



OPEN The downregulation of SASH1 expression promotes breast cancer occurrence and invasion accompanied by the activation of PI3K-Akt-mTOR signaling pathway

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SASH1 (SAM and SH3 domain containing 1) has been increasingly reported as a tumor suppressor gene. However, there is limited research on the role of SASH1 in breast cancer. This manuscript aims to investigate the mechanism of SASH1 in the occurrence, development, and prognosis of breast cancer. Firstly, we obtained RNA-sequencing data of the tumors from the Genomic Data Commons data portal website, along with the corresponding clinical information of patients. Pan-cancer analysis was performed to analyze the expression of SASH1 across all tumors. Univariate Cox regression analysis was used to assess the correlation between SASH1 expression and the prognosis of breast cancer patients. Then, immunohistochemistry was utilized to evaluate the expression levels of SASH1, p-Akt, p-PI3K, and p-mTOR in breast cancer tissue. Finally, a cell assay was employed to analyze the impact of SASH1 on the proliferation and invasion of breast cancer cells (MDA-MB-231). The results revealed that SASH1 expression is decreased in BRCA, LUSC, LUAD, CESC, ESCA, and COAD. Meta-analysis also found that SASH1 is downregulated in most tumor tissues, and the expression level of SASH1 in breast cancer was significantly lower than that in the control group (OR = 0.14, 95% CI = 0.08–0.25; $P < 0.001$). Further experimental validation showed that SASH1 expression is significantly downregulated in breast cancer tissue (38.33%, 23/60), and the overexpression of SASH1 can inhibit the proliferation and invasion of breast cancer cells accompanied by the suppression of PI3K-Akt-mTOR signaling pathway. Additionally, SASH1 overexpression can improve OS and RFS of breast cancer patients.

Keywords SASH1, PI3K, Akt, mTOR, Breast cancer, Prognosis

The development of tumors is a complex, multistage process influenced by various factors¹. Key among these are the activation of proto-oncogenes and the inactivation of tumor suppressor genes¹. Breast cancer, which originates from the epithelial tissue of the breast, is the most common malignancy affecting women, particularly those between the ages of 40 and 60 or around the peri-menopausal period². Statistics show that the incidence of breast cancer has been steadily increasing in recent years³. While numerous factors can contribute to its development, genetic factors account for less than 10% of cases, leaving the mechanisms behind the remaining 90% still largely unknown⁴.

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SAM and SH3 domain containing 1 (SASH1) is located on chromosome 6q24.3 and has a total length of 7709 bp⁵. It encodes 1247 amino acids and contains one SH3 domain and two SAM domains⁶. SASH1 belongs to the SLY family, which includes other protein members such as SAMS1 and SASH3⁶. SAMS1 primarily functions in B-cell activation, while SASH3 is involved in the specific immune response of organisms⁷. Notably, SASH1 expression is reduced in various malignant tumors, such as breast, lung, gastric, and thyroid cancers, and its levels are closely linked to tumor proliferation, migration, and prognosis⁸. Current studies highlight a significant connection between SASH1 and tumor progression⁹. Yang et al.¹⁰ confirmed that low expression of SASH1 not only promotes the occurrence of gliomas but also is closely related to the grading of gliomas. In liver cancer and thyroid tumors, the mRNA expression level of SASH1 is notably decreased¹¹. Studies have shown that SASH1 protein expression is significantly downregulated in 74% of breast cancer patients¹². Gong et al.¹³ demonstrated that, compared to the control group, the positivity rate of SASH1 protein in breast cancer patients is significantly reduced (63/186, 33.9%). Our previous studies have also confirmed that low expression of SASH1 is significantly associated with the occurrence of breast cancer⁴. These findings suggest that SASH1 may play a crucial role in the initiation and progression of breast cancer.

The phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt)-mammalian target of rapamycin (mTOR) signaling pathway relays signals from cell membrane receptors to the cytoplasm¹⁴. In-depth research on cancer molecular biology has revealed that the PI3K-Akt-mTOR signaling pathway is intimately linked with the initiation, progression, and metastasis of tumors¹⁴. Abnormal expression of this pathway has been detected in various tumors, including squamous cell carcinoma, prostate cancer, and liver cancer¹⁵. Activation of PI3K triggers the activation of Akt, which becomes phosphorylated to form p-Akt, subsequently regulating downstream gene expression, and mediating tumor cell proliferation and apoptosis¹⁶. Miao et al.¹⁷ discovered that PI3K-Akt-mTOR expression levels are significantly elevated in breast cancer, facilitating tumor proliferation and drug resistance. Pan et al.¹⁸ found that SASH1 can inhibit the proliferation and migration of pancreatic cancer cells by blocking the expression of the PI3K-Akt-mTOR signaling pathway. Nonetheless, there is currently insufficient research to confirm whether an interaction exists between SASH1 and the PI3K-Akt-mTOR signaling pathway in breast cancer. Examining the function and molecular mechanisms of the SASH1 gene could not only shed light on its role in tumor initiation and progression but also lay the groundwork for targeting it in breast cancer treatment^{9,19}. Consequently, this study aims to investigate the role of SASH1 in the onset, development, and prognosis of breast cancer through multiple approaches, including clinical samples, cell experiments, and meta-analysis.

Materials and methods

Reagents

Immunohistochemical detection reagents were purchased from OriGene Biotechnology Co., Ltd. (Wuxi, China). PCR primers synthesis and sequencing were performed by GenScript Biotechnology Co., Ltd. (Nanjing, China). Rabbit anti-human SASH1 polyclonal antibodies (cat. no. NBP-26650) were purchased from Novus (USA). Mouse monoclonal GAPDH antibody (cat. no. 97166), Akt total protein mouse monoclonal (cat. no. 2920) and Akt phosphoprotein rabbit monoclonal (cat. no. 4060) antibodies, PI3K total protein mouse monoclonal (cat. no. 13666) and PI3K phosphoprotein rabbit monoclonal (cat. no. 4228) antibodies, mTOR total protein mouse monoclonal (cat. no. 4517) and mTOR phosphoprotein rabbit monoclonal (cat. no. 5536) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). TRIzol[®] reagent was purchased from Takara Biotechnology Co., Ltd. (Dalian, China); Cell protein extraction and western blot detection reagents RIPA lysis and extraction buffer were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China).

