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Original Article

ETS1 is a prognostic biomarker of triple-negative breast cancer and promotes the triple-negative breast cancer progression through the YAP signaling

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Abstract: E26 transcription factor-1 (ETS1) is involved in extracellular matrix remodeling, migratory infiltration and angiogenesis in tumors and known to play an important role in tumor progression. However, the mechanism by which ETS1 promotes tumor progression remains elusive. In this report, we show that ETS1 is highly expressed in breast tumor tissues and specifically associated with the tumor metastasis and poor survival in triple negative breast cancer (TNBC) tumors, upon analysis by immunohistochemical (IHC) staining of tumor samples from 240 breast cancer cases. Depletion of ETS1 in TNBC cells by shETS1 significantly inhibited the cell proliferation and migration. Mechanistically, knockdown of ETS1 in TNBC cells dramatically reduced expression of YAP and the YAP target genes, and overexpression of YAP in the ETS1 knockdown cells restored the cell proliferation and migration. These data indicate that YAP is a downstream effector mediating the ETS1-promoted TNBC cell proliferation and migration. Taken together, our results suggest that ETS1 promotes TNBC progression through the YAP signaling.

Keywords: Breast cancer, triple negative breast cancer, ETS1, YAP, prognosis, metastasis

Introduction

Breast cancer is one of the most populous diseases and a major threat to women's health worldwide [1]. Based on expression status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), breast cancer can be classified as triple-negative breast cancer (TNBC, i.e. ER-/PR-/HER2-) and non-triple-negative breast cancer (N-TNBC). Because of lacking effective therapeutic targets, TNBC has the highest death rate among the breast cancer subtypes. Despite significant progress in exploring the molecular mechanism underlying TNBC progression and discovering new therapeutic targets, treatment for TNBC patients remains unsatisfactory with poor prognosis due to frequent recurrence and metastasis. Thus, identification of the new effective therapeutic targets and pathways becomes an important and urgent task for developing new drugs for treatment of TNBC.

The transcription factor ETS1 (c-ets-1, or Tpl-1) is a member of the ETS transcription factor family. ETS1 is a 54 kDa nuclear protein, possesses a highly conserved DNA-binding domain that recognizes the GGAA/T sequence, and is involved in progression of many types of cancer [2]. It has been found that ETS1 is overexpressed in multiple aggressive cancers and is essential for tumor angiogenesis [2-5]. Expression of ETS1 in tumor-associated vessels or avascular mesenchyme is associated with poor prognosis [6]. ETS1 enhances expression of a number of oncogene products, including urokinase-type fibrinogen activator (uPA), matrix metalloproteinases (MMPs) such as MMP-1, MMP-3 and MMP-9, and vascular endothelial growth factor (VEGF) [7-10]. Previous studies have shown that ETS1 is involved in several sig-

naling pathways in cancer cells, such as the PI3K/AKT and the RAS/RAF/MEK/ERK1/2 pathways [11, 12]. It has been found that ETS1 is overexpressed in breast cancer [13]. However, association of ETS1 expression with TNBC pathology and the patient survival has been poorly investigated and the down-stream signaling of ETS1 in TNBC remains incomplete.

The YAP (Yes-associated protein) is a transcriptional co-activator [14]. It shuttles between cytoplasm and nucleus and activates gene transcription by interacting with other transcription factors, especially members of the TEA domain family (TEAD) [15]. YAP participates in the Hippo signaling pathway and facilitates oncogenic process in cancer cells [16-18]. Our previous studies have found that YAP mediates the geranylgeranylation signaling in TNBC and promotes proliferation and migration of TNBC cells [19]. It has been shown that high expression and nuclear localization of YAP is closely associated with malignancy and poor prognosis and is a known driver for cancer progression and metastasis [20-22].

In this report, we have shown that ETS1 is highly expressed in breast tumor, particularly in TNBC tumor by IHC staining of the breast tumor tissue-array samples from 240 breast cancer patients. Statistical analysis indicates expression of ETS1 is positively associated with local metastasis (N category) in TNBC, but not in N-TNBC. Expression of ETS1 is significantly higher in TNBC than in N-TNBC when compared in all the pathological categories. Expression of ETS1 is correlated with poor survival only in TNBC patients, but not in N-TNBC, suggesting that ETS1 specifically promotes TNBC progression and is a prognostic biomarker for TNBC. In consistent with results of the IHC staining of TNBC, knockdown of ETS1 in the TNBC cell line MDA-MB-231 markedly inhibits cell proliferation and migration. Furthermore, we found that *YAP* is the target gene of ETS1. Knockdown of ETS1 significantly reduced expression of *YAP* and the known YAP-targeted genes in TNBC cells. Overexpression of YAP in the ETS1 knockdown TNBC cell line partially recovered the cell proliferation and migration. These data indicate that ETS1 promotes proliferation and migration of TNBC cells partially, if not totally, through the YAP signaling.

