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Original Article

WW45 inhibits breast cancer cell proliferation by the Hedgehog signaling pathway

Anwen Xiong[1,2], Lin Li[2], Wei Li[2], Jia Yu[2], Zhengjun Lian[3], Ziyu Zhang[4], Rongcheng Luo[1,5]

[1]Cancer Center, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong, People's Republic of China; [2]Department of Oncology, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, People's Republic of China; [3]Department of Oncology, Chest Hospital of Xinjiang Uygur Autonomous Region, Xinjiang, People's Republic of China; [4]Key Laboratory of Women's Reproductive Health of Jiangxi, Jiangxi Maternal & Child Health Hospital, Nanchang 330006, Jiangxi, People's Republic of China; [5]Cancer Institute, Southern Medical University, Guangzhou 510515, Guangdong, People's Republic of China

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Abstract: WW45 is a recently-discovered tumor suppressor gene. Overexpression of WW45 was found to significantly weaken proliferation and colony formation in a human breast cancer cell line, but the molecular mechanism of WW45's inhibitiory effect on proliferation was uncertain. It is a key transcription factor of the Hedgehog signaling pathway. In particular, the mechanism of Gli1's upstream proteins in regulating Gli1's nuclear import was not clear. We collected different breast cancer cell lines and detected WW45 and Gli1 expression by western blot. Gli1 expression was detected after WW45 was overexpressed in breast cancer cells. Gli1 and WW45 were transfected into breast cancer cells, and co-immunoprecipitation was used to detect whether the two proteins had physical interaction. We confirmed Gli1 blocks WW45-induced growth inhibition and colony formation in ZR-75-30 cells through cell functional experiments. Expression of WW45 negatively correlated with Gli1 expression in breast cancer cells. WW45 affected Gli1 intracellular localization though ww-PPxY/PsP interaction. Gli1 blocked WW45-induced growth inhibition and colony formation in ZR-75-30 cells. Our results strongly suggest that expression of WW45 negatively correlates with Gli1 expression in breast cancer cells. direct physical interaction occurred between WW45 and Gli1, and WW45 affected Gli1 intracellular localization though WW-PPxY/PsP interaction. Furthermore, Gli1 blocked WW45-induced breast cancer cell growth inhibition.

Keywords: Breast cancer, WW45, Gli1, hedgehog

Introduction

Breast cancer is the leading cause of cancer death among Chinese women. About 2.6% of Chinese women die of breast cancer [1]. Abnormality of tumor-related genes is an important cause of breast cancer, including genetic and epigenetic changes [2, 3]. Despite advances in hormone therapy, chemotherapy, and immunotherapy, one-third of patients with breast cancer relapse and die. It is significant to study the molecular mechanism of breast cancer occurrence and proliferation.

WW45 is a core component of conserved Hippo (mammalian sterile 20-like kinase, Mst1 and Mst2) signaling, which controls the organ size and suppresses tumorgensis [4]. Because its C-terminus contains two highly conserved WW domains with a molecular weight of 45 kDa, it was named WW45 [5]. Activation of hippo signaling causes Mst1 and Mst2 to recruit WW45, a tumor suppressor gene. Its activation causes cell cycle blockage and apoptosis [6, 7]. In recent years, genomic experiments have shown that WW45 is low or even absent in many tumors such as colon cancer, ovarian cancer, renal cancer, and malignant mesothelioma. Cell biology experiments showed that WW45 had a significant inhibitory effect on the proliferation of liver cancer cells and lung cancer cells [8, 9]. Song performed a phenotypic analysis of WW45 systemic knockout mice: WW45-/- mice were unable to survive due to embryonic lethality, whereas WW45+/- mice had a 74% incidence of liver cancer at 12

months of age. This indicates that WW45 plays an important role in the occurrence and development of tumors [10].

