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Trichostatin A alleviates the process of breast carcinoma by downregulating LPAR5

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Abstract. – OBJECTIVE: To elucidate the role of histone deacetylase inhibitor Trichostatin A (TSA) in affecting metastasis of breast carcinoma, and its molecular mechanism.

PATIENTS AND METHODS: LPAR5 levels in breast carcinoma tissues and paracancerous tissues were detected by quantitative real-time polymerase chain reaction (qRT-PCR), and its expression pattern was further verified in breast carcinoma cell lines. The relationship between LPAR5 and prognosis of breast carcinoma patients was analyzed. After TSA induction (100-400 nmol/L) for 6-48 h, the proliferative and migratory abilities of SKBR3 and MDA-MB-231 cells in overexpressing LPAR5 were examined by cell counting kit-8 (CCK-8), transwell and wound healing assay. By constructing a xenograft model in nude mice, the influences of TSA and LPAR5 on *in vivo* growth of breast carcinoma were examined.

RESULTS: LPAR5 was upregulated in breast carcinoma samples. High level of LPAR5 predicted higher rates of lymphatic metastasis and distant metastasis, as well as lower overall survival and progression-free survival in breast carcinoma patients. LPAR5 level was dose-dependently downregulated in TSA-induced SKBR3 and MDA-MB-231 cells. In addition, TSA induction dose-dependently declined proliferative ability, and time-dependently attenuated migratory ability in breast carcinoma cells. *In vivo* overexpression of LPAR5 in nude mice reversed the inhibitory effect of TSA on breast carcinoma growth.

CONCLUSIONS: TSA induction can suppress proliferative and migratory abilities in breast carcinoma by downregulating LPAR5.

Key Words:
Trichostatin A, LPAR5, Breast carcinoma, Metastasis.

Introduction

Breast carcinoma is the third most-common tumor in the world, and it is the mostly diagnosed tumor in women[1,2]. In 2018, the number of breast carcinoma patients reached 1.7 million, including 500,000 deaths in the world[3,4]. It is estimated that about 1/10-1/8 women with breast diseases would deteriorate into breast carcinoma. The mortality of breast carcinoma has declined in North America and Europe, whereas it is elevated in South America, Africa and Asia[1]. Genetic and environmental factors are vital regulators in epidemiology of breast carcinoma[1-4]. Symptoms and signs of early stage breast carcinoma are atypical and hardly to be diagnosed. Therapeutic efficacy of advanced or recurrent breast carcinoma is disappointed[5,6]. So far, cisplatin and paclitaxel are the most effective chemotherapy drugs for breast carcinoma[7]. Although the chemotherapy response rate of breast carcinoma is up to 70%, the curative effect cannot be last long. Patients with breast carcinoma usually experience relapse within 18 months after the first-line chemotherapy[8,9]. It is necessary to develop effective chemotherapy strategies aiming to prolong survival of breast carcinoma patients[10].

Multiple genes are interacted and involved in the complicated process of breast carcinoma, in which histone acetylation is a key event[11,12]. Imbalanced histone acetylation/deacetylation will result in gene dysfunction and thus pathological lesions. Notably, the imbalanced histone acetylation/deacetylation is a reversible process that can be corrected[12]. Trichostatin A (TSA) is an antifungal antibiotic derived from streptomycetes, which can inhibit histone deacetylase in mammals and thus restore the balance between histone acetylation and deacetylation. In addition, TSA is able to arrest G1 and G2 phase during the progression of cell cycle, and it is now utilized as a potential anti-cancer drug[13,14]. TSA is a kind of hydroxamic acid type histone deacetylase inhibitor which has been well concerned because of the anti-metastasis effect on breast carcinoma[14].

Wu et al[15] demonstrated the role of LPAR5 in affecting the progression of papillary thyroid cancer. In this paper, we collected 52 cases of breast

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carcinoma tissues and paracancerous tissues. Differential expression of LPAR5 and its clinical significance in breast carcinoma patients were firstly examined. In addition, the regulatory effects of TSA on the malignant progression of breast carcinoma were assessed *in vitro* and *in vivo*.