Patients and Cell lines

Breast cancer tissue specimens were obtained from 60 patients who underwent surgical resection at Yongchuan People's Hospital of Chongqing from April 2023 to April 2024. All specimens were confirmed by pathological examination (HE staining) for diagnosis. The patients' ages ranged from 37 to 75 years old. This study received approval from the Ethics Committee of the Yongchuan People's Hospital of Chongqing. MDA-MB-231 breast cancer cells were purchased from the Cell Resource Center, Shanghai Institute for Biological Sciences (Chinese Academy of Sciences).

Cell culture and infection

MDA-MB-231 cells were cultured in L15 culture medium containing 10% fetal bovine serum (FBS) (including 1% penicillin and streptomycin) at 37 °C, in the absence of CO₂. MDA-MB-231 cells in the exponential phase of growth were seeded at a density of 2×10^6 /culture flask. After 24 h, the cells were subsequently infected with adenovirus vehicles that express pcDNA3.1 and pcDNA3.1-SASH1.

Immunohistochemistry

The breast cancer tumor tissues and adjacent normal tissues were embedded in paraffin and cut into 5 μm thick sections. Drip 3% H₂O₂ deionized water to cover the tissue and inactivate endogenous enzymes. Normal goat serum blocking solution was then added dropwise (at room temperature, 30 min), followed by the addition of rabbit anti-human SASH1, p-PI3K, p-Akt, and p-mTOR polyclonal antibodies (at 37 °C, 1 h), and incubated overnight at 4 °C. Biotin-labeled ready-to-use goat anti-rabbit IgG was added dropwise (at 37 °C, 30 min). Horseradish peroxidase-labeled streptavidin working solution was added dropwise (at 37 °C, 15 min), followed by DAB chromogenic staining. Finally, neutral resin was used for sealing, and positive results were observed and photographed under an optical microscope²⁰.

Cell proliferation assay, wound-closure assay and transwell invasion assay

Cell proliferation was analyzed by Cell Counting Kit-8 (CCK-8) assay as described previously. The absorbance was measured daily for 5 days at 450 nm using a microplate reader. Each experiment was done in quintuplicate, and repeated three times. Then, a growth curve was delineated²¹.

The wound closure assay was performed as previously described. Cell migration was monitored by microscopy 24 h later and reported as wound healing rate. Wound healing rate = (initial scratch area – 24 h initial scratch area) / initial scratch area. The experiment was repeated three times²².

The transwell invasion assay was performed as previously described. After 24 h, the cells migrating through the filter were dried for 10 min, stained with hematoxylin and eosin. Cells on the lower chamber were counted under a microscope. The experiment was repeated three times²².

RT-qPCR

When the cells cover approximately 80% of the culture dish area, total RNA will be extracted from the breast cancer cells in each group using the TRIzol® method. Subsequently, the mRNA expression levels of SASH1 in each group will be detected using qPCR. Specifically, 2 µg of total RNA will be used to synthesize cDNA using a reverse transcription kit. Then, 1 µL of cDNA will be used as a template for PCR amplification. The following primer sequences were used: SASH1, sense, 5'-TCCCCTCACAGGAAGAAACG'-3 and antisense, 5'-GATACCATCACGTCGGTCC'-3'.

Western blotting

Different recombinant cells were collected, and the western blotting assay was performed as usual. The ECL chromogenic solution was used for imaging. GEL EQ (Bio-Rad Laboratories, Hercules, CA, USA) was used in the gel imager, and Quantity One® software (Bio-Rad Laboratories, Inc.) was used to analyze the relative optical densities. The relative expression level of the target gene protein was calculated according to the gray value of the target gene band/the gray value of the internal reference band²⁰.

Data acquisition and pre-processing

To download and organize the RNA-sequencing data of the TCGA-ALL project with the STAR pipeline from the TCGA database (<https://portal.gdc.cancer.gov>) and extract the data in FPKM format for corresponding paired tumor and normal samples. The following tumor types were included in this study: bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical and endocervical cancer (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), rectal adenocarcinoma (READ), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), thyroid carcinoma (THCA), thymoma (THYM), uterine corpus endometrial carcinoma (UCEC)²³.

Expression level and prognostic evaluation

R software was used for statistical analysis. We downloaded two breast cancer datasets (GSE22820 and GSE42568) from the GEO database. The 'normalizeBetweenArrays' function from the limma package was used to normalize the data. Patients were divided into a breast cancer group and a healthy control group. A linear model combined with the Empirical Bayes approach in the limma package was used to perform differential analysis between the two groups. To assess the relationship between SASH1 gene expression and various survival outcomes in breast cancer, univariate Cox regression analysis was employed. Specifically, this analysis evaluated the correlation between SASH1 gene expression and four key survival metrics: overall survival (OS), distant metastasis-free survival (DMFS), recurrence-free survival (RFS), and post-progression survival (PPS). According to the median value of SASH1 gene expression, the patients were divided into SASH1 high expression group and SASH1 low expression group. The Kaplan–Meier method was employed to construct survival curves, providing a visual representation of survival outcomes. Subsequently, the log-rank test was utilized to analyze the differences in survival status between groups. To assess the expression of the SASH1 gene between tumor and normal tissue, a t-test was employed. $P < 0.05$ was considered statistically significant.

Statistical methods

SPSS version 25.0 (IBM Corp.) was used for processing clinical and experimental data. For the analysis of immunohistochemical results, all data were divided into the breast cancer tissue group and the adjacent normal tissue group. The chi-square test was used to analyze whether there is differential expression of SASH1 in breast cancer tissues. Spearman's correlation analysis was used to evaluate the correlation between SASH1 and PI3K, Akt, and mTOR. For cell experiments, we categorized the MDA-MB-231 breast cancer cells into three groups: Blank, pcDNA3.1, and pcDNA3.1-SASH1, unpaired student's t-test was employed to perform the statistical analysis. For meta-analysis, the STATA 12.0 software (StataCorp LLC) was used to perform statistical analysis. When two or more articles examined the association between SASH1 expression and the risk of cancers, statistical analyses were performed. In cases where there were fewer than two relevant articles, descriptive analyses were conducted instead. The Chi-square test and I^2 test were used to assess heterogeneity among studies. To pool the data, when $I^2 \geq 50$ or $q \leq 0.1$, significant heterogeneity between studies is considered, and random effects model (REM) is adopted. When $I^2 < 50$ or $q > 0.1$, there was no significant heterogeneity between the studies and fixed effects model (FEM) is adopted. $P > 0.05$ was considered to have no publication bias.