Materials and methods

Materials

Anti-ETS1 (14069S, #14069, Rabbit) was purchased from Cell Signaling Technology; anti-YAP1 (ab245286) from Abcam; anti-ACTIN (100166-MM10) from Sino Biological; anti-MYC (MMS-150R) from Covance; The ETS1 shRNA oligos were synthesized by Shenggong Company. The PCR primers were synthesized by Shenggong Company. Migration assay chambers were purchased from Corning Company. RNA and DNA purification reagents were purchased from Sangon Biotech Company. The Prime Script RT Reagent kit (Takara Bio) for Quantitative RT-PCR on the Bio-Rad CFX96 system. Quantitative RT-PCR was performed by SYBR Green PCR Master Mix (Takara Bio). All the cell lines were purchased from ATCC.

Cell culture

The cell lines were cultured in DMEM, supplemented with 10% FBS, in 100 U/mL penicillin, and 100 mg/mL streptomycin, at 37°C environments containing 5% CO_2.

Construction of the ETS1 and YAP plasmids and the lentiviral packaging and infection

Two ETS1 shRNA oligos were synthesized: shETS1-1 (Forward: 5'-CCGGAGGTGGTAGACTTCCATTCCACTCGAGTGGAATGGAAGTCTACCACCTTTTTTG-3' and Reverse: 5'-AATTCAAAAAAGGTGGTAGACTTCCATTCCACTCGAGTGGAATGGAAGTCTACCACCT-3') and shETS1-2 (Forward: 5'-CCGGCTGGAATTACTCACTGATAAACTCGAGTTTATCAGTGAGTAATTCCAGTTTTTG-3'; Reverse: 5'-AATTCAAAAACTGGAATTACTCACTGATAAACTCGAGTTTATCAGTGAGTAATTCCAGGGGAGCCCCACCCTTCCCCC-3') and cloned into the lentiviral shRNA expression vector TETO-pLKO.1-TRC. The oligos were inserted into the Age I/EcoR I sites of the vector.

The human YAP cDNA was subcloned into the FUW-MYC lentiviral expression vector (Addgene, Inc.) to establish stable YAP-overexpressing MDA-MB-231 cell lines with ETS1 knocked down. The YAP cDNA was amplified from a human cDNA library with primers for hYAP-forward-BamH1 (5'-GCCCAACCCTTAAACTGAATG-3') and for hYAP-reverse-EcoR1 (5'-TCCCCCCCAACCGAGCTTTAC-3') by PCR and

inserted into the BamHI/EcoR1 sites of the FUW-MYC lentiviral expression vector.

The lentiviral shRNA plasmid was co-transfected with psPAX2 (Addgene) and pMD2. G (Addgene) in HEK293T cells for 8-10 hours, then the transfection medium was replaced 2 ml of culture medium. After 24 h, the culture medium containing viral particles was collected every day for three days. The collected medium was cleared by centrifugation at 1250 rpm for 5 min and stored at 4°C for use. To infect MDA-MB-231 cells, appropriate amount of 6 μg/ml polybrene was added along with the viral particle-containing medium and selected with puromycin. ETS1 expression was assayed by western blotting of SDS PAGE gels.

Western blotting

The cells were washed with precooled PBS, then added the appropriate amount of precooled mammalian cell lysis buffer containing protease inhibitors (10 μg/ml leupeptin and 10 μg/ml aprotinin) and placed on a shaking table for 60 minutes at 4°C. Collect it in a precooled EP tube, 12000 rpm, 4°C, centrifuged for 15 minutes. Mix the supernatant with 5 × SDS PAGE buffer and cook at 100°C for 8 minutes. Prepare 10% SDS-PAGE gels, add protein sample, electrophoresis, transferred to a PVDF membranes (EMD Millipore), blocked in 1% BSA for 1 hour, incubated with primary anti-bodies and 4°C overnight, wash with 1 × TBST, set secondary anti-bodies for 1-2 hour at room temperature, wash, exposed by the Western Lightning ECL Detection Kit (Viber).

Cell proliferation and migration assays

MDA-MB-231 cells (shRNA control or shETS1 cells, 2×10^4) were induced by DOX for 48 hours and then seeded in a 12-well culture plate. After every 24 hours, the cells were digested with trypsin and counted under a phase contrast microscope with a hemocytometer. The cell proliferation ability is evaluated based on the increased number of cells, and the test is repeated three times.

(1) The wound healing assay. The cells at a concentration of 2×10^5 cells/mL were plated in a 12-well cell culture medium. After 24 hours, the cell density reached about 90%. Use a small pipette tip to draw a straight line in the center of the cell monolayer and replace it with new DMEM medium. After 24 hours, the images were taken and analyzed with ImageJ software. (2) The transwell assay. Pour 200 μl of serum-free cells with a concentration of 2×10^5 cells/mL in the upper chamber of Transwell, and add 500 μl of 10% fetal bovine serum medium to the lower chamber. Incubate at 37°C, 5% CO_2 for 24 hours, wipe the inside of the chamber, fix the bottom of the chamber with 4% paraformaldehyde at room temperature for 20 minutes, stain with 0.1% crystal violet, wash three times with PBS, and randomly select three areas under the microscope to observe and take pictures. Statistics, Prism8.0 mapping. The test needs to be repeated three times.