Patients and Methods

Breast Carcinoma Samples

Breast carcinoma and paracancerous ones were collected during biopsy or surgery from 52 patients, which were stored at -80°C. Patients were diagnosed by two experienced doctors independently. Tumor node metastasis (TNM) staging and histological classification of breast carcinoma were defined according to the criteria proposed by UICC/AJCC (the 8th edition), and none of patients had preoperative chemotherapy, endocrine or molecular targeted therapy. Inclusion criteria: patients with no severe diseases in other organs and those undergoing no post-operative radiotherapy. Exclusion criteria: patients with distant metastasis or lung metastasis of tumors, those complicated with other malignancies, those with mental disease, those complicated with myocardial infarction, heart failure or other chronic diseases, or those previously exposed to radioactive rays. This investigation was approved by the research Ethics Committee of PLA General Hospital and complied with the Helsinki Declaration. Informed consent from patients was obtained before collecting samples and clinical data. Follow-up was conducted after discharge by telephone or outpatient review, including general conditions, clinical signs and symptoms, and imaging examinations.

Cell Lines and Reagents

Breast carcinoma cell lines (MCF-7, SKBR3, MDA-MB-231) and the mammary epithelial cell line (MCF-10A) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (except for SKBR3 cell lines in Roswell Park Memorial Institute-1640 (RPMI-1640)) (HyClone, South Logan, UT, USA) at 37°C with 5% CO_2. 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin were added in culture medium. Cell passage was conducted every 2-3 days.

Transfection

Cells were cultured in serum-free medium and cultivated to 50-70% confluence. Transfection was conducted using RNAi MAX transfection kit (Invitrogen, Carlsbad, CA, USA). Transfection efficacy was detected at 24 or 48 h.

Transwell Migration Assay

Transfected cells were prepared to serum-free suspension at 3×10^4 /mL. In the transwell chamber, 200 μL of suspension and 500 μL of complete medium were added on the upper and bottom chamber, respectively. After 24 h of incubation, cells in the bottom were fixed in methanol for 15 min, dyed with crystal violet for 30 min and counted using a microscope (Olympus, Tokyo, Japan).

Wound Healing Assay

Cell suspension in serum-free medium was prepared at 5×10^5 /mL, and implanted in 6-well plates. Until 90% of cell attachment, an artificial scratch was made using a sterilized pipette tip. Cells were washed in phosphate-buffered saline (PBS) for 2-3 times and cultured in the medium containing 1% FBS. 24 hours later, wound closure was captured for calculating the percentage of wound healing.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cellular or tissue RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Qualified RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using AMV reverse transcription kit (TaKaRa, Komatsu, Japan). SYBR®Premix Ex Taq™ (TaKaRa, Komatsu, Japan) was used for qRT-PCR with cDNA as the template. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. LPAR5: Forward: 5'-GAGGTCTCTGCTGCTGAT-3', Reverse: 5'-AGAACTGTTGGTTGAGGAG-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Forward: 5'-GGTCGGAGTCAACGGATTTG-3', Reverse: 5'-ATGAGCCCCAGCCTTCTCCAT-3'.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) on ice for 30 min, and centrifuged at 4°C, 14000 × g for 15 min. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples

were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and loaded on a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). The membrane was cut into small pieces according to the molecular size and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure and grey value analyses using alpha SP.

In Vivo Xenograft Model

This study was approved by the Animal Ethics Committee of PLA General Hospital Animal Center. Ten female nude mice with 8 weeks old were administrated with SKBR3 cells transfected with pcDNA3.1-NC (n=5) or pcDNA3.1-LPAR5 (n=5) following 400 nM TSA induction for 24 h in the armpit. Tumor width and length were recorded once a week at a fixed time point. Mice were sacrificed at the 6th week for collecting tumor tissues. Tumor volume was calculated using the formula: Tumor volume = Tumor width^2 × tumor length / 2.

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used to statistical analysis. The Student's *t*-test was applied to compare the differences between two groups. Each experiment was repeated in triplicate and data were expressed as mean±SD (standard deviation). Chi-square test was applied for analyzing the relationship between LPAR5 and clinical indicators of breast carcinoma. $p<0.05$ was considered as statistically significant.

