

Sperm-associated antigen 9 (SPAG9) promotes the survival and tumor growth of triple-negative breast cancer cells

Nirmala Jagadish¹ · Namita Gupta¹ · Sumit Agarwal¹ · Deepak Parashar¹ · Aditi Sharma¹ · Rukhsar Fatima¹ · Amos Prashant Topno¹ · Vikash Kumar¹ · Anil Suri¹

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Abstract Recently, we demonstrated the association of sperm-associated antigen 9 (SPAG9) expression with breast cancer. Among breast cancer, 15 % of the cancers are diagnosed as triple-negative breast cancers (TNBC) based on hormone receptor status and represent an important clinical challenge because of lack of effective available targeted therapy. Therefore, in the present investigation, plasmid-based small hairpin (small hairpin RNA (shRNA)) approach was used to ablate SPAG9 in aggressive breast cancer cell line model (MDA-MB-231) in order to understand the role of SPAG9 at molecular level in apoptosis, cell cycle, and epithelial-to-mesenchymal transition (EMT) signaling. Our data in MDA-MB-231 cells showed that ablation of SPAG9 resulted in membrane blebbing, increased mitochondrial membrane potential, DNA fragmentation, phosphatidyl serine surface expression, and caspase activation. SPAG9 depletion also resulted in cell cycle arrest in G₀–G₁ phase and induced cellular senescence. In addition, in in vitro and in vivo xenograft studies, ablation of SPAG9 resulted in upregulation of p21 along with pro-apoptotic molecules such as BAK, BAX, BIM, BID, NOXA, AIF, Cyto-C, PARP1, APAF1, Caspase 3, and Caspase 9 and epithelial marker, E-cadherin. Also, SPAG9-depleted cells showed downregulation of cyclin B1, cyclin

D1, cyclin E, CDK1, CDK4, CDK6, BCL2, Bcl-xL, XIAP, cIAP2, MCL1, GRP78, SLUG, SNAIL, TWIST, vimentin, N-cadherin, MMP2, MMP3, MMP9, SMA, and β -catenin. Collectively, our data suggests that SPAG9 promotes tumor growth by inhibiting apoptosis, altering cell cycle, and enhancing EMT signaling in in vitro cells and in vivo mouse model. Hence, SPAG9 may be a potential novel target for therapeutic use in TNBC treatment.

Keywords Triple-negative breast cancer · SPAG9 · Senescence · Cell growth · Cellular motility · Apoptosis · Tumor growth

Introduction

Breast cancer is the most common cancer among women and is the second leading cause of cancer-related death worldwide [1]. Recent studies have documented that early detection and diagnosis of breast cancer result in increased survival rate [2]. In developing countries, the mortality rate is even higher because of limited awareness and lack of infrastructure [2]. The intraductal carcinoma (IDC) of breast cancer is the most diagnosed among various histotypes of breast cancer [3]. Among breast cancer, 15 % of the cancers are diagnosed as triple-negative breast cancer (TNBC) based on the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2) receptor status [4]. TNBC is most difficult to treat because of non-responsiveness to hormone therapy, and hence, the prognosis is poor [4]. Thus, there is a need to identify novel candidate molecule for developing therapeutic target for TNBC for better cancer management. Recently, we have demonstrated that cancer testis (CT) antigens could be promising molecules for early detection and diagnosis for breast cancer [5]. Our recent studies

Namita Gupta and Sumit Agarwal contributed equally to this work.

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✉ Anil Suri
anil@nii.res.in

¹ Cancer Research Program, Cancer Microarray, Genes and Proteins Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110067, India

demonstrated abundant expression and association of sperm-associated antigen 9 (SPAG9) in IDC breast cancer specimen in various stages and grades [6]. Furthermore, we also reported the involvement of SPAG9 in TNBC cell line MDA-MB-231 in cellular proliferation and tumor growth in in vivo human breast cancer xenograft mice model [7].

In the present investigation, attempts were made to delineate the various molecular pathways involved in malignant properties of TNBC cells. Plasmid-based small hairpin (small hairpin RNA (shRNA)) approach was used to ablate SPAG9 in aggressive breast cancer cell line model (MDA-MB-231) to understand the role of SPAG9 at molecular level involved in cell cycle, apoptosis, and epithelial-to-mesenchymal transition (EMT). Our data demonstrated that ablation of SPAG9 resulted in reduced expression of cyclins and cyclin-dependent kinases during cell cycle. Furthermore, SPAG9 protein was also shown to be involved in apoptosis and EMT pathways. For the first time, we have put forth evidence that SPAG9 expression is involved in TNBC cell growth and may be a potential therapeutic target for TNBC patients.

Materials and methods

Cell culture

Four breast cancer cell lines of various histotypes BT-474 (luminal-B, ER⁺ PR⁺ Her2⁺), MCF-7 (luminal-A, ER⁺ PR⁺ Her2⁻), MDA-MB-231 (highly metastatic basal, triple-negative ER⁻ PR⁻ Her2⁻), and SK-BR-3 (HER2 overexpressing, ER⁻ PR⁻ Her2⁺) were used in this study. The cell lines were procured from American Type Culture Collection (ATCC, Manassas, VA) and were cultured according to the recommended growth medium and culture conditions. The cell lines were revived within a month of procurement and checked for mycoplasma contamination prior to their use for further experiments. MDA-MB-231 cell line was used to study the effect of SPAG9 gene and protein ablation on various properties of TNBC.

Indirect immunofluorescence

Indirect immunofluorescence (IIF) was carried out to study localization of SPAG9 in various sub-cellular organelles in different breast cancer cell lines as described earlier [8]. The cells were probed with rat anti-SPAG9 antibody followed by incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG antibody (Jackson ImmunoResearch Laboratories, Inc., Baltimore, USA). Subsequently, cells were probed with antibodies raised in mouse for different organelles: endoplasmic reticulum (calnexin, 6D195, sc-70481; Santa Cruz Biotechnology, Santa Cruz, CA), Golgi bodies (GM130, sc-55591; Santa Cruz Biotechnology),

mitochondria (MTCO2; ab3298; Abcam), and nuclear envelope (lamin A/C, sc-7292; Santa Cruz Biotechnology). Subsequently, cells were probed with goat anti-mouse Texas Red secondary antibody (Jackson ImmunoResearch Laboratories, Inc., Baltimore, USA). Photo micrographs were captured using the Carl Zeiss LSM 510 Meta confocal microscope (Zeiss, Oberkochen, Germany) in central confocal microscopy facility.