The WW45 gene has five exons, of which the third exon encodes two WW domains and the fifth exon encodes the SARAH domain [10]. The WW domain refers to two glycosylated tryptophan regions, that contain approximately 40 amino acids, and are involved in protein-protein interaction. Studies have shown that the WW domain is closely related to the following five groups of proline-containing motifs: PPxY motif, PPLP motif, PPI motif, PGM motif, and pSP motif [11-14]. In recent years, there have been many studies on the biologic function of the WW domain by binding to the PPxY motif of the target protein. For example, AMOTL1, SCAMP3, LATS1, and U24 were all confirmed to contain a conserved PPxY motif [11, 15, 16].

Gli1 is a transcription factor of the Sonic Hedgehog (Shh) signaling pathway [17]. In recent years, our research confirmed that Gli1 contains a conserved PPxY domain [18]. Gli1 regulates a wide variety of developmental processes, and malfunction of this pathway has been linked to numerous human disorders including cancer [19]. Kubo et al. detected high levels of Shh and Gli1 expression in 52 breast cancer specimens by immunohistochemistry, while low expression of Shh and Gli1 was detected in normal breast tissue [20]. Yang et al. found that patients with high expression of Gli1 had high malignant behavior and rapid proliferation by analyzing the expression of Gli1 in 284 breast cancer patients [21]. Our previous studies have also shown that overexpression of Gli1 in breast cancer cells reverses tumor growth inhibition caused by the tumor suppressor protein Wwox [18].

In this work, expression of WW45 negatively correlated with Gli1 expression in breast cancer cells. Direct physical interaction was found between WW45 and Gli1. WW45 affected Gli1 intracellular localization though WW-PPxY/PsP interaction. Furthermore, Gli1 blocked WW45-induced breast cancer cell growth inhibition.

Materials and methods

Cell culture

Breast cancer-derived cell lines were a gift from Dr. Wu Jia-xue (Fudan University, Shanghai, China) and Dr. Shao Zhi-min (Fudan University, Shanghai, China). Breast cancer cell lines MDA-MB-361, HCC1937, MCF7, ZR-75-30, MDA-MB-231, and SK-BR-3 were all cultured using 10% fetal calf serum. These DMEM culture media contain double antibiotics and amino acids. The cells were adhered as a single layer in a 5% CO_2 concentration and a 37°C incubator.

Plasmids

Human Gli1 expression vector pLVX-AcGFP and pRK5 was kindly provided by Dr. Steven Y. Cheng (Nanjing Medical University, China). pLVX-AcGFP-WW45, pEGFP-WW45, pRK5-HA-Gli1, pRK5-Myc-WW45, pRK5-Gli1-Flag, pRK5-Gli1(PPxY)-Flag, pRK5-Gli1(PsP)-Flag, Gli1(TM)-Flag were constructed by us.

Transfections

Cells were plated in 6-well plates 24 h before transfection and cotransfected with the indicated amounts of expression constructs using FuGENE®HD Transfection Reagent (Promega) per manufacturer's instructions.

Western blot

Breast cancer cell lines were lysed with RAPI lysate. Total protein was extracted and quantified with BCA kit (Beyotime). After SDS-PAGE electrophoresis, the protein was transferred to PVDF membrane by electric current, and then added into 5% skim milk for 2 h. Anti-Gli1 antibody (1/1000 dilution, Abcam) and anti-WW45 antibodies (1/1000 dilution, LifeSpan Biosciences) were incubated overnight. After washing, the membrane was incubated with secondary antibody for 2 h, and imaging was performed with enhanced chemiluminescence (ECL, Beyotime). ZR-75-30 cells were transfected either with pLVX-AcGFP or pLVX-AcGFP-WW45 lentivirus and harvested 24 to 30 hours after transfection. Following preclearing for 1 hour at 4°C, we did immunoprecipitation by incubating 1 mg of whole-cell extracts with the Anti-Gli1 antibody (Abcam) with rocking overnight at 4°C.

Co-immunoprecipitation

The experimental plasmid was transferred into ZR-75-30 cells, and after 24 hours, the cells were lysed with RIPA buffer (sc-24948). Cell

debris was pelleted by centrifugation at 10,000 × g for 10 minutes at 4°C. The supernatant was used as the experimental protein. Cell lysates were immunoprecipitated (IP) with anti-Myc antibodies followed by immunoblotting (IB) with HRP-conjugated antibody to HA.

