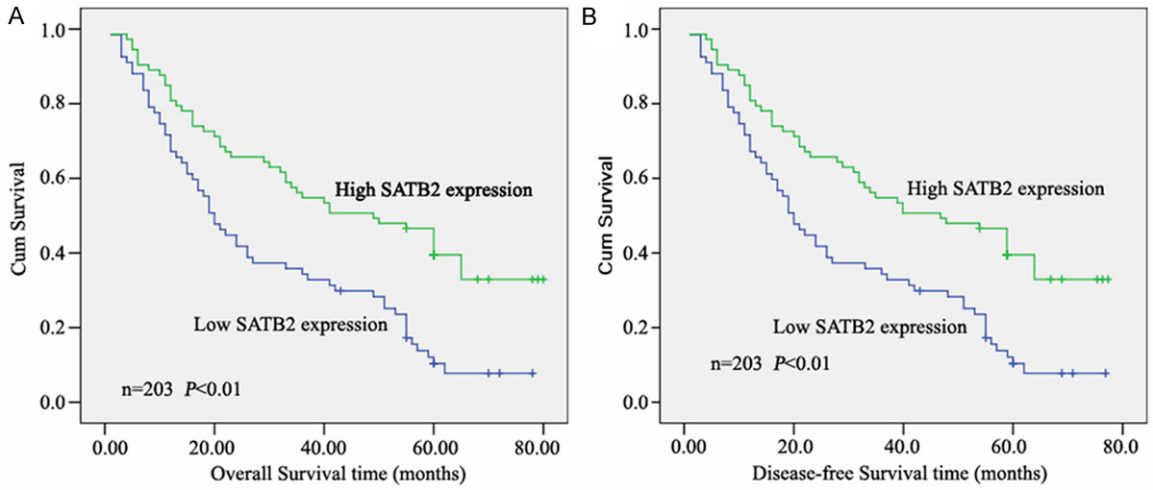

Figure 3. Survival analysis of primary ESCC patients (n = 203). Kaplan-Meier analysis of (A) Overall and (B) disease-free survival duration in all patients according to SATB2 expression. The survival rate for patients in the SATB2 low group was significantly lower than that for patients in the SATB2 positive group (log-rank test, $P < 0.01$).

log-rank test. Univariate and multivariate regression analyses were performed with the Cox proportional hazards regression model to analyze independent factors affecting prognosis. *P*-value of less than 0.05 was considered to be statistically significant.

Results

Expression of SATB2 in ESCC tissues

To determine the expression of SATB2 in esophageal carcinoma biopsies, quantitative RT-PCR was performed in 10 esophageal cancer and nontumorous tissue samples obtained from the same patients. In 10 tumor samples, the mRNA level of SATB2 was significantly lower than that in nontumorous samples ($P < 0.01$, **Figure 1A**). To determine whether the lower level of SATB2 mRNA expression revealed by Real time PCR analysis was linked to decreased level of SATB2 protein expression, we performed western blot analysis with protein extracts from matched samples of tumor (T) and adjacent normal tissue (N). As shown in **Figure 1B**, SATB2 expression was found to be decreased in 8 of 10 cases of primary esophageal carcinoma, whereas high SATB2 expression was found in the normal esophageal tissues. There was no significant difference in other two pairs of esophageal carcinoma biopsies, which showed similar expression of SATB2 in both normal and tumor tissues (Data not shown). Taken together, these data demonstrate that SATB2 is low expressed at both mRNA and protein levels in most of the esophageal cancer tissues.

Expression of SATB2 in archival esophageal cancer tissues

We measured SATB2 protein expression levels and subcellular localization in archived paraffin-embedded esophageal cancer and normal esophageal tissue samples using Immunohistochemical staining. We observed the subcellular location of SATB2 was nuclei and cytoplasm of tumor cells, but mainly nuclei. Also, we found 48 of 203 (23.6%) paraffin-embedded esophageal cancer tissues showed negative staining of SATB2 protein, while 55 of 203 (27.1%) esophageal cancer tissues showed mainly moderate SATB2 staining and 100 of 203 (49.3%) showed strong staining in tumor cells (**Figure 2**).

Relationship between SATB2 protein expression and clinicopathological parameters

The correlation between the expression of SATB2 and clinical characteristics are listed in **Table 1**. The expression of SATB2 was closely associated with stage of esophageal cancer patients ($P = 0.023$). Higher staging on correlated with higher SATB2 expression. However, there was no significant correlation between the expression level of SATB2 and age, gender, N classification, or T classification of esophageal cancer patients.