RNA extraction and quantitative RT-PCR

Total RNA was extracted by the RNAiso plus following the specification, RNA was reverse transcribed to cDNA. Comparative cycle threshold (CT) method for Quantitative RT-PCR to measure RNA expression and relative RNA amounts were calculated by using the comparative cycle threshold method (2-ΔΔCT) and normalised to GAPDH.

Primer sequences used in Quantitative RT-PCR. ETS1: forward: 5'-AGGGACAGAGCGGAACTCAAC-3', reverse: 5'-AATTGGTCCGCTTCCTGTGTAG-3'; YAP: forward: 5'-ATGGATCCCGGGCAGCAGC-3', reverse: 5'-AGCTCCCAACTGCAGAGAAG-3'; TAZ: forward: 5'-ATCACCACATGGCAAGACCC-3', reverse: 5'-TCTGGATTCTCTGAAGCCGC-3'; AURK1: forward: 5'-AATACAGTCCCACCTTCGGC-3', reverse: 5'-GGAGCATGTACTGACCACCC-3'; BUB1: forward: 5'-GCTGCACAACTTGCGTCTAC-3', reverse: 5'-TGGAGCCCAGCAATAGCATC-3'; CTGF: forward: 5'-CCAGTGTAGCAGCAGCCTGAA-3', reverse: 5'-CGCATCTTCACAGTCCTGGT-3'; CEMPM: forward: 5'-GTGGAAGGCTTTAGGGCCAC-3', reverse: 5'-CATGAGAAGTATGACAACAGCCT-3'; GAPDH: forward: 5'-AAGCCCTGACTGGACATCCT-3', reverse: 5'-AGTCCTTCCACGATACCAAAGT-3'.

Beast tumor samples

In this retrospective study, the paraffin-embedded specimens from 240 patients with breast cancer were treated at the Department of Breast Surgery of The Affiliated People's Hospital of Jiangsu University. Including 105 TNBC and 135 non-TNBC (N-TNBC) tissues. As well as

145 paired adjacent normal tissues (ANTs) as negative controls. Each patient's age at diagnosis, tumor grade, tumor size, clinical stage and nodal status were obtained from medical records. The histological type was based on the TNM system. Overall survival (OS) was the time from surgery until death from any cause. All patients were regularly followed up through medical appointments or by telephone before the follow-up deadline. All the tissue specimens were collected after obtaining informed patient consent. The Affiliated People's Hospital of Jiangsu University Institutional Review Board approved the use of the breast cancer specimens.

Preparation of breast tumor tissue microarrays

Breast cancer surgical specimens were used for donor tissue wax blocks, and two representative areas were selected in each case. Each block has a pore diameter of 2 mm. All specimens were fixed in 10% neutral buffered formalin in a refrigerator at 4°C for 4 to 48 h and used for sectioning and making the tissue microarray slides.

Immunohistochemical analysis

Immunohistochemistry (IHC) was performed to examine the expression of ETS1. First, paraffin-embedded breast cancer tissue microarray sections were baked at 65°C for 1 to 2 h. Specimens were then dewaxed by immersion in xylene for 30 min, followed by hydration using gradients of alcohol (concentrations of 100%, 95%, 80%, 70%, 50%) for 5 min each. The specimens were completely immersed in 800 ml of sodium citrate buffer (pH 6.0) for antigen repair, boiled in a microwave oven, and then continued to boil for 20 min and cooled naturally to room temperature. Incubation with 3% hydrogen peroxide at room temperature for 10 min was used to block endogenous peroxidase activity and non-specific protein interactions. The anti-ETS1 antibody was incubated overnight at 4°C and the biotinylated goat anti-rabbit secondary antibody working solution (Bio derivatives) was incubated at 37°C for 30 min, followed by color development with diaminobenzidine (DAB) and observed under light microscopy and terminated immediately when a yellow pellet appeared. The staining was repeated with hematoxylin for 30 s. The specimens were then dehydrated in a gradient of alcohol (50%, 70%, 80%, 95%, 100%) for 5 min each, followed by immersion in xylene for 30 min for transparency. Finally, the slides were sealed with resin glue. Phosphate buffer (5% BSA) was used as negative controls.

IHC scoring

The stained tumor or normal tissues were scored semi-quantitatively by two independent observers. Immunostaining scores were determined based on the percentage positivity and staining intensity. The following scoring system was used: 0 point (≤5%), 1 point (5-25%), 2 point (25-50%), 3 point (50-75%) and 4 point (≥75%). Dyeing intensity is divided into 0 points (No staining), 1 point (light yellow), 2 point (yellow) or 3 point (Brown yellow). The ETS1 immunostaining score were calculated as (positive percentage score) × (staining intensity score). In this experiment, a score of ≥4 points were considered positive.

