OncoTargets and Therapy

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ORIGINAL RESEARCH

SRC Promotes Tamoxifen Resistance in Breast Cancer via Up-Regulating SIRT1

This article was published in the following Dove Press journal:
OncoTargets and Therapy

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Background: Endocrine therapy plays a key role in estrogen receptor-positive breast cancer patients; but, tamoxifen resistance could be a real difficulty for these patients. Several attempts have been made to explore the mechanism and new therapies for these patients. We intend to clarify the expression change of SRC and SIRT1 in tamoxifen-resistant breast cancer cells and explore their functions on tamoxifen resistance.

Methods: SRC and SIRT1 expressions were analyzed by RNA sequencing, qPCR and Western blotting. Loss and gain of function of SRC and SIRT1 were utilized to indicate their oncogenic roles in tamoxifen resistance in vitro and in vivo. Kaplan–Meier analysis and receiver operating characteristic curve were used to evaluate the survival and the predicted effects of SRC and SIRT1 on patients' prognosis.

Results: High expressions of SRC and/or SIRT1 were found in tamoxifen-resistant cells and related to poor overall survival ($p<0.05$ for SRC, $p<0.001$ for SIRT1, $p<0.001$ for SRC and SIRT1) and cancer-specific survival ($p<0.05$ for SRC, $p<0.01$ for SIRT1, $p<0.01$ for SRC and SIRT1) of tamoxifen-treated breast cancer patients. Down-regulation of SRC ($p<0.01$) or SIRT1 ($p<0.05$) separately reversed the resistance to tamoxifen and the minimal concentration of SRC inhibitor KX-01 ($p<0.05$) or SIRT1 inhibitor EX527 ($p<0.001$) could also suppress cell proliferation. The expression level of SIRT1 was positively correlated with that of SRC. Overexpression of SRC significantly promotes the cell resistance to tamoxifen inhibited by SIRT1 ($p<0.01$). In vivo experiments confirmed the effects of SRC on tumor growth by over- or down-regulating SRC expression ($p<0.001$ and $p<0.001$, respectively).

Conclusion: SRC and SIRT1 are both up-regulated in tamoxifen-resistant breast cancer cells and related to a poor prognosis in tamoxifen-treated breast cancer. Moreover, SRC could promote tamoxifen resistance by up-regulating SIRT1. SRC and SIRT1 might be novel therapeutic targets in tamoxifen-resistant breast cancer and the interaction between SRC and SIRT1 needs to be further explored.

Keywords: breast cancer, tamoxifen resistance, SRC, SIRT1, inhibitor

Introduction

Breast cancer accounts for 24.2% of new-onset female cancers in 2018,[1] which is the most common malignancy in females. More than 60% of breast cancer could be detected to have positive estrogen receptor (ER)[2] and recommended to receive endocrine therapy. However, one-third of hormone receptor-positive breast cancer patients could suffer recurrence in fifteen years after the five-year treatment of tamoxifen.[3] Based on these situations, researches in endocrine therapy resistance has been investigated profoundly. There is an increasing number of studies focused on the aspect of ER and its downstream pathways, cell cycle, intratumoral inflammation, stem cells and

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the cross-talk between different pathways[4–7]. However, the underlying molecular mechanisms of tamoxifen resistance remain unknown and more research on this topic needs to be undertaken deeply.

SRC is one of the non-receptor tyrosine kinases, participating in cell proliferation, differentiation, survival, and invasion through PI3K, MAPK, STAT3, FAK signaling pathways.[8] ER-SRC axis was reported to closely relate to tamoxifen resistance,[9] but few findings explained the mechanism of SRC mediating in tamoxifen resistance. Sirtuin type 1 (SIRT1), a member of Silent Information Regulator 2 Superfamily, is a histone deacetylase and associated with many kinds of tumors.[10] There have been a lot of studies on SIRT1 over the decades, but the role of SIRT1 in breast cancer remains a subject of debate and its tumor-suppressive or promoting function has not been confirmed yet.[11]

In this study, we aimed to identify potential predictive biomarkers of tamoxifen resistance and explore the function of SRC and SIRT1 in the process of tamoxifen resistance. Using multiple gene expression analyses, we illustrated that expression levels of SRC and SIRT1 were both associated with the prognosis in tamoxifen-treated breast cancer patients and upregulated in tamoxifen-resistant breast cancer cells. Since no research has yet elucidated the association between SRC and SIRT1, we explored SRC and SIRT1 expressions and employed knockdown and overexpression strategies to investigate the phenomenon of SRC mediating tamoxifen resistance via up-regulating SIRT1 for the first time.

Materials and Methods

Cell Culture and Transfection

Human breast cancer cell line T47D (tamoxifen-sensitive) was purchased from the American Type Culture Collection (ATCC, USA). T47D cells were cultured in RPMI/1640 medium (Gibco, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (Beyotime, China). T47DR (tamoxifen-resistant) cell line was established using 1 μmol/L 4-Hydroxytamoxifen (H7904, Sigma, USA) for more than 6 months. All cell lines were maintained at 37°C in a 5% CO_2 humidified incubator. T47DR cells were cultured in the medium without tamoxifen for more than 7 days before all the following assays.

Lipofectamine 3000 (Thermo Fisher, USA) was used for transient transfection and polybrene (Biosharp, China) was used for lentivirus infection according to the manufacturer's protocol. siRNAs for SRC (si-SRC, 5′-CCAAGGGCCUCAACGUAA-3′), SIRT1 (si-SIRT1, 5′-CCAUCUCUCUGUCACAAAUTT-3′) and their corresponding negative controls (si-NC) were purchased from Ribobio (Ribobio, China). Overexpression plasmids for SRC (OE-SRC) and the corresponding negative controls (OE-NC) were purchased from Genechem (Genechem, China). T47DR cells were respectively transfected with recombinant OE-SRC lentivirus, sh-SRC lentivirus or sh-NC and OE-NC lentivirus (Genechem, China). Stable clones (T47DR/LV-OE-SRC, T47DR/LV-sh-SRC, and T47DR/LV-NC) were selected with 8 μg/mL puromycin (Biosharp, China) and then used for the following studies.

The combination of endocrine therapy and SRC inhibitors has been suggested as a novel treatment option to overcome endocrine resistance.[12] Unlike the inhibitors used in other studies,[13,14] such as Dasatinib, we first used KX-01 for SRC and EX527 for SIRT1 to explore their effects on tamoxifen resistance. To explore whether combined treatment of tamoxifen with inhibitors could suppress cell growth in the tamoxifen-resistant T47DR cells, we administrated of the cells with tamoxifen (0 μM, 0.1 μM, 1 μM, 5 μM, 10 μM), KX-01 (0 nM, 10 nM, 20 nM, 50 nM) and EX527 (0 μM, 1 μM, 5 μM, 10 μM) alone or in combination for 48 hours. Inhibitors (KX-01 for SRC and EX527 for SIRT1) were purchased from Selleck (Selleck, China).