Results

SASH1 expression is decreased in cancer

We observed that SASH1 is commonly downregulated across various tumor types. Among the 23 types of tumors related to the expression level of SASH1 gene in the TCGA database, 16 types of tumors have statistically significant differences in SASH1 gene expression compared with normal tissues (all $P < 0.05$). SASH1 showed low expression levels in BLCA, BRCA, COAD, ESCA, HNSC, KICH, KIRC, KIRP, LUAD, LUSC, READ, STAD, THCA, and UCEC, whereas it exhibited higher expression levels in CHOL and PRAD (Fig. 1). In addition, we conducted a meta-analysis to explore the role of SASH1 in tumorigenesis. A total of 253 articles were identified. Following the elimination of duplicate studies and the application of the specified criteria, 16 articles were dedicated to exploring the correlation between the expression levels of SASH1 and eight distinct types of cancer^{11,13,24-37} (Fig. 2; Table 1). Specifically, among these 16 articles, 5 studies examined the correlation between

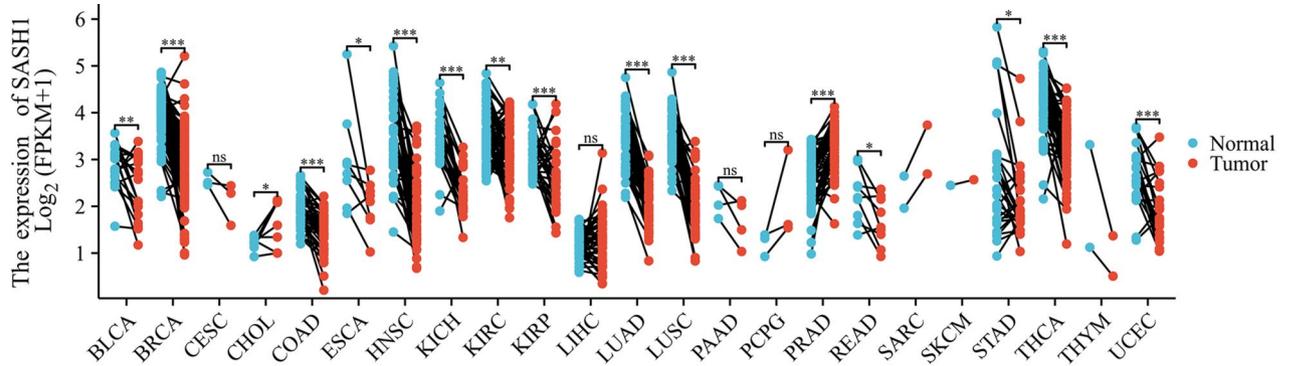


Fig. 1. The expression level of SASH1 gene in tumors and their corresponding normal tissues. bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical and endocervical cancer (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), rectal adenocarcinoma (READ), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), thyroid carcinoma (THCA), thymoma (THYM), uterine corpus endometrial carcinoma (UCEC).

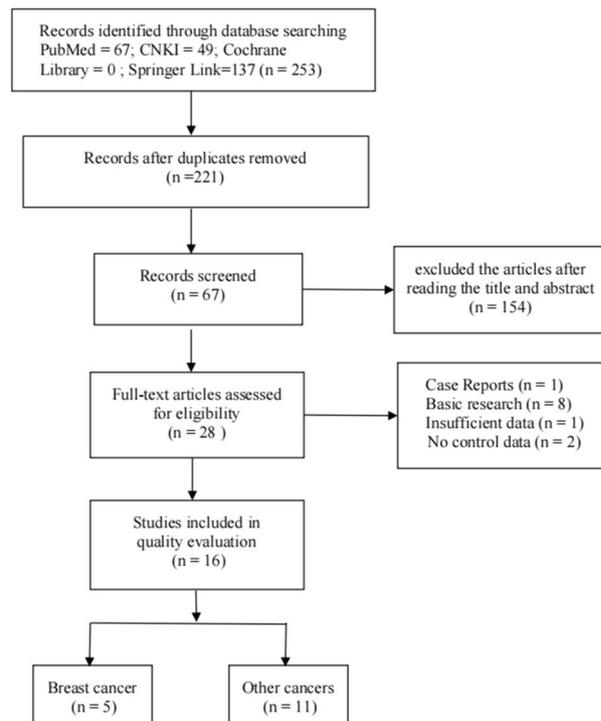


Fig. 2. The flow diagram of study selection. CNKI, China National Knowledge Infrastructure.

Author	Year	Country	Experience group		Control group		Method	Ages	Cancer types	Refs.	NOS
			+	-	+	-					
Qinwen	2019	China	13	67	22	7	IHC	-	Breast cancer	24	8
Lin	2019	China	31	69	32	8	IHC	26-75	Breast cancer	25	8
Gong	2017	China	63	123	31	19	IHC	49.32 ± 5.78	Breast cancer	13	9
Binbin	2015	China	16	34	40	10	IHC	-	Breast cancer	26	8
Jing	2014	China	13	28	24	7	IHC	-	Breast cancer	27	7
Xiaoli	2021	China	13	17	26	4	IHC	59.20 ± 2.12	Lung cancer	28	7
Nan	2017	China	72	130	69	41	IHC	63.19 ± 9.16	Lung cancer	29	9
Zhou	2018	China	1	7	4	4	WB	-	Gastric cancer	30	6
Qihong	2017	China	59	93	22	8	IHC	-	Gastric cancer	31	9
Yudi	2017	China	48	21	79	0	Q-PCR	-	Ovarian cancer	32	8
Ren	2016	China	48	31	79	0	Q-PCR	-	Ovarian cancer	33	8
Xie	2017	China	66	63	129	0	IHC	-	Cervical cancer	34	9
Xiaoying	2014	China	27	49	41	35	IHC	43-74	Esophageal cancer	35	8
Qiujun	2013	China	30	42	32	8	IHC	-	Esophageal cancer	36	8
Meng	2013	China	11	25	16	2	Q-PCR	11-36	Osteosarcoma	37	6
Rimkus	2006	Germany	65	48	3	12	Q-PCR	-	Colon cancer	11	8

Table 1. Basic characteristics of included literature. IHC, immunohistochemistry; Q-PCR, quantitative PCR; -, not mentioned. NOS, Newcastle-Ottawa Scale.