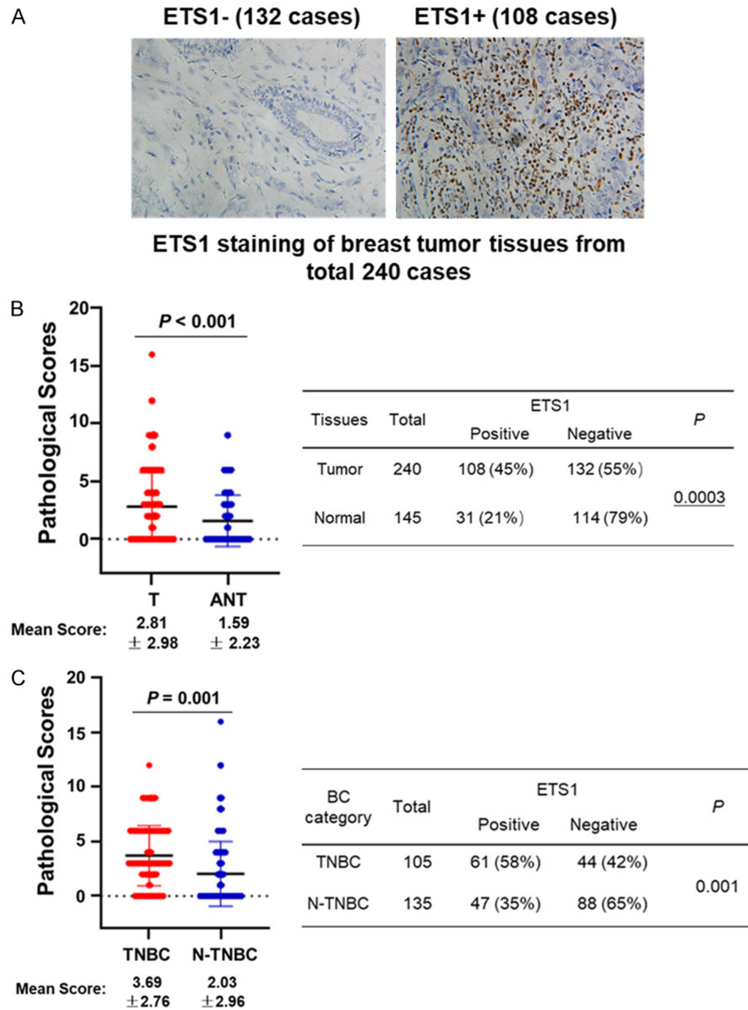
Statistical analysis

IHC score data were analyzed using IBM SPSS Statistics version 25. The relationships between the expression levels of ETS1 and clinicopathological characteristics were analyzed using a Chi-square test and Fisher exact probability method. The log-rank test and Kaplan-Meier method were also used, and the survival analysis was depicted graphically. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ETS1 is overexpressed in breast cancer tissues, especially in triple negative breast cancer tissues

We examined expression of ETS1 by IHC staining with an anti-ETS1 antibody in breast tumor tissues from 240 breast cancer cases, including 105 triple-negative breast cancer (TNBC) and 135 non-triple-negative breast cancer (N-TNBC), and along in 145 adjacent normal tissue samples. As shown in **Figure 1A** and **1B**, expression of ETS1 was detected in 108 or 45% of the 240 total breast tumor samples, comparing to that in 31 or 21% of the 145 adjacent normal tissue samples. The difference in expression of ETS1 between tumor and adjacent normal tissues is statistically significant ($P<0.001$) (**Figure 1B**), indicating that ETS1 is preferentially expressed in breast tumors.



Tissues	Total	ETS1 Positive	ETS1 Negative	P
Tumor	240	108 (45%)	132 (55%)	0.0003
Normal	145	31 (21%)	114 (79%)	

BC category	Total	ETS1 Positive	ETS1 Negative	P
TNBC	105	61 (58%)	44 (42%)	0.001
N-TNBC	135	47 (35%)	88 (65%)	

Figure 1. ETS1 is overexpressed in breast cancer, particularly in TNBC. A. The images (× 400) showing positive and negative IHC staining with anti-ETS1 in breast tumor tissues. B. The dot-plot (left panel) and statistical data (right panel) of IHC staining of ETS1 in 240 breast tumor tissue samples (T) and 145 adjacent normal tissue samples (ANT). C. The dot plot (left panel) and statistical data (right panel) of IHC staining of ETS1 in 105 TNBC and 135 N-TNBC tumor tissue samples.

We also examined the difference in expression of ETS1 between TNBC and N-TNBC tumor tissues. As shown in **Figure 1C**, expression of ETS1 was detected in 61 or 58% of total 105 TNBC tumor samples and in 47 or 35% of total 135 N-TNBC tumor samples. Difference in expression of ETS1 between TNBC and N-TNBC samples is significant (P = 0.001), suggesting that ETS1 is preferentially expressed in TNBC tumor tissues.

Expression of ETS1 is positively correlated with lymph node metastasis of TNBC

We next statistically analyzed association of expression of ETS1 with breast cancer clinicopathological characteristics, including age, tumor size, T category, N category, and TNM stages, in total breast tumor samples or separately in TNBC and N-TNBC tumor samples. As shown in **Table 1**, expression of ETS1 in breast cancer is approximately correlated with lymph node metastasis (P = 0.07), and has no correlation with other categories. In TNBC, expression of ETS1 is significantly associated with N category (P = 0.029), while not associated with other categories (**Table 2**). However, expression of ETS1 in N-TNBC has no correlation with any of the clinicopathological categories (**Table 3**). These results suggest that ETS1 may be primarily involved in promoting local metastasis of TNBC.