RNA Sequencing Assay

High-throughput sequencing was performed using T47D and T47DR cell lines. Total RNA of 5×10^6 cells was isolated using RNAiso Plus reagent (TaKaRa, Japan). Library preparation and transcriptome sequencing on the Illumina HiSeq™ platform were performed by Sangon Biotech Co. to generate 150-bp paired-end reads. Transcripts per million (TPM) of each gene were calculated for relative quantitative analysis. Sequencing data have been uploaded in Gene Expression Omnibus (GEO) database (accession code GSE129544).

Bioinformatic Analysis

We obtained the information of gene expression levels between breast cancer cell lines MCF7 (tamoxifen-sensitive) and MCF7R (tamoxifen-resistant) from the microarray dataset GSE31831 in GEO database. We also analyzed the standardized gene expression levels in 155 patients' primary tumors from another microarray dataset GSE9893,[15] in which all of the patients with breast

cancer were treated with tamoxifen. The median follow-up time was 65.9 months. During the follow-up, 52 patients had recurrences (48 distant metastases and 4 local recurrences) with a median relapse time of 37.1 months. Time-dependent receiver operating characteristic (ROC) curve analyses were performed using "survivalROC" package in R and the area under the ROC curve (AUC) was calculated to determine the optimal cutoff values of SRC and SIRT1 expression levels for survival analysis. Patients were divided into two groups showing high or low levels of SRC and SIRT1 according to the cut-off values. Cox proportion hazards model and Kaplan–Meier analyses were used to assess the association between SRC and SIRT1 subgroups and survival.

Western Blot Analysis

The total protein of cells was isolated with protein extraction reagent RIPA buffer (Beyotime, China) and quantified by the BCA Protein Assay Kit (Beyotime, China). Equal amounts of protein were subjected to 10% SDS-PAGE and then transferred to a PVDF membrane (Millipore, USA). The immunoblots were incubated with primary antibodies against SRC (1: 2000 dilution, Abcam, USA), SIRT1 (1: 3000 dilution, Abcam, USA), and GAPDH (1: 3000 dilution, Cell Signaling Technology, USA) as the internal control. The protein signals were determined with the ChemiDoc XRS+ System (Bio-Rad, USA) using the ECL detection kit (Beyotime, China).

Quantitative Reverse Transcription-PCR

The total RNA was extracted from cultured cells using RNAiso Plus reagent (TaKaRa, Japan) according to the manufacturer's protocol. mRNAs were reverse-transcribed to cDNAs with a PrimeScript RT Master Mix Kit (TaKaRa, Japan). Quantitative real-time PCR (qRT-PCR) was performed in triplicate using synthesized primers (Tsingke, China) with an SYBR Premix Ex Taq II Kit (TaKaRa, Japan) to detect the mRNA levels. The primer sequences are as follows:

SRC forward: 5′-GAGCGGCTCCAGATTGTCAA-3′;

SRC reverse: 5′-CTGGGGATGTAGCCTGTCTGT-3′;

SIRT1 forward: 5′-TAGCCTTGTCAGATAAGGAAGGA-3′;

SIRT1 reverse: 5′-ACAGCTTCACAGTCAACTTTGT-3′;

GAPDH forward: 5′-GGAGCGAGATCCCTCCAAAAT-3′;

GAPDH reverse: 5′-GGCTGTTGTCATACTTCTCATGG-3′.

Cell Counting Kit-8 (CCK8) Assay

A cell viability assay was analyzed by the CCK8 (Dojindo, Japan) method according to the manufacturer's protocol. A total of 5000 cells were seeded into 96-well plates with different concentrations of tamoxifen or other drugs. After 48 hours, the medium was removed and 100 μL fresh medium with 10% CCK8 solution inside was added to each well of the plate and incubated at 37°C for 2 hours. The spectrometric absorbance of the samples at 450 nm was measured on a microplate reader (Thermo Fisher, USA). Each assay was replicated at least three times.

Tumor Xenograft Model

Athymic female BALB/c nude mice (4–6 weeks old) were obtained from Beijing Vital River Laboratory Animal Technology Co. and maintained under specific pathogen-free conditions. All of the procedures of animal experiments were approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology (approval number: S2124), and carried out following NIH Guidelines for the Care and Use of Laboratory Animals. 5×10^6 cells were suspended in 0.1 mL of a 1:1 mixture of Matrigel (356234, BD, USA) and PBS, and then injected into the flanks of mice. Health conditions of mice and tumor sizes were checked every 3 days, and tumor volume was estimated by the formula: length × width × width × 0.5. 15 days after tumor cell implantation, all the mice were treated with tamoxifen in a dose of 10 mg/kg per day through oral gavage for 24 days. Tumor tissues excised from euthanized mice were paraffin-embedded for Immunohistochemistry (IHC) staining. IHC assays were respectively performed using the antibodies against SRC and SIRT1 as described above.

Statistical Analysis

In this study, GraphPad Prism 7.0 (GraphPad Software, USA), R 3.5.3 (Lucent Technologies, USA) and SPSS 23.0 (IBM, USA) were used. In our RNA sequencing assay, $|\text{Fold Change}| > 2$ and q value < 0.05 between two cell lines were selected to represent statistical significance. Spearman and Kendall correlation analyses were used to investigate the correlation between SRC and SIRT1 expression levels. Unless stated otherwise, the Student's *t*-test, two-way analysis of variance, or Chi-square test was performed to compare the differences between different groups, respectively. The Log rank test and Cox proportion hazards model were used to assess the survival difference

and hazard ratio. Results of $p < 0.05$ were considered significant: NS means not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Results

Identification of SRC and SIRT1 as Prognostic Markers for Tamoxifen-Treated Patients

We cultured T47D cells exposed to 1 μmol/L 4-Hydroxytamoxifen for more than 6 months to establish the T47DR cell line with resistance to tamoxifen as shown in Figure 1. The identification of tamoxifen resistance in T47DR cells was performed using a CCK8 assay and 4-hydroxytamoxifen caused a concentration-dependent decrease in the cell viability of both T47D and T47DR cells. The results showed that the T47DR cells exhibited significantly less sensitivity to tamoxifen treatment compared to the control cells (Figure 1A). Microscopic analysis was used to assess the morphological changes between these two cells, and we found T47DR cells showed more branches and became spindle-shaped (Figure 1B). RNA sequencing assay was then performed, and 5123 up-regulated and 5229 down-regulated mRNAs (|Fold Change| > 2 and q value < 0.05) were found in T47DR cells compared to T47D cells (Figure 1C). Our RNA sequencing data (GSE129544) were analyzed in combination with other GEO datasets (GSE9893 and GSE31831[16]) to identify SRC and SIRT1 (Figure 1D), which are up-regulated in tamoxifen-resistant breast cancer cells and related to the outcomes of tamoxifen-treated breast cancer.