Sperm-associated antigen 9 gene silencing and transient transfection

In order to study the role of SPAG9 at molecular level in various malignant properties of breast cancer cells, transient transfection was carried out in MDA-MB-231 cells by two shRNA constructs (shRNA1 and shRNA2) against SPAG9 along with scrambled shRNA (NC shRNA) as described earlier [9]. Scrambled shRNA (NC shRNA) was used as a negative control. The transient transfection was carried out with 6 µg of shRNA plasmid using Lipofectamine (Invitrogen, Thermo Fisher Scientific, MA, USA) in optiMEM media (Gibco, Thermo Fisher Scientific, MA, USA). Cells were harvested post 48-h transfection for Western blot analysis and all in vitro assays.

Western blotting

SPAG9 protein expression in MDA-MB-231 cells transfected with NC shRNA, SPAG9 shRNA1, and shRNA2 was assessed by Western blotting as described earlier [8]. Post 48 h of transient transfection, lysates were prepared using cell lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 1 % Triton X-100). Protein lysates (10 µg/lane) were resolved on 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. To check the SPAG9 protein expression, rat anti-SPAG9 antibody was used as a primary antibody and goat anti-rat IgG horseradish peroxidase (HRP) as a secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The immunoblots were developed with the Immobilon Western Chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA) according to the manufacturer's protocol. Similarly, Western blotting was also carried out for various molecules involved in cell cycle, apoptosis, and EMT. Western blotting was repeated in three independent experiments.

Antibodies

The following antibodies were used for apoptosis studies: [AIF (ab89583), Cyto-C (sc-13560), Caspase 3 (sc-56052), Caspase 7 (sc-81654), Caspase 9 (sc-56077), PARP1 (CST9532), BCL-2 (B3170), Bcl-x_L (B9429), cIAP2 (sc-

7944), BAX (B8554), BIM (sc-11425), BAK (sc-7873), BAD (sc-8044), BID (sc-11423), XIAP (ab2541), MCL-1 (ab32087), NOXA (sc-56169), and GRP78 (sc-166490)]; for cell cycle studies: [cyclin B1 (sc-7393), cyclin D1 (sc-8396), cyclin E (sc-56310), p21 (sc-817), CDK1 (ab18), CDK4 (sc-23896), and CDK6 (sc-7961)]; for EMT studies [E-cadherin (ab1416), N-cadherin (ab 76011), SNAIL (ab85931), SLUG (ab51772), SMA (ab7817), MMP2 (ab92536), MMP3 (ab52915), MMP9 (ab119906), TWIST (ab50887), vimentin (ab92547), and β -catenin (sc-57535)]; and for senescence studies: DcR2 (ab2019).

Cellular senescence assay

Cellular senescence was visualized in cells transfected with NC shRNA, SPAG9 shRNA1, and shRNA2 as described earlier [8]. β -Galactosidase activity was assessed using Senescence kit (Sigma-Aldrich, St. Louis, MO, USA) as per manufacturer's protocol. Transfected cells were fixed and stained with X-gal. The images were captured using ELWTD1-SNCP camera (Nikon, Fukuoka, Japan). The three independent experiments were performed in triplicates.

Cell cycle analysis by propidium iodide staining

NC shRNA-, SPAG9 shRNA1-, and shRNA2-transfected MDA-MB-231 cells were harvested by trypsinization, ethanol fixed, and treated with propidium iodide (PI) and RNAase solution for 30 min. The acquisition and analysis were done using BD-FACS VERSE (BD Biosciences, CA, USA). The three independent experiments were performed in triplicates.

Mitochondrial membrane potential

MDA-MB-231 cells transfected with NC shRNA, SPAG9 shRNA1, and shRNA2 were stained with JC-1 (BD MitoScreen Kit) for 2 min at 37 °C. Mitochondrial membrane potential was analyzed using BD-FACS VERSE (BD Biosciences, CA, USA). The three independent experiments were performed in triplicates.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

DNA damage in MDA-MB-231 cells due to SPAG9 knock-down was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using In-Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, GmbH, Mannheim, Germany). NC shRNA-, SPAG9 shRNA1-, and shRNA2-transfected cells were harvested and processed as per manufacturer's instructions. The cells were analyzed at 488 nm using BD-FACS VERSE (BD

Biosciences, CA, USA). The three independent experiments were performed in triplicates.

Annexin V staining

Annexin V staining was done to determine the surface expression of phosphatidyl serine, a key biochemical feature of apoptosis, in the cells transfected with SPAG9 shRNA1 and shRNA2 as compared to NC shRNA. Staining was done using annexin V-FITC kit as per manufacturer's protocol. The samples were analyzed with BD-FACS VERSE (BD Biosciences, CA, USA). The three independent experiments were performed in triplicates.

M30 assay

M30 assay was carried out to assess the caspase activation as described earlier [8]. Cells transfected with NC shRNA, SPAG9 shRNA1, and shRNA2 were incubated with M30 antibody (Roche Diagnostics, GmbH, Mannheim, Germany) followed by incubation with anti-mouse FITC (Jackson ImmunoResearch Laboratories, Inc., Baltimore, USA). The acquisition was done using BD-FACS VERSE (BD Biosciences, CA, USA). The three independent experiments were performed in triplicates.

Scanning electron microscopy

The effect of SPAG9 shRNA1 and shRNA2 on the phenotypic characteristics of MDA-MB-231 cells was demonstrated using scanning electron microscopy (SEM). The cells were fixed and processed as described earlier [8]. The SEM images were captured using electron microscope (EVO LSM10 Zeiss, Germany) at 20 kV using SmartSEM software in central microscope facility.