Expression of ETS1 in association with clinicopathological categories in TNBC is significantly higher than that in N-TNBC

We further compared expression of ETS1 protein expression in TNBC with N-TNBC within each of pathological categories. As shown in **Table 4**, except TNM stage I, expression of ETS1 in each of pathological categories in TNBC is significantly more frequent than that in N-TNBC, particularly in the categories including tumor size larger than 3 cm, T2/T3, N1-3, and TNM stages II and III (P<0.01 in all of these categories). These data indicate that expression of ETS1 is more closely associated with pathological categories in TNBC than in N-TNBC, suggesting a specific role of ETS1 in TNBC progression.

Expression of ETS1 is reversely correlated with the survival rate of TNBC patients

To assess the prognostic value of ETS1, we determined the correlation of expression of ETS1 with cumulative survival of postoperative patients by the Kaplan-Meier analysis. As

Table 1. Association of expression of ETS1 with pathological categories in all BC tumor samples

Pathological category		Total	ETS1		P
			Positive	Negative	
Age	≤45	48	20	28	0.604
	>45	192	88	104	
Tumor size (cm)	≤3	193	82	111	0.113
	>3	47	26	21	
T category	T1	124	56	68	0.959
	T2/T3	116	52	64	
N category	N0	157	64	93	0.07
	N1-3	83	44	39	
TNM stage	I/II	211	95	116	0.984
	IIIA/B	29	13	16	

Table 2. Association of expression of ETS1 with pathological categories in TNBC tumor samples

Pathological category		Total	ETS1		P
			Positive	Negative	
Age	≤45	19	12	7	0.621
	>45	86	49	37	
Tumor size (cm)	≤3	71	38	33	0.17
	>3	34	23	11	
T category	T1	36	21	15	0.972
	T2/T3	69	40	29	
N category	N0	66	33	33	0.029
	N1-3	39	28	11	
TNM stage	I/II	89	52	37	0.871
	IIIA/B	16	9	7	

Table 3. Association of expression of ETS1 with pathological categories in N-TNBC tumor samples

Pathological category		Total	ETS1		P
			Positive	Negative	
Age	≤45	29	8	21	0.356
	>45	106	39	67	
Tumor size (cm)	≤3	122	44	78	0.542
	>3	13	3	10	
T category	T1	88	35	53	0.098
	T2/T3	47	12	35	
N category	N0	91	31	60	0.793
	N1-3	44	16	28	
TNM stage	I/II	122	43	79	0.747
	IIIA/B	13	4	9	

shown in **Figure 2A**, expression of ETS1 is not significantly associated with the cumulative survival in total breast cancer patients ($P>0.05$). However, when analyzed in TNBC or N-TNBC patients, we found that expression of ETS1 is reversely associated with cumulative survival in TNBC patients ($P = 0.003$) (**Figure 2B**), but no significant association in N-TNBC patients ($P = 0.117$) (**Figure 2C**), indicating that ETS1 is a specific prognostic biomarker for TNBC patients. Furthermore, the ETS1 positive TNBC patients had a much worse survival rate than the ETS1 positive N-TNBC patients ($P = 0.004$) (**Figure 2D**). Taken together, ETS1 is a poor prognostic biomarker specific for TNBC.

ETS1 promotes proliferation and migration of TNBC cells

To verify the results of the IHC studies that ETS1 plays a role in metastasis and poor survival of TNBC patients, we examined expression of ETS1 in three breast cancer cell lines and found that ETS1 highly expressed in the TNBC cell line MDA-MB-231 cells, but not in the N-TNBC cell lines T47D and MCF7 (**Figure 3A**). Thus, we utilized MDA-MB-231 cells to investigate if ETS1 has a promoting effect on TNBC cell proliferation and migration. Two ETS1 knockdown cell lines in MDA-MB-231 cells were established using the lentiviral vector-loaded ETS shRNAs (**Figure 3B**). The cell proliferation assay of these two shETS1 cell lines indicates that depletion of ETS1 significantly reduced proliferation of MDA-MB-231 cells (**Figure 3C**). The transwell assay of these two shETS1 cell lines showed that knockdown of ETS1 markedly inhibited migration of MDA-MB-231 cells (**Figure 3D**). These results support the data of IHC staining studies and demonstrate that ETS1 is driver protein for TNBC cell proliferation and migration.

The effect of ETS1 on TNBC cell proliferation and migration is mediated by YAP signaling

Our previous studies have shown that the YAP signaling promotes proliferation and migration of TNBC cells [19]. We suspect that YAP may be downstream of ETS1 and mediate the effect of ETS1 on promotion of the TNBC cell proliferation and migration. Therefore, we detected the effect of ETS1 knock-

Table 4. Comparison of expression of ETS1 in TNBC with that in N-TNBC within pathological categories

