Suppression of Tumorigenicity 5 Ameliorates Tumor Characteristics of Invasive Breast Cancer Cells via Integrating ERK/JNK Pathway

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Research

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Abstract

Background: Suppression of tumorigenicity 5 (ST5) has been considered as a tumor suppressor gene in HeLa tumor cells. However, there is no report of ST5 expression or function in the progression of breast cancer.

Methods: ST5 expression in different subtypes and pathological stages of breast cancer was determined by Oncomine database, Breast Cancer Gene-Expression Miner v4.4 (bc-GenExMiner v4.4) analysis and immunohistochemistry. Cell viability was measured by CCK8 assay and metastatic behavior was assessed using scratch wound model and Transwell. Flow cytometry was employed for cell cycle and apoptosis detection, and methylation-specific PCR (MSP) was used to detect methylation level.

Results: ST5 was expressed at low level in different subtypes of breast cancer specimens compared to normal breast and there was a negative association between ST5 status and pathological stages of breast cancer patients. Additionally, ST5 was lower in cases of recurrent and invasive breast cancer than that in non-recurrent and non-invasive patients. In *in vitro* experiment, ST5 status was also negatively associated with the invasive capability of breast cancer cells, showing lower in MDA-MB-231 and SKBR3 cell lines than that in MCF-7 cells. ST5-downregulation promoted, while ST5-upregulation inhibited the tumour characteristics of MDA-MB-231 cells including cell viability, cell cycle and migration. And exogenous ST5 also elevated, but ST5 depletion limited the proportion of apoptotic cells in MDA-MB-231 cells. However, the alteration of ST5, no matter upregulation or downregulation, had no impact on tumour behaviors of MCF-7 cells. Mechanistically, ST5 protein ablation activated, while ST5-upregulation repressed the activities of phosphorylated JNK and ERK1/2, and subsequently the expression of c-Myc. Of note, low level of ST5 in breast cancer cells was possibly related with the aberrant methylation of ST5 promoter region.

Conclusion: Our findings suggest that ST5 potentially acts as a tumor suppressor gene in invasive breast cancer through regulating ERK/JNK signaling pathway and provide a novel insight for breast cancer treatment.

Background

Breast cancer is the leading cause of cancer deaths among women worldwide (1). According to the statistics, more than one million women are diagnosed with breast cancer every year in the world (2). Based on the status of estrogen receptor (ER), progesterone receptor (PR) and receptor tyrosine-protein kinase erbB-2 (HER2), breast cancer is classified as Luminal A, Luminal B, HER2-positive and triple-negative breast cancer (TNBC) subtypes (3). Although the endocrine therapy is the preferred treatment and has a better therapeutic effect for hormone receptor-positive breast cancer, quite a few hormone receptor-positive patients will develop either primary or secondary drug resistance (4). Additionally, most metastatic breast tumor patients, such as patients with TNBC subtype, has poor prognosis and high

recurrence rate (5). Currently, there remains plenty of challenges for treating highly malignant and invasive breast cancer due to lacking of well-defined molecular targets.

Suppression of tumorigenicity 5 (ST5), also known as DENND2B, is a member of DENN protein family which is localized to chromosome 11p15.2 (6). DENN protein family presents significantly differential expression between normal and tumor cells, suggesting it plays a critical role in the development of cancer (6). As a tumor suppressor, *ST5* gene can encode cleavage ST5 protein P70 (70kD) and P82 (82kD), and the full length of ST5 protein (126kD, P126) (7). In comparison to normal ovarian epithelial cells, ST5 is down-regulated in ovarian carcinoma cells (8). Exogenous ST5 suppresses tumor formation and growth of Hela cells-bearing mice in *vivo* (9), and this inhibition effect is correlated with the elevation of cleavage ST5 (P70), but not P126 (10). Moreover, combined with microarray gene chip study, the significant elevation of ST5 (p126) in uterine leiomyoma is observed compared to that in normal myometrium (11). It is confirmed that ST5 could interact with Rab13, leading to the promotion on migration, invasion and tumorigenicity phenotype of normal breast epithelial cells MCF-10A (12). The results suggest that ST5 may be involved in the regulation of gynecological oncology process, but the specific function and regulation mechanism of ST5 in breast cancer still need more deeply investigations.

Herein, we explored the expression of ST5 in breast cancer specimens and its biological function in ER-positive or ER-negative breast cancer cells. ST5-evoked the changes of downstream signaling pathway was also determined. Additionally, we demonstrated the association between ST5 expression and DNA methylation. Collectively, our data illustrate ST5 potentially acts as a potent tumor suppressor in the progression of ER-negative invasive breast cancer, but not ER-positive patients.

Materials And Methods

Online database analysis

The differential expression of ST5 in different subtypes of breast cancer tissues, and the association between ST5 status and tumor stage (SBR standard classification), were determined using the Breast Cancer Gene-Expression Miner v4.4 (bc-GenExMiner v4.4). The expression of ST5 in patients with no recurrence and recurrence, patients with ductal breast cancer and invasive breast cancer, was assessed by using Oncomine database.

Cell culture and treatment

The normal mammary epithelial cell line (MCF-10A), ER-positive breast cancer cell (MCF-7), adriamycin resistant MCF-7 cells (MCF-7/ADR), ER-negative cell line (MDA-MB-231), and ER/PR-positive and Her2-negative breast cancer cell lines (SKBR3) were purchased from American Type Culture Collection (ATCC). MDA-MB-231 and SKBR3 cells were incubated with RPMI 1640 (SH30809.01; HyClone, USA), MCF-10A and MCF-7 were cultured in dulbecco's modified eagle medium (DMEM) (SH30022.01; HyClone, USA) and supplemented with 10% foetal bovine serum (FBS; SH30070.03; HyClone, USA). MCF-7/ADR cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 1 μM doxorubicin. All cells were

incubated in a humidified incubator containing 5% CO2 at 37 °C. For the treatment of methylation inhibitor, MDA-MB-231 cells were exposed to 10 μM and 20 μM of 5-aza-2'-deoxycytidine (5-Aza) (Sigma-Aldrich, USA) in DMSO or DMSO alone for 72 h. After the initial 5-aza-dC treatment, cells were subjected to total protein.