SASH1 expression levels and the risk of breast cancer^{13,24-27} (Fig. 3; Table 1). After statistical analysis, it was found that the expression level of SASH1 in tumor tissue was significantly lower than that in the control group (OR = 0.16, 95% CI = 0.09-0.28; $P < 0.001$), with the difference being statistically significant (Fig. 3). We conducted a stratified analysis according to the type of tumor. The results showed that only breast cancer related SASH1 studies were relatively more, and other tumor related SASH1 studies were few (less than or equal to 2 studies) (Fig. 3). So, the association between the SASH1 expression level and the risk of breast cancer was analyzed in five datasets. It was found that the expression level of SASH1 in breast cancer was significantly lower than that in control group (OR = 0.14, 95% CI = 0.08-0.25; $P < 0.001$), with the difference being statistically significant (Fig. 3; Table 2).

Furthermore, the analysis also revealed that elevated expression levels of SASH1 in lung cancer, ovarian cancer, gastric cancer, esophageal cancer, cervical cancer, and osteosarcoma were associated with a suppression of tumor occurrence compared to normal tissues. Conversely, high expression of SASH1 in colon cancer was

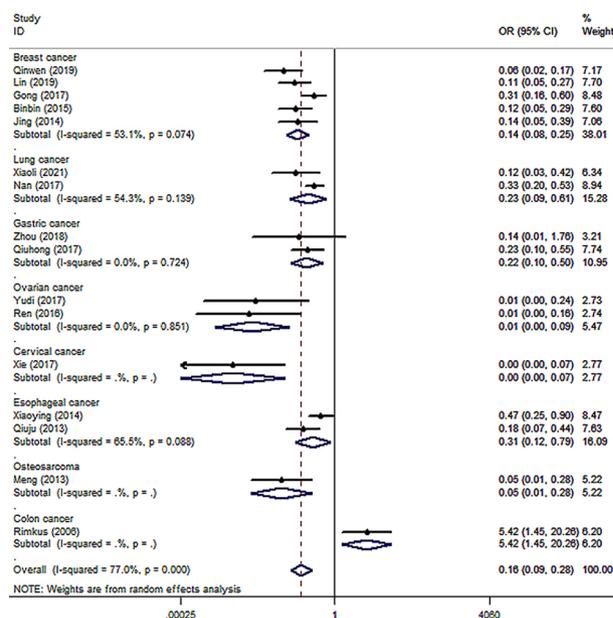


Fig. 3. The relationship between the expression level of SASH1 and the risk of tumor. OR, odds ratio; CI confidence interval; SASH1, SAM—and SH3 domain containing 1.

Cancer	Z	P	Cancer	Z	P
Breast cancer	6.68	<0.001	Cervical cancer	3.86	<0.001
Lung cancer	3.00	0.003	Esophageal cancer	2.46	0.014
Gastric cancer	3.61	<0.001	Osteosarcoma	3.48	<0.001
Ovarian cancer	4.37	<0.001	Colon cancer	2.51	0.012

Table 2. The significance test of the expression level of SASH1 in tumor.

associated with the promotion of tumor occurrence compared to normal tissues (Fig. 3; Table 2). It is exciting to note that this finding is consistent with the conclusion of our pan-cancer. However, it's noteworthy that the available clinical information on the role of SASH1 in these malignancies is currently insufficient. Consequently, additional research is warranted to delve deeper into the role of SASH1 in these types of cancer.

SASH1 is differentially expressed in breast cancer

The cBioPortal was employed to assess the frequency of the genetic alteration of SASH1 in breast cancer patients. The findings indicated that genetic alterations of SASH1 were observed in 6% of breast cancer patients. Interestingly, the genetic alterations of SASH1 is primarily characterized by decreased mRNA levels. This further indicates that the expression of SASH1 is down in breast cancer (Fig. 4A).

We downloaded two breast cancer datasets (GSE22820 and GSE42568) from the GEO database. After obtaining the differentially expressed genes from both datasets and taking the intersection, we found 985 common differentially expressed genes. Additionally, SASH1 showed differential expression in both datasets (Fig. 4B). ROC analysis of SASH1 expression data yielded an AUC (Area Under the Curve) value of 0.915, demonstrating that SASH1 is highly accurate in predicting the risk of breast cancer (Fig. 4C).

The expression levels of SASH1 are negatively correlated with p-Akt, p-PI3K, and p-mTOR in breast cancer tissues

In the 60 cases of breast cancer tissues and their paracancerous tissues, immunohistochemical results revealed that the expression level of SASH1 in tumor tissues was 38.33% (23/60), while in paracancerous tissues, it was 70% (42/60). The expression level of SASH1 in tumor tissues was significantly lower than that in paracancerous tissues ($P < 0.05$). Furthermore, compared to paracancerous tissues, the expression levels of p-Akt (39/60, 65.00%), p-PI3K (44/60, 73.33%), and p-mTOR (46/60, 76.67%) were significantly increased in tumor tissues (Fig. 5A–C). We performed a correlation analysis on the expression levels of SASH1, p-Akt, p-PI3K, and p-mTOR in tumor tissues. The results showed a significant negative correlation between SASH1 and p-Akt ($r = -0.447$, $P < 0.01$), p-PI3K ($r = -0.385$, $P < 0.01$), and p-mTOR ($r = -0.390$, $P < 0.01$) (Fig. 5B).

SASH1 can inhibit the proliferation and invasion of MDA-MB-231 cells

The wound closure assay and transwell invasion assay were utilized to explore the impact of SASH1 on the migration and invasion of MDA-MB-231 cells. The results indicated, that compared to MDA-MB-231/Blank cells (80.15%) and MDA-MB-231/pcDNA3.1 cells (80.34%), the wound healing rate of MDA-MB-231/pcDNA3.1-SASH1 cells (17.31%) was significantly reduced ($P < 0.05$) (Fig. 6A,B). Additionally, the invasion assay revealed that the invasive ability of MDA-MB-231/pcDNA3.1-SASH1 cells was significantly lower compared to that

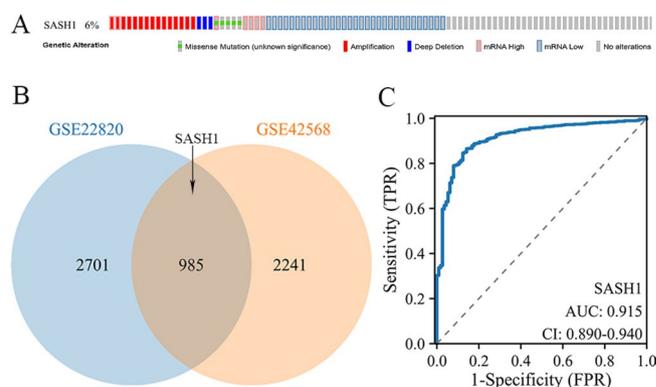


Fig. 4. The rate of SASH1 mutations in breast cancer. (A), the mutation rate of SASH1 is relatively high in breast cancer. (B), the differential expression of SASH1 in breast cancer was observed in both breast cancer datasets. Patients were divided into a breast cancer group and a healthy control group. A linear model combined with the Empirical Bayes approach in the limma package was used to perform differential analysis between the two groups. (C), SASH1 has a high predictive value for the risk of breast cancer occurrence. SASH1, SAM—and SH3 domain containing 1.