Pathological category		Total	ETS1		*P*
			Positive	Negative	
Tumor size >3 cm	TNBC	34	23 (68%)	11 (32%)	<0.01
	N-TNBC	13	3 (23%)	10 (77%)	
Tumor size ≤3 cm	TNBC	71	38 (54%)	33 (46%)	0.011
	N-TNBC	122	44 (36%)	78 (64%)	
T1	TNBC	36	21 (58%)	15 (42%)	0.011
	N-TNBC	88	35 (40%)	53 (60%)	
T2/T3	TNBC	69	40 (58%)	29 (42%)	<0.01
	N-TNBC	47	12 (26%)	35 (74%)	
N0	TNBC	66	33 (50%)	33 (50%)	0.015
	N-TNBC	91	31 (34%)	60 (66%)	
N1-3	TNBC	39	28 (72%)	11 (28%)	<0.01
	N-TNBC	44	16 (36%)	28 (64%)	
TNM I	TNBC	24	11 (46%)	13 (54%)	0.196
	N-TNBC	70	26 (37%)	44 (63%)	
TNM II	TNBC	65	41 (63%)	24 (37%)	<0.01
	N-TNBC	52	17 (33%)	35 (67%)	
TNM IIIA/B	TNBC	16	9 (56%)	7 (44%)	<0.01
	N-TNBC	13	4 (31%)	9 (69%)	

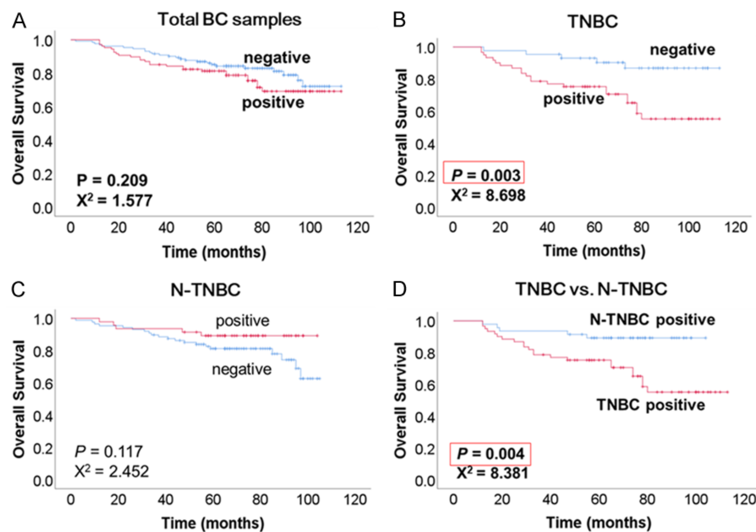


Figure 2. Expression of ETS1 is reversely associated with overall survival of TNBC. A. Association of expression of ETS1 with overall survival of total breast cancer patients; B. Association of expression of ETS1 with overall survival of TNBC patients; C. Association of expression of ETS1 with overall survival of N-TNBC patients; D. Comparison of the overall survival of the ETS1 positive TNBC patients with the ETS1 positive N-TNBC patients.

down on the mRNA and protein level of *YAP* in the shETS1 cell lines. As shown in **Figure 4A** and **4B**, knockdown of ETS1 in MDA-MB-231 cells significantly reduced both the mRNA and protein level of *YAP*. We also examined the effect of ETS1 knockdown on the mRNA level of previously identified target genes of YAP, such as *CTGF*, *BUB1*, and *CENPM* [23] and found that the mRNA level of these YAP target genes was dramatically reduced upon knockdown of ETS1 (**Figure 4C**). These results suggest that *YAP* is a downstream target gene of ETS1. To further demonstrate that YAP mediates the effect of ETS1, we overexpressed YAP in the ETS1 knockdown cell lines using the lentiviral expression system (**Figure 4B**). Upon resuming the YAP level in the ETS1 knockdown cell lines, both cell proliferation and migration capacity were partially recovered (**Figure 4D** and **4E**), indicating that YAP is one of the major downstream target proteins of ETS1 that mediates the promoting effect of ETS1 on TNBC cell proliferation and migration.

Discussion

Here we systematically assessed the relationship between expression of ETS1 and breast cancer progression/prognosis. Our studies have shown that ETS1 is highly expressed in breast cancer and expression of ETS1 is associated with poor prognosis in TNBC patients and reversely correlated with TNBC patient survival. Expression of ETS1 in TNBC is correlated with clinicopathological N-category and TNM stages. Furthermore, knockdown of ETS1 in TNBC cells inhibits cell proliferation and migration. These data suggest a role of ETS1 in TNBC metastasis. Our studies have also demonstrated that YAP is one of the major downstream effector of ETS1 in TNBC progression. Taken together, our

