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Original Article

Downregulation of SETBP1 promoted non-small cell lung cancer progression by inducing cellular EMT and disordered immune status

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Abstract: Purpose: SET binding protein 1 (SETBP1) has involved in cancer pathogenesis like leukemic malignancies and breast cancer. But the role and the underlying mechanism in NSCLC remain unclear. Methods: RT-PCR and western blotting were used for determining the expression level of SETBP1 in NSCLC. The clinical values of SETBP1 expression were evaluated by tissue microarray and immunohistochemistry. CCK-8, transwell and Matrigel assays were used to assess NSCLC cells proliferation, migration and invasion ability. The analysis of EMT markers was carried out by RT-PCR, western blotting and immunofluorescence. Bioinformatics analysis revealed the relationship between SETBP1 expression and tumor-associated immune cells. Results: SETBP1 expression was significantly downregulated in NSCLC tissues than matched peri-tumors and NSCLC patients with the decreased level of SETBP1 had worse OS. Downregulation of SETBP1 expression induced EMT to promote NSCLC cells proliferation, migration and invasion by the activation of ERK1/2 signal pathway. Aberrant SETBP1 expression was companied by disordered immune status of NSCLC patients and might be involved in regulation of polarization of tumor-associated macrophages. Conclusion: SETBP1 can act as a tumor suppressor to reduce the progression of NSCLC and can be used for a prognostic biomarker in NSCLC. Aberrant SETBP1 expression was companied by disordered immune status of NSCLC patients.

Keywords: SETBP1, NSCLC, EMT, ERK1/2, prognosis, tumor-associated lymphocytes

Introduction

Lung cancer is the most common cancer of human beings worldwide, with an estimated 1.7 million deaths and 2.1 million news in 2018 [1]. Non-small cell lung cancer (NSCLC) accounts for 85% patients of lung cancer and lung adenocarcinomas (LUAD) and lung squamous cell carcinomas (LUSC) are most common sub-types [2]. NSCLC is heterogeneous disease which is a challenge for cure. Surgical resection is still the major method to cure NSCLC, although immunological therapy, (neo) adjuvant chemotherapy and targeted therapy have embraced huge advances [3, 4]. However, the 5-year survival rate of NSCLC is still low [5]. Therefore, it is of vital importance to disclose the underlying mechanism of NSCLC progression.

SET binding protein 1 (SETBP1), a 170-KDa protein localized in the cell nucleus and cytoplasm, was assigned to chromosome 18q21 [6]. Previous studies reported that SETBP1 overexpression promoted the proliferation of leukemic cells and older patients of acute myeloid leukemia with SETBP1 expression had a significantly shorter overall survival rates (OS) [7]. Makishima et al demonstrated that somatic mutations in SETBP1 were associated with myeloid leukemic transformation and poor prognosis in chronic myelomonocytic leukemia and myelodysplastic syndromes [8]. Another research illustrated miR-211-5p suppressed the triple-negative breast cancer progression via directly targeting SETBP1 [9]. Several studies has suggested the aberrant SETBP1 expression in lung cancer but the function and the molecular mechanism of SETBP1 in NSCLC development remains unclear [10-12].

Here, we analyzed the expression level of SETBP1 in human NSCLC and determined the relationship between SETBP1 expression and

OS in NSCLC patients. Moreover, we tried to unveil the roles of SETBP1 in NSCLC patients and the underlying mechanisms of decreased SETBP1 level in promoting NSCLC cell proliferation, migration and invasion. In addition, the correlation between SETBP1 expression and tumor-infiltrating immune cells level was also assessed.

Material and methods

Patients and samples

A total of 208 pairs of NSCLC patients who received standard lobectomy and mediastinal lymph node dissection were randomly collected in 2005 at Zhongshan Hospital of Fudan University (Shanghai, China). All these NSCLC patients included our study were diagnosed by two pathologists independently. Tumors and matched peri-tumors were obtained after surgery and were used for constructing tissue microarray (TMA, see below). All follow-up procedures were completed in July 2010 and the median follow-up was 43 (range: 1-66) months. Twelve fresh NSCLC and their adjacent peritumors were randomly obtained from other NSCLC patients in 2018. Ethical approve was obtained by Zhongshan Hospital Research Ethics Committee and all patients included this study written informed consent.

Tissue microarray, immunohistochemistry and evaluation

The method of construction of TMA was reported in our previous report [13]. In total, the TMA included 110 cases of LUAD, 85 LUSC and 13 other types of NSCLC.

After dewaxing at 60°C about 5 hours and rehydration, the paraffin sections were blocked by 3% H_2O_2 for 30 minutes. Next, the sections were put into citric acid retrievals at 100°C for 15 minutes and allowed to cool to room temperature. Then, five percent bovine serum albumin was used to block the sections for 1 hour and incubated with primary antibody overnight in 4°C. The slides were incubated with HRP-labeled secondary antibody (YESEN, Shanghai, China) for 1-2 hours at room temperature. Then, the slides were stained with diaminobenzidine (DAB, Gene Tech, Shanghai, China) and observed under a microscope. Finally, the sections stained cell nucleus with hematoxylin and sealed with neutral resin.

SETBP1 staining was evaluated based on intensity scores (0, negative; 1, weak; 2, moderate; and 3, strong staining) and the percentage scores (1, ≤ 25%; 2, 25%-50%; 3, 50%-75%; 4, > 75%). Final scores were evaluated using a combination of intensity and percentage scores (range 1-7): scores ≤ 3 were considered the low expression group and those > 3 were defined as the high expression group.

Cell lines and cultures

The NSCLC cell lines A549, H1299 and H460 were purchased from the Institute of Biochemistry and Cell Biology of Chinese Academy of Science. The cells were maintained in DMEM or RPMI1640 with 10% fetal bovine serum and 1% penicillin and streptomycin in humidified incubator at 37°C.

RNA isolation and RT-PCR

Total RNA was extracted from tissue and cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The procedure of reverse transcription of RNA was followed the instruction of kit (YESEN, Shanghai, China). Each quantitative polymerase chain reaction was performed in Applied Biosystem with triplicate samples in total reaction volume of 10 ul (SYBR Green Real-Time PCR Master Mix; YESEN, Shanghai, China). The gene GAPDH was used as an internal parameter. All experiments were performed in triplicate. The primers included in this study were showed in Table S1.

Western blotting

The protein of tissue or cells were extracted with radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China) on ice and centrifuged for 15 minutes at 12,000 rpm at 4°C. The supernatants were transferred into other fresh 1.5 ml tube and the protein concentration was measured by bicinchoninic (BCA) kit (Beyotime, Shanghai, China). Protein samples were separated by dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membrane. After being blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST) (Sangon Biotech, Shanghai, China), the membranes were incubated at 4°C over-

night with primary antibody. Next, the membranes were washed three times with TBST and incubated with HRP-labeled secondary antibody for 2 hours at room temperature. Finally, the membranes were detected with chemiluminescent HRP substrate (Share-bio, Shanghai, China) in an electrogenerated chemiluminescence imaging system (Tanon, Shanghai, China). The primary antibodies included in this study were in Table S2 and all experiments were performed in triplicate.