Immunohistochemistry

Tumor growth experiments of human tumor xenograft of breast cancer MDA-MB-231 cells were carried out in 16 athymic nude mice. Animals were subcutaneously injected with MDA-MB-231 cells. When the tumor size reached between 50 and 100 mm³, animals were divided in two groups: control animals ($n = 8$) injected with NC shRNA and experimental animals injected with SPAG9 shRNA2 ($n = 8$ mice) as described earlier [7]. Animals were sacrificed for immunohistochemistry (IHC) as explained earlier [7]. IHC analysis was performed on 4- μ m-thick sections of tumor tissue specimens of mice treated with NC shRNA and SPAG9 shRNA2 as described earlier [7]. Briefly, serial sections were deparaffinized and rehydrated using decreasing gradients of ethanol. Serial sections were probed with primary antibody as detailed earlier followed by incubation with secondary

antibody. Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO, USA). Slides were counterstained with hematoxylin solution and mounted and images captured under a Nikon Eclipse E400 microscope (Nikon, Fukuoka, Japan).

Statistics

The statistical significance of the results of in vitro and in vivo data was determined by the Student's *t* test using the SPSS version 20.0 statistical software package (SPSS Inc., Chicago, IL). A *p* value of less than 0.05 was considered statistically significant. All experimental data are presented as mean \pm standard error.

Results

Sperm-associated antigen 9 protein localization in breast cancer cell lines

SPAG9 protein localization was analyzed in different breast cancer cell lines using confocal microscopy. Confocal microscopy images showed a cytoplasmic distribution of SPAG9 protein in cancer cell lines BT-474, MCF7, MDA-MB-231, and SK-BR-3 with prominent localization in endoplasmic reticulum, Golgi bodies, and mitochondria. However, SPAG9 did not localize with nuclear envelope (Fig. 1).

Plasmid-based gene silencing ablates sperm-associated antigen 9 protein expression

Two shRNA targets against *SPAG9* gene and NC shRNA were used to find out the knockdown efficiency in MDA-MB-231 cells by Western blotting. Our analysis revealed that NC shRNA did not ablate the protein. In contrast, SPAG9 shRNA1 and shRNA2 were found to ablate SPAG9 protein in breast cancer cells (Fig. 2a).

Sperm-associated antigen 9 small hairpin RNA inhibits cellular proliferation and initiate cellular senescence

Previously, we have described the association of SPAG9 on cellular growth and colony formation ability [7]. To further validate our results, cell cycle analysis by PI staining was performed. Knockdown of SPAG9 by SPAG9 shRNA1 (67.03 %) and shRNA2 (65.14 %) resulted in accumulation of most of the cells in G1 phase as compared to NC shRNA (63.50 %)-transfected cells (Fig. 2b). Also, the percentage of G2-M phase cells showed decrease in SPAG9 shRNA [shRNA1 (23.08 %), shRNA2 (26.42 %)]-transfected cells as compared to NC shRNA (28.26 %)-transfected cells (Fig. 2b).

To study the molecules involved during cellular proliferation, Western blotting was carried out to study the various molecules in different phases of cell cycle, which showed that there was a significant decrease in cyclins and cyclin-dependent kinases such as cyclin B1, cyclin D1, cyclin E, CDK1, CDK4, and CDK6. Also, upregulation was observed in case of tumor suppressor protein, p21 (Fig. 2c). These results suggest that SPAG9 ablation results in cell cycle arrest of breast cancer cells and inhibited cellular proliferation by downregulation of cyclins and cyclin-dependent kinases.

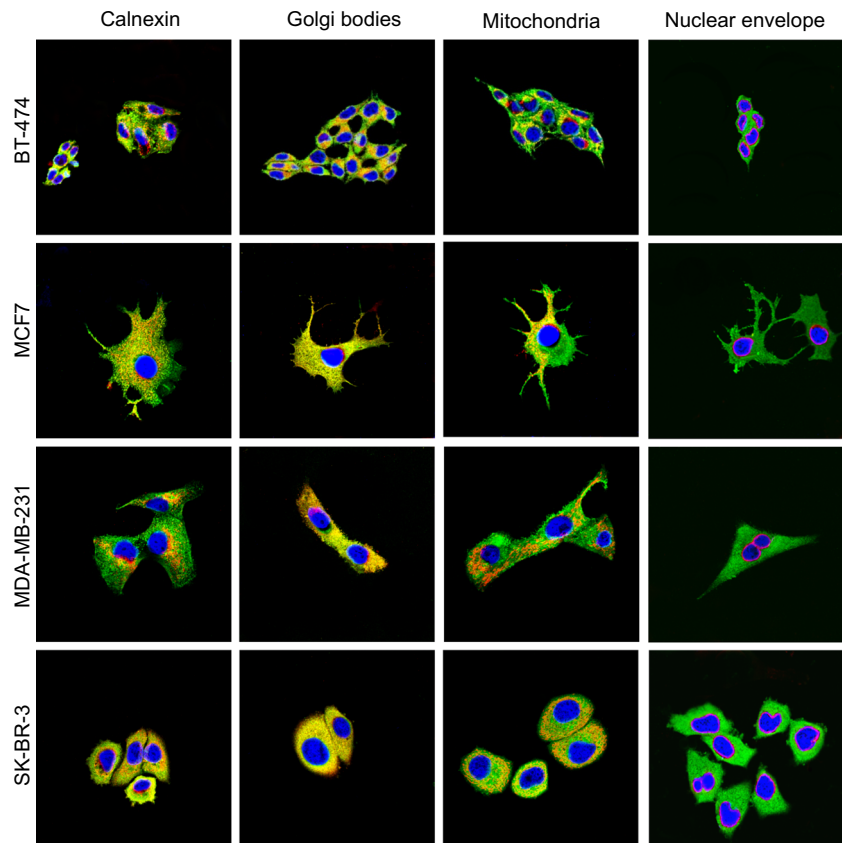
Reduction in cellular proliferation may also be as a result of cell entering into senescence state; therefore, breast cancer cells were transfected with SPAG9 shRNA, and cellular senescence was assessed by β -galactosidase staining. The percentage of senescent cells was significantly higher ($p < 0.0001$), 52.6 % and 67.0 %, when transfected with SPAG9 shRNA1 and shRNA2, respectively, as compared to 7.4 % when transfected with NC shRNA (Fig. 2d). Furthermore, enhanced expression of the level of DcR2 was observed after knockdown of SPAG9 by two SPAG9 shRNA targets as compared to NC shRNA-transfected cells. The results confirmed that ablation of SPAG9 protein contributes toward reduced cellular growth through onset of senescence.