Further analysis of GSE9893 indicated that expression levels of SRC and SIRT1 were related to clinicopathological

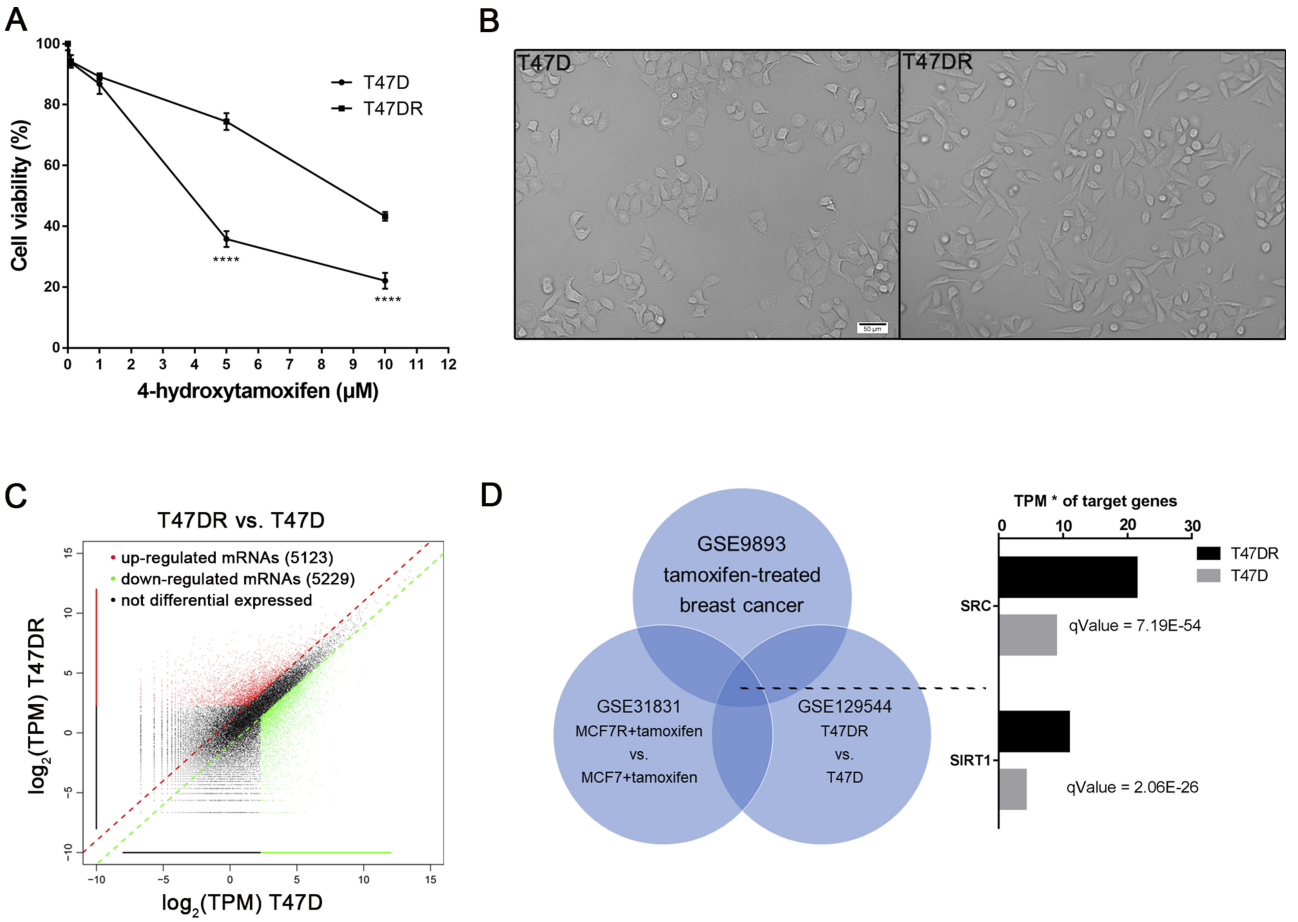

Figure 1 SRC and SIRT1 were upregulated in tamoxifen-resistant breast cancer cells.

Notes: (**A**) CCK-8 assay showed that the cell proliferation ability of T47DR was higher than that in T47D when they were treated with tamoxifen. The IC50 values of tamoxifen were 3.53μmol/L in T47D cells and 8.93 μmol/L in T47DR cells. (**B**) Morphological differences between T47D and T47DR cells. Scale bar, 50 μm. (**C**) Differentially expressed genes in T47D and T47DR cells. (**D**) The intersection of the three datasets (GSE9893, GSE31831, and GSE129544). SRC and SIRT1 were upregulated in tamoxifen-resistant breast cancer cells MCF7R and T47DR. Error bars represent means ± SD of triplicate. ****$p < 0.0001$. Every experiment was repeated three times.

Abbreviation: TPM*, transcripts per million

features in 155 breast cancer patients treated with tamoxifen. For patients with local recurrence or distant metastases after tamoxifen treatment, SRC (p = 0.0058, Figure 2A) and SIRT1 (p < 0.0001, Figure 2B) are both highly expressed. From the time-dependent ROC curve analyses (Figure 2C and D), we got the optimal cutoff values for SRC and SIRT1 in overall survival analyses and cancer-specific survival analyses, respectively. We chose 1.556713 for SRC (range from −0.57 to 4.69, unit: Log_2RPKM, 1.556713 at the 60% of all expressions) and −0.6445193 for SIRT1 (range from −2.12 to 3.98, unit: Log_2RPKM, −0.6445193 at the 60% of all expressions) as the cutoff values. Accordingly, patients were divided into two groups showing high or low levels of SRC and SIRT1 and their clinical characteristics of the patients are summarized in Table 1. Patients with high SRC or SIRT1 expression showed high clinical score in N category, local recurrence or distant metastases, overall death and cancer-specific death (p < 0.05). Kaplan–Meier curves indicated that high expression of SRC (Figure 2E and H) or SIRT1 (Figure 2F and I) predicted a poor prognosis in tamoxifen-treated patients in both overall survival and cancer-specific survival. As shown in Figure 2G and J, the combination of high SRC and high SIRT1 also predicted an inferior outcome and poor response to tamoxifen treatment. The hazard ratios (HRs) for overall survival and cancer-specific survival are listed in Table 2 to investigate the clinical significance. Cox univariate and multivariate regression analyses both showed that “high SRC”, “high SIRT1”, and “both high” were significant risk factors for the unfavorable outcome of overall survival and cancer-specific survival (HR > 1.0, p < 0.05). The Spearman correlation coefficient was 0.467 (p < 0.01, calculated according to the standardized expression level values) and the Kendall correlation coefficient was 0.341 (p < 0.01, calculated according to the high or low expression groups) between SRC and SIRT1 expression. As shown in Table 3, SIRT1 expression is positively correlated to SRC expression in these breast cancer samples.