Proliferation, migration and invasion

Cell proliferation was assessed by Cell Counting Kit-8 (CCK-8, YESEN, Shanghai, China) following the manufacture's instruction. Cell migration was detected by transwell assay and the method of transwell assays was reported as previously [13]. Matrigel invasion was performed in 24-well transwell plates (8-μm pore size, Corning, NY, USA). Twenty thousand cells were seeded into each well which had been pre-coated with 60 ul diluted Matrigel (1:6). DMEM or RPMI1640 with 30% FBS was added into low chambers while serum-free medium was added into upper ones. Then, the cells were cultured in thermostatic incubator with 5% CO_2 at 37°C for 48 hours. Finally, the cells were stained with 0.5% crystal violet after fixation of 4% paraformaldehyde and were observed under a microscope.

Small interfering RNA and plasmid construction

Small interfering RNA was designed and purchased from Genomeditech (Shanghai, China). Overexpression of plasmid of pLenti-h-SETBP1 and pLenti-Vector were designed and purchased from Shanghai Asia-vector Biotechnology. The siRNAs, the negative control (NC), pLenti-h-SETBP1 (-oe) and pLenti-Vector (Vector) were transfected into cells using lipofectamine™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacture's protocol. Moreover, the efficiency of silence or overexpression was evaluated after 48-72 hours of transfection via RT-PCR.

Immunofluorescence assays

Cells were seeded onto the cover glass in 6-well plates. When each glass reach 60-70% confluence, the cells were fixed with 4% paraformaldehyde about 15 minutes. After that, the cells were permeabilized using 0.3% Triton X-100 (Beyotime, Shanghai, China) for 20 minutes at room temperature. Then, the cells were blocked in 5% BSA for one hour and were incubated in primary antibody overnight at 4°C. Next, the cells were incubated in immunofluorescent-labelled secondary antibody (Yesen, Shanghai, China) in darkness for one hour at room temperature. The nucleus was stained by 4',6-diamidino-2-phenylindole (DAPI) (Yesen, Shanghai, China) for 15 minutes. Finally, the slides were photographed with a fluorescence microscope.

Bioinformatics analysis

The mRNA expression level in tumor and peri-tumor was investigated via Oncomine Datasets Platform (https://www.oncomine.org/resource/login.html) [14]. The correlation between SETBP1 expression and survival in NSCLC was evaluated by the PrognoScan database (http://dna00.bio.kyutech.ac.jp/PrognoScan/index.html) [15]. The genes related to SETBP1 expression were chose via LinkedOmics (http://www.linkedomics.org/login.php) [16] and UALCAN (http://ualcan.path.uab.edu/index.html) [17] using Pearson' correlation analysis ($|r| \geq 0.3$). Venn diagrams were mapped by website tools of Venn (http://bioinformatics.psb.ugent.be/webtools/Venn/). Gene ontology analysis and KEGG pathway analysis were based on the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 website (https://david.ncifcrf.gov/) [18, 19]. In addition, the relationship between SETBP1 expression and tumor-infiltrating immune cells using Spearman' correlation via TIMER (https://cistrome.shinyapps.io/timer/) and TISIB (http://cis.hku.hk/TISIDB/index.php) database [20-22].

Statistical analysis

The SPSS 23.0 software and Prim Software version 6.0 (GraphPad) were applied to statistical analysis. Statistical differences in clinicopathological parameters between different groups were evaluated via the student's t test or χ^2 test. Survival analysis was assessed by Kaplan-Meier methods and differences were analyzed using log-rank test. Cox proportional hazards model was used to analyze independent factors. A P-value < 0.05 was considered statistically significant.

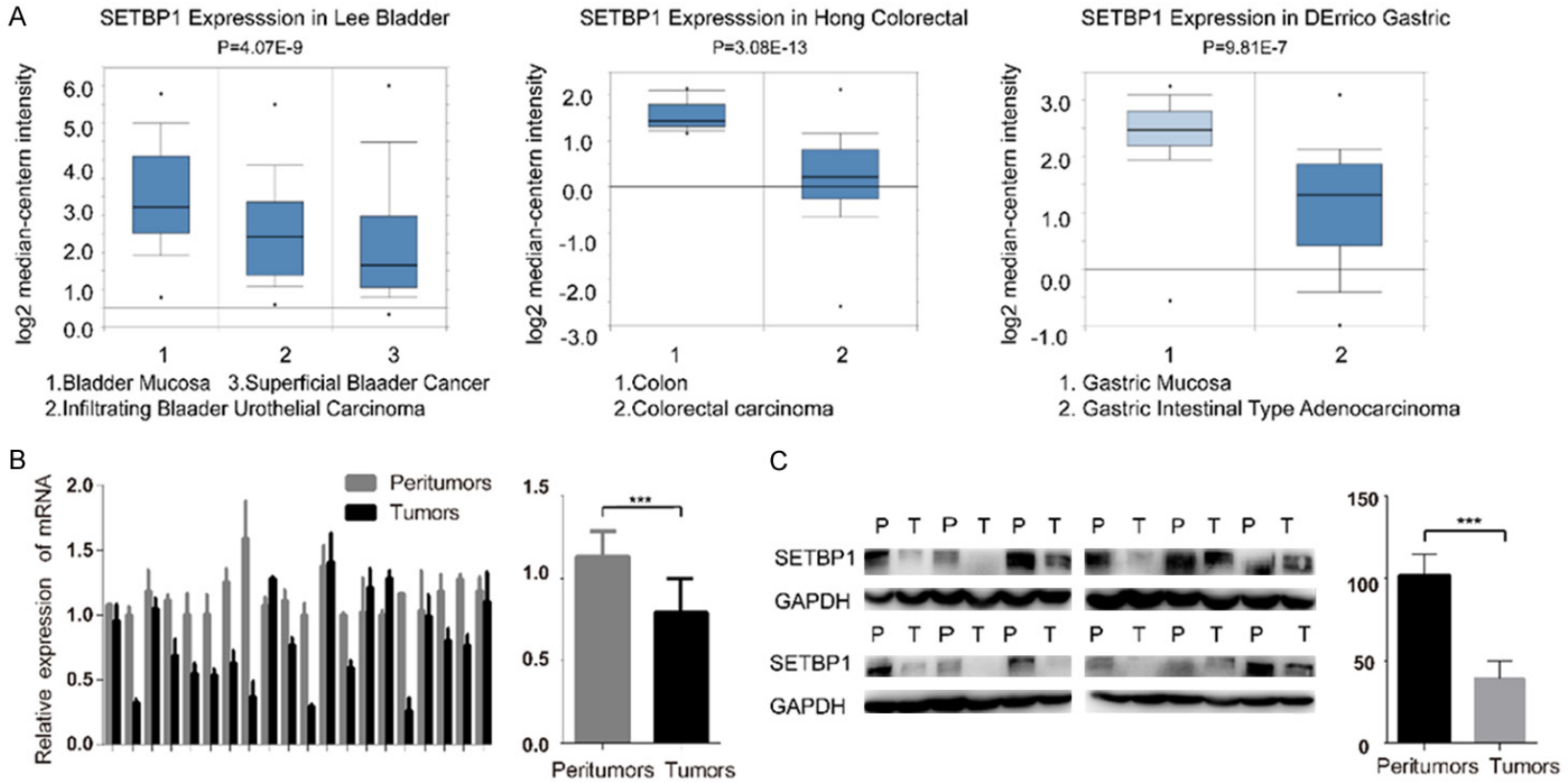


Figure 1. The SETBP1 expression in different types of cancer. A. SETBP1 expression level in bladder carcinoma (P=4.07e-9), colorectal carcinoma (P=3.08e-13) and gastric carcinoma (P=9.81e-7); B. Relative SETBP1 mRNA expression in 12 paired NSCLC tissues (T) and matched peri-tumors (P); C. Relative SETBP1 protein levels in 12 paired NSCLC tissues (T) and matched peri-tumors (P) (T: tumor, P: peritumor; ***P < 0.001).