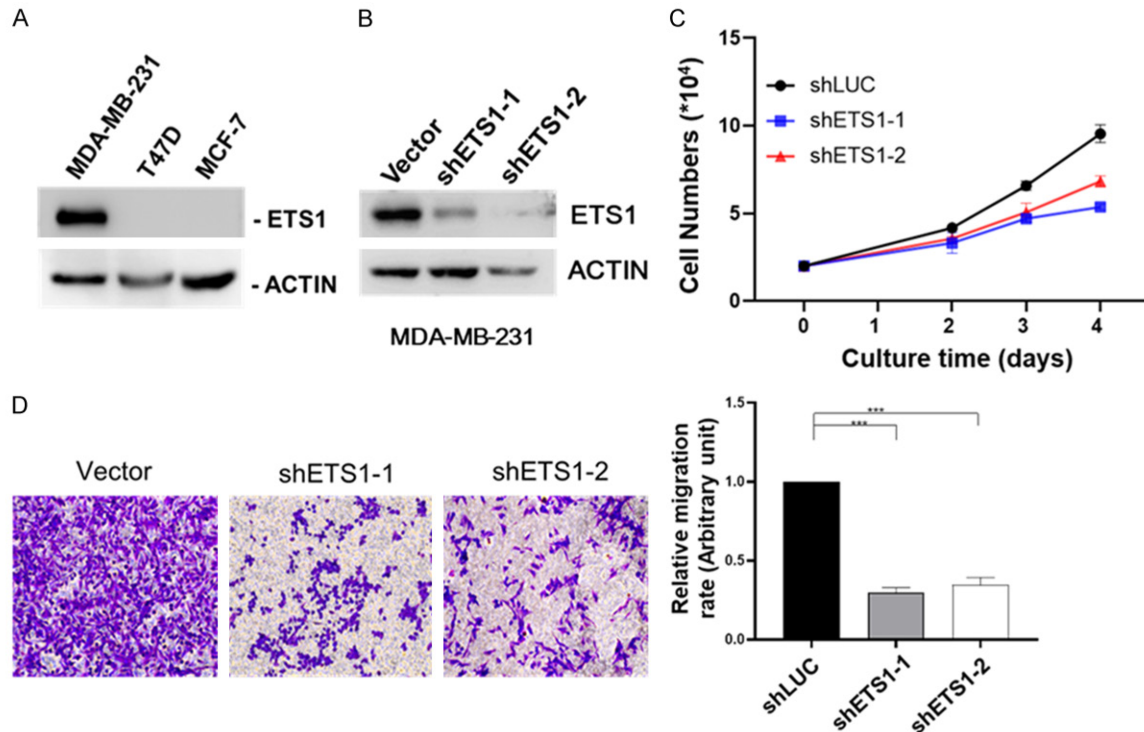


Figure 3. Knockdown of ETS1 inhibits proliferation and migration of the TNBC cells. (A) The Western blot images of the ETS1 protein level in breast cancer MDA-MB-231, T47D, and MCF7 cells. (B) The Western blot images of ETS1 knockdown in MDA-MB-231 cells by ETS1 shRNAs. (C) Knockdown of ETS1 inhibited cell proliferation of MDA-MB-231 cells. (D) Knockdown of ETS1 markedly impaired migration of MDA-MB-231 cells. All the quantifications in (B and C) were from three independent experiments. ****P*<0.001.

studies have identified a new ETS1 signaling pathway in TNBC that may be a potential target for TNBC therapy.

Our studies have shown that expression of ETS1 is a specific prognostic biomarker for TNBC patients, but not for N-TNBC or total breast cancer patients. However, the mechanism underlying the ETS1 specificity is not known. YAP is identified in our studies as one of ETS1 major downstream targets. Knockdown of ETS1 inhibits the mRNA level of YAP and the YAP downstream target genes in TNBC cells (**Figure 4**), suggesting that ETS1, as a transcription factor, may directly promote transcription of *YAP*. Our previous studies found that YAP is a downstream target of geranylgeranylation signaling that is a tumorigenic driving force specific to TNBC, but not N-TNBC cells [19]. Thus, the specificity of ETS1 promoting TNBC progression may be mediated by YAP signaling. To further understand the interaction between ETS1 and YAP in TNBC, we will investigate how ETS1 activates YAP, i.e. through direct transcriptional activation or indirect activation, and how YAP mediates the specific promoting effect of ETS1 on TNBC cell proliferation and migration. By demonstrating that ETS1 functions as a specific driver for TNBC progression, we may provide experimental basis for targeting ETS1 and YAP in TNBC therapy.

Our data indicate that expression of ETS1 is closely associated with metastasis of TNBC, suggesting that ETS1 is a potential target for anti-metastasis therapy in TNBC. Previous studies have shown that ETS1 is an upstream transcription factor of MMPs and abnormally high expression of ETS1 induces up-regulation of MMPs and enhances the invasiveness of cancer cells [8-10]. Expression of PAI-1, an inhibitor of uPA that regulates cell adhesion and promotes cell migration, is up-regulated by ETS1 [24]. Thus ETS1 may facilitate TNBC metastasis through elevating expression of MMPs and PAI-1. Interestingly, YAP is a known protein associated with metastasis of multiple cancers [20-22] and may activate expression