SRC and SIRT1 Promote Tamoxifen Resistance

Based on the results above, we predicted that both SRC and SIRT1 might play important roles in tamoxifen resistance. Other than the RNA sequencing results, we confirmed the higher expression levels of SRC and SIRT1 in T47DR cells compared to that in T47D cells. Western blot (Figure 3A) showed the overexpression of SRC and SIRT1 proteins in T47DR cells. Meanwhile, qRT-PCR indicated the elevated mRNA levels of SRC (Figure 3B, left, p<0.001) and SIRT1 (Figure 3B, right, p<0.05) in T47DR cells. As shown in Figure 3C and D, the cell viability appeared to be dependent on the concentration of SRC inhibitor KX-01 and SIRT1 inhibitor EX527, respectively. Next, CCK8 assay was used to determine cell viability in T47DR cell lines treated with tamoxifen after the transfection of siRNAs and plasmids. The concentration of 10 nM KX-01 started to inhibit T47DR cell growth (Figure 3C, p<0.05), while cell proliferation was significantly suppressed with the increase of KX-01 concentration (Figure 3C, p<0.01 for 20 nM KX-01 and p<0.001 for 50 nM KX-01). The same trend of SIRT1 inhibitor EX527 was demonstrated in Figure 3D. The concentration of 1μM, 5μM and 10μM EX527 had a gradually ascending influence on T47DR cell growth (Figure 3D, p<0.001, p<0.0001 and p<0.0001, respectively).

Knockdown of SRC could down-regulate the expression of SIRT1 in T47DR cells (Figure 3E), while SRC overexpression could up-regulate the expression level of SIRT1 compared to corresponding control cells (Figure 3G). After the establishment of T47DR SRC or SIRT1 knockdown cells, we further investigated their function on tamoxifen-resistant cells. Suppression of SRC enhanced the inhibition effect from tamoxifen on T47DR cells (Figure 3F p<0.01). The inhibition of SIRT1 showed similar effects on cell growth (Figure 3E, p<0.05). With the elevated expression levels of SRC and SIRT1, the resistance to tamoxifen of T47DR cells was strengthened (Figure 3H, p<0.05).

SRC Promotes Tamoxifen Resistance via Up-Regulating SIRT1

To confirm the regulating effect of SRC on SIRT1, we performed further studies by co-transfecting overexpressed plasmids of SRC and siRNA of SIRT1 in cultured T47DR cells. As shown in Figure 4A, SRC overexpression elevated SIRT1 expression from the decrease induced by si-SIRT1 siRNAs. Furthermore, the CCK8 assay indicated the weakening effect on tamoxifen resistance induced by SIRT1 knockdown (Figure 4B, p<0.01). This effect was effectively reversed by SRC overexpression (Figure 4B, p<0.01). Based on the results above, we considered that SRC promoted tamoxifen resistance via up-regulating SIRT1 in T47DR cells, and their roles in tamoxifen resistance were shown in the schematic diagram (Figure 4C). But how does SRC act on SIRT1? We used the website GeneMANIA (http://genemania.org)[17] to predict gene/protein interactions and