To validate the in vitro effects of SPAG9 ablation on cell cycle, in vivo MDA-MB-231 xenograft mouse model was established. Our studies demonstrated significant reduction in tumor size of shRNA2-treated tumor as compared to NC shRNA-treated tumor (Fig. 2e). IHC analysis of tumor serial sections revealed significantly enhanced immunoreactivity of p21 and decreased immunoreactivity of cyclin B1, cyclin D1, cyclin E, CDK4, and CDK6 in SPAG9 shRNA2-treated mice as compared to NC shRNA-treated mice (Fig. 2f).

Effect of sperm-associated antigen 9 small hairpin RNA on apoptosis

Various biochemical assays (JC1 staining, TUNEL, annexin V-FITC staining assay, and M30 assay) were performed to study the initiation of onset of apoptosis in SPAG9-depleted cells (Fig. 3). JC1 assay showed that the ratio of FL2 red and FL1 green was significantly reduced ($p < 0.005$) post SPAG9 shRNA1 and SPAG9 shRNA2 treatment indicating early apoptosis (Fig. 3a, b). TUNEL assay showed that apoptosis was induced in 49.9 % of SPAG9 shRNA1- and 30.8 % of SPAG9 shRNA2-transfected cells as compared to 1.33 % of NC shRNA-transfected cells (Fig. 3a, b). Similarly, annexin V-FITC staining showed 40.9 % of SPAG9 shRNA1- and 58.2 % of SPAG9 shRNA2-transfected cells as compared to 2.7 % of NC shRNA-transfected cells (Fig. 3a, b). M30 assay was also performed to further evaluate the effect of SPAG9 knockdown on apoptosis. The result showed a significant increase in apoptosis in SPAG9 shRNA1- and SPAG9 shRNA2-transfected cells as compared to NC shRNA-transfected cells.

Fig. 1 SPAG9 co-localization in various cell organelles in different breast cancer cells. The confocal images exhibit the co-localization (orange-yellow) of SPAG9 (green) in endoplasmic reticulum, Golgi bodies, and mitochondria (red), whereas no co-localization was observed in nuclear envelope. Nucleus was stained blue with DAPI. Original magnification $\times 630$, objective $\times 63$



As depicted in Fig. 3a, b, 40.1 and 43.7 % of cells were positive for M30 in SPAG9 shRNA1- and SPAG9 shRNA2-transfected cells as compared to only 2.03 % of NC shRNA-transfected cells.

Moreover, the morphological changes due to the onset of apoptosis process were studied by scanning electron microscopy (SEM) in MDA-MB-231-transfected cells with NC shRNA, SPAG9 shRNA1, and shRNA2. As depicted in Fig. 3c, the photomicrograph showed a significant increase in apoptosis in SPAG9 shRNA1- and shRNA2-transfected cells as compared to NC shRNA-transfected cells (Fig. 3c). Blebbing, holes, and apoptotic bodies were seen in cells post 24 and 48 h of transfection with SPAG9 shRNA1 and shRNA2. However, no morphological changes were observed in cells transfected with NC shRNA (Fig. 3c).

Further, to study the involvement of SPAG9 in apoptosis, various molecules of intrinsic apoptotic pathway including anti-apoptotic and pro-apoptotic molecules were investigated by Western blot analysis. Our results revealed that apoptotic molecules BAD, BAK, BAX, BIM, BID, AIF, Cyto-C, NOXA, PARP1, Caspase 3, Caspase 7, and Caspase 9 were upregulated. However, anti-apoptotic molecules cIAP2, BCL2, and Bcl-x_L were downregulated (Fig. 4a). Similarly, we also found

upregulation of apoptotic molecules BAD, BAX cyto-C, and Caspase 7 and downregulation of anti-apoptotic molecule, BCL-X_L, in SPAG9-depleted MCF7, BT-474, and SK-BR-3 cells (Supplementary Fig. 1).

In addition, to validate our in vitro findings, human MDA-MB-231 xenografts treated with SPAG9 shRNA2 were probed for molecules involved in apoptotic pathway. IHC analysis revealed increased expression of apoptotic proteins such as BAK, BAX, BIM, BID, NOXA, AIF, Cyto-C, PARP1, APAF1, Caspase 3, and Caspase 9, whereas reduction in BCL2, Bcl-x_L, XIAP, cIAP2, GRP78, and MCL1 was observed when treated with SPAG9 shRNA2 as compared NC shRNA-treated mice (Fig. 4b). These results suggest that SPAG9 is important for cell viability of MDA-MB-231 cells and is in contrast with our in vitro findings.

Sperm-associated antigen 9 gene silencing reduces cellular motility

Earlier, we have demonstrated the effect of SPAG9 knockdown on cellular motility by analyzing migration and invasion ability of MDA-MB-231 cells [7]. To evaluate the signaling pathways that may be involved in cellular motility, different molecules of epithelial-