Results

LPAR5 Was Highly Expressed in Breast Carcinoma Samples

Fifty-two cases of breast carcinoma tissues and paracancerous tissues were collected for detecting differential levels of LPAR5. Compared with paracancerous ones, LPAR5 was upregulated in breast carcinoma tissues (Figure 1A). Meanwhile, LPAR5 was highly expressed in breast carcinoma cell lines as well (Figure 1B). SKBR3 and MDA-MB-231 cells were selected in the following experiments because of their high abundances of LPAR5. The above data indicated that LPAR5 may be an oncogene involved in breast carcinoma process.

LPAR5 Expression Was Correlated with Metastasis in Breast Carcinoma

Chi-square analysis was conducted to assess the relationship between LPAR5 and clinical indicators of breast carcinoma patients we recruited. It is shown that LPAR5 was positively correlated to rates of lymphatic metastasis and distant metastasis (Table I). As expected, we detected higher abundance of LPAR5 in breast carcinoma cases with lymphatic metastasis or distant metastasis (Figure 1C). Furthermore, the prognostic potential of LPAR5 in breast carcinoma was examined by plotting Kaplan-Meier survival curves. High level of LPAR5 was favorable to the overall survival and progression-free survival in breast carcinoma (Figure 1D, 1E).

Table I. Association of LPAR5 expression with clinicopathologic characteristics of breast cancer.

		LPAR5 expression		
Parameters	No. of cases	Low (%)	High (%)	*p*-value
Age (years)				0.228
<60	20	14	6	
≥60	32	17	15	
Tumor size (cm)				0.080
<3	25	18	7	
≥3	27	13	14	
T stage				0.174
T1-T2	31	20	10	
T3-T4	21	10	11	
Lymph node metastasis				0.011
No	33	24	9	
Yes	19	7	12	
Distance metastasis				0.030
No	36	25	11	
Yes	16	6	10	