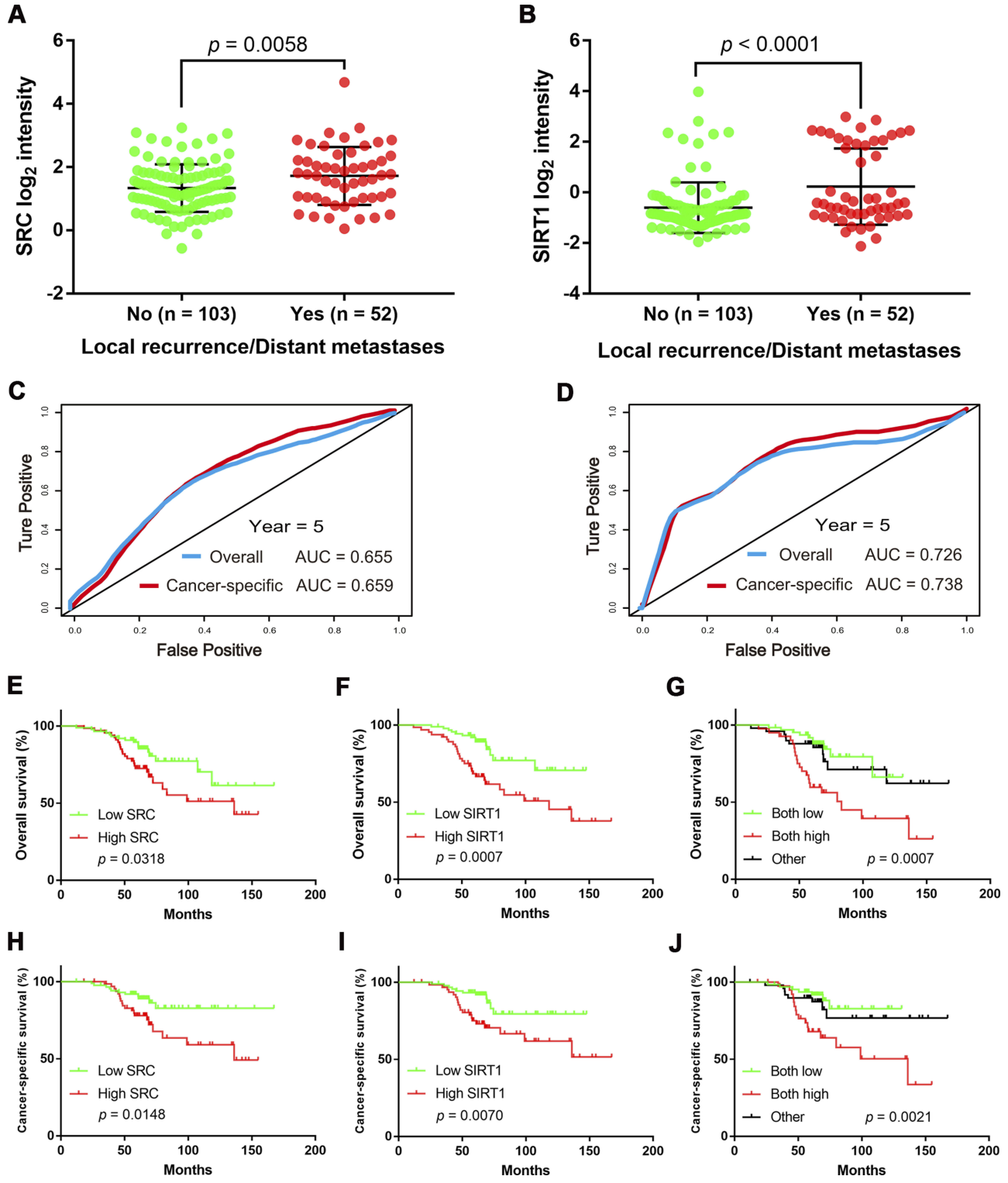

Figure 2 High expression of SRC and/or SIRT1 was correlated to poor outcomes in GSE9893 cohort.
Notes: SRC (**A**) and SIRT1 (**B**) were significantly higher in patients with local recurrence or distant metastases compared with others. ROC curves for five-year overall survival and cancer-specific survival according to SRC (**C**) and SIRT1 (**D**) expression, respectively. Cut-off values and AUC values are described in the figures. (**E–J**) Overexpression of SRC and/or SIRT1 was correlated to worse overall survival and cancer-specific survival.

functions of SRC and SIRT1. The GeneMANIA bioinformatic analysis for interaction among *SRC* and *SIRT1* with other genes is displayed in Figure 4D. The results showed that *SRC* and *SIRT1* interact with subunits of RNA polymerase II, *MCM10, JUND, RACK1*, etc. directly or indirectly.

Table 1 Correlation Between SRC and SIRT1 mRNA Expression and Clinicopathological Parameters of Breast Cancer Patients Treated with Tamoxifen

Characteristics	SRC			SIRT1		
	Low (n=88)	High (n=67)	*p*-value	Low (n=90)	High (n=65)	*p*-value
Age (years)	67.65±9.22	66.73±11.46	0.582	67.56±9.96	66.83±10.63	0.664
T category			0.704			0.524
T1	46	36		51	31	
T2	39	28		35	32	
T3/T4	1	0		1	0	
NA	2	3		3	2	
N category			**0.011**			**<0.001**
pN0	54	24		59	19	
pN1	23	24		18	29	
pN2/pN3	9	15		9	15	
NA	2	4		4	2	
SBR grade			0.865			0.682
1	12	9		14	7	
2	55	39		53	41	
3	18	15		20	13	
NA	3	4		3	4	
Adjuvant therapy			**0.033**			0.637
TAM	23	11		18	16	
X-ray + TAM	64	50		67	47	
X-ray + TAM + LHRH	1	6		5	2	
Local recurrence/Distant metastases			**0.010**			**<0.001**
No	66	37		70	33	
Yes	22	30		20	32	
Overall death	17	25	**0.013**	14	28	**<0.001**
Cancer-specific death	11	20	**0.007**	11	20	**0.004**

Note: Bold values show $p < 0.05$.
Abbreviations: SBR grade, Scarff-Bloom-Richardson grading system; TAM, tamoxifen; LHRH, luteinizing hormone-releasing hormone; X-ray, radiation therapy.

Table 2 Hazard Ratios for Overall Survival and Cancer-Specific Survival in Various Groups

Subgroups	Overall Survival				Cancer-Specific Survival			
	HR	*p*-value*	Adjusted HR[a]	*p*-value*	HR	*p*-value*	Adjusted HR[a]	*p*-value*
	(95% CI)		(95% CI)		(95% CI)		(96% CI)	
SRC (high vs. low)	1.945 1.048–3.610	0.035	2.031 1.066–3.872	0.031	2.432 1.162–5.087	0.018	2.626 1.214–5.681	0.014
SIRT1 (high vs. low)	2.892 1.518–5.509	0.001	3.363 1.721–6.570	<0.001	2.661 1.271–5.573	0.009	3.082 1.418–6.701	0.004
SRC+SIRT1 (both high vs. both low)	3.463 1.624–7.384	0.001	3.971 1.824–8.647	0.001	3.822 1.566–9.331	0.003	4.581 1.803–11.643	0.001

Notes: *p-values were calculated by univariate or multivariate Cox analysis. [a]Adjusted with age, T category, SBR grade, and adjuvant therapy.
Abbreviations: HR, hazard ratio; CI, confidential interval.

Table 3 Correlations Between SRC and SIRT1 Expression Levels

Correlation	Correlation Coefficient	*p*-value
Spearman*	0.467	<0.01
Kendall**	0.341	<0.01

Notes: *Calculated by the standardized expression level values. **Calculated by the expression levels (high or low).

Verification of SRC and SIRT1 in Tamoxifen Resistance in vivo

To explore the roles of SRC and SIRT1 in tamoxifen resistance in vivo, T47DR cells with stable overexpression of SRC (T47DR/LV-OE-SRC), stable knockdown of SRC (T47DR/LV-sh-SRC) and normal control (T47DR/LV-NC) were subcutaneously injected into the flanks of nude mice. The Western blot (Figure 5A) confirmed the expression levels of SRC and SIRT1 in the stable clones that SIRT1 expression could be regulated by SRC synchronously. The CCK8 results showed that the elevated expression of SRC and SIRT1 promotes cell resistance to tamoxifen (Figure 5B, $p<0.05$) and the decrease of SRC and SIRT1 significantly reduced cell viability (Figure 5B, $p<0.01$). The volume growth curves and weight of tumors were measured to evaluate the antitumor efficacy of tamoxifen. Tumors with overexpression of SRC were more resistant to tamoxifen, while tumors with knockdown of SRC were more sensitive to tamoxifen compared with the normal control tumors. As shown in Figure 5C, the volume of tumors increased upon time and overexpression of SRC enhanced tumor volume significantly after the administration of tamoxifen and vice versa. We excised tumors on the 39th day. Tumor sizes were obviously larger in SRC knock-in group (Figure 5D and E, $p<0.001$). Similarly, knock-down of SRC inhibited tumor growth after tamoxifen treatment (Figure 5D and E, $p<0.001$). IHC results also revealed the expression levels of SIRT1 varied with that of SRC (Figure 5F). According to the results above, we demonstrated that SRC could promote the resistance of breast cancer to tamoxifen via SIRT1 in vitro and in vivo.