Cell transfection

Briefly, the CDS fragment of ST5 was inserted into pEGFP-C1 plasmid. Control pEGFP-C1 vector and recombinant pEGFP-C1-ST5 plasmids were transfected into MDA-MB-231 and MCF-7 cells using the TurboFect transfection reagent (R0531; Thermo Fisher Scientific, USA). At 24 h post-transfection, cells were harvested for further investigation. For RNA interfere (RNAi)-mediated depletion of ST5, cells were transfected with negative control siRNA (siNC) or siST5 (sequence) using the TurboFect transfection reagent. At 72 h post-transfection, cells were used for subsequent experiments.

Human specimens and immunohistochemical staining

Human breast cancer tissues were collected from the First Affiliated Hospital of Xiamen University. Clinicopathological characteristics of Breast Cancer patients were presented in Table 1. Human breast cancer tissue chips or purchased from OUTDO BIOTECH (Shanghai, China). A signed informed consent form was obtained from all subjects, and this study was approved by the ethics committee of the First Affiliated Hospital of Xiamen University (2016XMU-BIO-77). Tissue chips and tissue sections were dewaxed in xylene solution, and hydrated in graded ethanol solutions. After antigen retrieval using high pressure method, the sections were incubated with 0.3% H_2O_2 for 20 min. Then, sections were incubated overnight with a primary antibody against ST5 (ab187759, Abcam, UK). The next day, the sections were hybridised with the secondary antibody and subjected to chromogenic reaction using DAB reagent (1:50). The staining intensity and the number of positive cells in the tissue chip, which were analysed using the Image Pro Plus software v6.0 (Media Cybernetics, Inc., Maryland, USA) (negative: –; weakly positive: +, < 20%; middling positive: ++, 20–50%; strongly positive: +++, > 50%). Tumours were categorised as "Low" or "High" grade based on relative ST5 expression according to the staining intensity (+ + and +++ were defined as High, - and + were defined as Low). Relative staining intensity was evaluated by three independent pathologists.

Table 1
Chi-squared analysis of contingency tables between ST5 status and clinicopathological characteristics of Breast Cancer patients

Characteristics	Relative ST5 expression		*P value*
	Low (n = 33)	High (n = 26)	
Gender			0.4407
Male	0	1	
Female	33	25	
Age			0.4096
≤ 50	9	10	
> 50	24	16	
Tumor grade			0.0397*
G1	2	8	
G2	12	8	
G3	19	10	
Tumor diameter (cm)			0.3716
≤ 5	29	25	
> 5	4	1	
*P < 0.05 was considered statistically significant.			

MTT assay

MDA-MB-231 and MCF-7 cells transfected with pEGFP-C1 vector, recombinant pEGFP-C1-ST5 and small interfering RNAs were seeded into 96-well plates at a density of 2×10^3 cells/well, and cultured for additional 24 h, 48 h and 72 h. After incubation, 10 μL MTT (Sigma-Aldrich, USA) was added to the medium and incubated for another 4 h. Then, OD values were measured at the wavelength of 492 nm using a Multiscan plate reader (MK3, Thermo, USA).

Flow cytometry

All group cells were seeded in 6-well plates at a density of 5×10^5 cells/well. For cell cycle detection, cells were digested with trypsin without Ethylene Diamine Tetraacetic Acid (EDTA). After fixing with 1 ml 70% ethanol overnight, cells were centrifugated and the cell pellets were stained with 2 μl 50ug/ml PI and 1 μl 100 μg/mL RNase A for 30 min at 4 ℃ in darkness. Subsequently, cells were analysed by Flow cytometry. For cell apoptosis assay, the digested cells were stained with Annexin V-APC Apoptosis Detection Kit

according to the manufacturer's protocols (KeyGEN, Nanjing, Jiangsu, China). Then the apoptosis rates were detected using a flow cytometer.

Migration assay

Wound healing and Transwell experiments were employed to determine the migration ability of MDA-MB-231 cells. For wound healing assay, a linear wound was sketched with the pipette tip after cells reached a confluency of 90%-95%. Then cells were incubated in serum-free medium for 24 h and 48 h. Cells were then photographed at different time points and the migration rate was calculated by measuring the wound area. The transwell assay was also used to assess migration ability. Cells were plated into Transwell upper chamber at a density of 1×10^5 cells/chamber (Millipore, Germany), and cultured with fresh medium without FBS. The lower chamber was surrounded with 600 μl of 10% FBS. After incubation for 12 h, cells on the upper chamber were wiped off and fixed for 30 minutes with ethanol. Then cells were stained with 0.1% crystal violet for 20 minutes after washing with PBS buffer for twice. Then, the migrated cells was calculated using a microscope (OLYMP®S, IX51, Japan).

Quantitative real-time PCR

Total RNA was isolated utilizing TRIzol reagent (Roche, Indianapolis, IN, USA) according to manufacturer's protocol. 1 μg total RNA was reversely transcribed into cDNA with All-in-One First-Strand cDNA Synthesis Kit (Transgene, China). Then, quantitative real-time PCR was performed using the SYBR Green kit (Roche, Indianapolis, IN, USA). GAPDH was served as the internal control. Relative gene levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Total protein was extracted from MDA-MB-231 and MCF-7 cells using RIPA buffer. Primary antibodies against ERK1/2 and c-Myc were obtained from Santa Cruz Biotechnology (California, USA). Phosphorylated ERK1/2 and c-Jun N-terminal kinase (JNK)/p-JNK antibodies were purchased from Cell Signaling Technology (Boston, USA). ST5 antibody was purchased from Abcam BioTech. GAPDH (Sangon Biotech, Shanghai, Chian) and Tublin were served as the internal control. Grey scale intensity of the immunoblots was analysed using Quantity One analysis software (Bio-Rad, California, US).