Immunofluorescence

We transfected the experimental plasmids in ZR-75-30 cells. After 48 hours of transfection, the cells were gently washed three times with PBS for at least three minutes each. Exogenous Gli1 was labeled with rabbit-derived anti-Flag antibody overnight. WW45-conjugated GFP was observed directly under a fluorescent microscope. Exogenous Gli1 was labeled with DyLight 649 goat anti-mouse IgG secondary antibody (red) for one hour. Nuclei were stained with DAPI stain. Localization of WW45 and Gli1 was seen in the cells under a fluorescent microscope.

Cell growth assays

We transfected the experimental plasmid into breast cancer ZR-75-30 cells. Cells in the logarithmic growth phase were collected and seeded in 100 μl at a concentration of 3 * 10^4 in 96-well plates and cultured for 3-5 days. 200 μl MTT solution was added to each well and incubated for 4 more hours. Culture was stopped, the culture supernatant was carefully aspirated from the well, and 200 μl DMSO was added to each well, and shaken at room temperature for 10 min to fully dissolve the crystals. The wavelength of 490 nm was selected to measure the light absorption value of each well on the enzyme-linked immunosorbent monitor. The results were recorded. The cell growth curve was plotted with time as the abscissa and light absorption value as the ordinate.

Cell cycle analysis

We transfected the experimental plasmid into breast cancer cell ZR-75-30 cells. The cells were washed 3 times with ice-cold 1 × PBS. Cells were pelleted by centrifugation, and the supernatant was discarded. Cells were resuspended in 0.5 ml 1 × PBS, then resuspended with 1 ml PI/Triton X-100 staining solution (20 μg PI/0.1% Triton X-100) containing 0.2 mg RNase A, and stained at 37°C for 15 min. Cell cycle was measured by flow cytometry.

Colony formation

A total of 2 × 10^5 cells were plated in 6-well plates and were transfected with indicated plasmids. 72 hours after treatment, 500 cells were plated in a p60 plate and allowed to grow until visible colonies appeared. Colonies were stained with Giemsa and counted.

Statistical analysis

All the data were analyzed by Student's t-test. During the analysis, the data were examined by SPSS 20.0, and shown as mean ± SD. Statistical significance was defined as $P < 0.05$.

Results

Expression of WW45 negatively correlates with Gli1 expression in breast cancer cells

To investigate the effect of WW45 on the expression of Gli1 in breast cancer cells, we first detected the protein expression levels of WW45 and Gli1 in several human breast cancer cell lines by western blotting (**Figure 1A**, **1B**). We detected the expression of WW45 and Gli1 proteins in six breast cancer cell lines, MDA-MB-361, HCC1937, MCF7, ZR-75-30, MDA-MB-231, and SK-BR-3, respectively. The results suggest: MDA-MB-361, HCC1937, and SK-BR-3 breast cancer cells had relatively high WW45 expression levels, while Gli1 expression levels were relatively low. MDA-MB-231 breast cancer cells had a relatively high Gli1 expression level, while WW45 was almost not expressed.

Since Gli1 is a target of the Shh pathway, it can enhance the activity of Gli1 by positive feedback [22]. We transfected pLVX-AcGFP and pLVX-AcGFP-WW45 lentiviral plasmids into breast cancer cell line MDA-MB-231, respectively. The expression level of exogenous WW45 increased significantly after transfection of pLVX-AcGFP-WW45 lentiviral plasmid. After the introduction of exogenous WW45, the expression level of Gli1 decreased by 32.1% compared with the control group (**Figure 1C**, **1D**).