Discussion

Over the past decades, major advances have been reached in the mechanism of endocrine therapy resistance in breast cancer patients with positive hormone receptors. Recurrence could occur in one-third of ER-positive breast cancer patients even if they finished endocrine therapy.[3] Based on this situation, researches in endocrine therapy resistance (ETR) has been investigated profoundly in the aspect of ER and its downstream pathways, cell cycle and the cross-talk between different pathways. ER mutations, changes in the expression of ER transcriptional modulators have been shown to have a direct adverse impact on patients' survival.[4] Alterations of cell cycle-related genes are also contributed to the loss of endocrine responsiveness according to researches on cyclin-dependent kinase (CDK) pathways.[5] Besides, multiple signaling pathways such as PI3K/AKT/mTOR pathway are also investigated deeply. Moreover, several reports have shown that intratumoral inflammation[6] and breast cancer stem cells[7] play critical roles in the acquiring of ETR. However, there is still abundant room for further progress in determining the mechanisms of tamoxifen resistance.

SRC is a non-receptor tyrosine kinase which has been found to mediate in cell growth, migration and angiogenesis through PI3K, MAPK, STAT3, and FAK signaling pathways.[8,18,19] SRC was reported to interact with ERα and regulate cell proliferation and cell cycle by activating ERK1/2 and AKT phosphorylation[20,21] and promote tumor growth and metastasis by activating YAP/TAZ axis.[22] Besides, there are a few researches focusing on the effect of SRC in ETR. SRC kinase activity has been reported to enhance the metastasis of tamoxifen-resistant breast cancer and blocking its activity could prevent metastasis of tamoxifen-resistant breast cancer.[23] Recent literature has implicated the participation of SRC in breast cancer stem cells to develop resistance to endocrine therapy.[7] A previous study reported that SRC inhibitor KX-01 could be used alone or in combination with tamoxifen to treat triple-negative breast cancer but its role in ER-positive breast cancer was not explored.[24] In our study, we found that KX-01 and knock-down of SRC could reverse tamoxifen resistance and SRC might be a potential therapeutic target in the treatment of tamoxifen-resistant breast cancer.

SIRT1 is a highly conserved NAD^+-dependent protein deacetylase and involved in many biological processes including cell proliferation, tumorigenesis, apoptosis, and angiogenesis[25–27]. It is revealed to regulate the acetylation status of ER and inhibit the transcriptional activity of ERα, consequently suppress cell proliferation in vitro.[28] But on the other hand, SIRT1 could co-activate ERα[29] and it is required in the process of estrogen-induced breast cancer proliferation.[30] We demonstrated the increased expression of SIRT1 in T47DR cells for the first time. These results are coordinated with previous researches. Multidrug resistance-associated protein 2 (MRP2) expression was found

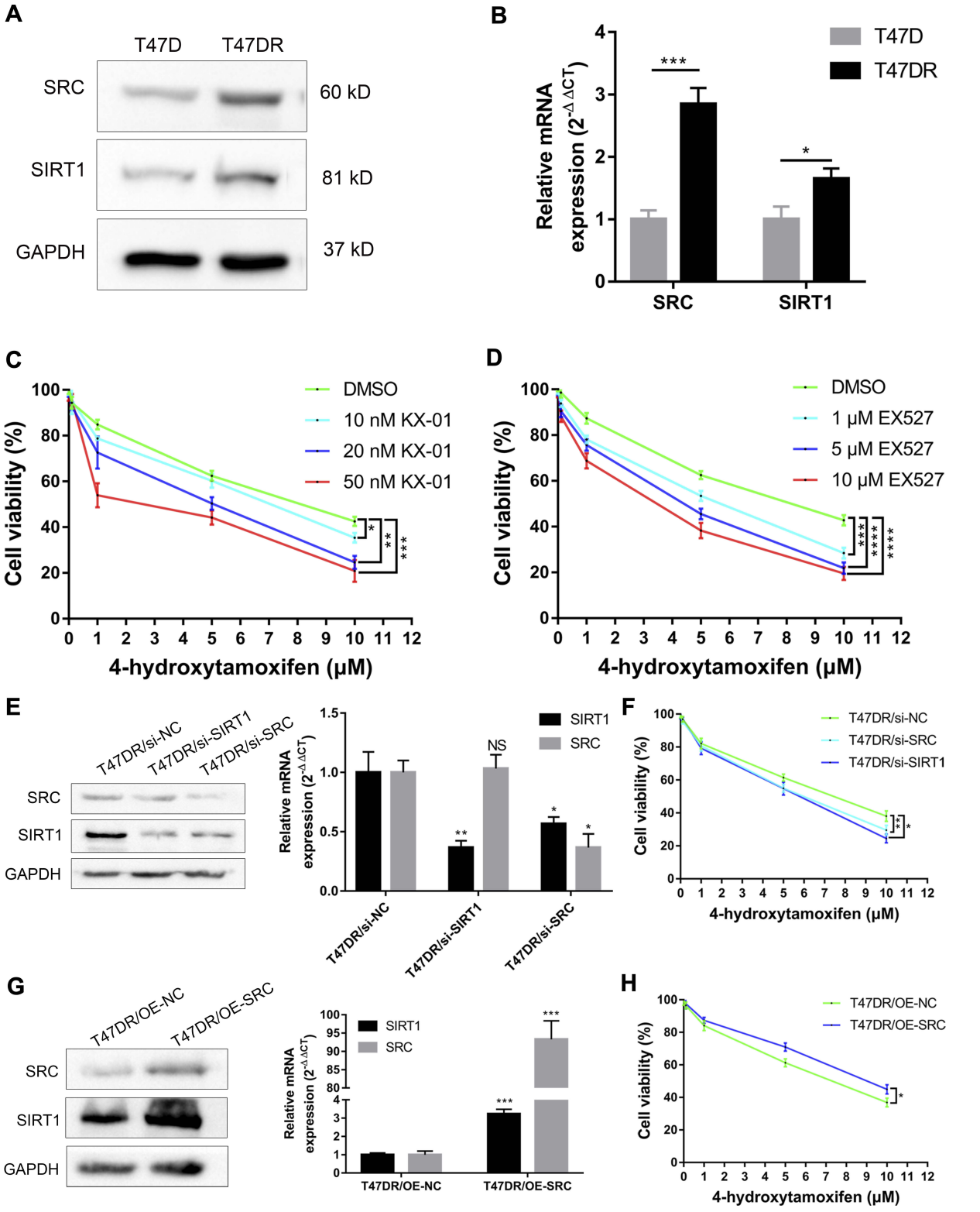

Figure 3 SRC and SIRT1 could promote tamoxifen resistance.