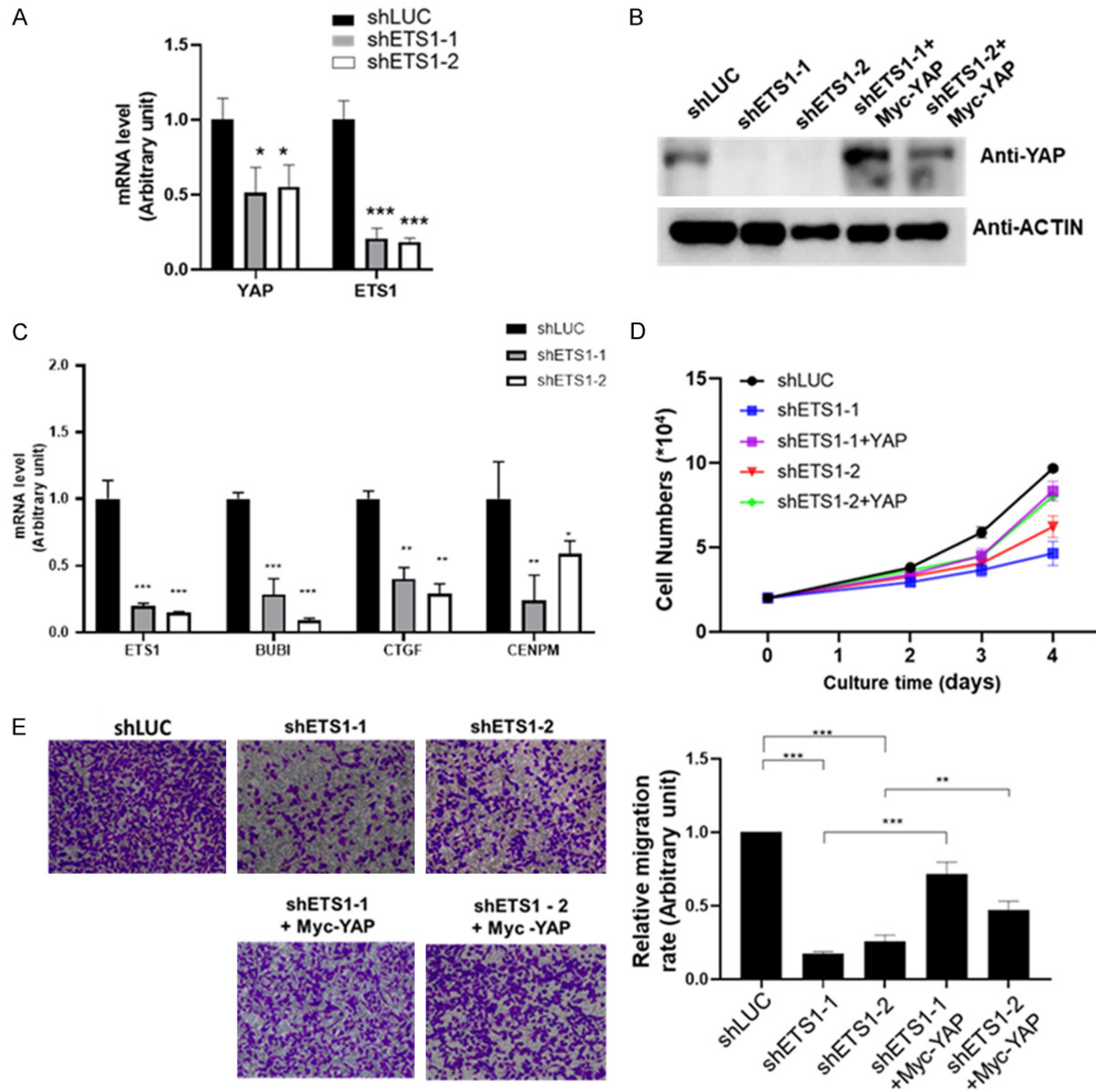


Figure 4. YAP is one of the downstream effectors of ETS1 and mediates the effect of ETS1 on proliferation and migration of MDA-MB-231 cells. (A) Knockdown of ETS1 in MDA-MB-231 cells reduced the mRNA level of *YAP*. (B) Western blot image of YAP protein levels in the ETS1 knockdown and the exogenously expressed YAP in ETS1 knockdown cells. (C) Knockdown of ETS1 reduced the mRNA level of the YAP target genes *BUB1*, *CTGF*, and *CENPM*. (D) Exogenous expression of YAP in the ETS1 knockdown cells partially restored proliferation of the ETS1 knockdown cells. (E) Exogenous expression of YAP in the ETS1 knockdown cells partially restored migration of the ETS1 knockdown cells. Right panel, images (× 200) of cell migration by the transwell assay; left panel, quantification of cell migration by the transwell assay. All the quantifications in (A, C-E) were from three independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

of MMPs and PAI-1 [25-27]. It is plausible to propose that YAP mediates ETS1 signaling to regulate expression of MMPs and PAI-1, thus promotes metastasis of TNBC. As metastasis is the major cause behind poor prognosis of TNBC, investigation of the specific role of ETS1 and/or YAP in promoting metastasis and the connection to poor prognosis in TNBC is particularly important for targeted therapy for advanced TNBC.

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Disclosure of conflict of interest

None.

Abbreviations

ER, Estrogen Receptor; ETS1, E26 Transcription Factor-1; HER2, Human Epidermal Growth Factor Receptor 2; IHC, Immunohistochemical; MMP, Matrix Metalloproteinases; N-TNBC, Non-TNBC; PR, Progesterone Receptor; TAZ, Transcriptional Coactivator With PDZ-Binding Motif; TEAD, TEA Domain; TNBC, Triple Negative Breast Cancer; VEGF, Vascular Endothelial Growth Factor; YAP, Yes-Associated Protein.

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