Direct physical interaction between WW45 and Gli1

To examine whether WW45 binds to Gli1 directly, we transiently co-transfected ZR-75-30 cells with Myc-WW45 and HA-Gli1 expression con-

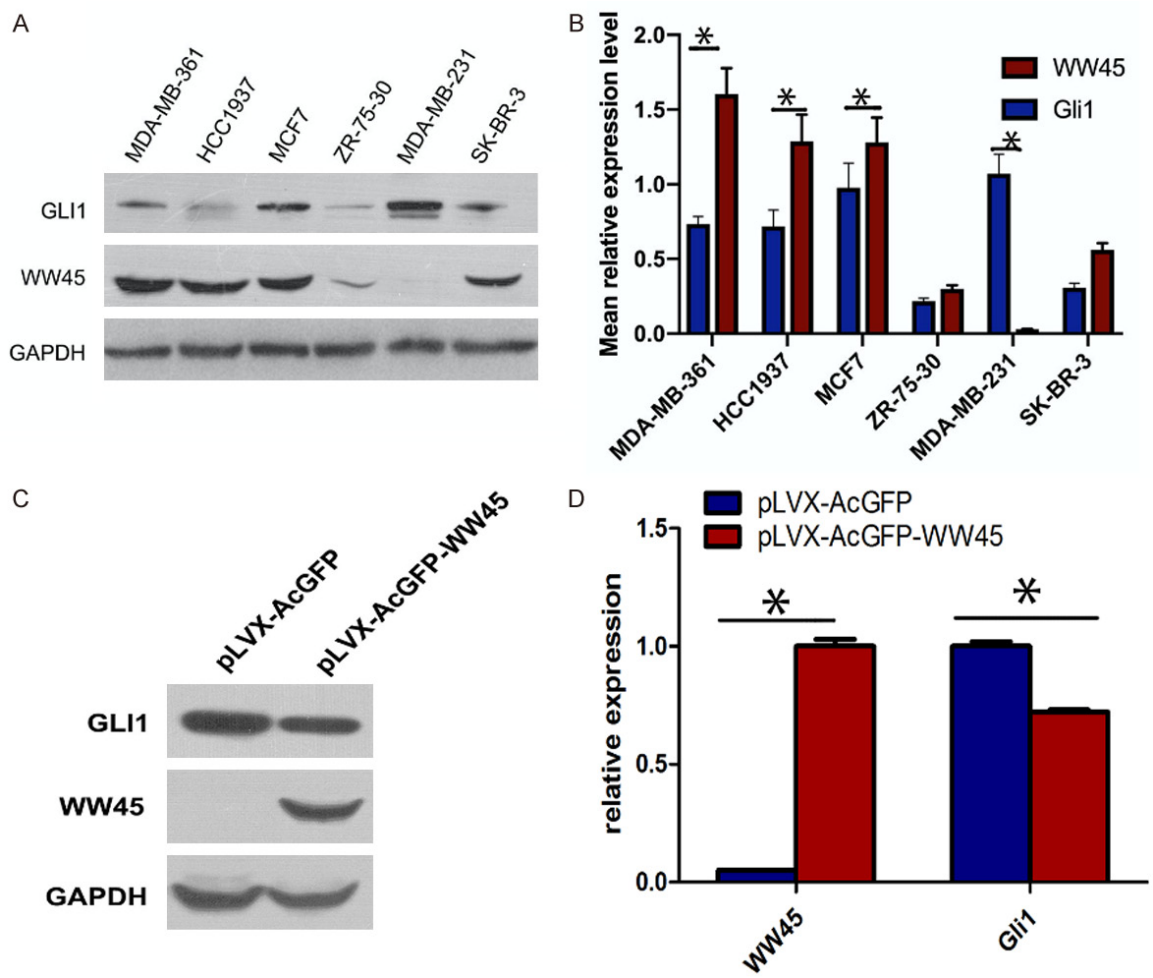

Figure 1. Expression of WW45 negatively correlates with Gli1 expression in breast cancer cells. A. Endogenous expression of WW45 and Gli1 in human breast cancer cells lines. GAPDH served as control. B. Densitometry analysis was performed and data presented as mean ± SD of three independent experiments (n=3). Western blot result was quantified using Image J. C. Effects of WW45 on Gli1 protein levels in MDA-MB-231 cells determined by western blotting. MDA-MB-231 cells were transfected with pLVX-AcGFP or pLVX-AcGFP-WW45 vector, and kept in growth medium for 48 h before protein isolation. D. Densitometry analysis was performed and data presented as mean ± SD of three independent experiments (n=3). Western blot result was quantified using Image J.

structs. Cell lysates were immunoprecipitated (IP) with anti-Myc antibodies and then immunoblotted (IB) with HRP-conjugated HA antibodies. Our results indicate that WW45 interacts with Gli1 by immunoprecipitation with anti-Myc and immunoblotting with anti-HA antibody (**Figure 2A**). Gli1 was determined by immunoprecipitation with anti-HA and anti-Myc antibodies interacting with WW45 (**Figure 2B**).