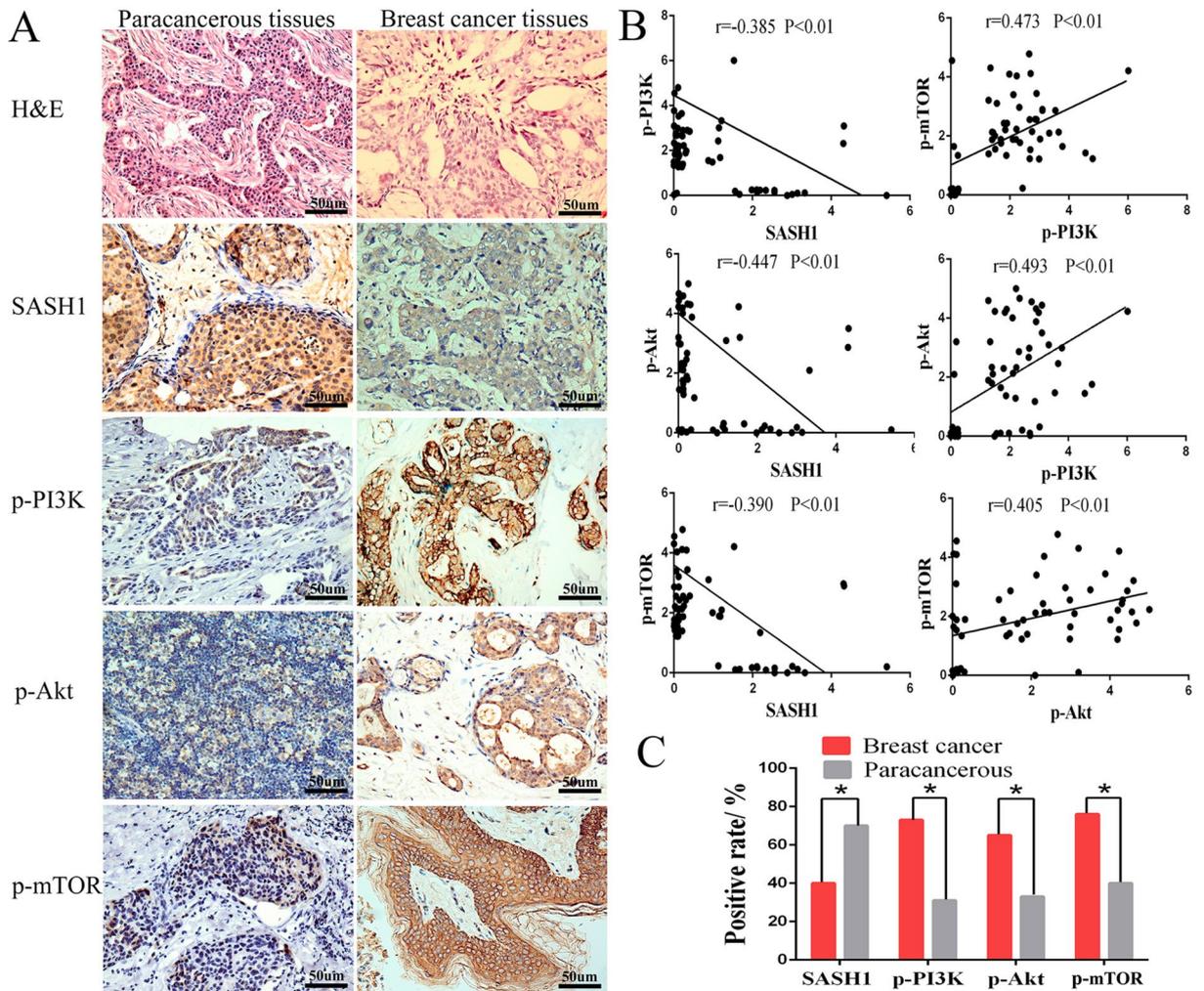


Fig. 5. The expression levels of SASH1 are negatively correlated with p-Akt, p-PI3K, and p-mTOR in breast cancer tissues. (A, C), The expression levels of SASH1, p-Akt, p-PI3K, and p-mTOR in breast cancer tissues. (B), The correlation analysis between SASH1 and p-Akt, p-PI3K, and p-mTOR. SASH1, SAM—and SH3 domain containing 1; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin.