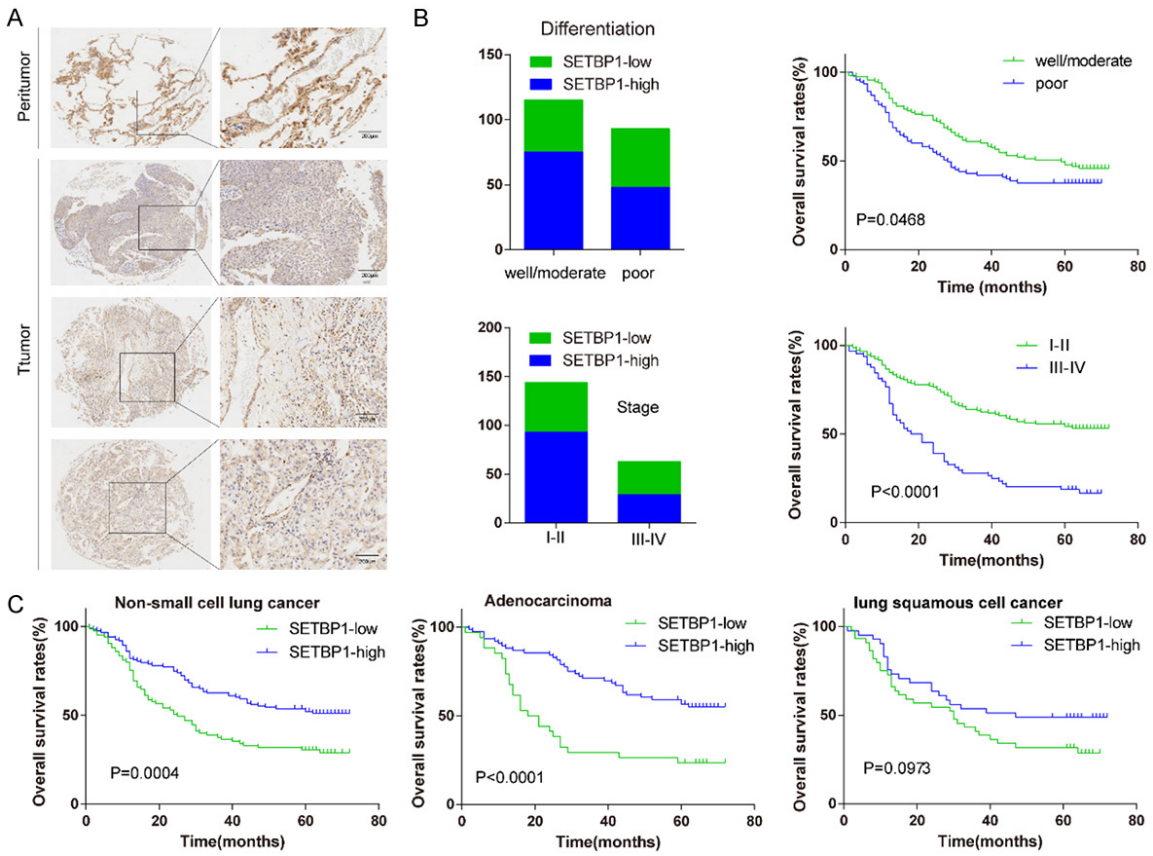


Figure 2. Decreased level of SETBP1 expression in NSCLC patients was associated with poor prognosis. A. Representative photos of SETBP1 staining in 208 paired NSCLC tissues and matched adjacent normal tissue. B. Low level of SETBP1 correlated with poor tumor differentiation and OS was also found; Low level of SETBP1 correlated with high TNM stage and OS was also found; C. Kaplan-Meier plots showed the OS associated with SETBP1 expression in NSCLC, adenocarcinoma and lung squamous cell cancer (SETBP1-high: high expression of SETBP1, SETBP1-low: low expression of SETBP1).

Results

SETBP1 was down-regulated in NSCLC

Firstly, we analyzed the expression level of SETBP1 in three different types of cancer from Oncomine database, which contained cancer and matched peri-tumor tissues in mRNA expression level. And we found the mRNA level of SETBP1 was significantly decreased in bladder cancer [23], colorectal cancer [24] and gastric carcinoma [25] (**Figure 1A**).

Then, we analyzed the mRNA and protein expression level in NSCLC by RT-PCR and western blotting, respectively. As shown in **Figure 1B** and **1C**, the mRNA and protein expression level were both apparently down-regulated, which was in line with the results of other cancers from Oncomine database. Therefore, we can safely conclude SETBP1 was significantly decreased in many types of cancer including NSCLC.

Decreased expression of SETBP1 was associated with more aggressive clinical characteristics and poor prognosis of NSCLC patients

We next focused on the clinical characteristics of SETBP1 in NSCLC patients since the expression level of SETBP1 was low in cancerous tissue. According to the evaluation methods of IHC above, the cohort of 208 NSCLC tissues was divided into SETBP1-low and SETBP1-high two groups. The expression of SETBP1 was greatly diverse in NSCLC tissues (**Figure 2A**).

As shown in **Table 1**, low expression of SETBP1 was significantly correlated with aggressive characteristics of NSCLC patients, including tumor stage (P=0.016) and differentiation (P=0.047). Down-regulated expression of SETBP1

Table 1. Correlation between SETBP1 and clinicopathological characteristics in 208 NSCLCs

Variables	No. of patients	SETBP1 expression level		
		low	high	*P*
Age				0.349
< 60	102	45	57	
≥ 60	108	40	66	
Gender				0.159
Male	148	65	83	
Female	60	20	40	
Smoking status				0.291
Smokers	84	38	46	
Non-smokers	124	47	77	
Histological type				**0.003***
LUSC	85	44	41	
LUAD	110	34	76	
Other[a]	13	7	6	
Tumor stage				**0.016**
I-II	144	51	93	
III-IV	64	34	30	
Lymph node metastasis				0.229
Yes	90	41	49	
No	118	44	74	
Tumor size				0.094
< 3 cm	70	23	47	
≥ 3 cm	138	62	76	
Differentiation				**0.047**
Well/moderate	115	40	75	
Poor	93	45	48	

NOTE: LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; Bold values are statistically significant ($P < 0.05$). [a]Other including adenosquamous carcinoma, large-cell carcinoma, mucoepidermoid carcinoma and carcinosarcoma. **P* value was analyzed by squamous cell carcinomas vs. adenocarcinomas.

was more common in poor differentiation (48.39%, 45/93) than well/moderate differentiation (34.78%, 40/115). Low SETBP1 expression was more related with advanced stage III-IV (53.97%, 34/63) than stage I-II (35.42%, 51/144) (**Figure 2B**). Additionally, the patients with poor tumor differentiation or advanced tumor stage had a more unsatisfied OS (**Figure 2B**). Moreover, we found the expression of SETBP1 was associated with histological type (**Table 2**).