Previous co-immunoprecipitation experiments showed that WW45 specifically binds Gli1. Gli1, as a nuclear transcription factor needs to enter the nucleus to perform its biologic function, while WW45 is a cytoplasmic protein. The question thus arises: where do WW45 and Gli1 interact? To answer this question, we studied the localization of two proteins with the help of a confocal microscope. Transient expression of pEGFP-WW45 alone or with ZR-75-30 was done in breast cancer cells transfected with Gli1-Flag. The localization of the Flag-tagged protein was then determined by immunofluorescence staining using appropriate antibodies as described in Materials and Methods. The results suggested that Gli1 was mostly localized in the nucleus of breast cancer ZR-75-30 cells. In contrast, exogenous WW45 was mainly detected in the cytoplasm (**Figure 2C**). Interestingly, in cells co-transfected with pEGFP-

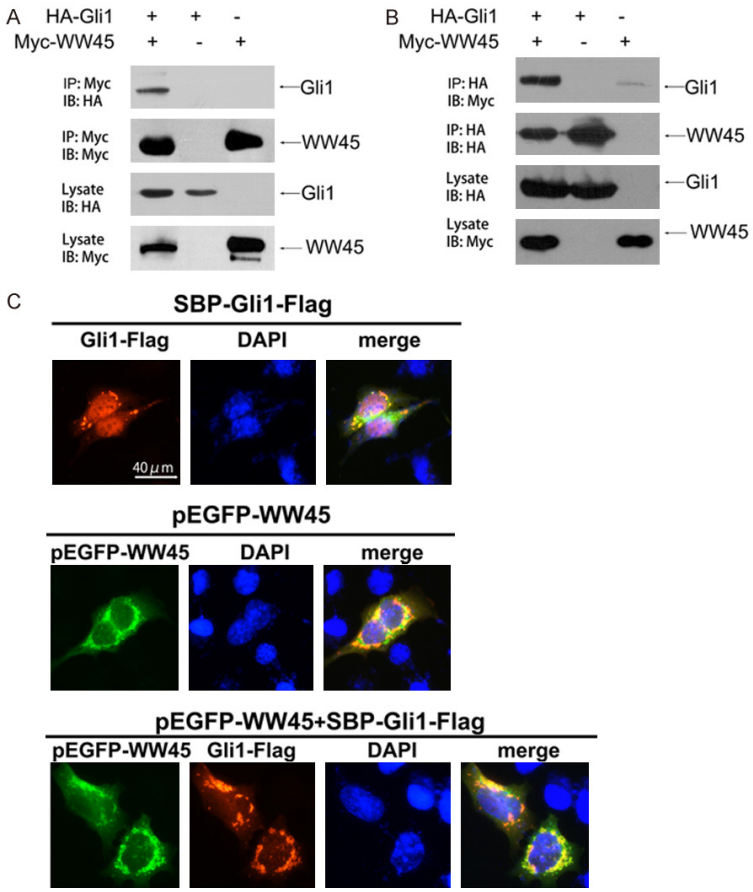

Figure 2. WW45 physically interacts with Gli1 in vivo. ZR-75-30 cells were transiently transfected with expression plasmids encoding HA-Gli1 with Myc-WW45. A. Whole cell lysates were immunoprecipitated (IP) with anti-Myc antibodies 36 hours after transfection. The immunoprecipitates were analyzed by immunoblotting (IB) with anti-HA or anti-Myc antibodies. B. Whole-cell lysates were immunoprecipitated (IP) with anti-HA antibodies 36 hours after transfection. The immunoprecipitates were analyzed by immunoblotting (IB) with anti-HA or anti-Myc antibodies. C. ZR-75-30 cells were transfected with pEGFP-WW45 and SBP-Gli1-Flag. 36 hours later, cells were fixed, permeabilized, and immunostained with monoclonal mouse anti-HA and polyclonal rabbit anti-Myc antibodies, followed by secondary goat anti-mouse Alexa Fluor 488 and secondary goat anti-rabbit Alexa Fluor 568.