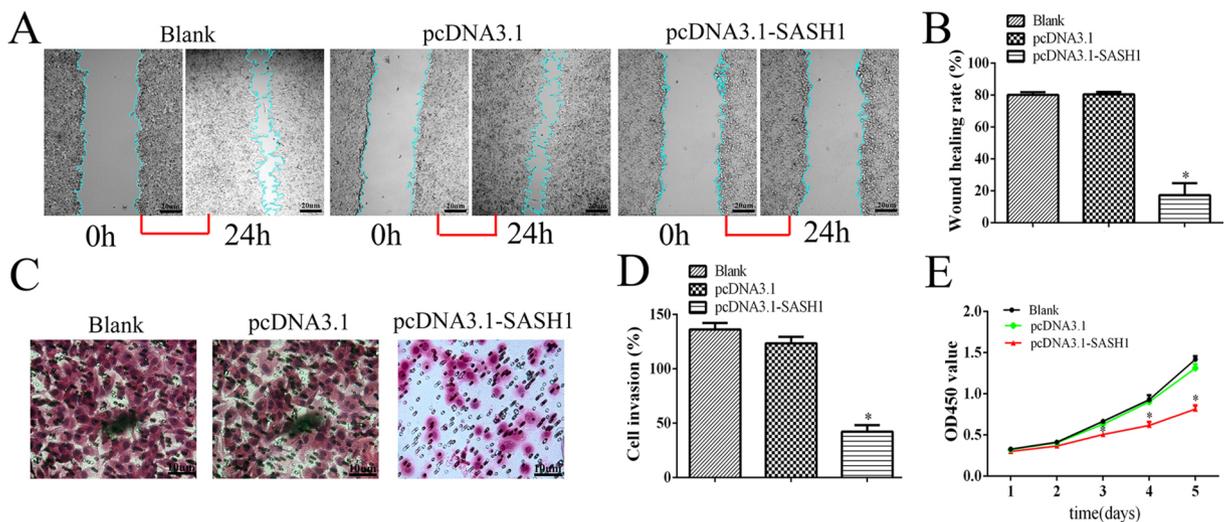


Fig. 6. The overexpression of SASH1 can inhibit the proliferation, migration, and invasion abilities of MDA-MB-231 cells. (A–D), The wound-closure assay and transwell invasion assay shown that SASH1 can inhibit the migration and invasion of MDA-MB-231 cells. (E), CCK8 assay shown that SASH1 can inhibit the proliferation of MDA-MB-231 cells. SASH1, SAM—and SH3 domain containing 1.

of MDA-MB-231/pcDNA3.1 cells ($P < 0.05$) (Fig. 6C,D). These findings suggest that SASH1 can inhibit the migration and invasion of breast cancer cells.

The CCK-88 assay was utilized to explore the impact of SASH1 on the proliferation of MDA-MB-231 cells. The results revealed that, starting from the 3rd day, the proliferative capacity of MDA-MB-231/pcDNA3.1-SASH1 cells was significantly lower compared to that of MDA-MB-231/Blank cells and MDA-MB-231/pcDNA3.1 cells. Overexpression of SASH1 significantly inhibited the proliferation of breast cancer MDA-MB-231 cells ($P < 0.05$). The proliferative capacity did not show a significant difference between MDA-MB-231/Blank cells and MDA-MB-231/pcDNA3.1 cells (Fig. 6E).

The overexpression of SASH1 can inhibit the PI3K-Akt-mTOR signaling pathway

In breast cancer tissues, we found that the expression levels of SASH1 were negatively correlated with the levels of p-Akt, p-PI3K, and p-mTOR, indicating that the overexpression of SASH1 can inhibit the PI3K-Akt-mTOR signaling pathway. To further validate this conclusion, we conducted cell experiments. Q-PCR results showed the successful construction of SASH1 overexpression cells (MDA-MB-231/pcDNA3.1-SASH1) (Fig. 7A). Western blotting results revealed that, compared to MDA-MB-231/Blank cells and MDA-MB-231/pcDNA3.1 cells, the expression levels of p-Akt, p-PI3K, and p-mTOR were significantly decreased in breast cancer cells with SASH1 overexpression (MDA-MB-231/pcDNA3.1-SASH1). This further supports the notion that SASH1 may regulate the progression of breast cancer via the PI3K-Akt-mTOR signaling pathway (Fig. 7B).

High expression of SASH1 can improve OS, RFS, and reduce PPS of breast cancer patients

Through TCGA database, the data on the relationship between the expression level of SASH1 and the prognosis of breast cancer were downloaded, including three datasets (213236_at, 226022_at, and 41644_at) (Fig. 8). According to the median value of SASH1 gene expression, breast cancer patients were divided into a SASH1 high expression group and a SASH1 low expression group. Survival analysis found that high expression of SASH1 could increase OS (213236_at: HR = 0.75, 226022_at: HR = 0.75, and 41644_at: HR = 0.81) and RFS (213236_at: HR = 0.82, 226022_at: HR = 0.78, and 41644_at: HR = 0.83) of patients, and the difference is statistically significant (all $P < 0.05$) (Fig. 8). However, the high expression of SASH1 could reduce PPS (213236_at: HR = 1.45 and