Notes: Western blot (**A**) and qRT-PCR (**B**) confirmed the overexpression of SRC and SIRT1 in tamoxifen-resistant breast cancer cells T47DR. CCK8 assay showed that SRC inhibitor KX-01 (**C**) and SIRT1 inhibitor EX527 (**D**) could reverse tamoxifen resistance. Knockdown of SRC or SIRT1 by siRNAs (**E**) could enhance the sensitivity of T47DR cells to tamoxifen (**F**). Overexpression of SRC by plasmids (**G**) could enhance the resistance of T47DR cells to tamoxifen (**H**). Knockdown of SRC could down-regulate SIRT1 (**E**) and SRC overexpression could up-regulate SIRT1 (**G**) compared to corresponding control cells. NS means not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Every experiment was repeated three times.

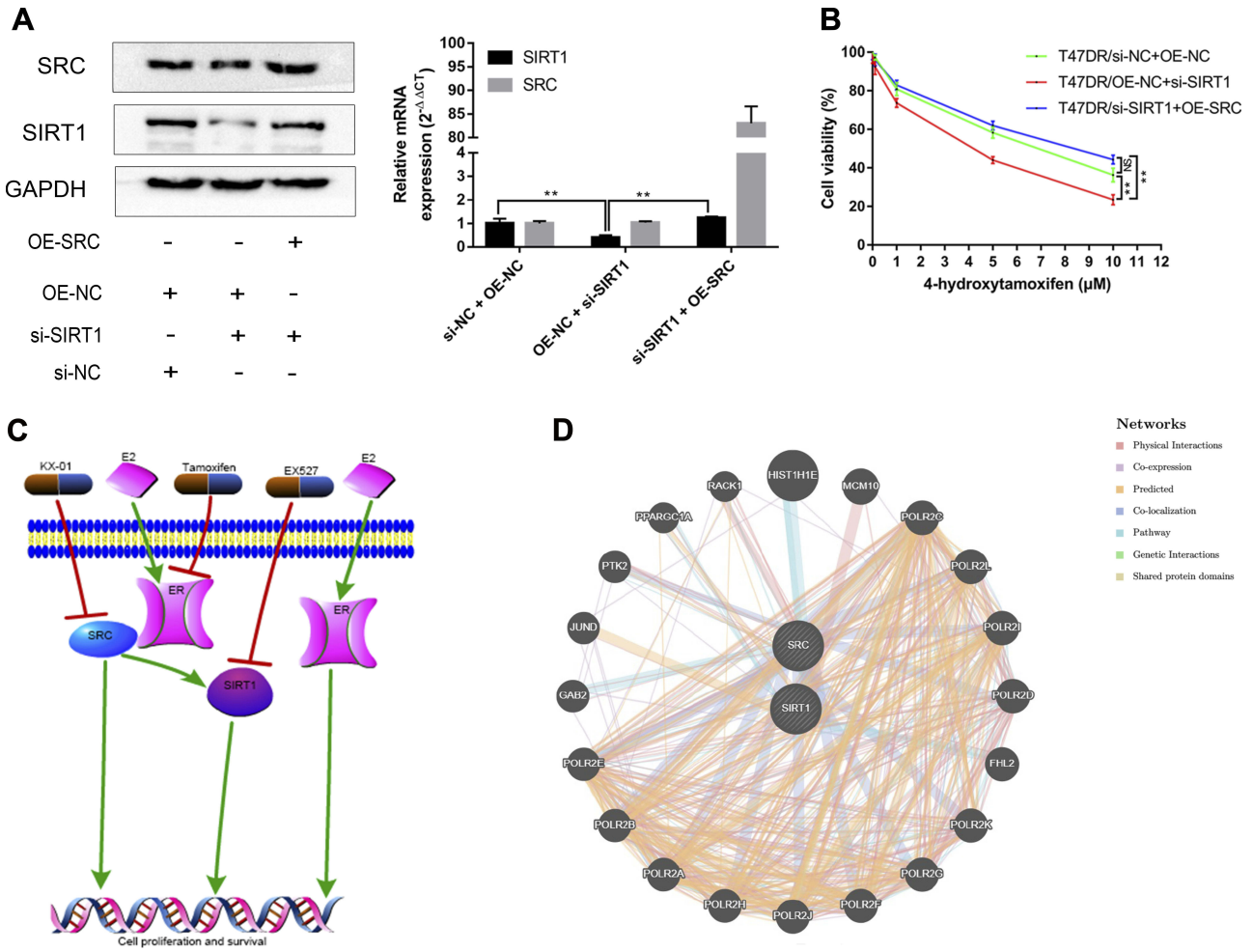

Figure 4 SRC promotes tamoxifen resistance via up-regulating SIRT1.

Notes: (**A**) SRC overexpression rescued SIRT1 expression from the decrease induced by si-SIRT1 siRNAs. (**B**) CCK8 assay verified that the weakening effect on tamoxifen resistance induced by SIRT1 knockdown was effectively reversed by SRC overexpression. (**C**) Schematic diagram of the roles of SRC and SIRT1 in tamoxifen resistance. (**D**) The potential interaction among *SRC* and *SIRT1* with other genes according to GeneMANIA. NS means not significant, $^{**}p < 0.01$. Every experiment was repeated three times.

to overexpress in tamoxifen-resistant MCF-7 cells and SIRT1 reduced MRP2 expression via SIRT1-mediated forkhead box-containing protein, O subfamily1 FoxO1 deacetylation.[31] Besides, SIRT1 was considered as a target of Brachyury to promote tamoxifen resistance in breast cancer.[32] In our study, the role of SIRT1 as a prognosis predictor of tamoxifen-treated patients was suggested and our discovery of the function of EX527 on reversing tamoxifen resistance is the basis for further study.

Our previous study has shown that SRC and SIRT1 both play pivotal roles in the progression of luminal breast cancer.[33] We identified the regulatory effect of SRC on SIRT1 in this study, but how SIRT1 mediates the effect of SRC in tamoxifen-resistant cells remains unknown. Thus, we used the GeneMANIA website to predict SRC and SIRT1 interaction. Though twenty related genes were summarized to be interacted, co-localized, co-expressed or modulate the same pathway, we have limited progression studying these genes. More detailed studies are required to confirm the direct or indirect regulatory mechanism of SRC on SIRT1 in tamoxifen resistance. Their potential roles as therapy targets for tamoxifen-resistant breast cancer deserves further validation in a larger cohort of in vitro and in vivo studies.