DNA Methylation Determination

Bisulphite conversion of DNA samples from MDA-MB-231 cells was performed using the EZ DNA Methylation™ Gold Kit (ZYMO RESEARCH, USA) according to the manufacturer' s instructions. After that, 2 μL of the eluted DNA was subjected to PCR under the following conditions: 98 °C for 10 min, 53 °C for 30 min, 53 °C for 6 min, followed by 8 cycles at 37 °C for 30 min, and 4 °C storage. The primer sequences for methylation-specific PCR (MSP) were presented in **Table S1**. Then, the reaction products were detected by agarose gel electrophoresis.

Statistical analyses

All experiments were independently performed at least three times using independent samples. The number of ST5-positive cells and migrated cells were measured on the basis of at least five horizons by three independent researcher. Quantitative analysis of western blotting data, wound healing rate and apoptotic rate were performed using the GraphPad Prism 6.0 software with a two-tailed Student's *t*-test. MTT assay data was analysed by GraphPad Prism using two-way analysis of variance (ANOVA). Statistical analysis of clinical correlation was performed by the Cochran-Mantel-Haenszel and Chi-squared tests. Values have been presented as mean ± standard error of mean (SEM). Statistically significant differences were received at $P < 0.05$.

Results

ST5 is reduced in breast cancer specimens

To investigate the role of ST5 in breast cancer, *ST5* gene expression was firstly analyzed in different subtypes of human breast cancer tissue and normal breast tissue using the bc-GenExMiner v4.4. Results demonstrated a lower level of *ST5* expression in multiple molecular subtypes of breast cancer tissue, including Luminal B subtype (n = 663), Luminal A subtype (n = 1,596), HER2-E subtype (n = 642) and Basal-like subtype (n = 624), than in normal breast tissue (n = 840) ($P < 0.01$) (Fig. 1A). Based on the immunohistochemical staining (IHC) results, a higher expression of ST5 in para-carcinoma tissues than in paired tumour tissues was observed (Fig. 1B). After assessing the difference in protein level of ST5 between human breast cancer tissues and their corresponding normal breast tissues, results showed that ST5 level was observably declined in tumour samples compared to that in paired normal breast tissue samples (Fig. 1C). Among these clinical tissue samples (n = 12), mRNA expression approximately 58% of the tumour tissues (7/12) presented extremely low level of *ST5* mRNA as compared with their matching normal tissues (Fig. 1D). Taken together, these findings demonstrate that ST5 status is decreased in human breast cancer specimens.

ST5 presents lower in invasive breast cancer specimens

Next, the correlation between the expression of ST5 and tumor stage (SBR standard classification) was analysed. Combined with the results from the bc-GenExMiner v4.4 database, a significant negative correlation between *ST5* status and tumor stage was verified, where patients with a higher tumor grade (SBR3) had lower *ST5* expression (Fig. 2A). Uniformly, in the results of IHC assay, patients with advanced grade (grade II and grade III) always presented low ST5 expression compared to patients with early grade tumours (grade I) (Fig. 2B). Based on the staining intensity of ST5 using microarray, 33 out of 58 breast cancer patients presented low protein level of ST5 (- and +) and 26 out of 58 presented high level of ST5 (++ and +++). Correlation analysis indicated that ST5 status had no correlation with the clinicopathological characteristics of patients, including sex, age and tumor diameter. However, patients with advanced grade (grade 2: G2 and grade 3: G3) always showed low ST5 expression compared to patients with early grade tumours (G1) ($P = 0.0397$) (Table 1). Additionally, in comparison to patients with no recurrence (n = 89), lower ST5 was also authenticated in recurrent breast cancer patients (Fig. 2C). Of

note, there showed a notable decline in ST5 status in invasive ductal/lobular breast carcinoma (n = 3 and 105) as compared to non-invasive ductal/lobular breast cancer patients (n = 105 and 9) (Fig. 2D-E). In the database of Nikolsky Breast, the decrease of ST5 copy number was also observed in invasive ductal breast carcinoma (n = 5) by contrast to non-invasive ductal breast cancer specimens (n = 133) (Fig. 2F). Additionally, ST5 level was very low in breast cancer cells, such as MCF-7, MCF-7/ADR, MDA-MB-231 and SKBR3 cells compared to that in normal breast cells (MCF-10A), and ST5 status showed more lower in cell lines with a high invasive capability (SKBR3 and MDA-MB-231) (Fig. 2G). Moreover, the mRNA expression of *ST5* was notably decreased in breast cancer cells compared to that in normal MCF-10A cells, indicating lower ST5 levels in invasive cell lines (MDA-MB-231: a 50% fall; SKBR3: a 85% fall, P = 0.0001) than those in MCF-7 cells with a lower invasive capability (a 25% fall) (Fig. 2H). The data indicate low level of ST5 may most likely be observed in invasive breast cancer specimens.

ST5 affects cell viability and migration of invasive breast cancer cells

To assess the cellular functions of ST5, cell proliferation and migration assays were performed in MDA-MB-231 (invasive cell line) and MCF-7 cells (cells with a lower invasive capability) transfected with exogenetic ST5 and siRNAs targeting ST5. Results showed a robust suppression of cell viability in the ST5 overexpressing group compared with that in the control vector group from days 3 to 4 (Fig. 3A), while RNAi-mediated knockdown of ST5 resulted in 35% (P = 0.036) and 30% (P < 0.001) upregulation in cell viability in the ST5 knockdown group compared to that in the cells transfected with negative control siRNA group on days 3 to 4, respectively (Fig. 3B). In the Wound Healing experiment, the wound area and healing rate notably reduced by 25% at 48 h (P < 0.001) and 35% at 48 h (P < 0.001) post-wounding in the ST5 group compared to that in the vector group (Fig. 3C, 3E). Moreover, ST5 depletion increased by 45% at 48 h (P < 0.0001) and 42% at 48 h (P < 0.0001) in wound healing rate as compared to that in the control cells (Fig. 3C, 3E). Additionally, in the Transwell assay, a 50% reduction (P < 0.001) in migration rate following overexpression of ST5, in contrast to a 30% increase (P < 0.0001) in migration rate observed in ST5-depleted cells, as compared with the corresponding control cells (Fig. 3F-G). However, forced expression of ST5 or ablation of ST5 had no effect on cell viability and migration in MCF-7 cells compared to the corresponding control cells (**Fig. S1A–D**). Thus, these results indicate a potential anti-neoplastic effects of ST5 on invasive breast cancer cells.