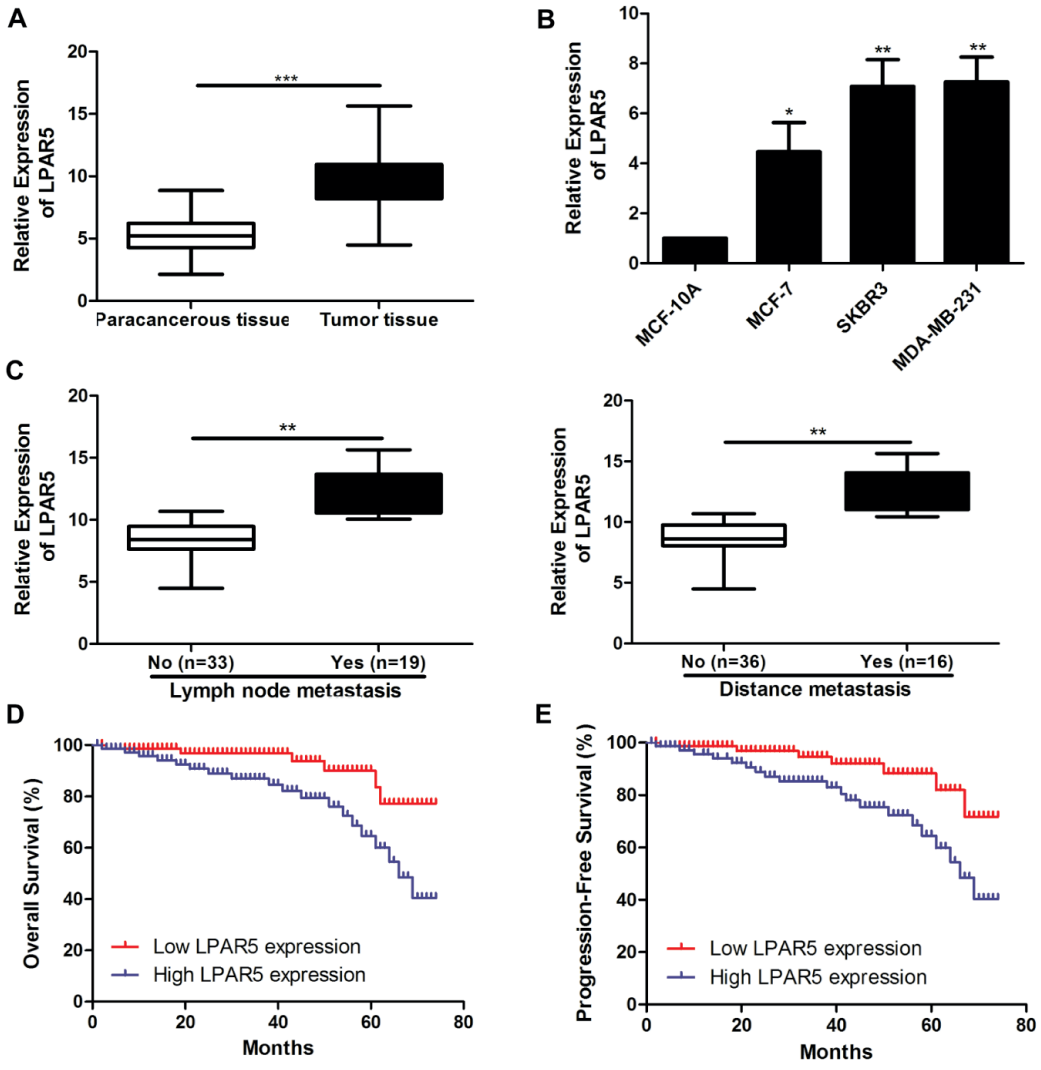

Figure 1. LPAR5 was highly expressed in breast carcinoma samples. **A,** Differential level of LPAR5 in breast carcinoma and paracancerous tissues; **B,** LPAR5 levels in breast carcinoma cell lines; **C,** LPAR5 levels in breast carcinoma cases either with lymphatic metastasis, distant metastasis or not; **D,** Overall survival in breast carcinoma patients based on LPAR5 expression; **E,** Progression-free survival in breast carcinoma patients based on LPAR5 expression. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

TSA Induction Dose-Dependently Declined LPAR5 Level and Proliferative Rate in Breast Carcinoma Cells

SKBR3 and MDA-MB-231 cells were induced with 100-400 nmol/L TSA for 6-48 h. QRT-PCR revealed that LPAR5 was dose-dependently downregulated in TSA-induced breast carcinoma cells (Figure 2A). Besides, proliferative rate was dose-dependently declined as well (Figure 2B).

TSA Suppressed Migratory Ability in Breast Carcinoma Cells

To elucidate the influence of TSA on breast carcinoma cell phenotypes, SKBR3 and MDA-MB-231 cells were either induced with 400 nM TSA or not. TSA induction remarkably reduced the migratory cell number in breast carcinoma cell lines (Figure 3A). Furthermore, wound closure assay consistently suggested the inhibitory effect of TSA induction on the migratory ability of breast carcinoma cells (Figure 3B).

LPAR5 Reversed the Inhibitory Effect of TSA on Breast Carcinoma Migration

We next explored the involvement of LPAR5 in breast carcinoma the malignant progression. Overexpression of LPAR5 markedly elevated the migratory cell number in TSA-induced SKBR3