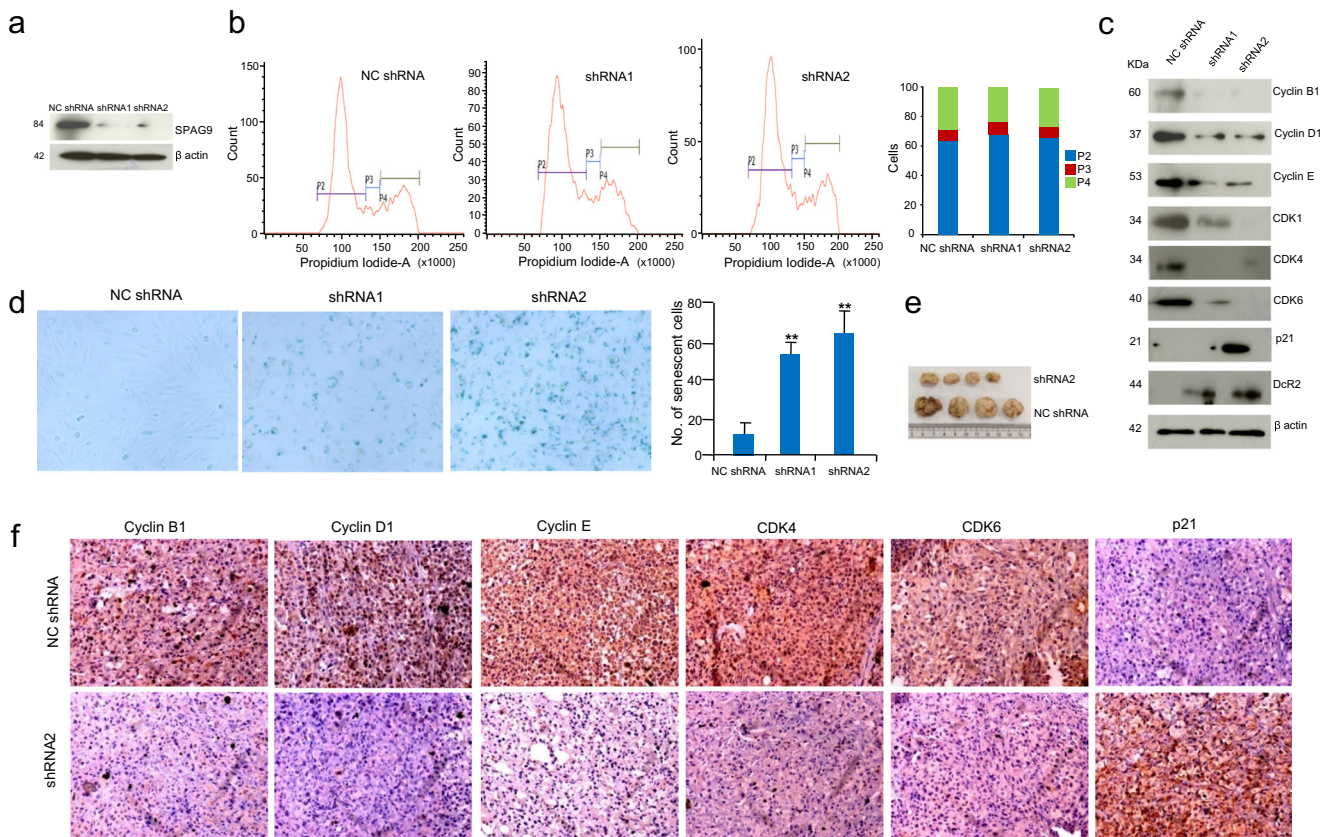


Fig. 2 SPAG9 depletion arrests cell cycle and induces cellular senescence. **a** Western blot analysis shows significant SPAG9 protein ablation in shRNA1- and shRNA2-transfected cells as compared to NC shRNA-transfected cells. **b** Histogram and bar diagram depict accumulation of cells in G0–G1 phase when transfected with shRNA1 and shRNA2 as compared to when transfected with NC shRNA (P2: G0–G1, P3: S, P4: G2–M phase). **c** Western blot data reveals downregulation of cyclin B1, cyclin D1, cyclin E, CDK1, CDK4, and CDK6 and upregulation of p21 and senescence marker: DcR2 in shRNA1- and shRNA2-transfected cells as compared to NC shRNA-transfected cells. **d** Phase contrast images and bar diagram show increased percentage of

cells undergoing senescence (*blue staining*) in shRNA1- and shRNA2-transfected cells as compared to NC shRNA-transfected cells. Original magnification $\times 100$, objective $\times 10$. **e** Tumor size when compared on scale shows significantly reduced size when treated with shRNA2 as compared to NC shRNA-treated tumor. **f** Representative photographs of IHC analysis show reduced immunoreactivity of cyclin B1, cyclin D1, cyclin E, CDK4, and CDK6 and increased immunoreactivity of p21 in shRNA2-treated tumor sections as compared to NC shRNA-treated tumor sections. Original magnification $\times 200$, objective $\times 20$. The three independent experiments were performed in triplicates for each assay. ** $p < 0.0001$

mesenchymal transition (EMT) were studied in in vitro and in vivo models. As shown in Fig. 5a, epithelial marker, E-cadherin, was upregulated, while mesenchymal markers SLUG, SNAIL, TWIST, vimentin, N-cadherin, MMP2, MMP3, MMP9, SMA, and β -catenin were downregulated in SPAG9 shRNA1- and shRNA2-transfected cells. These results suggested that SPAG9 may be involved in cellular motility via EMT pathway.

Furthermore, the xenograft tumor specimen sections were also probed for EMT pathway molecules. IHC analysis demonstrated increased immunoreactivity of E-cadherin in SPAG9 shRNA2-treated mice and reduction in SNAIL, TWIST, vimentin, N-cadherin, MMP2, MMP3, MMP9, SMA, and β -catenin as compared to NC shRNA treatment (Fig. 5b). Our findings indicate that SPAG9 plays an important role in cellular motility of MDA-MB-231 cells via EMT pathway.

Discussion

Breast cancer is the most frequently diagnosed cancer in developing countries and accounts for 15 % of total cancer-related death in women worldwide [1]. Among all diagnosed breast cancers, about 15 % patients are diagnosed with triple-negative breast cancer (TNBC). TNBC patients are difficult to treat timely because of limited treatment options [4]. Various studies are being conducted worldwide to identify and characterize cancer-associated molecules, which may have therapeutic potential for better cancer management. In this context, cancer testis (CT) antigens are the unique class of antigens which are abundantly expressed in various malignancies and not in normal tissues except testis [5]. Hence, CT antigens could be the ideal targets for the development of early detection and diagnosis and therapeutic use [5, 10, 11]. So far, only few CT antigens have been reported in TNBC patients [7,