ST5 induces cell cycle arrest and apoptosis in invasive breast cancer cells

Next, flow cytometry analysis was employed to determine the impact of ST5 on cell cycle and programmed cell death. As shown in Fig. 4A, forced expression of ST5 in MDA-MB-231 cells decreased the number of S phase cells by 25% (P < 0.01) (Fig. 4A-B), while ST5 ablation increased the proportion of S phase cells by 21% (P < 0.01) compared to that in siNC group (Fig. 4C-D). Based on the apoptosis assay, approximately a 70% increase in the proportion of apoptotic cells was observed in (P < 0.01) in

ST5-overexpressing cells (Fig. 4E, 4G). Conversely, knockdown of ST5 markedly reduced the proportion of apoptotic cells by 49% ($P < 0.01$) (Fig. 4F, 4H). In MCF-7 cells, genetically modified ST5 did not affect cell cycle progression (**Fig. S2A–B**) and programmed cell death (**Fig. S2C–F**). Accordingly, our data demonstrate that ST5 moderately abrogates the DNA synthesis, resulting in cell cycle arrest and a subsequent increase in apoptosis.

The ERK/JNK pathway is suppressed by ST5 upregulation

Consequently, the signalling cascade affected by ST5 in invasive breast cancer cells was verified. As expected, overexpression of ST5 restrained the phosphorylation of ERK1/2 and JNK by 50% ($P < 0.01$) and 30% ($P < 0.05$), respectively, with a decrease of c-Myc by 55% (Fig. 5A-B). However, ablation of ST5 promoted the activation of ERK1/2 ($P < 0.05$) and JNK ($P < 0.05$), and the expression of c-Myc by 2-fold (Fig. 5C-D). Overall, ERK/JNK signaling pathway potentially was negatively regulated by ST5 in invasive breast cancer cells.

ST5 hypermethylation affects cell viability and migration of invasive breast cancer cells

Gene expression is always regulated by epigenetic changes. Then, the methylation level of the ST5 promoter region in MDA-MB-231 cells was determined by methylation-specific PCR with five pairs of primers designed using the MethPrimer method (**Table S1**). MSP data indicated that hypermethylated ST5 was found in the MDA-MB-231 cells (Fig. 6A). To investigate whether promoter methylation affected the expression of ST5, the protein level of ST5 in breast cancer cells was detected using 5-aza-dC, an inhibitor of DNA methylation. An increase in 5-aza-dC dosage (10 μM and 20 μM) affected the expression of ST5 in MDA-MB-231 cells (Fig. 6B). In addition, the cell viability and migration of MDA-MB-231 cells also were inhibited by the exposure of methylation inhibitor. Possibly, promoter methylation-mediated the decline of ST5 was involved in the progression of tumor metastasis in breast cancer cells.

Discussion

Currently, a combination of surgery and chemotherapy or radiation therapy is the most common treatment for breast cancer; however, poor prognosis and relapse still occur frequently in patients with distant metastases (13). Although advanced targeted cancer therapy and neoadjuvant therapy are constantly emerging, they are associated with several limitations for blocking cellular migration (14–17). ST5 shows differentially expression in tumorigenic and nontumorigenic somatic cell hybrids of Hela cells, implying ST5 might affect tumorigenesis (18). In the present study, ST5 upregulation abolished tumor migration of breast cancer cells and hypermethylated ST5 might implicated in this progression. Probably, ST5 acts as a potential therapeutic target for metastatic breast cancer.

ST5 is differential expression in several gynecological oncology (8, 11). ST5 (P70kD) decreases tumorigenicity in HeLa cells, implying it may serve as a cancer suppressor gene (10, 19). Here, the decline

of full length ST5 was observed in human breast cancer, particularly in invasive breast cancer cells. The finding firstly demonstrates ST5 (P126kD) shows differential expression among normal mammary epithelial cells, non-invasive and invasive breast cancer cells. However, the data does not detect the alterations of the other two variants among these cell types. Interestingly, the high invasive capability cell line SKBR3, which is ER- and progesterone receptor (PR)-negative but HER2-positive, also presented lower ST5 levels than TNBC cells (MDA-MB-231). On the basis of analysis results from bc-GenExMiner v4.4, ST5 status also appeared a significant decline in HER2-positive breast cancer patients (n = 661) compared to that in HER2-negative patients (n = 3582) (**Fig. S3**). The data imply a potential association between HER2 and ST5 in breast carcinogenesis which still needs to study further. Additionally, ST5 (P70kD) is correlated with decreased tumorigenic phenotype in mammalian cells, with a restoration in their transformed phenotype and contact-dependent growth (9, 20). Based on our present data, full length of ST5 (P126kD) also possessed the inhibition effect on biological behavior of invasive breast tumor cells, though previous study has indicated that ST5 (P126kD) promoted metastatic behavior of normal breast epithelial cell (12). Of note, in our data, we did not exclude the impact of ER that was expressed in MCF-7 cells, but not in MDA-MB-231 cells. Whether overexpression of ST5-mediated antitumor effect is prevented by the presence of ER in MCF-7 cells remains unclear.