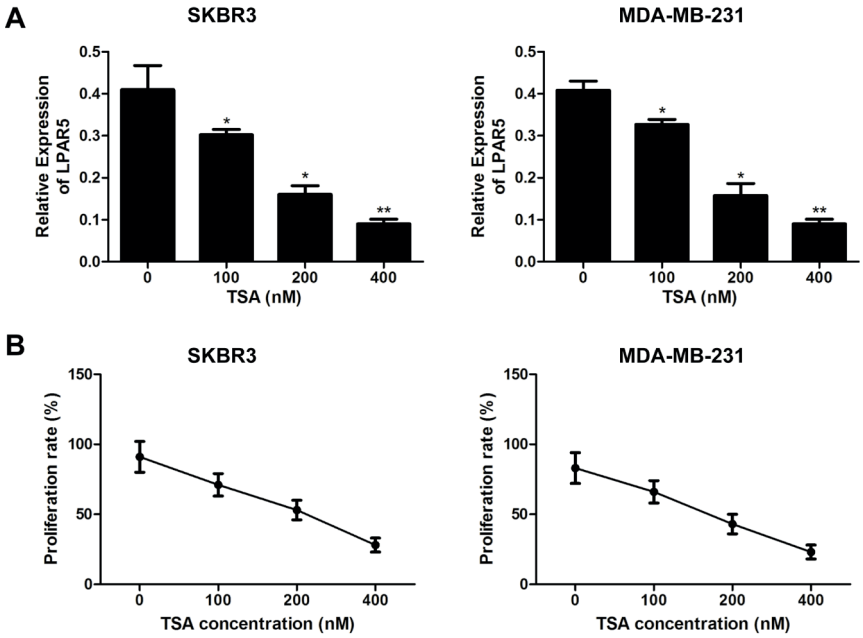

Figure 2. TSA induction dose-dependently declined LPAR5 level and proliferative rate in breast carcinoma cells. **A,** LPAR5 level in SKBR3 and MDA-MB-231 cells induced with 0, 100, 200 and 400 nM TSA; **B,** Proliferative rate in SKBR3 and MDA-MB-231 cells induced with 0, 100, 200 and 400 nM TSA. *$p < 0.05$, **$p < 0.01$.

and MDA-MB-231 cells (Figure 4A). Besides, wound closure percentage was higher in TSA-induced breast carcinoma cell lines after overexpressing LPAR5 (Figure 4B). It is suggested that overexpression of LPAR5 reversed the role of TSA induction in inhibiting breast carcinoma migration ability.

LPAR5 Reversed the Inhibitory Effect of TSA on In Vivo Growth of Breast Carcinoma

A xenograft model was constructed by administrating SKBR3 induced with 400 nM TSA and transfected with either pcDNA3.1-NC or pcDNA3.1-LPAR5 in nude mice. Notably, *in vivo* overexpression of LPAR5 markedly stimulated the growth of breast carcinoma in nude mice, as the average tumor volume and tumor weight were higher than those of controls (Figure 5A, 5B). As expected, protein level of LPAR5 was upregulated in harvested breast carcinoma tissues from mice after overexpressing LPAR5, suggesting a satisfactory *in vivo* transfection (Figure 5C).

Discussion

Histone acetylation is a vital link involved in tumor progression. The balance between histone acetylation and deacetylation maintains homeostasis in the body. Once the balance breaks, it will lead to carcinogenesis of normal cells. Luckily, the imbalanced histone acetylation and deacetylation can be corrected, which is conducive to alleviate pathological conditions[11,12]. TSA is a non-competitive reversible inhibitor of HDAC, which is responsible for regulating histone acetylation and deacetylation. Here, TSA induction not only dose-dependently decreased LPAR5 level and proliferative rate in SKBR3 and MDA-MB-231 cells, but also time-dependently attenuated the migratory ability in breast carcinoma cell lines.

Lysophosphatidic acid receptor (LPAR) is a member of class A GPCRs (G-protein-coupled receptors), also known as endothelial differentiation gene (EDG). LPAR is responsible for transmitting lipid signals[16]. It contains six members, LPAR1-LPAR6, and among them, LPAR1-3 exert high affinity for LPA[16,17]. Currently, the potential function of LPAR5 remains largely unclear[15,16]. The three-dimensional structural information of the human LPAR5 can provide a molecular basis for revealing its function, which also provides references for the development of targeted drugs[18,19]. However, the association of LPAR5 and TSA, as well as the biological functions in breast carcinoma are unclear. Therefore, we aimed to elucidate the role of TSA in affecting the metastasis of