WW45 and Gli1-Flag, the distribution of Gli1 in 60%-65% of breast cancer cells was changed to the cytoplasm and co-localized with WW45 (**Figure 2C**).

WW45 affects Gli1 intracellular localization though ww-PPxY/PsP interaction

To examine whether WW45 binds to Gli1 though ww-ppxy/psp interaction, ZR-75-30 cells were transiently cotransfected with pEGFP-WW45 and Gli1-Flag/Gli1(PPxY)-Flag/Gli1(PsP)-Flag/Gli1(TM)-Flag expression constructs. The Gli1(TM)-Flag vector has both PPxY and PsP mutation. Cell lysates were immunoprecipitated (IP) with anti-Flag antibodies followed by immunoblotting (IB) with HRP-conjugated antibody to GFP. Our results showed that when Gli1 is mutated, the ability of WW45 to bind Gli1 is significantly reduced (**Figure 3A**).

Our data showed that binding of WW45 to Gli1 prevented Gli1 from entering the nuclei. The next question that arose was: If Gli1 is mutated, does WW45 still prevent Gli1 from entering the nucleus? To answer this question, we studied the localization of both proteins with the aid of confocal microscopy. pEGFP-WW45 and Gli1-Flag/Gli1(PPxY)-Flag/Gli1(PsP)-Flag expression constructs were transiently expressed in ZR-75-30 cells. Interestingly, in cells cotransfected with pEGFP-WW45 and Gli1-Flag, 60 to 65% of cells showed cytoplasmic staining of Gli1 where it colocalized with WW45. When gli1 was mutated, most of the mutated gli1 could be successfully nucleated (**Figure 3B**).

Gli1 blocks WW45-induced growth inhibition and colony formation in ZR-75-30 cells

Flow cytometry was used to detect the effect of WW45 on the cell cycle of breast cancer cells ZR-75-30. After repeated tests, the results showed that overexpression of WW45 in breast cancer cell line ZR-75-30 could significantly increase its G1/G0 arrest. Moreover, this blockage could be partially reversed by Gli1 transferred from external sources (**Figure 4A**). The effect of WW45 on the proliferation and activity of breast cancer cells ZR-75-30 was detected by MTT method. The results showed that the overexpression of WW45 in breast cancer cell line ZR-75-30 cells could significantly inhibit proliferation.The inhibition could be partially reversed by Gli1 (**Figure 4B**). Furthermore, colony