Next, we observed the prognostic role of SETBP1 in NSCLC patients and found low SETBP1 expression group had worse OS compared with high SETBP1 expression group (P=0.0004) (**Figure 2C**). Strikingly, the OS of low expression of SETBP1 group was more unfavorable than the high group in adenocarcinomas (P < 0.0001) but lung squamous cell cancer (P=0.0973). Univariable analysis illustrated larger tumor size, lymph node metastasis, advanced stage and low SETBP1 expression were significant indicator of OS in NSCLC patients (**Table 2**). The expression of SETBP1 (P=0.023) was independent indicator of OS in NSCLC patients according to the multivariable analysis (**Table 2**). Furthermore, we investigated the correlation between SETBP1 expression level and overall survival via PrognoScan database. NSCLC patients with low SETBP1 expression level had worse OS (Figure S1), which was line with our observation.

To sum up, our data demonstrated low level of SETBP1 in NSCLC patients had worse OS and decreased expression of SETBP1 can predict the worse prognosis.

Down-regulated SETBP1 promoted NSCLC cells proliferation, migration and invasion

To understand the effect of SETBP1 in NSCLC development, we first investigated the basic expression level of SETBP1 in three types of NSCLC lines via RT-PCR (**Figure 3A**). Then, we chose A549 and H460 cells to interfere the expression of SETBP1 by siRNAs and overexpression of plasmids were transfected into H1299 cells to overexpress the target gene. Then, we analyzed the efficiency of silence of siRNAs (**Figure 3B**) and the effect of plasmids of overexpression (**Figure 3C**) after 48 hours by RT-PCR. Next, we chose siRNA2 (S2) and siRNA3 (S3) to assess the ability of SETBP1 to influence the progression of NSCLC in A549 and H460 cells. CCK-8 assays of A549 and H460 cells both revealed that down-regulated SETBP1 promoted the proliferation of NSCLC cells (**Figure 3D**). Moreover, transwell assay and Matrigel invasion assay illustrated that down-regulated SETBP1 accelerated the migration and invasion of A549 and H460 cells (**Figure 3E**). Simultaneously, CCK-8, transwell and Matrigel assays of overexpression of plasmids in H1299 illustrated elevated SETBP1 compromised the ability of proliferation, migration and invasion of NSCLC cell (**Figure 3F**, **3G**). Taken together, these data demonstrated decreased SETBP1 promoted the progression of NSCLC cells.

Table 2. Univariate and multivariate analysis of factors associated with OS

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
Gender (male *vs*. female)	0.789	0.526-1.183	0.251			
Smoking status (no *vs*. yes)	0.779	0.543-1.118	0.175			
Differentiation (well/moderate *vs*. poor)	1.431	1.000-2.049	0.050			
Tumor size (≥ 3 cm *vs*. < 3 cm)	2.639	1.695-4.106	0.000	1.971	1.249-3.111	0.004
Lymph node metastasis (yes *vs*. no)	0.329	0.227-0.476	0.000	0.471	0.305-0.727	0.001
Tumor stage (III-IV *vs*. I-II)	2.771	1.922-3.993	0.000	1.474	0.956-2.272	0.079
SETBP1 level (low *vs*. high)	0.531	0.371-0.761	0.001	0.655	0.454-0.944	0.023

Abbreviations and note: OS, overall survival; 95% CI, 95% confidence interval; multivariate analysis, Cox proportional hazards regression model. Variables were adopted for their prognostic significance by univariate analysis with forward stepwise selection (forward, likelihood ratio). Variables were adopted for their prognostic significance by univariate analysis ($P < 0.05$).

Low level of SETBP1 induced EMT in NSCLC cells

It's reported that epithelial-mesenchymal transition (EMT) can promote NSCLC progression [26]. Here, we tried to investigate the EMT markers like e-cadherin and vimentin in different NSCLC cells. RT-PCR and western blotting demonstrated that E-cadherin was significantly reduced in A549-S2 and A549-S3 cells compared to A549-NC, which was consistent with the changes of EMT markers in H460 cells (**Figure 4A**, **4C**). However, the expression of vimentin was increased in A549-S2, A549-S3 and H460-S2, H460-S3 compared with A549-NC and H460-NC, respectively (**Figure 4A**, **4C**). Then, we overexpressed SETBP1 to check whether it can reverse the expression changes of EMT markers. The results of western blotting and RT-PCR confirmed that e-cadherin was up-regulated and vimentin was down-regulated in H1299-oe cells compared to H1299-vector cells (**Figure 4B**, **4C**). Furthermore, immunofluorescence assays of EMT marker also confirmed the outcomes above (**Figure 4D**, **4E**). These data together illustrated that down-regulated SETBP1 fostered the progression of NSCLC by modulating EMT in NSCLC cells.

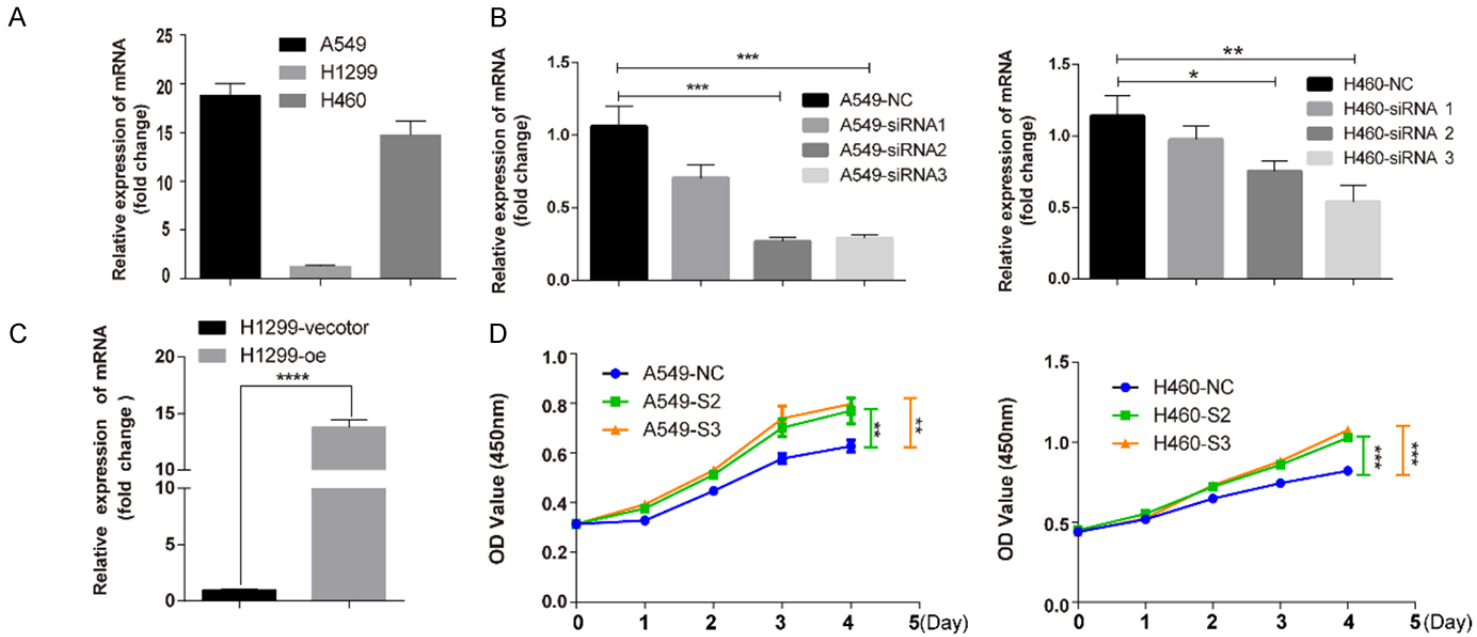
SETBP1 regulated NSCLC cell EMT by ERK1/2 signaling pathway

To elucidate the mechanism of SETBP1 to regulate cell EMT, we searched for the genes associated with SETBP1 via LinkedOmics and UALCAN in NSCLC by Pearson' correlation analysis ($|r| \geq 0.3$). Then, three hundred and forty genes were chose in the intersection of Venn diagram (**Figure 5A**). GO and KEGG pathway analysis were conducted by DAVID. We found that SETBP1 was associated with several biological process including intracellular signal transduction, cellular response to starvation and angiogenesis (**Figure 5B-D**). Simultaneously, KEGG pathway analysis revealed that SETBP1 was involved in many signal pathway like cancer (**Figure 5E**).