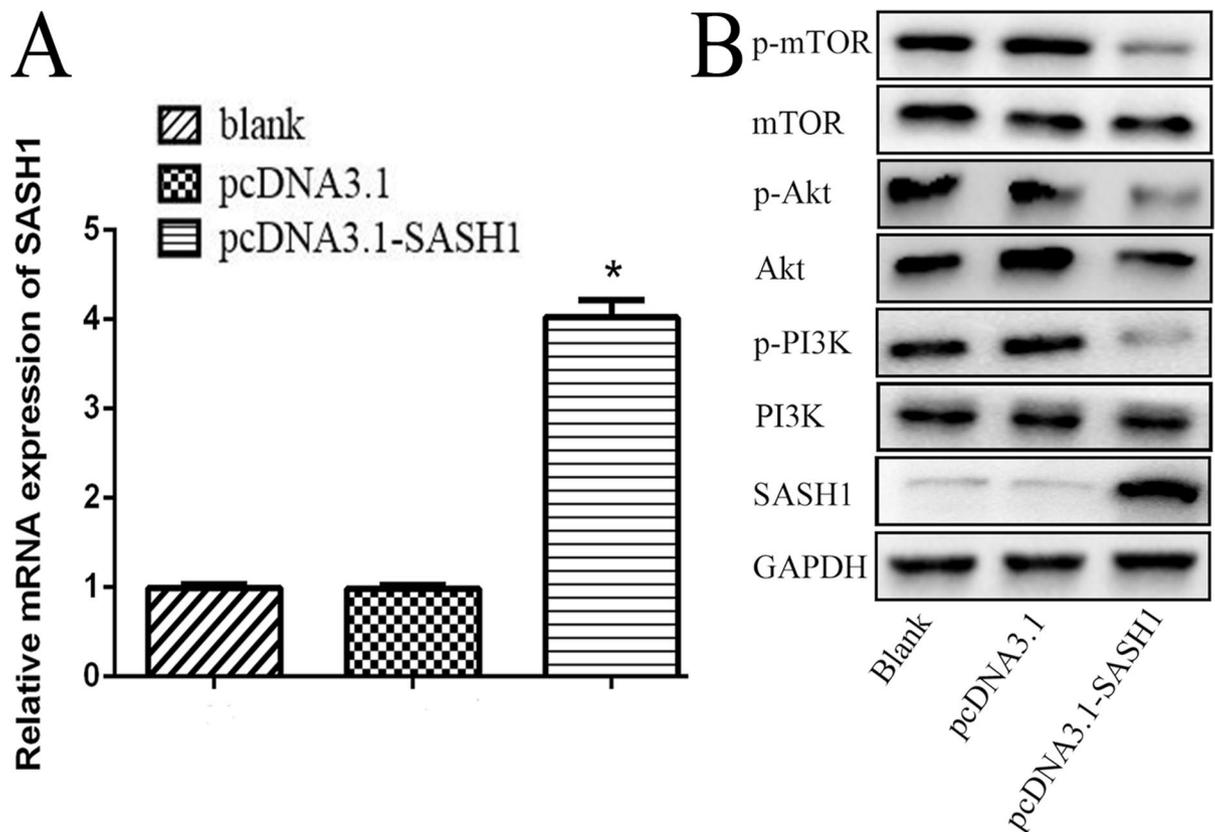


Fig. 7. The overexpression of SASH1 can inhibit the PI3K-Akt-mTOR signaling pathway. **(A)**, Q-PCR showed successful construction of SASH1 overexpression cells (MDA-MB-231/pcDNA3.1-SASH1). **(B)**, The expression levels of p-Akt, p-PI3K, and p-mTOR were significantly decreased in breast cancer cells of SASH1 overexpression (MDA-MB-231/pcDNA3.1-SASH1). SASH1, SAM— and SH3 domain containing 1; PI3K, phosphoinositide 3-kinase (This study explores PIK3 p110); Akt, protein kinase B (This study explores AKT-Thr308 phosphorylation sites); mTOR, mammalian target of rapamycin (This study explores mTORC1).

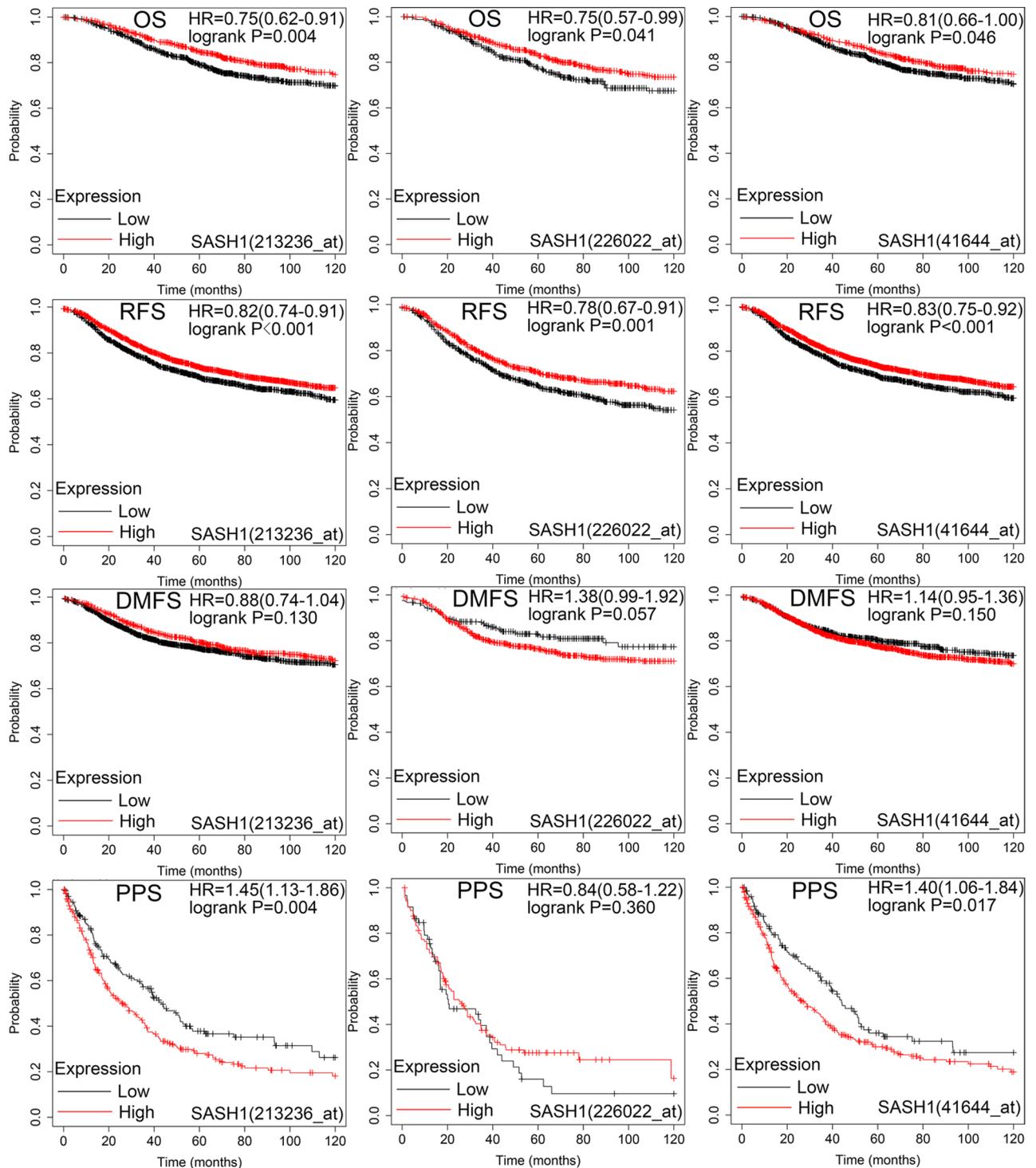


Fig. 8. The relationship between SASH1 expression levels and the prognosis of breast cancer patients. OS, overall survival; DMFS, distant metastasis free survival; RFS, recurrence free survival; and PPS, post-progression survival; SASH1, SAM—and SH3 domain containing 1.

41644_at: HR = 1.4) of patients, and the difference is statistically significant (all $P < 0.05$) (Fig. 8). High expression of SASH1 has no effect on the DMFS of patients (Fig. 8).

The relationship between the expression level of SASH1 and clinicopathological parameters in breast cancer patients

In the Breast Cancer Gene Expression Miner v4.9 database, clinicopathological data of breast cancer patients are available, including information from three datasets (213236_at, 226022_at, and 41644_at)³⁸. We found that the expression level of SASH1 was related to age and SBR grading. The expression level of SASH1 increased in

patients older than 51 years. The finding shows that SASH1 might improve the prognosis of elderly patients (> 51 years old). With the increase of SBR grading, the expression level of SASH1 gradually decreased, indicating that the higher the level of SASH1, the better the prognosis of breast cancer patients (Supplementary figs. 1, 2, 3).