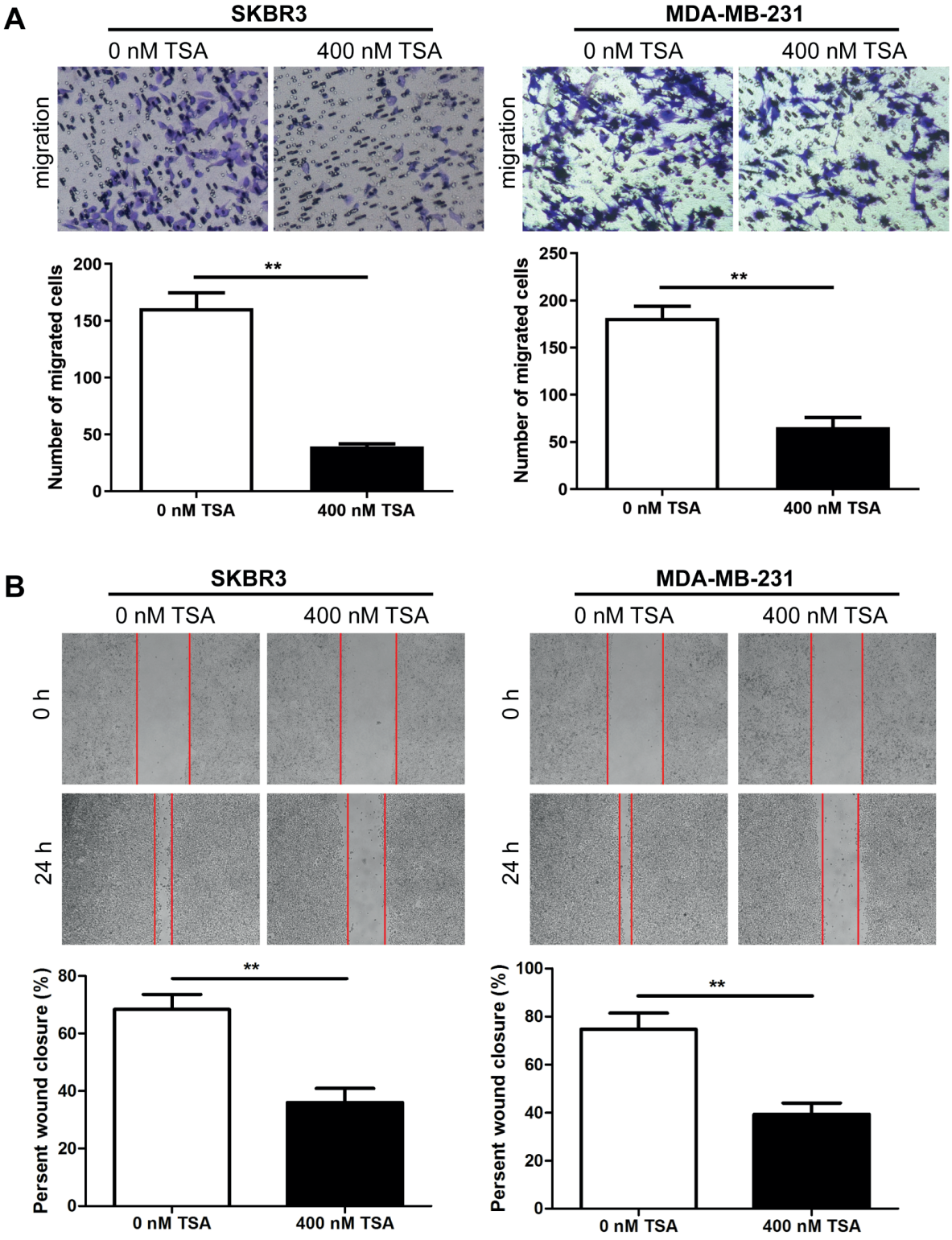

Figure 3. TSA suppressed migratory ability in breast carcinoma cells. **A,** Migration in SKBR3 and MDA-MB-231 cells either induced with 400 nM TSA or not (magnification: 40×); **B,** Wound closure in SKBR3 and MDA-MB-231 cells either induced with 400 nM TSA or not (magnification: 40×). $^{**}p < 0.01$.