Previous study reported SETBP1 might associate with AKT/NF-κB regulatory pathway [10]. Next, we chose several classic acknowledged signal pathway including AKT/NF-κB to determine the potential mechanism of SETBP1. The results showed p-65, p-p65, AKT and p-AKT were not obviously affected by SETBP1 expression in NSCLC cells (**Figure 5F**). However, we found interfered SETBP1 could upregulated the phosphorylation level of ERK1/2 both in A549 and H460 cells (**Figure 5F**), which was always changed in cancers [27, 28]. Moreover, overexpression of SETBP1 also decreased the phosphorylation level of ERK1/2. To further examine whether down-regulated SETBP1 affected the NSCLC proliferation, migration and invasion by ERK1/2 signal pathway, we treated H460-S2(3) with AZD6244, a MEK/ERK inhibitor (10 μmol/L). The concentration of AZD6244 was referred to literature [29]. As shown in **Figure 5G** and **5H**, the AZD6244 inhibited the proliferation, migration and invasion of H460 cells with decreased SETBP1 level. These results suggested SETBP1 regulated NSCLC cell EMT by ERK1/2 signaling pathway.

SETBP1 expression was associated with tumor-infiltrating immune cells level

The strong infiltration of tumor-infiltrating lymphocytes are positively associated with clinical outcome in NSCLC [30]. First, we assessed the


A
Relative expression of mRNA (fold change)
A549
H1299
H460
B
Relative expression of mRNA (fold change)
A549-NC
A549-siRNA1
A549-siRNA2
A549-siRNA3
H460-NC
H460-siRNA 1
H460-siRNA 2
H460-siRNA 3
C
Relative expression of mRNA (fold change)
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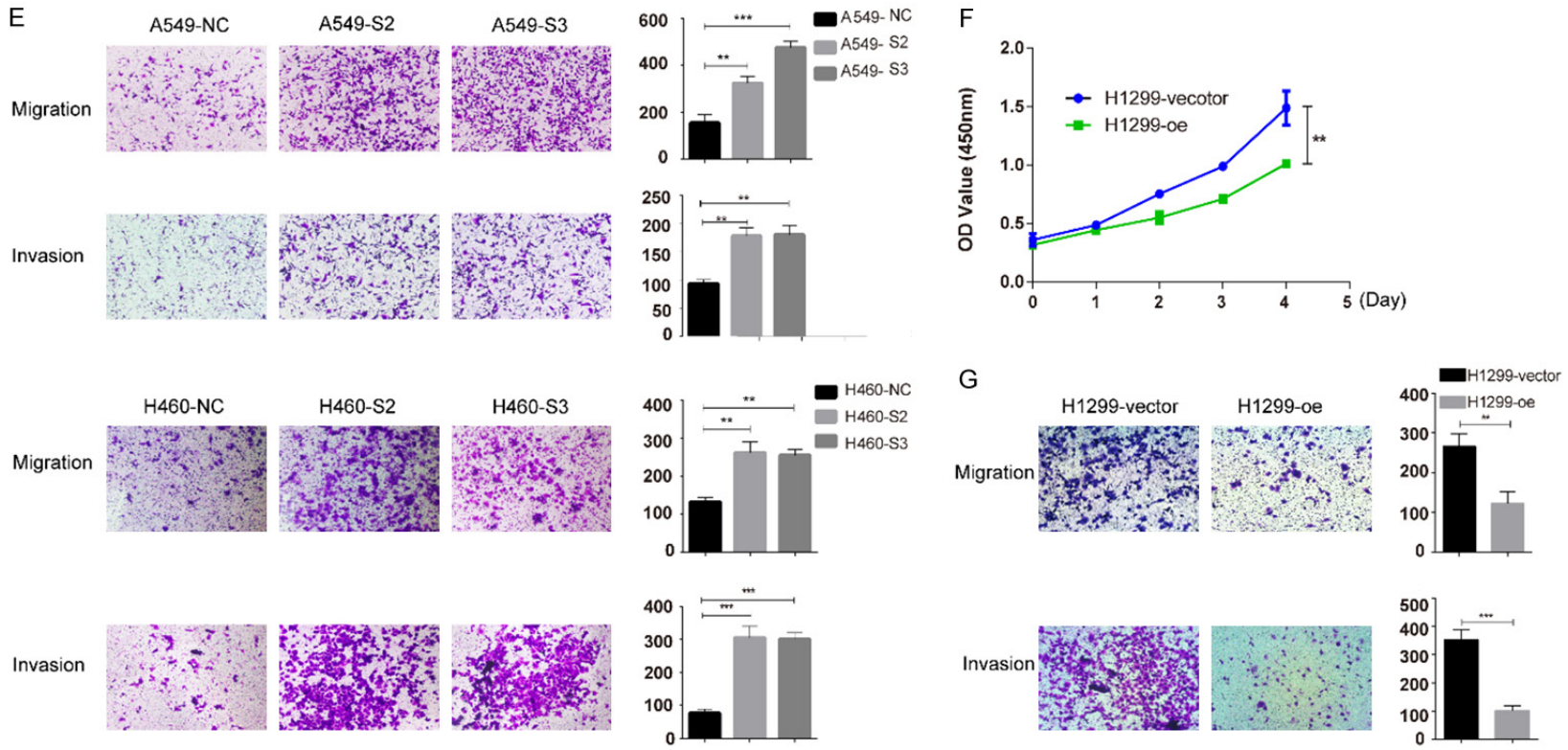

Figure 3. Down-regulation of SETBP1 promoted the migration, invasion and proliferation of NSCLC cells. A. Relative SETBP1 mRNA levels in NSCLC cells lines. B, C. Efficiency of SETBP1 down-regulation and overexpression in NSCLC cells; D, F. CCK8 assays were used to detect NSCLC cells proliferation in SETBP1 down-regulation and overexpression; E, G. Transwell assays were used to measure the effects of SETBP1 down-regulation and overexpression on migration and invasion in NSCLC (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001).