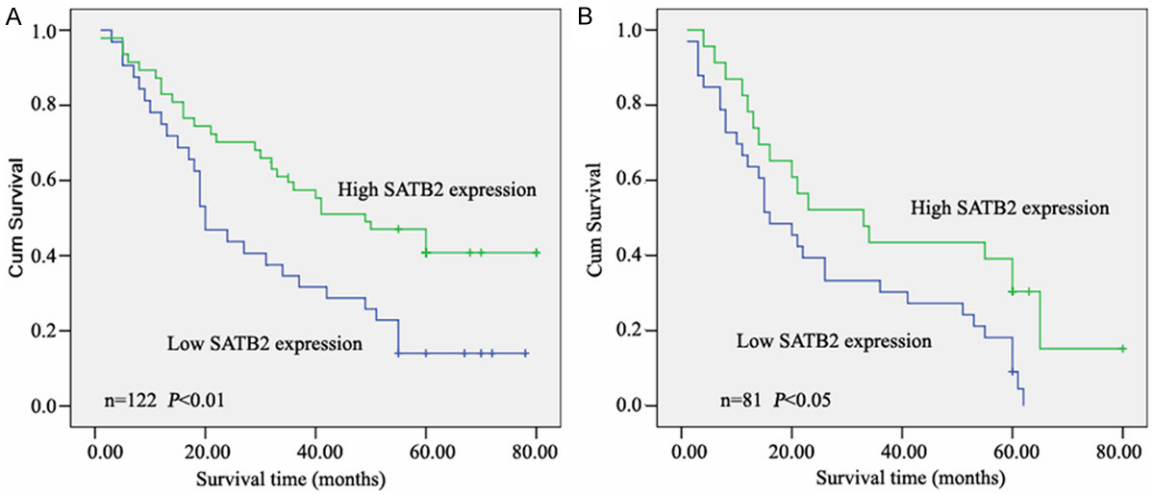

Figure 4. Kaplan-Meier analysis showing the overall survival of esophageal carcinoma patients categorized according to the Pathological stage and status of SATB2 expression. The statistical significance of the difference between curves of SATB2 high-expressing and low-expressing patients was compared in I-II (A) and III-IV (B) patient subgroups. Plot shows the survival rate for patients with low SATB2 expression versus high SATB2 expressing tumors. *P* values were calculated by the log-rank test.

Correlation of SATB2 expression with overall survival

To investigate the prognostic value of SATB2 for ESCC, we evaluated the association between SATB2 expression and survival duration using Kaplan-Meier analysis with the log-rank test. We next evaluated whether the level of SATB2 expression was associated with patient prognosis. We observed that expression of SATB2 was significantly correlated with overall survival (**Figure 3A**) and disease-free survival (**Figure 3B**) in patients with ESCC. The low SATB2 expression group had shorter survival, whereas the high SATB2 expression group had better survival. The median survival of patients with low SATB2 expression was much shorter (21 months) than those with high SATB2 expression (41 months). Furthermore, the relationship of SATB2 expression with prognosis was determined in 203 patients, which were divided into 2 subgroups depending on the pathologic stage. Patients with tumors exhibiting high SATB2 expression had significantly longer overall survival than those with low expression of SATB2 either in the stage I plus II subgroup (n = 122; log-rank, $P = 0.007$; **Figure 4A**), the stage III plus IV subgroup (n = 81; log-rank, $P = 0.022$; **Figure 4B**).

In addition, univariate Cox regression analysis showed that gender, stage and N classification were also significantly correlated with overall survival (for stage, $P = 0.014$ and for gender, $P = 0.016$; for N classification, $P = 0.009$). We thereafter did multivariate survival analysis, which included SATB2 expression level, stage, N classification and gender, to determine whether SATB2 expression level is an independent prognostic factor for outcomes. In this analysis, N classification and SATB2 expression were recognized as independent prognostic factors (**Table 2**). Thus, our findings indicate that SATB2 expression has an obvious correlation with poor prognosis of ESCC patients.

Discussion

The SATB family proteins have various functions due to their capacity to regulate gene expression and higher arrange chromatin structure. Until recently, the majority of studies on SATB2 were limited to roles in development of the nervous and immune systems and roles for SATB2 in cancer had not been well described. Chung et al. first reported that SATB2 is highly expressed in advanced HNSCC where it promotes survival and chemoresistance [6]. They now have showed a novel role for SATB2 in promoting OS invasion and migration by regulating the expression of EPLIN and other genes involved in motility, cytoskeletal organization and adhesion [10]. In contrast, Zeng et al. demonstrated that SATB2 might involve in the

Table 2. Univariate and multivariate analysis of different prognostic variables in patients with ESCC by cox

	Univariate analysis		Multivariate analysis	
	P	HR (95% CI)	*P*	HR (95% CI)
Gender				
Female vs. Male	0.016	1.059-2.021	0.057	1.011-2.153
Clinical stage				
III-IV vs I-II	0.014	1.024-2.071	0.078	0.955-1.495
pN classification				
Yes vs no	0.009	1.037-2.156	0.006	1.023-2.371
Expression of SATB2				
high vs low	0.017	0.503-0.914	0.042	0.397-0.739

development and progression of LSCC as a tumor suppressor [8]. Up to now, little has been known about the expression of SATB2 in ESCC tissue or cell line, although in other types of carcinoma, such as breast, colorectal and oral cancer, SATB2 seems to be an oncogene [11, 12]. This is the first study to report that the status of SATB2 expression in carcinoma tissues is much lower than that in paracarcinoma tissues in ESCC, and SATB2 is an independent prognostic marker for patient survival in tumors.