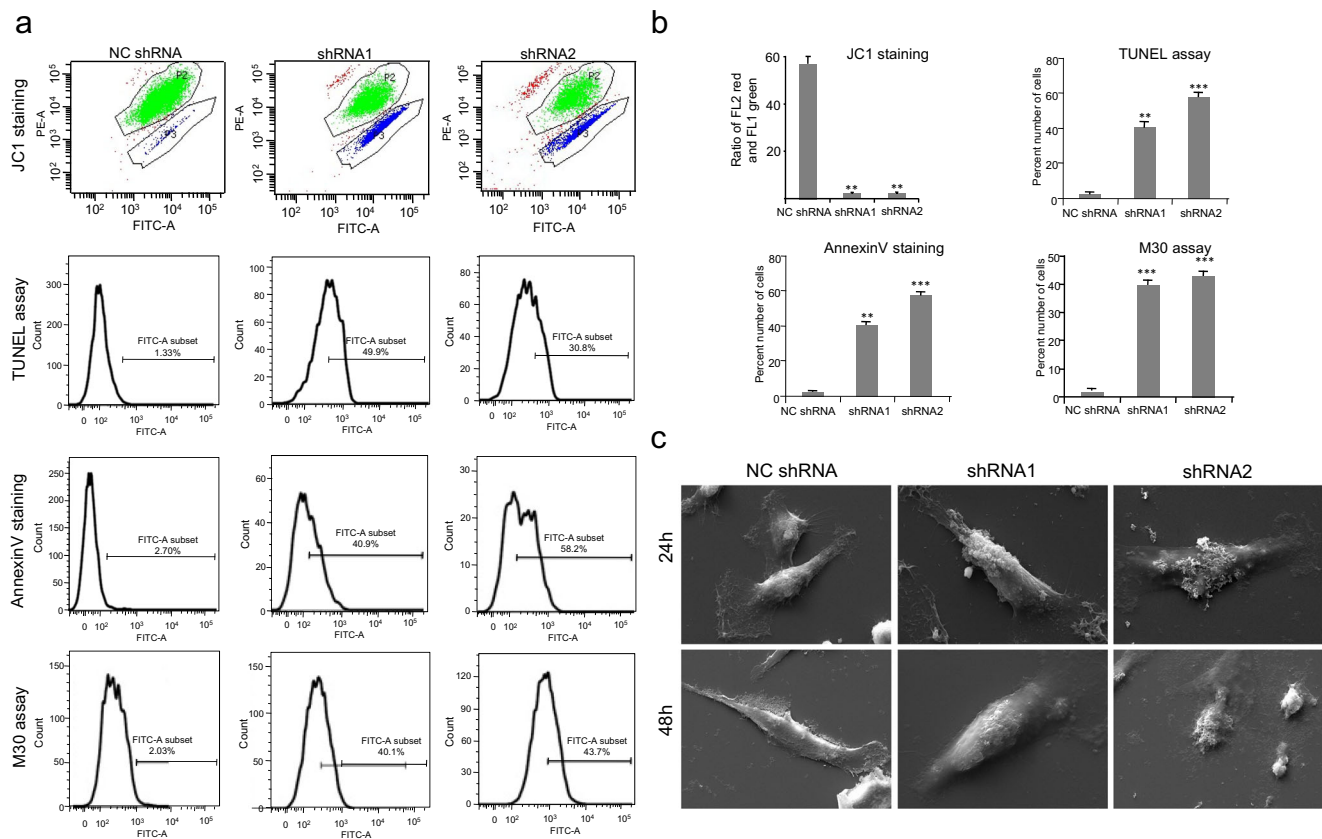


Fig. 3 *SPAG9* gene ablation initiates apoptosis. **a** Flow cytometric analysis demonstrates the effects of *SPAG9* ablation on onset of apoptosis in MDA-MB-231 cells by JC1 staining (mitochondrial membrane potential), TUNEL assay (DNA damage), annexin V staining (phosphatidyl serine expression), and M30 assay (caspase activation). **b** Histogram depicts percentage of cells with decreased mitochondrial membrane potential, increased DNA damage, enhanced phosphatidyl serine expression, and increased caspase activation in *SPAG9* shRNA1 and shRNA2 cells transfected with NC shRNA. **c**

Photomicrographs of scanning electron microscope (SEM) show MDA-MB-231 cells undergoing apoptosis after shRNA1 and shRNA2 treatments. Blebbing and disordered structure of cells are present in images taken at 24 and 48 h post-transfection by shRNA1 and shRNA2, whereas NC shRNA group cells are unaffected. The experiments were done three times in triplicates, and the results are from representative experiments. Magnification $\times 6000$, WD = 6 mm, EHT = 20.00 kV

12–14]. Recently, our group reported an association of *SPAG9* in 90 % of breast cancer patients [6]. Further, we demonstrated the involvement of *SPAG9* in colony formation, migration and invasion, cellular motility, and tumor growth in TNBC cell line [7]. In the present investigation, we examined the role of *SPAG9* in the various molecular pathways involved in TNBC development. Plasmid-based gene silencing approach was employed to study the effect of *SPAG9* at molecular level in *in vitro* and *in vivo* human breast cancer xenograft mouse models.

Tumor growth is an outcome of alteration in expression of various gene products, which are involved in cell cycle regulation *viz.*, cyclin-dependent kinase and cyclin complexes [15]. Our earlier studies demonstrated that knockdown of *SPAG9* resulted in reduced cellular proliferation and colony-forming ability of triple-negative MDA-MB-231 cells [7]. This led us to further investigate the expression pattern of different cell cycle regulators in *SPAG9*-depleted cells. Interestingly, we found that

knockdown of *SPAG9* in TNBC cells showed decreased expression of cyclin B1, cyclin D1, and cyclin E along with kinases CDK1, CDK4, and CDK6 and thus inhibited cellular proliferation. Similar observations were shown in CT antigen CAGE, which revealed that knockdown of CAGE inhibited cellular proliferation and showed reduced levels of cyclin D1 and cyclin E [16]. Yet, another study showed that silencing of testis-restricted dual-specificity phosphatase 21 (DUSP1, a CT antigen) resulted in cell cycle arrest in G1 phase with increased levels of p21, p27, and p53 [17]. These findings support our data that *SPAG9* gene silencing results in increased levels of cyclin-dependent kinase inhibitor molecule p21. Cell cycle arrest could result in cellular senescence; in this context, we found upregulation of DcR2, a putative marker of senescence. Further similar to our study, knockdown of Brother of the regulator of imprinting sites (BORIS), CT antigen, reduced cellular proliferation by inducing cellular senescence [18].