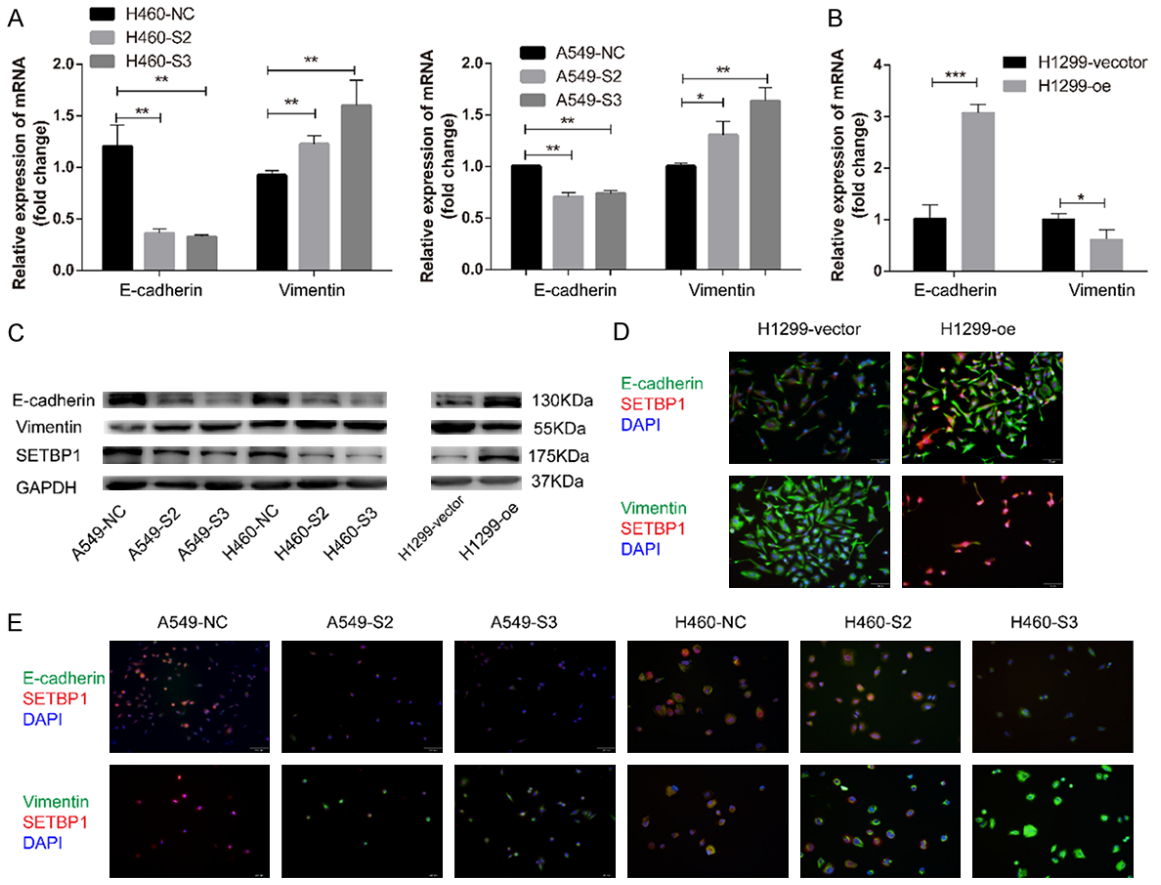

Figure 4. Down-regulation of SETBP1 promoted NSCLC progression by inducing EMT. A, B. Relative mRNA level of EMT markers after down-regulation and overexpression of SETBP1 in H460, A549 and H1299 cells. C. Changes in key molecules of EMT determined by western blotting after overexpression and downregulation of SETBP1. D, E. Effects of SETBP1 overexpression and down-regulation on epithelial and mesenchymal makers using immunofluorescence staining (*P < 0.05, **P < 0.01 and ***P < 0.001).

correlation between SETBP1 expression and different immune cells by TISIB website tool. SETBP1 expression level was negatively associated with activated CD8+ T cell, activated dendritic cells and monocyte both in LUAD and LUSC (**Figure 6A**). Moreover, we investigated the relationship between SETBP1 expression and diverse immune cell markers to further verify the results from TISIB databases. Therefore, we analyzed the markers of diverse functional cells in the tumor immunity, including neutrophils, macrophages, monocytes, NK cells, B cells, DCs and CD8+ T cell in TIMER database (**Table 3**).

Surprisingly, we found partial markers of CD8+ T cell, DCs and monocytes were significantly correlated with SETBP1 expression, which was partly in line with the outcome of TISIB. In addition, we found SETBP1 expression was distinctly associated with B cell, CD4+ cell, macrophage infiltration level while tumor purity was not significant correlations with SETBP1 level (**Figure 6B**). But more importantly, SETBP1 expression has significant correlations with M1 macrophage but M2 both in LUAD and LUSC, which suggested that SETBP1 may regulate the polarization of macrophage in LUAD and LUSC (**Table 3**). These findings demonstrated that aberrant SETBP1 expression was significantly associated with regulation of tumor-infiltrating immune cells especially M1 macrophage.

Discussion

NSCLC is one type of carcinoma with high progression potential, which causes the low OS and high mortality of patients. In this study, we determined tumor SETBP1 expression was often downregulated in NSCLC tissues, which was consistent with other cancers in the analysis of Oncomine. Moreover, the expression of