Discussion

Despite being a member of the SLY family, SASH1's expression is limited in mature lymphocytes, indicating that it may have functions beyond the immune system³⁹. Recent studies provide growing evidence that SASH1 acts as a tumor suppressor gene^{30,33}. It is expressed in a variety of human tissues, but its expression is notably reduced or lost in solid tumors^{33,40}. Reintroducing SASH1 has been shown to significantly suppress tumor cell growth, proliferation, and migration, suggesting that SASH1 could be a valuable therapeutic target with potential clinical applications in cancer therapy^{40,41}.

Studies have demonstrated that SASH1 can inhibit cell proliferation⁴¹. An increase in SASH1 expression leads to a rise in cell adhesion proteins, which in turn inhibits cell migration⁵. On the other hand, reduced SASH1 expression diminishes cell adhesion capabilities and facilitates tumor cell migration^{19,42}. Gong et al.¹³ discovered that the expression level of SASH1 in tumor tissue (33.9%, 63/186) was significantly lower than that in patients with breast ductal hyperplasia (62.0%, 31/50), and the expression of SASH1 was associated with edge burr sign and lymph node metastasis. In our study, we conducted a comprehensive analysis of SASH1 expression in various tumors. We found that SASH1 expression levels were decreased in tumors (OR = 0.16, 95% CI = 0.09–0.28; $P < 0.001$), indicating its role as a tumor suppressor gene. These findings were also supported by the results of the pan-cancer analysis and immunohistochemistry assay. Moreover, we further analyzed the impact of SASH1 on the progression of breast cancer and found that the expression level of SASH1 in breast cancer tissue was significantly reduced. Cell experiments also revealed that overexpression of SASH1 can significantly reduce the proliferation, migration, and invasion of breast cancer cells. We examined the correlation between SASH1 and clinical pathological parameters in breast cancer patients, uncovering a relationship between SASH1 expression and both age and SBR grade. Low SASH1 expression was correlated with a higher malignancy level in breast cancer. These studies collectively suggest that SASH1 is downregulated in tumor tissues and is associated with tumor cell proliferation and metastasis.

As a scaffold protein, SASH1 is located in both the cytoplasm and nucleus of cells, recruiting numerous signaling molecule proteins^{4,8}. SASH1 can enhance LPS-induced cell migration by binding to TRAF6/TAK1/IKK/IKK, and it can also bind to MAP2K2/MAP4K4, thereby inhibiting ERK1/2 phosphorylation and suppressing tumor cell proliferation and migration^{8,27}. The overexpression of SASH1 may involve multiple cellular signaling pathways, necessitating further research to explore its spatiotemporal expression characteristics²⁷. In our prior studies, we discovered that SASH1 is downregulated in breast cancer⁴. SASH1 interacts with IQGAP1, and its decreased expression leads to reduced E-cadherin levels, which promotes epithelial-mesenchymal transition (EMT), lowers cell adhesion between tumor cells, and facilitates tumor cell migration⁴. SASH1 can influence tumor progression through various signaling pathways, with the PI3K-Akt-mTOR pathway being particularly crucial¹⁸. The abnormal activation of the PI3K-Akt-mTOR pathway in various tumors results in the dysregulation of downstream proteins related to the cell cycle, proliferation, and apoptosis, thereby contributing to tumor initiation and progression⁴³. The overexpression of the PI3K-Akt-mTOR pathway has been observed in cervical cancer tissues, potentially driving the occurrence, progression, and metastasis of cervical cancer⁴⁴. SASH1 can directly or indirectly interact with PI3K, inhibiting its kinase activity and reducing the production of PIP3⁶. This limits the membrane localization and activation of AKT⁶. By inhibiting PI3K, SASH1 reduces the phosphorylation of AKT⁴⁰. Additionally, SASH1 may directly interact with AKT or indirectly affect its activation through upstream negative regulatory proteins (such as PTEN)⁴⁰. Since the activities of mTORC1 and mTORC2 depend on the phosphorylation state of AKT, SASH1 further influences downstream mTOR signaling by regulating AKT activity, thereby affecting cell growth, survival, and metabolism⁶. However, whether SASH1 can exert its anticancer effects through the PI3K-Akt-mTOR signaling pathway in breast cancer remains understudied.

In this manuscript, we found through immunohistochemistry that the expression levels of SASH1 in breast cancer tissues are negatively correlated with the levels of p-Akt, p-PI3K, and p-mTOR, suggesting that overexpression of SASH1 can regulate the proliferation of breast cancer cells through the PI3K-Akt-mTOR signaling pathway. Our western blotting results further confirmed this conclusion. However, due to the lack of follow-up data from breast cancer patients, we downloaded data on the relationship between SASH1 and the prognosis of breast cancer patients from the TCGA database. The Kaplan–Meier method was used to plot the survival curve. The results revealed that SASH1 overexpression can improve the OS, RFS, and reduce the PPS of breast cancer patients.

In conclusion, our manuscript found that the expression level of SASH1 is downregulated in multiple types of tumors, suggesting a potential tumor-suppressive role for SASH1. The downregulation of SASH1 can promote the occurrence, proliferation, and metastasis of breast cancer. Overexpressing SASH1 can suppress the proliferation and invasion of breast cancer accompanied by the inhibition of the PI3K-Akt-mTOR signaling pathway. Moreover, SASH1 overexpression may improve OS and RFS in breast cancer patients. Therefore, SASH1 may serve as a novel therapeutic target in the treatment of tumors. However, our study also has certain limitations. For instance, we only validated that SASH1 regulates breast cancer proliferation and metastasis through the PI3K-Akt-mTOR signaling pathway in a single cell line. Additionally, we did not follow up on the survival time of breast cancer patients to investigate the impact of SASH1 on patient prognosis. These issues will need to be addressed in future research.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

SL conceptualized the study, visualized the data, developed the methodology used, applied the software, performed the statistical analysis, wrote the original draft, and reviewed and edited the manuscript. YS applied the software and performed the statistical analysis. XW and LD developed the methodology used. JT and KW visualized the data. CL conceptualized the study, visualized the data, developed the methodology, and wrote, reviewed and edited the manuscript. SL and CL confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

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Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

The study was performed in accordance with the Declaration of Helsinki, and all patients signed the informed consent form. This study received approval from the Ethics Committee of the Yongchuan People's Hospital of Chongqing (YCQRM.Y2023008).

Additional information

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