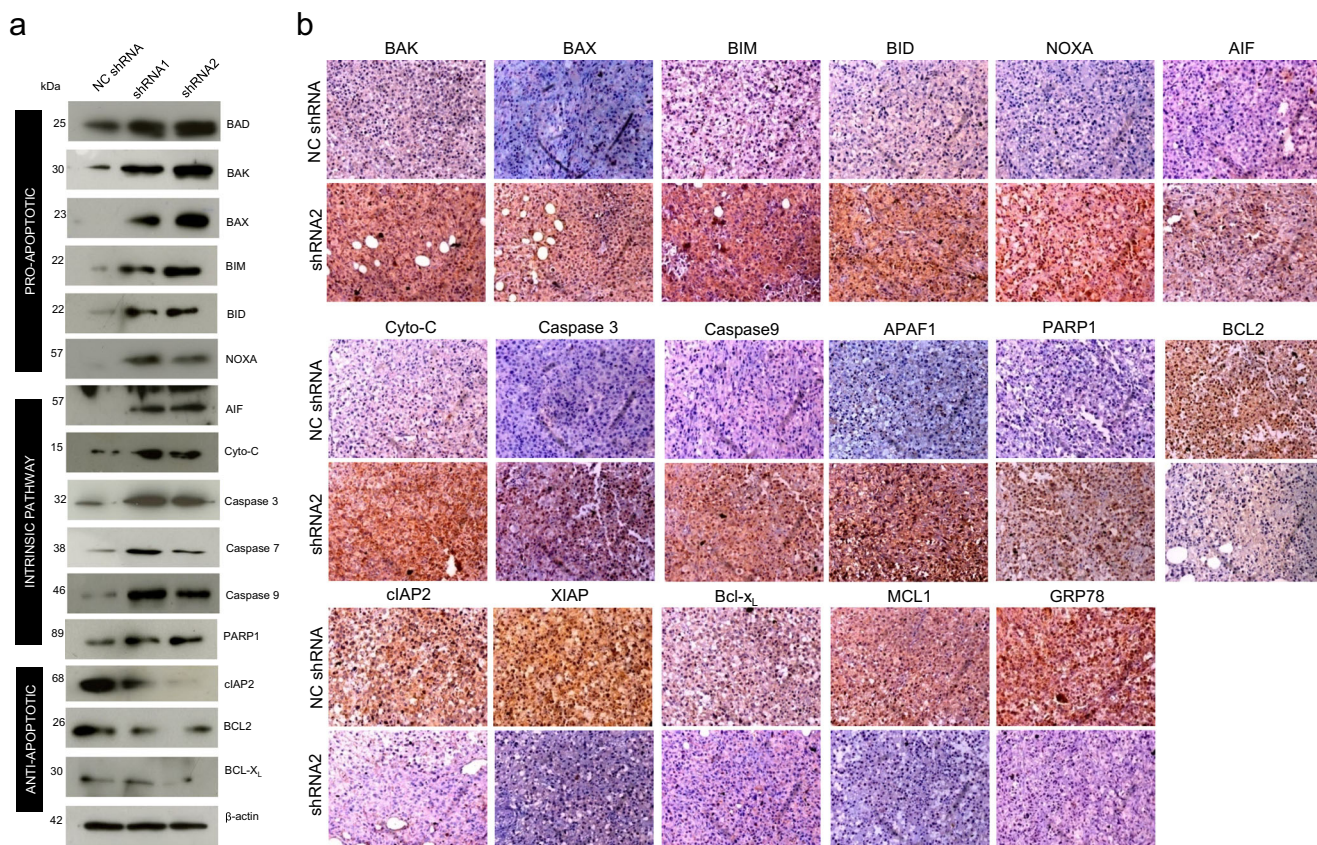


Fig. 4 *SPAG9* knockdown alters the expression of various molecules involved in apoptosis. **a** Western blot analysis demonstrates that *SPAG9* knockdown by shRNA1 and shRNA2 resulted in increased level of BAD, BAK, BAX, BIM, BID, NOXA, AIF, Cyto-C, caspase 3, caspase 7, and caspase 9 and decreased expression of PARP1, cIAP2, BCL2, and Bcl-x_L. β-Actin was used as internal loading control. **b** Representative images of

IHC analysis of tumor serial sections show increased immunoreactivity of BAK, BAX, BIM, BID, NOXA, AIF, Cyto-C, caspase 3, caspase 9, and APAF1 and reduced immunoreactivity of XIAP, cIAP2, BCL2, Bcl-x_L, MCL1, and GRP78 in shRNA2-treated tumor as compared to NC shRNA-treated tumor. The three independent experiments were performed in triplicates. Original magnification $\times 200$, objective $\times 20$

Therefore, our study suggested that *SPAG9* plays an important role in apoptosis and also induces cellular senescence process.

Conventional chemotherapy in various cancers is toxic and has adverse effect on normal somatic tissue [19]. In this context, many studies have suggested that CT antigens could be a potential target for cancer therapy based on the fact that these are expressed only in tumors, and hence, normal cells would be unaffected [20]. Previously, it has been shown that CT antigens particularly CAGE, XAGE, and MAGE proteins play an important role in apoptosis [16, 21, 22]. Our previous study demonstrated that ablation of *SPAG9* inhibited cellular proliferation and colony formation ability in MDA-MB-231 cells [7]. In addition, our studies in *in vivo* breast cancer cell [MDA-MB-231] xenograft mouse model further demonstrated that downregulation of *SPAG9* resulted in reduced tumor growth, which may be due to the cell death [7]. In the present study, we demonstrated that ablation of *SPAG9* in MDA-MB-231 cells induces apoptosis by increasing pro-apoptotic molecules and decreasing anti-

apoptotic molecules. Similar results were reported for MAGE where ablation of MAGE resulted in increase of pro-apoptotic molecules [22]. We further demonstrated onset of apoptosis at phenotypic level by capturing SEM images, which showed that *SPAG9*-depleted cells revealed blebbing, holes, and apoptotic bodies, which are signature features of cells undergoing apoptosis. It is important to mention that ours is the first study to show this phenotypic change through SEM imaging in TNBC cells.