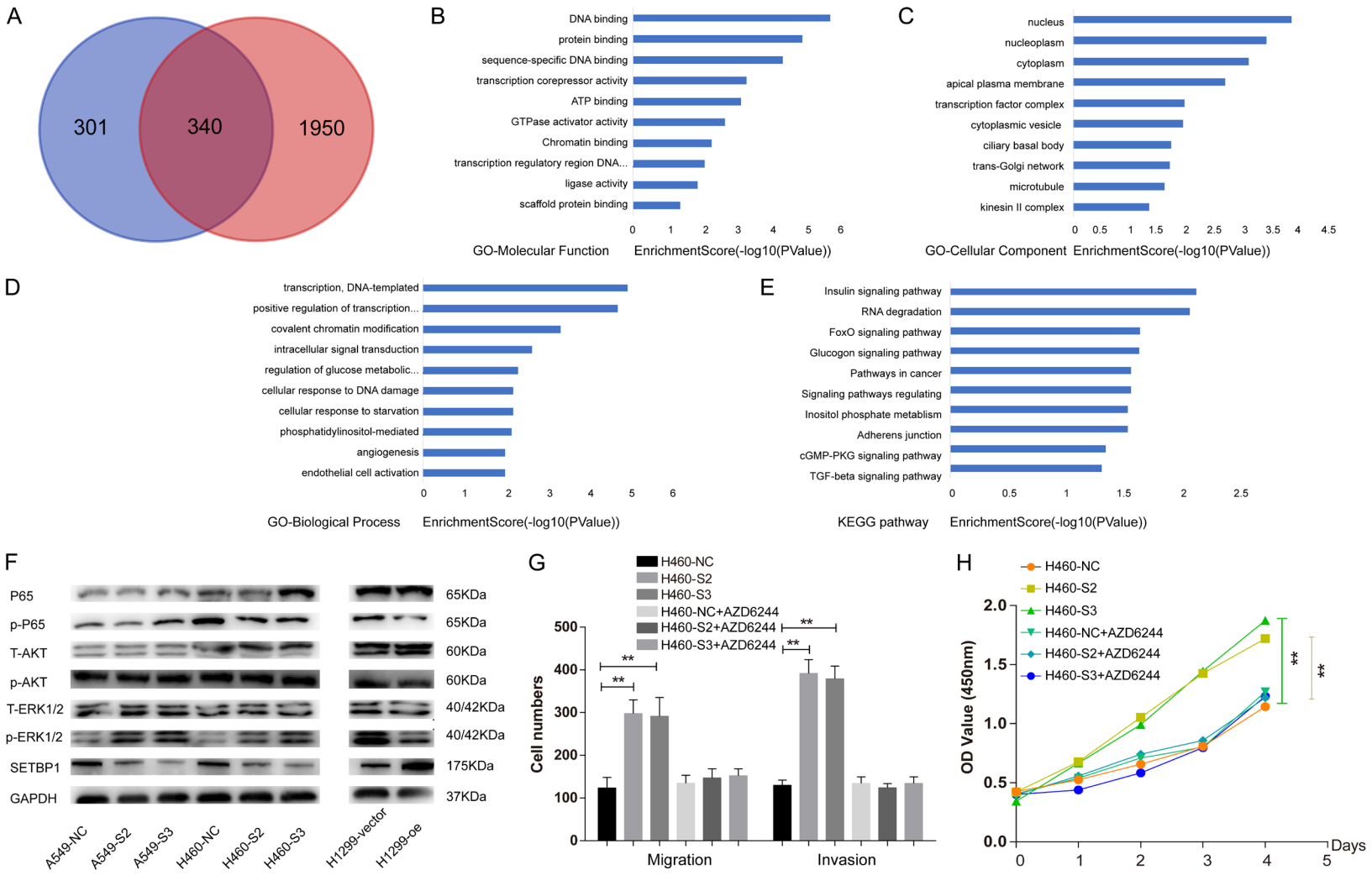

Figure 5. Correlation between SETBP1 downregulation and ERK1/2 signaling activity. A. Venn chart showed the number of genes associated with SETBP1. B-E. GO and KEGG analysis were performed for those up or down correlated genes of SETBP1. F. Western blotting detection of p65, p-p65, AKT, p-AKT, ERK1/2 and p-ERK1/2 in NSCLC cells. G. Transwell assays were performed to investigate changes in H460 cells after treatment with AZD6244 (AZD6244, a MET/ERK inhibitor). H. Proliferation of H460 cells was measured by CCK8 after treatment with AZD6244 (**P < 0.01).

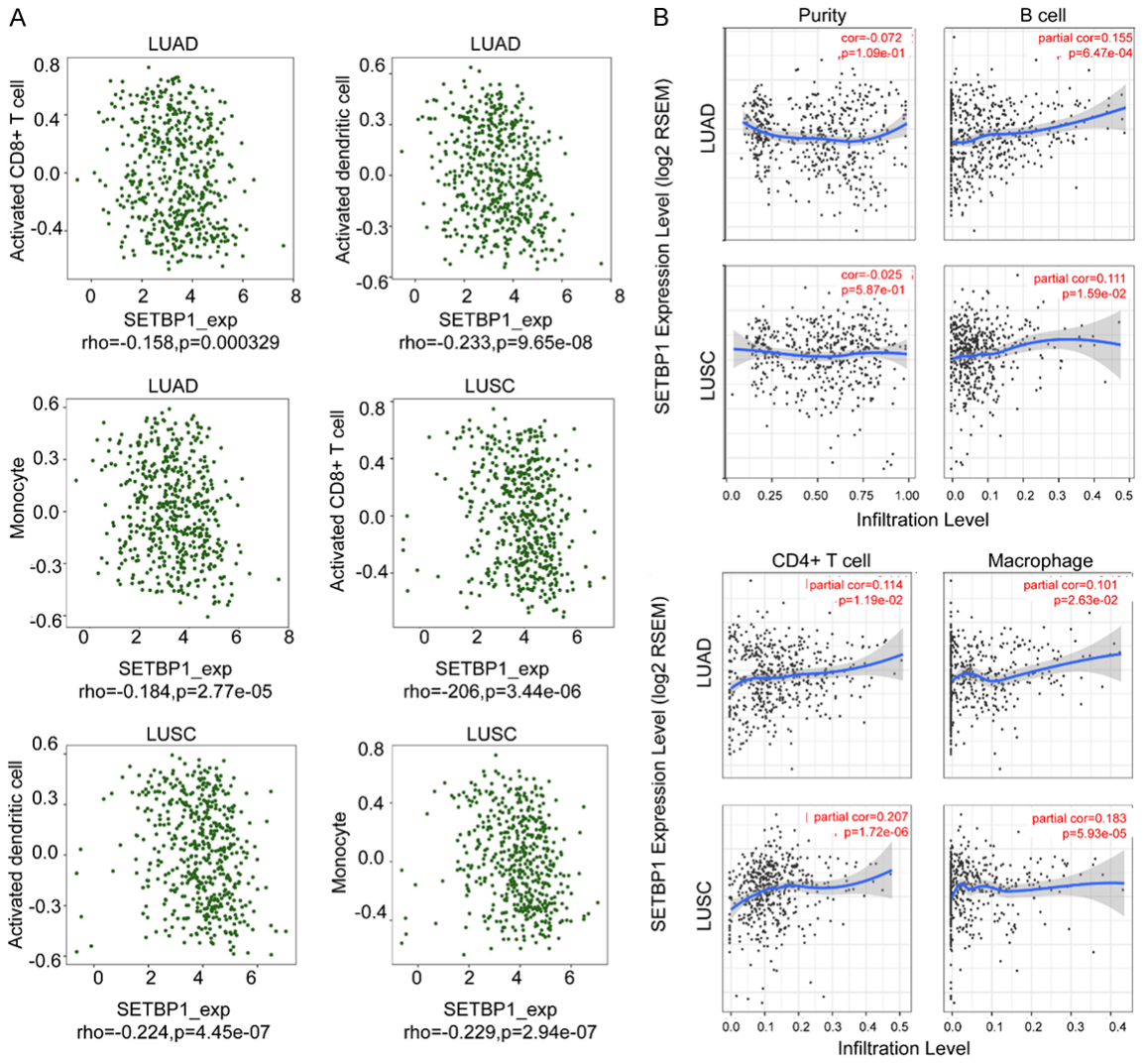

Figure 6. Association of SETBP1 expression with the abundance of immune cell and infiltration level. A. SETBP1 expression associated with activated CD8+ T cell, activated dendritic cells and monocytes in LUAD and LUSC. B. The relation between immune cell infiltration level (B cell, CD4+ T cell and macrophage) and SETBP1 expression.

SETBP1 was statistically associated with aggressive clinical features of NSCLC patients, including tumor stage and differentiation. Previous studies has claimed aberrant SETBP1 expression was correlated with acute myeloid leukemia and Schinzel-Giedion syndrome [7, 8, 31]. To our knowledge, it's first time to report that SETBP1 expression as a prognosis predictor of NSCLC patients and low tumor level of SETBP1 expression was significantly associated with worse OS in NSCLC.

Then, we demonstrated the anti-oncogenic roles of SETBP1 in NSCLC. Decreased expression of SETBP1 contributed to the progression in NSCLC cells (A549 and H460) via promoting cell proliferation, migration and invasion. On the other hand, the aggressive ability of H1299 were weaken when SETBP1 was overexpressed. In addition, it was reported that SETBP1 was directly targeted by microRNA-211-5p to suppresses triple-negative breast cancer cell proliferation, invasion, migration and metastasis [9]. Moreover, we observed that knockdown of SETBP1 in NSCLC cells contributed to the downregulation of e-cadherin and upregulation of vimentin. These changes suggested us that EMT might occur in the process of progression of NSCLC cells and this is the first report of SETBP1 in EMT induction.