ST5 is important for cellular signal cascades and the regulation of the transformed phenotype (21). Structurally, ST5 contains a group of GDP/GTP exchange region for Rab protein family and the mitogen-activated protein (MAP) kinase-activating death region which is verified as a interactant of TNF-alpha receptor (22, 23). ST5 can activate the MAP kinase ERK1/2 in response to epidermal growth factor (EGF) in cos-7 African green monkey fibroblast cells (24). The proline-rich region of ST5 isoform can recognize Src homology 3 (SH3) binding domains and MAP kinase phosphorylation sites (25–27), and participated in RAS/MEK2/ERK2 signaling cascade (20). Additionally, DENN/MADD, a multifunctional domain protein, interacts with JNK, activating MAPK/JNK pathway in Alzheimer's disease (AD) pathogenesis (28, 29). Therefore, ST5-mediated intracellular signaling transduction has the involvement of ERK1/2/JNK pathway. In the present study, we observed that ERK1/2/JNK activation was obviously inhibited, followed by the decrease of c-Myc in MDA-MB-231 cells transfected with exogenous ST5, vice versa. Possibly, ERK1/2/JNK signal axis was the downstream of ST5 during the migration process of breast tumor cells. However, whether ST5-mediated anti-tumor effect depends on ERK1/2/JNK pathway needs more investigation.

Low expression of tumor suppressors was regulated by diverse of factors, such as microRNAs, transcription factors, and DNA methylation (30, 31). Similar to other cancer types, human breast cancer also exhibits a high number of epigenetic alterations in the genome (32). Methylation profiles can be used as clinical biomarkers to predict drug response and prognosis of breast cancer (33, 34). DNA methylation alterations associated with cancer-related genes have become more pronounced and acted as the effective biomarker for early diagnosis and disease progression monitoring in the invasive breast tumors (35–37). The previous research from our group has revealed hypermethylated ST5 in gastric poorly differentiated adenocarcinoma (38). Herein, ST5 was also highly methylated in invasive breast cancer cells. And methylation inhibitor not only promoted ST5 expression, but also rescued tumor

characteristics of MDA-MB-231 cells. Probably, the promoter methylation of ST5 regulated ST5 decline-evoked cell viability and migration enhancement.

Conclusion

In summary, we elaborated the anti-tumor role of ST5 in cell proliferation and migration of invasive breast cancer cells possibly through regulating ERK1/2/JNK signaling pathway. DNA methylation was a underlying cause of low level of ST5 in invasive breast tumor cells. Possibly, methylation inhibitor-mediated the upregulation of ST5 and/or exogenous ST5 contributes to blocking the proliferation and the metastasis of breast cancer.

Abbreviations

ST5, Suppression of tumorigenicity 5; MSP, methylation-specific PCR; ER, estrogen receptor; PR, progesterone receptor; HER2, receptor tyrosine-protein kinase erbB-2; TNBC, triple-negative breast cancer; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; AD, Alzheimer's disease.

Declarations

Ethics approval and consent to participate

We declare that the study includes a statement on ethics approval and consent.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Conflicting Interests

The authors declare that there have no competing interests.

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Author Contributions

[Shuai Chen] and [Xingchun Gou] contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Jianghong Cheng] and [Mingli Li]. IHC staining was performed by [Mingli Li] and analyzed by [Chi-Meng Tzeng]. The first draft of the manuscript was written by [Shuai Chen] and [Xingchun Gou], and all authors commented on previous versions of the manuscript. All authors have read and approved the final manuscript.

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Figures

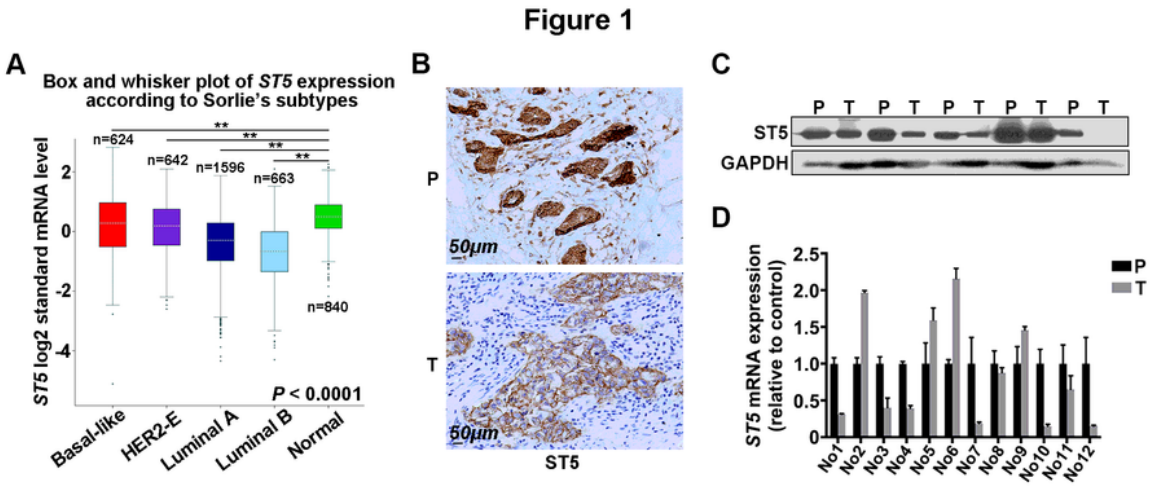

Figure 1

Evaluation of ST5 expression in human breast cancer specimens. (A) ST5 gene expression as analyzed by using the bc-GenExMiner v4.4 in different subtypes of human breast cancer tissue and normal breast tissue (n=840). The subtypes of human breast cancer included Luminal B subtype (n=663), Luminal A subtype (n=1,596), HER2-E subtype (n=642) and Basal-like subtype (n=624). (B) Immunohistochemical staining of ST5 in human breast cancer samples and its corresponding adjacent tissues. Scale bar:

50μm. Protein expression (C) and mRNA level (D) of ST5 in human breast cancer tissues (n=12) and its paired para-carcinoma tissue were measured by Western blotting and qRT-PCR, respectively. **, P < 0.01.