Cancer cells acquire migration and invasion abilities as a result of epithelial-mesenchymal transition (EMT), which is a reversible process and is facilitated by down-regulation of cell-cell junction and single-cell detachment [23]. In EMT, upstream signals including Wnt, TGF β , FGF, and EGF lead to upregulation of transcription repressor SLUG, SNAIL, TWIST, vimentin, N-cadherin, SMA, and β -catenin as well as MMP2, MMP3, and MMP9, which are involved in extracellular matrix breakdown [24, 25]. Our earlier studies demonstrated that ablation of *SPAG9* resulted in reduced migration, invasion,

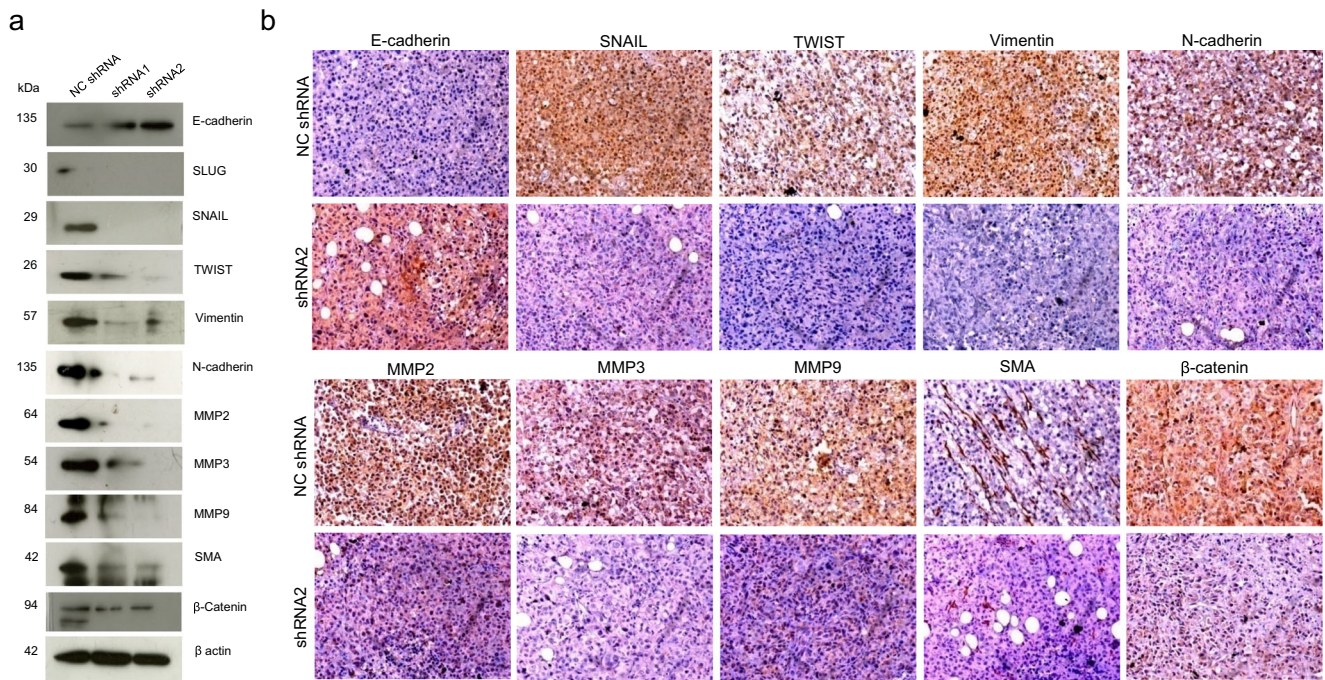


Fig. 5 Ablation of SPAG9 inhibits epithelial-to-mesenchymal transition in MDA-MB-231 cells in vitro and in vivo models. **a** Western blots depicting the level of E-cadherin was upregulated in shRNA1- and shRNA2-transfected cells as compared to NC shRNA-transfected cells, whereas SLUG, SNAIL, TWIST, vimentin, N-cadherin, MMP2, MMP3, MMP9, SMA, and β-catenin were decreased. **b** IHC analysis

of tumor serial sections demonstrates increased immunoreactivity of E-cadherin, while decreased immunoreactivity of SLUG, SNAIL, TWIST, vimentin, N-cadherin, MMP2, MMP3, MMP9, SMA, and β-catenin in shRNA2-transfected cells as compared to NC shRNA-transfected cells. The three independent experiments were performed in triplicates. Original magnification $\times 200$, objective $\times 20$

and cellular motility in TNBC cells [7]. Interestingly, in the present in vitro investigation, we found that ablation of SPAG9 resulted in downregulation of N-cadherin and SLUG, which are essential molecules for mesenchymal transformation, whereas E-cadherin and MMP2 were upregulated, which opposes the initiation of EMT. In consistent with our finding, other studies on CT antigens such as SSX, MAGE-D4B, CAGE, piwil2, and CT45A1, also demonstrated enhanced EMT by promoting upregulation of metastasis and mesenchymal marker proteins [26]. Furthermore, another study suggested that SSX promotes EMT pathway by upregulating the mesenchymal marker MMP2 [27]. Furthermore, our in vitro studies corroborated the finding in TNBC tumor xenograft mice model by immunohistochemical (IHC) analysis, which showed that SPAG9 ablation affected various molecules involved in cell cycle, senescence, apoptosis, and EMT. Hence, our detailed study indicated that SPAG9 promotes cellular growth and various hallmarks of TNBC cells.

In summary, for the first time, we have put forth a comprehensive study suggesting that SPAG9 is involved in multistep process contributing toward malignant properties of TNBC cells in in vitro and in vivo. Ablation of SPAG9 critically altered molecular pathways involved in cellular proliferation, senescence, apoptosis, and EMT. Furthermore, IHC analysis

in tumor specimens of TNBC human xenograft studies validated our in vitro findings: cell proliferation, senescence, apoptosis, and EMT. Collectively, we demonstrated that SPAG9 protein is a key molecule which plays an important role at molecular level in tumor growth by regulating apoptosis, cell cycle, and EMT pathways. Our study has laid the foundation wherein SPAG9 could be a potential molecule for developing as therapeutic target for TNBC treatment.

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Authors' contributions NJ, NG, SA, APT, DP, RF, AS, and VK carried out all the experiments, prepared the figures, and drafted the manuscript. NJ participated in data analysis and interpretation of results. AS designed the study and participated in data analysis and interpretation of results. All authors read and approved the manuscript.

Compliance with ethical standards

Conflicts of interest None

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