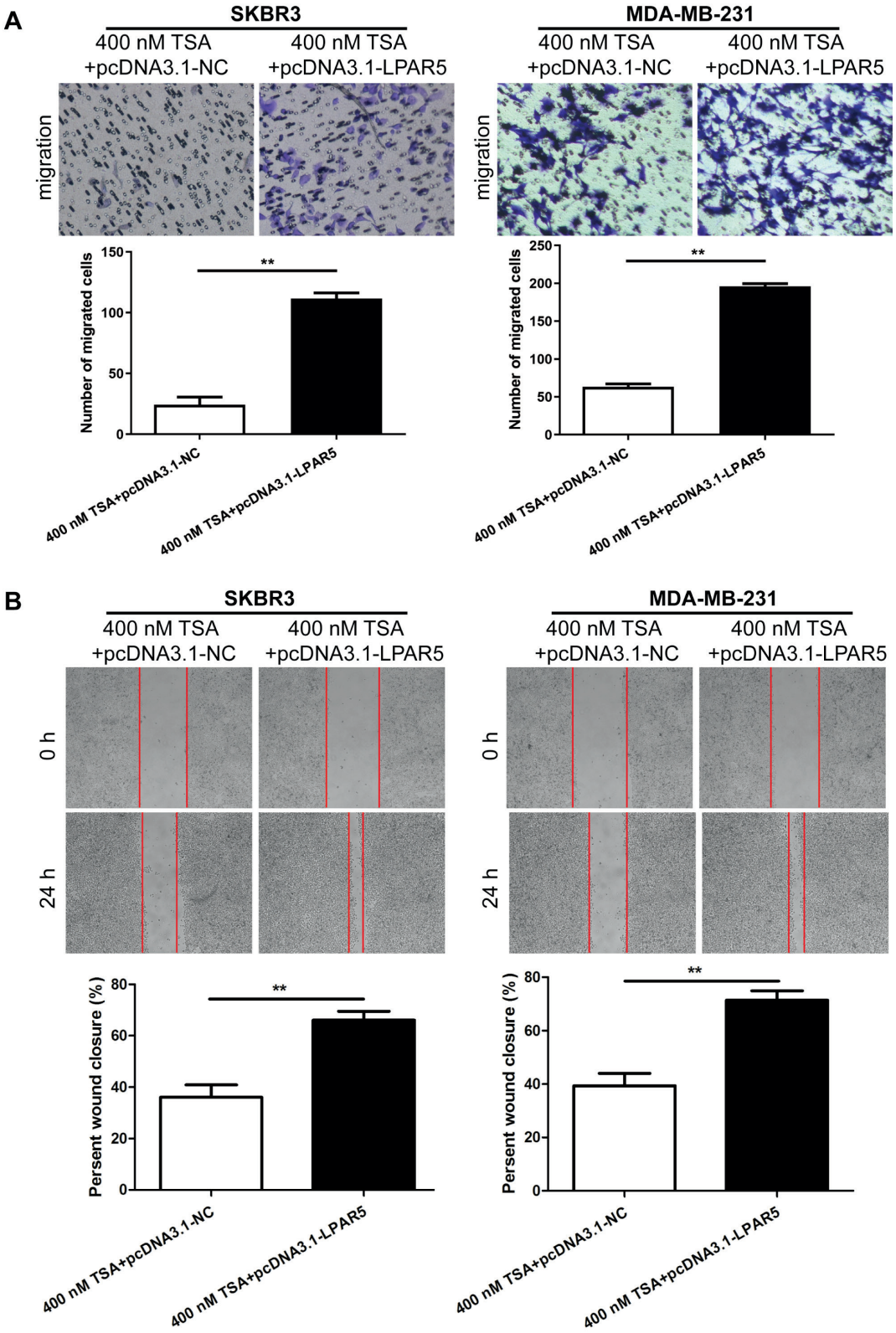

Figure 4. LPAR5 reversed the inhibitory effect of TSA on breast carcinoma migration. **A,** Migration in TSA-induced SKBR3 and MDA-MB-231 cells transfected with pcDNA3.1-NC or pcDNA3.1-LPAR5 (magnification: 40×); **B,** Wound closure in TSA-induced SKBR3 and MDA-MB-231 cells transfected with pcDNA3.1-NC or pcDNA3.1-LPAR5 (magnification: 40×). $^{**}p < 0.01$.