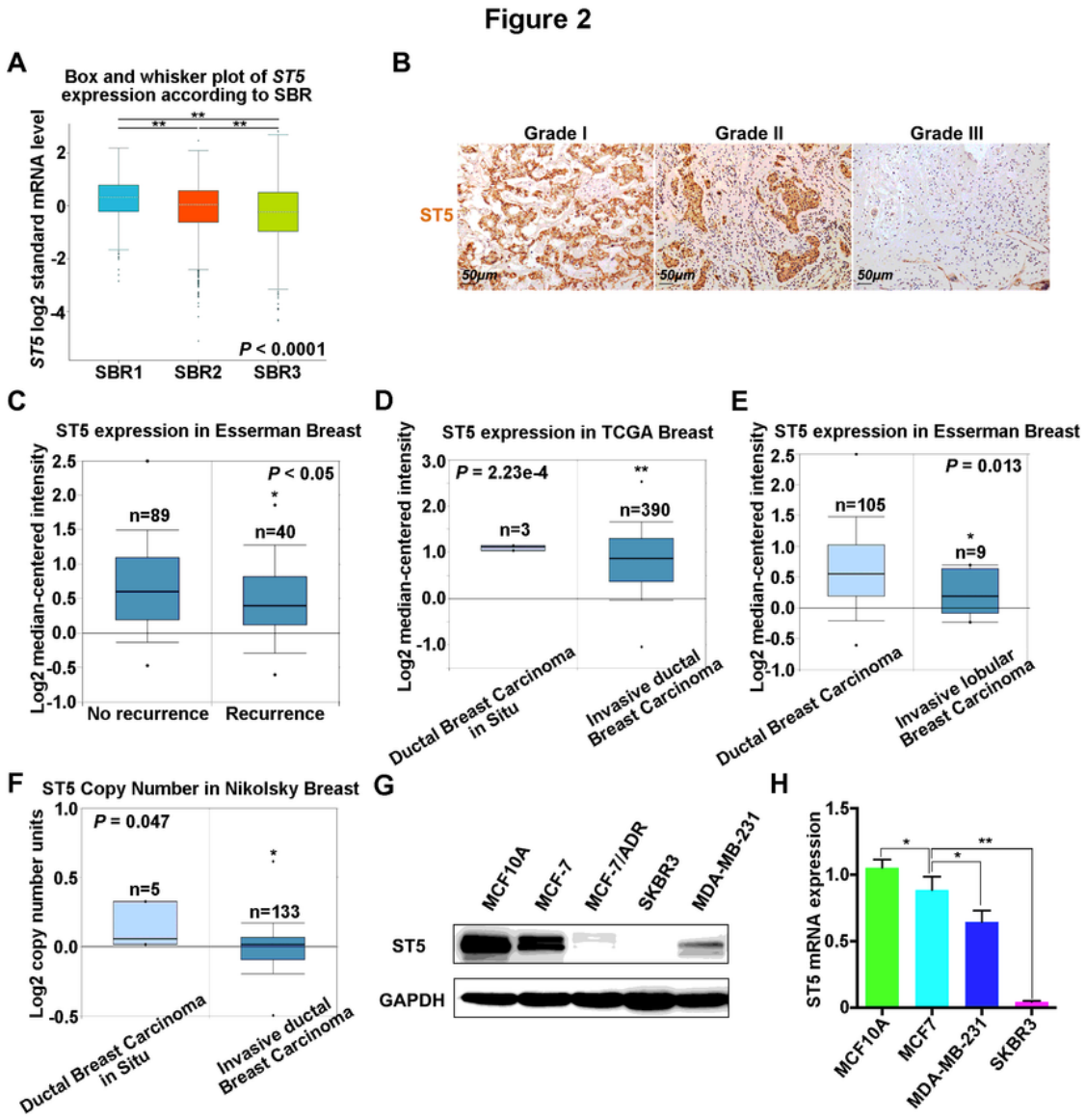

Figure 2

Analysis of ST5 differential expression in invasive and non-invasive breast cancer. (A) Association between ST5 expression status and SBR classification as determined by bc-GenExMiner v4.4. (B) Immunohistochemical staining using ST5 primary antibody in different pathological grades of human

breast cancer tissue (tissue microarray). Scale bar, 50μm. ST5 differential expression between non-recurrent and recurrent breast cancer patients (C), between invasive ductal breast carcinoma and non-invasive ductal/lobular breast cancer patients in TCGA breast database (D), between invasive lobular breast carcinoma and non-invasive lobular breast cancer patients in Esserman breast database (E). (F) The difference in ST5 copy number between invasive ductal breast carcinoma and non-invasive ductal breast cancer specimens. (G) Protein level of ST5 in MCF-7, MCF-7/ADR, MDA-MB-231, SKBR3 cells and normal breast cells (MCF-10A) were determined by Western Blotting. (H) Transcriptional level of ST5 in MCF-7, MDA-MB-231, SKBR3 cells and normal breast cells (MCF-10A) were determined by qRT-PCR. *, $P < 0.05$; **, $P < 0.01$.