Table 3. Correlation analysis between SETBP1 and markers of immune cells in TIMER

Description	Gene markers	LUAD				LUSC			
		None		Purity		None		Purity	
		Cor	P	Cor	P	Cor	P	Cor	P
Neutrophils	CD66b	0.119	**	-0.126	**	0.014	0.747	-0.013	0.780
	CD11b	-0.025	0.571	-0.06	0.181	0.085	0.058	0.11	*
	CCR7	0.182	****	0.154	***	0.046	0.309	0.049	0.283
M1 Macrophage	CD68	-0.073	0.099	-0.105	*	-0.025	0.576	-0.021	0.654
	CXCL10	-0.221	****	-0.276	****	-0.102	*	-0.101	*
	NOS2	0.217	****	0.21	****	0.119	**	0.112	*
	IRF5	-0.285	****	-0.336	****	-0.108	*	-0.114	*
M2 Macrophage	CD163	0.047	0.289	0.021	0.683	0.048	0.287	0.06	0.188
	CCL18	0.044	0.319	0.021	0.645	0.027	0.055	0.034	0.463
	VSIG4	-0.077	0.082	-0.105	*	-0.01	0.831	-0.006	0.889
Monocyte	CD86	-0.07	0.115	-0.124	**	-0.021	0.64	-0.021	0.645
	CD115	0.031	0.483	-0.009	0.84	0.109	*	0.134	**
NK cell	NKP46	0.084	0.058	0.05	0.264	0.082	0.065	0.086	*
	KIR2DL1	0.161	***	0.156	***	-0.038	0.396	-0.038	0.404
	KIR2DL4	-0.111	*	-0.126	**	-0.052	0.249	-0.052	0.259
B cell	CD19	0.2	****	0.18	****	0.083	0.065	0.097	*
	CD79A	0.229	****	0.22	****	0.101	*	0.12	**
Dendritic cell	CD1C	0.102	*	0.072	0.112	0.097	*	0.117	*
	CD11C	0.008	0.862	-0.023	0.607	0.024	0.588	0.038	0.408
	CD83	0.026	0.555	-0.003	0.98	0.142	**	0.143	**
	CD141	0.323	****	0.312	****	0.037	0.4	0.039	0.396
CD8+ T cell	CD8A	-0.03	0.493	-0.077	0.087	-0.034	0.444	-0.037	0.417
	CD8B	-0.038	0.394	-0.075	0.095	-0.032	0.48	-0.033	0.473
	CD103	-0.047	0.289	-0.068	0.131	-0.116	**	-0.127	**

LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NK cell, natural killer cell; Cor, R value of Spearman's correlation; None, correlation without adjustment; Purity, correlation adjustment by purity. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

We further to uncover the underlying signal pathway of SETBP1 in NSCLC and found that SETBP1-mediated NSCLC progression might be due to the activation of ERK1/2 signal pathway, which was always changed in cancers [32, 33]. By using MEK/ERK signal inhibitors, we further determined SETBP1 regulated NSCLC cell EMT by ERK1/2 signaling pathway. Tumor-infiltrating immune cells in NSCLC are determinants of prognosis [34]. Here, we evaluated the correlation between SETBP1 expression and immune cells markers and found CD8+ T cell, CDs and monocyte was negatively associated with SETBP1 expression level. These data suggested aberrant SETBP1 expression was often accompanied by disordered immune state. M1 macrophages generally have been associated with better prognoses, while M2 macrophages have been associated with poorer prognosis in lung cancer [35] and the M1/M2 polarization is a potential prognostic predictor of NSCLC [36]. In our study, SETBP1 expression was negatively correlated with M1 macrophage but M2 type and this suggested that the expression level of SETBP1 might be involved in regulation of polarization of tumor-associated macrophages.

Conclusions

In summary, SETBP1 was downregulated in NSCLC and decreased expression level of SETBP1 promoted NSCLC cells proliferation, migration and invasion by inducing EMT via ERK1/2 signal pathway. In addition, aberrant SETBP1 expression was companied by disor-

dered immune status of NSCLC patients and might be involved in regulation of polarization of tumor-associated macrophages. Finally, SETBP1 could be a novel marker of poor prognosis for NSCLC.

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Disclosure of conflict of interest

None.

Abbreviations

NSCLC, non-small cell lung cancer; OS, overall survival; IHC, immunohistochemistry; TMA, tissue microarray; RT-PCR, real time-quantitative polymerase chain reactions; SETBP1, SET-binding protein 1; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; GO, gene, ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DAVID, database for annotation, visualization and integrated discovery.

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Table S1. Lists of primers sequence analyzed by RT-PCR

Genes	Chain	Sequence (5'-3')
SETBP1	Forward	GCCAGCCGCAGTTGACAGTG
	Reverse	CCGCCGCTTGAACCTCTTCTTC
E-cadherin	Forward	GAACGCATTGCCACATACAC
	Reverse	GAATTCGGGCTTGTTGTCAT
Vimentin	Forward	TTGCCGTTGAAGCTGCTAACTACC
	Reverse	AATCCTGCTCTCCTCGCCTTCC
GAPDH	Forward	CAGGAGGCATTGCTGATGAT
	Reverse	GAAGGCTGGGGCTCATTT

Table S2. List of primary antibodies used in the study

Antibody	Applications	Company (CAT#)
SETBP1	WB, IF, IHC	Abs117738; Abclonal A7212
E-cadherin	WB, IHC, FC	EM0502
Vimentin	WB, IHC, ELISA	Abs132076
ERK	WB, IHC, FC, IF, IP	CST #4695
p-ERK	WB, IHC, FC, IF, IP	CST #4370
AKT	WB, IHC, FC, IF, IP	CST #4685
p-AKT	WB, IHC, FC, IF, IP	CST #4060
p65	WB, IHC, FC, IF, IP	CST #8424
p-p65	WB, FC, IF, IP	CST #3033
GAPDH	WB	Abs830030

Abbreviations: WB: western blot; IHC: immunohistochemistry; IF: immunofluorescence; IP: immunoprecipitation; ELISA: enzyme-linked immunosorbent assay; FC: flow cytometric analysis.

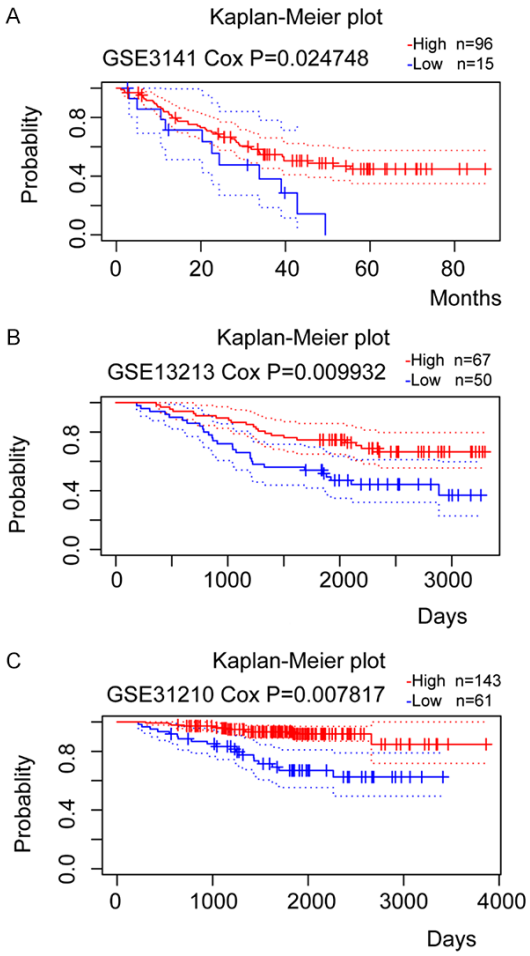

Figure S1. Kaplan-Meier survival curves comparing the high and low expression of SETBP1 in NSCLC in the PrognoScan. A. The Kaplan-plot of GSE3141 in NSCLC; B. The Kaplan-plot of GSE13213 in LUAD; C. The Kaplan-plot of GSE31210 in LUAD.