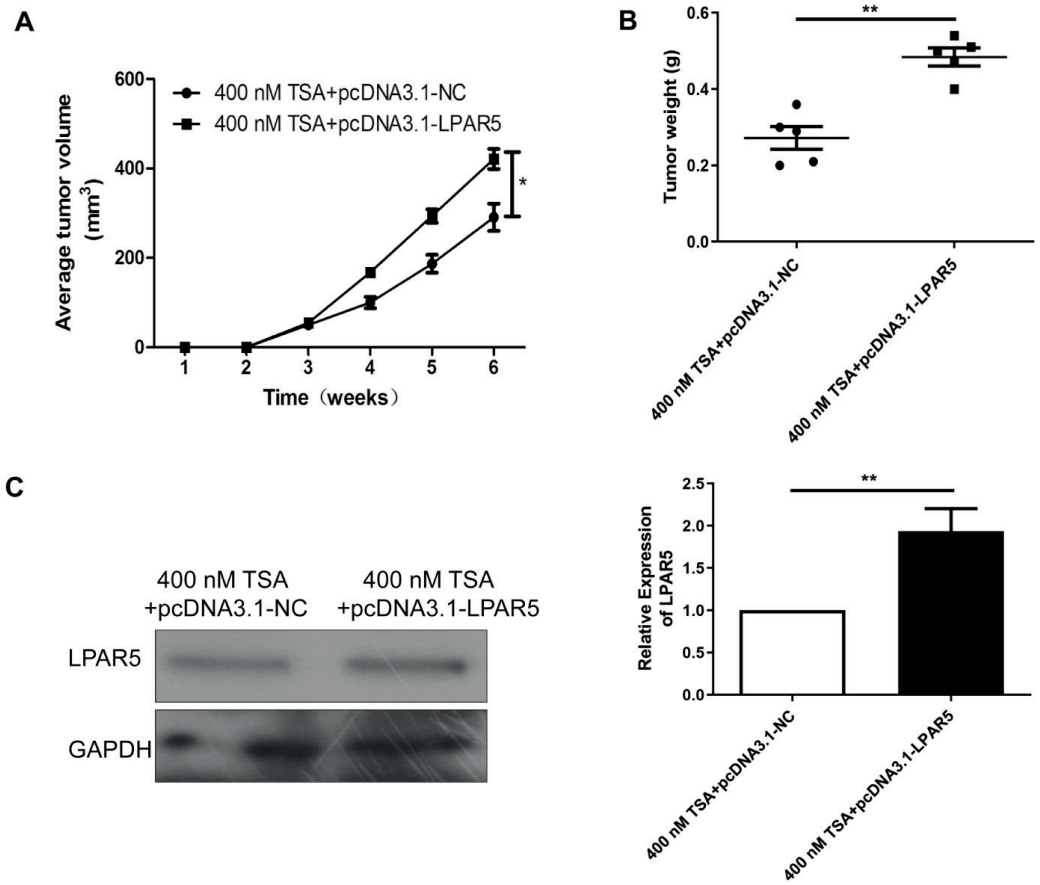

Figure 5. LPAR5 reversed the inhibitory effect of TSA on *in vivo* growth of breast carcinoma. **A,** Average tumor volume in nude mice administrated with TSA-induced SKBR3 cells transfected with pcDNA3.1-NC or pcDNA3.1-LPAR5; **B,** Tumor weight in nude mice administrated with TSA-induced SKBR3 cells transfected with pcDNA3.1-NC or pcDNA3.1-LPAR5; **C,** Protein level of LPAR5 in breast carcinoma tissues of nude mice administrated with TSA-induced SKBR3 cells transfected with pcDNA3.1-NC or pcDNA3.1-LPAR5. *$p < 0.05$, **$p < 0.01$.

breast carcinoma, and its molecular mechanism. In this paper, LPAR5 was upregulated in breast carcinoma tissues than paracancerous ones. By analyzing clinical data of breast carcinoma patients, it is found that LPAR5 level was positively correlated to rates of lymphatic metastasis and distant metastasis of breast carcinoma. Furthermore, survival analysis demonstrated that LPAR5 was an unfavorable factor for the prognosis of breast carcinoma. We thereafter speculated that LPAR5 could be an oncogene involved in breast carcinoma process. Of note, overexpression of LPAR5 reversed the inhibitory effect of TSA induction on migratory ability of breast carcinoma cells. Consistently, overexpression of LPAR5 largely accelerated tumor growth of breast carcinoma in nude mice. To sum up, TSA contributed to suppressing the proliferative and migratory abilities of breast carcinoma by downregulating LPAR5. Its molecular mechanism, however, remained to be explored in the future.

Conclusions

Altogether, these data revealed that TSA induction is able to suppress proliferative and migratory abilities in breast carcinoma by downregulating LPAR5.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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