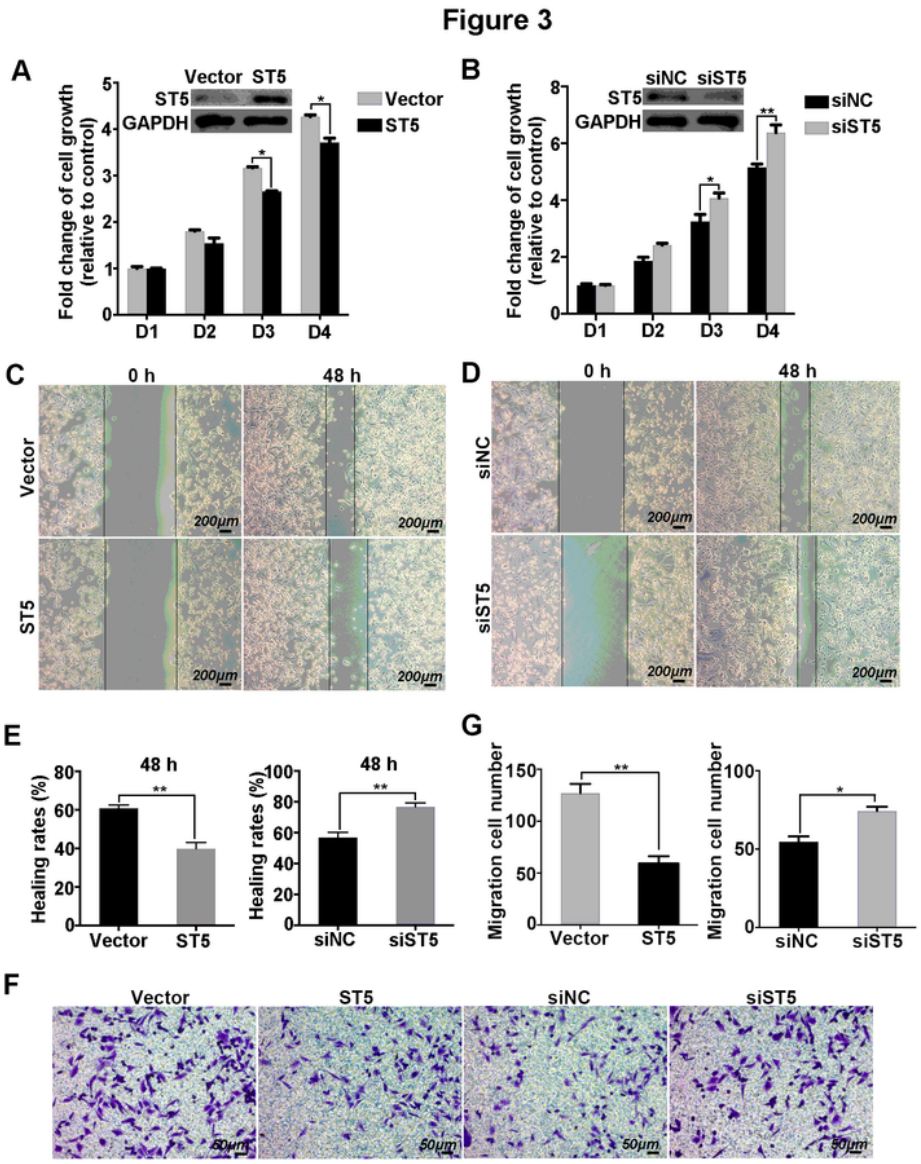

Figure 3

Effect of ST5 on cell proliferation and migration of MDA-MB-231 cells. MTT assay was performed to measure the cell growth ability of MDA-MB-231 cells transfected with control plasmid (Vector) and ST5-overexpressing plasmid (ST5) (A) and cells transfected with negative control siRNA (siNC) and siRNA targeting ST5 (siST5) (B). The verification of transfection efficiency as detected by Western Blotting and showed above the corresponding histograms. Images of wound healing in Vector and ST5 group (C) and

siNC and siST5 group (D). Scale bar, 200μm. (E) Healing rates in the above four groups as analysed by using Image Pro Plus software (version 6.0). (F) Cell migration was determined using Transwell assay and the migrated cells were stained by crystal violet. Scale bar, 50μm. (G) The number of migration cells as calculated by using Image Pro Plus software. *, P < 0.05; **, P < 0.01.