There are also some limitations in this study. As with many other previous studies[34–36], we established only one tamoxifen-resistant cell line, which might make our conclusion less general. Another limitation is that the potential signaling pathways in which SRC and SIRT1 might be involved were not explored, which deserves further validation in a larger cohort of in vitro and in vivo studies. As

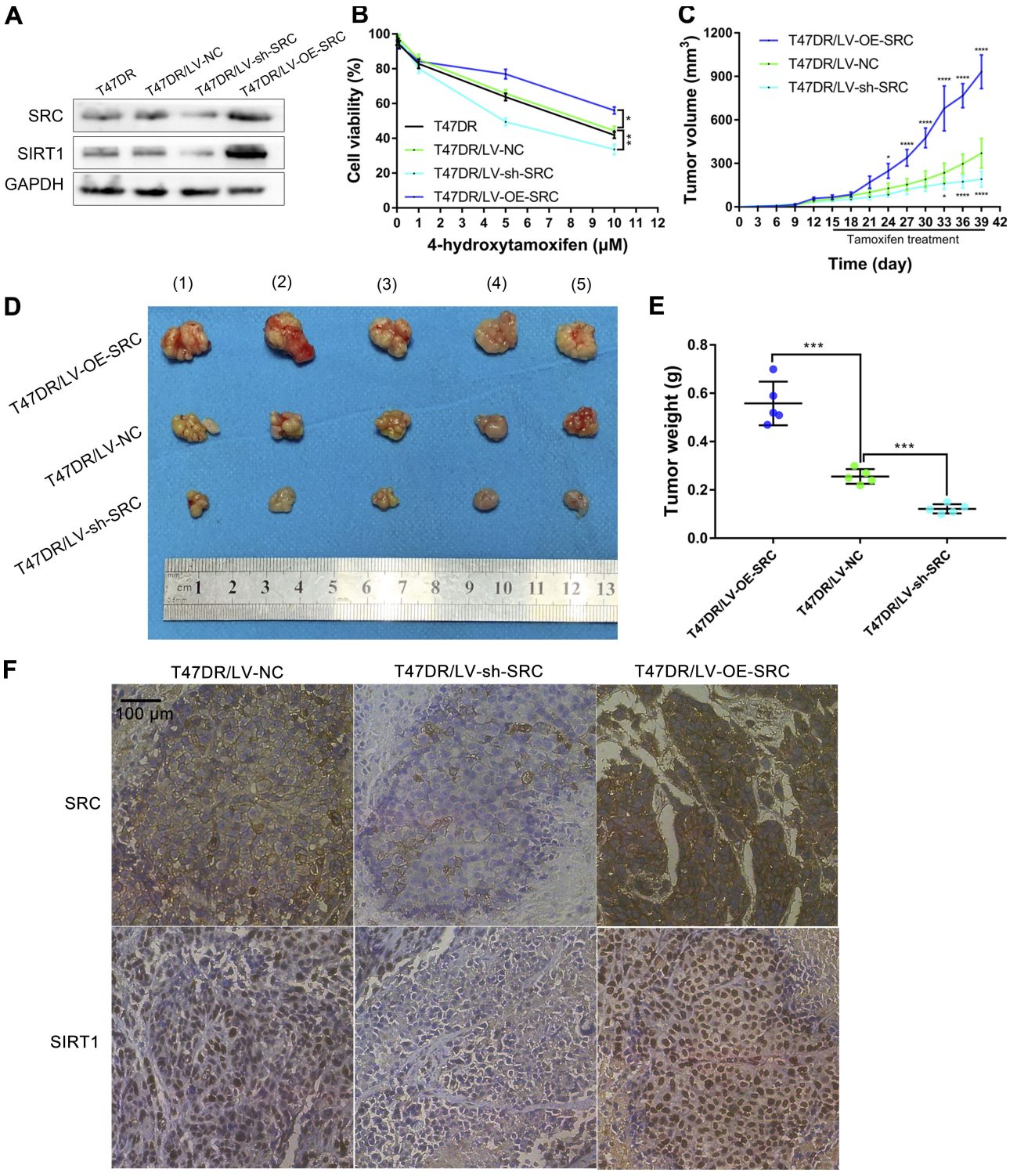

Figure 5 Tamoxifen resistance is positively correlated to expression levels of SRC and SIRT1 in vivo.

Notes: (**A**) Western blot indicating the expression levels of SRC and SIRT1 in T47DR cells transfected with OE-SRC lentivirus, sh-SRC lentivirus or their corresponding controls. (**B**) CCK8 assays verified the sensitivity of these three cell lines to tamoxifen in vitro. The IC50 values of tamoxifen were 7.95 μmol/L in T47DR cells, 8.68 μmol/L in T47DR/LV-NC cells, 4.78 μmol/L in T47DR/LV-sh-SRC cells, and 13.96 μmol/L in T47DR/LV-OE-SRC cells, respectively. (**C**–**E**) Tumor xenograft model verified the sensitivity of these three cell lines to tamoxifen in vivo. N=5 for every group. (**F**) IHC of SRC and SIRT1 in tumor tissues and the role of SRC in promoting tamoxifen resistance via SIRT1 was verified again. Scale bar, 100 μm. NS means not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Every experiment was repeated three times. The xenograft model had 5 replicates in every group.

shown in Supplementary Table, several genes might also be involved in tamoxifen resistance, but they have not been thoroughly studied.

Conclusion

According to our study, we found that SRC and SIRT1 are both up-regulated in tamoxifen-resistant breast cancer cells compared with tamoxifen-sensitive breast cancer cells and related to a poor prognosis in tamoxifen-treated breast cancer. Besides, SRC could promote tamoxifen resistance by up-regulating SIRT1. Taken together, we considered that the SRC and SIRT1 had the possibility to be novel therapeutic targets in tamoxifen-resistant breast cancer.

Data Sharing Statement

The high-throughput sequencing data (GSE129544) used to support the findings of this study have not been made available before it is released in GEO database. "Reviewer access" to GSE129544: ityxssamvbqbvct. The other data used to support the findings of this study are included within the article or GEO database.

Acknowledgment

This study is supported by National Natural Science Foundation of China (Grant 81672611 to JM and Grant 81602350 to HG).

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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