In this study, we found that low levels of SATB2 were identified at both the transcriptional and translational levels in ESCC tissues in comparison with adjacent normal tissue samples. Furthermore, immunohistochemical analysis showed that SATB2 expression was reduced in a large number of human clinical ESCC samples. SATB2 expression seems to be less extensive in poorly differentiated ESCC, as these cases in general showed slightly lower levels of SATB2 protein compared with well-differentiated and moderately differentiated primary tumors. SATB2 protein may be acts as a tumor suppressor, and down-regulates esophageal carcinoma carcinogenesis. The finding between SATB2 expression and tumor differentiation has been noted in various malignancies. In skeletal osteosarcomas and soft-tissue tumors, SATB2 was specific for osteoblastic differentiation. Immunoreactivity was primarily limited to areas of heterologous differentiation within either dedifferentiated sarcomas or malignant peripheral nerve sheath tumors, whereas tumors without morphologically clear heterologous elements were nearly always negative for SATB2 [13]. In colon cancer, SATB2 expression is important for cell differentiated. SATB2 expression also strongly correlated with the histopathological differentiation in human laryngeal carcinoma. However, another study showed that the mRNA expression of SATB2 and SATB1 were higher in malignant breast tissue compared to normal breast tissue. Since the measurement of transcript levels may not always linked to protein level, mRNA level might not match the important function of SATB2 correctly.

Our study suggests that the decreased expression of SATB2 was correlated with pathologic stage, recurrence, and prognosis. Patients with lower SATB2 expression had shorter survival time, and those with higher SATB2 expression had a longer survival time. In addition, the relationship of SATB2 expression with prognosis was determined in the patients, which were divided into 2 subgroups depending on the pathologic stage. We found that SATB2 could be a valuable prognostic marker for ESCC patients at all disease stages. Our results also indicated that the level of SATB2 protein expression was closely correlated with overall and disease-free survival. In multivariate analyses, a low level of expression of SATB2 protein was associated with a bad prognosis for ESCC. This result indicated that SATB2 is a potential favorable prognostic factor for ESCC. However, the number of patients in our study is small; further studies are still needed to verify these findings and establish SATB2 as a reliable clinical predictor of outcome of ESCC.

These observations highlight the important role of SATB2 in the development and progression of ESCC. As study in knockout mouse models showed that SATB2 can indirectly affect gene transcription by augmenting the activity of Runx2 and activating transcription factor 4 [4]. Rrwin et al. reported that SATB2 might promote chemoresistance by augmenting DNp63a engagement to p53-family responsive elements in HNSCC [6]. The SATB2 homologue, SATB1, has been shown to promote tumor growth and metastasis by altering gene profiles. This homologue obviously change the expression of over 1000 breast cancer genes including metastasis-associated genes and tumor suppressor genes, and high SATB1 levels are connected with poor prognosis in breast tumors [14]. Furthermore, recent studies that SATB1 upregulated the expression of multiple

genes associated with migration, invasion, angiogenesis, and metastasis including the small calcium binding protein S100A4 [15, 16], VEGF-B [17], the type IV collagenase MMP-9 [18], the cytokine TGFb1 [19], and CTGF [20]. Similar to SATB1, SATB2 binds to AT-rich sequences in nuclear matrix attachment regions and regulates gene expression by modulating chromatin packing and organization [3, 4]. Therefore, further studies are needed to clarify the mechanisms by which SATB2 is involved in the development and progression of ESCC as a tumor suppressor and its exact role in the regulation of gene expression in ESCC.

In conclusion, this is the first study highlighting the clinical significance of SATB2 in ESCC, and lower SATB2 expression is also a significant prognostic marker of poor survival in ESCC patients.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jie Jiang, Department of Thoracic Surgery, The First Affiliated Hospital of Xiamen University, 55 Zhenhai Road, Xiamen 361003, China; Tel: +86-(592)-2137270; Fax: +86-(592)-2137189; E-mail: Jiejiangfy@163.com; Dr. Xian-En Fa, Department of Thoracic Surgery, The Second Affiliated Hospital, Zhengzhou University, 2 Jingba Road, Zhengzhou 450014, China. Tel: +86-(371)-63934118; Fax: +86-(371)-63910120; E-mail: faxianen@126.com

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