Figure 4

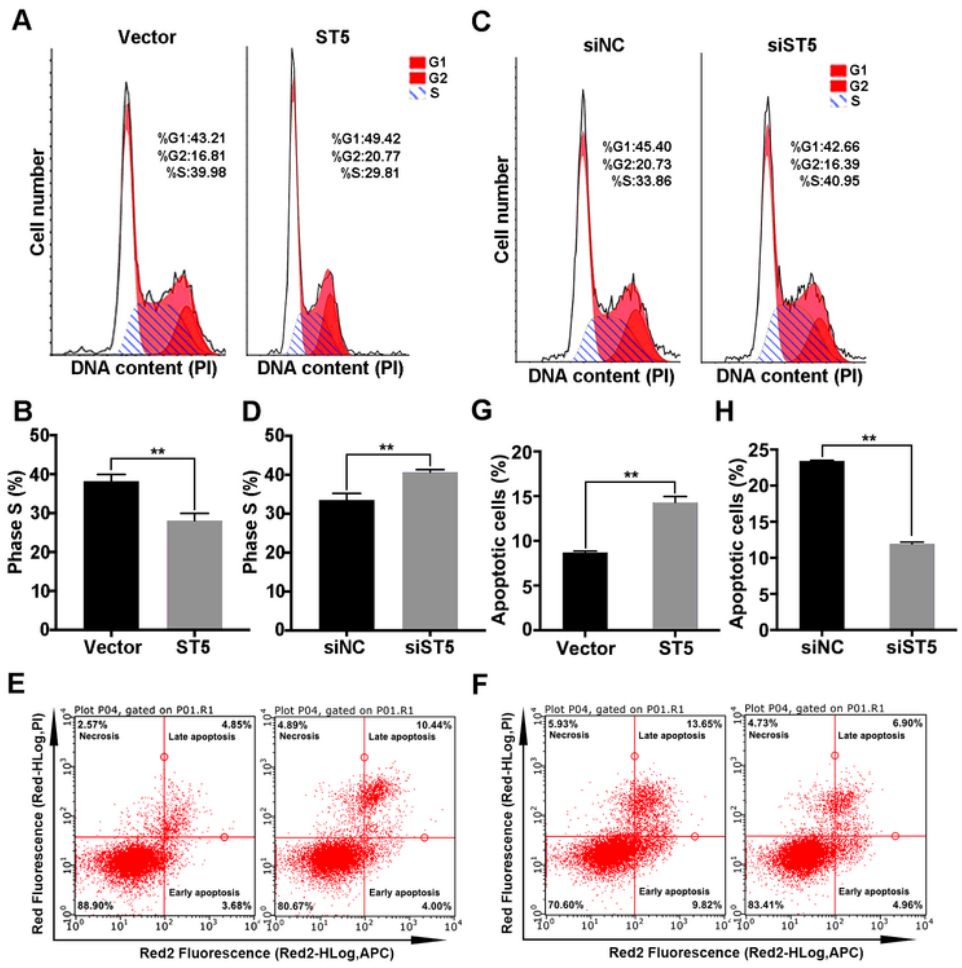

Figure 4

Effect of ST5 on cell cycle and apoptosis of MDA-MB-231 cells. Cell cycle progression was analysed in MDA-MB-231 cells transfected with vector and ST5 plasmids (A). (B) Quantitative analysis of the proportion of phase S cells in panel A. (C) Cell cycle progression was analysed in MDA-MB-231 cells transfected with negative control and ST5-siRNA. (D) Quantitative analysis of the proportion of phase S cells in panel C. The effect of ST5-upregulation on cell apoptosis (E) or ST5-downregulation on cell apoptosis (F) were analysed by flow cytometry. The proportion of apoptotic cells was measured in the vector and ST5 groups (G) and in the siNC and siWDR41 groups (H). * P < 0.05, ** P < 0.01.

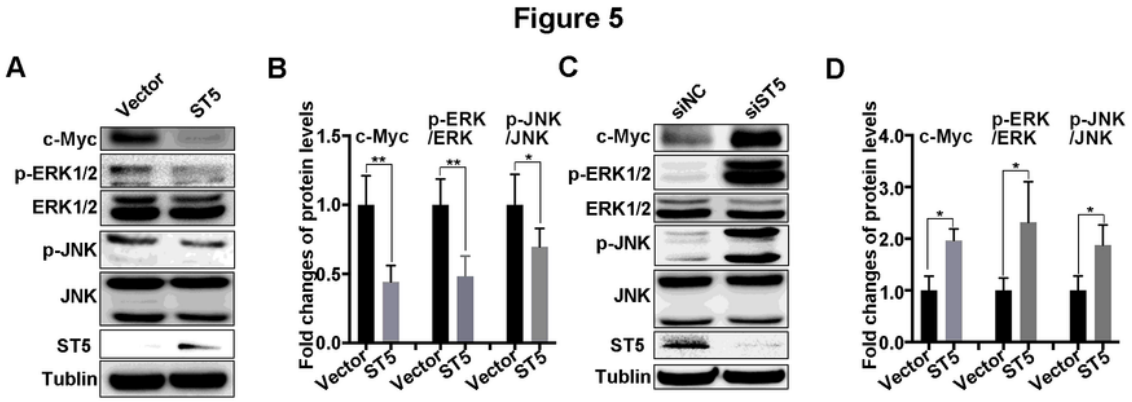

Figure 5

Regulatory mechanism of ST5-mediated tumour inhibition. (A) The activation of ERK1/2 and JNK, and the expression of c-Myc were detected by western blotting after knockdown of ST5 in MDA-MB-231 cells. (B) Relative quantification of phosphorylated ERK1/2 and JNK and c-Myc in the vector and ST5 groups. (C) The activation of ERK1/2 and JNK, and the expression of c-Myc were detected by western blotting after upregulation of ST5 in MDA-MB-231 cells. (D) Relative quantification of phosphorylated ERK1/2 and JNK and c-Myc in the siNC and siWDR41 groups. * P < 0.05, ** P < 0.01.

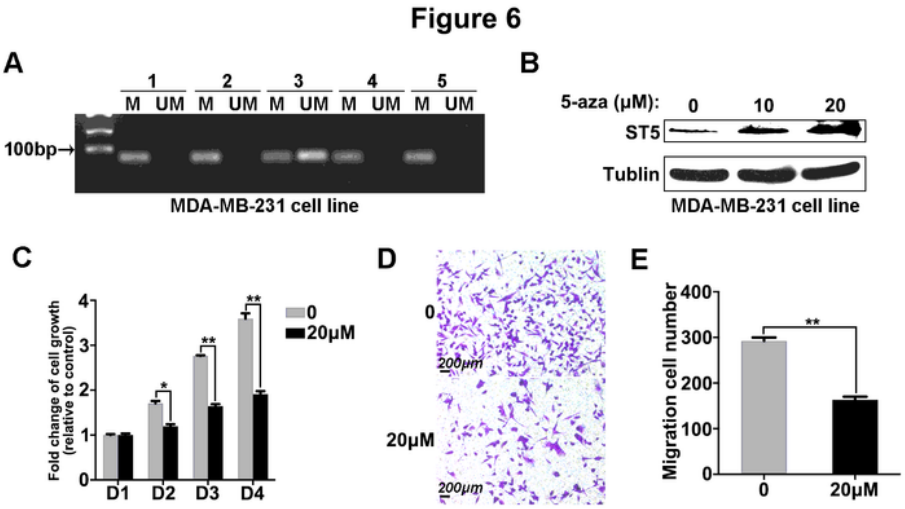

Figure 6

Regulatory mechanism of ST5-mediated tumour inhibition. (A) The activation of ERK1/2 and JNK, and the expression of c-Myc were detected by western blotting after knockdown of ST5 in MDA-MB-231 cells. (B) Relative quantification of phosphorylated ERK1/2 and JNK and c-Myc in the vector and ST5 groups. (C) The activation of ERK1/2 and JNK, and the expression of c-Myc were detected by western blotting after upregulation of ST5 in MDA-MB-231 cells. (D) Relative quantification of phosphorylated ERK1/2 and JNK and c-Myc in the siNC and siWDR41 groups. * $P < 0.05$, ** $P < 0.01$.

Supplementary Files

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