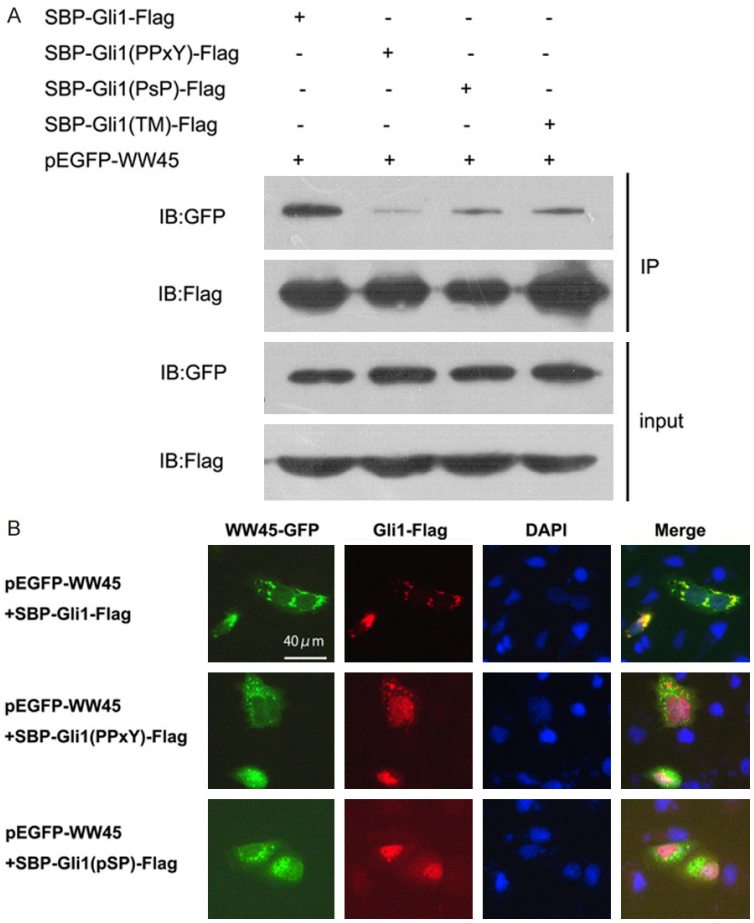

Figure 3. Subcellular localization of WW45 and Gli1. A. ZR-75-30 cells were transfected with pEGFP-WW45 and SBP-Gli1-Flag/SBP-Gli1(PPxY)-Flag/SBP-Gli1(pSP)-Flag/SBP-Gli1(TM)-Flag. 36 hours later, cell lysates were immunoprecipitated (IP) with anti-Flag antibodies and then immunoblotted (IB) with HRP-conjugated EGFP antibodies. B. ZR-75-30 cells were transfected with pEGFP-WW45 and SBP-Gli1-Flag/SBP-Gli1(PPxY)-Flag/SBP-Gli1(pSP)-Flag. 36 hours later, cells were fixed, permeabilized, and immunostained with monoclonal mouse anti-HA and polyclonal rabbit anti-Myc antibodies, followed by secondary goat anti-mouse Alexa Fluor 488 and secondary goat anti-rabbit Alexa Fluor 568.

formation showed significant inhibition in pGFP-WW45 transfected cells compared to pGFP transfected cells, and WW45-induced growth inhibition was reversed by Gli1 (**Figure 4C**).

Discussion

The function of WW45 in the development and progression of malignant tumors has been a hot topic in recent years, but the function of WW45 in breast cancer was rarely studied. We first discovered that WW45 could inhibit breast cancer cell proliferation. However, the specific mechanism by which WW45 inhibited Gli1 entry into the nucleus remained unclear.

It has been reported that the WW45 physically and functionally interacts with Gli1 in lung cancer cells [9]. A previous study revealed that BCL2 Associated Athanogene 3 (BAG3) interacted with PPxY-containing proteins though WW domains [11]. Because WW45 also contains two distinct WW domain-containing proteins, recent data demonstrate that Itch physically interacts with either PPxY or pSP motifs of Gli1. Our previous research found that Wwox regulates the hedgehog signaling pathway by binding to Gli1 [18]. We hypothesized that WW45 might physically associate with Gli1 though ww-ppxy/psp interaction (**Figure 5**).

In the current study, we first demonstrate that WW45 down-regulates Gli1 in breast cancer cells. In this study we clearly show that expression of Gli1 was very low in HCC1937 and SK-BR-3 but high in MDA-MB-231 and MCF-7. In contrast, expression of WW45 was high in HCC1937 and SK-BR-3 but low in MDA-MB-231. Because Gli1 is a target of the Shh pathway that acts in a positive feedback manner to reinforce Gli activity [23], we next set out to determine whether WW45 might down-regulate Gli1 in breast cancer cell lines. To test this hypothesis, ZR-75-30 cells with low levels of WW45 were transfected either with pLVX-AcGFP or pLVX-AcGFP-WW45. Gli1 in the pLVX-AcGFP-WW45 transfectants was tested to compare with Gli1 in the pLVX-AcGFP transfectant cells. Our data indicated that Gli1 was down-regulated by overexpression of WW45. Our study has determined that Gli1 binds to WW45 protein in breast cancer cells and is anchored in the cytoplasm and cannot be successfully inserted into the nucleus. Further studies have shown that Gli1 binds to WW45 protein through its PPxY/pSP motif.

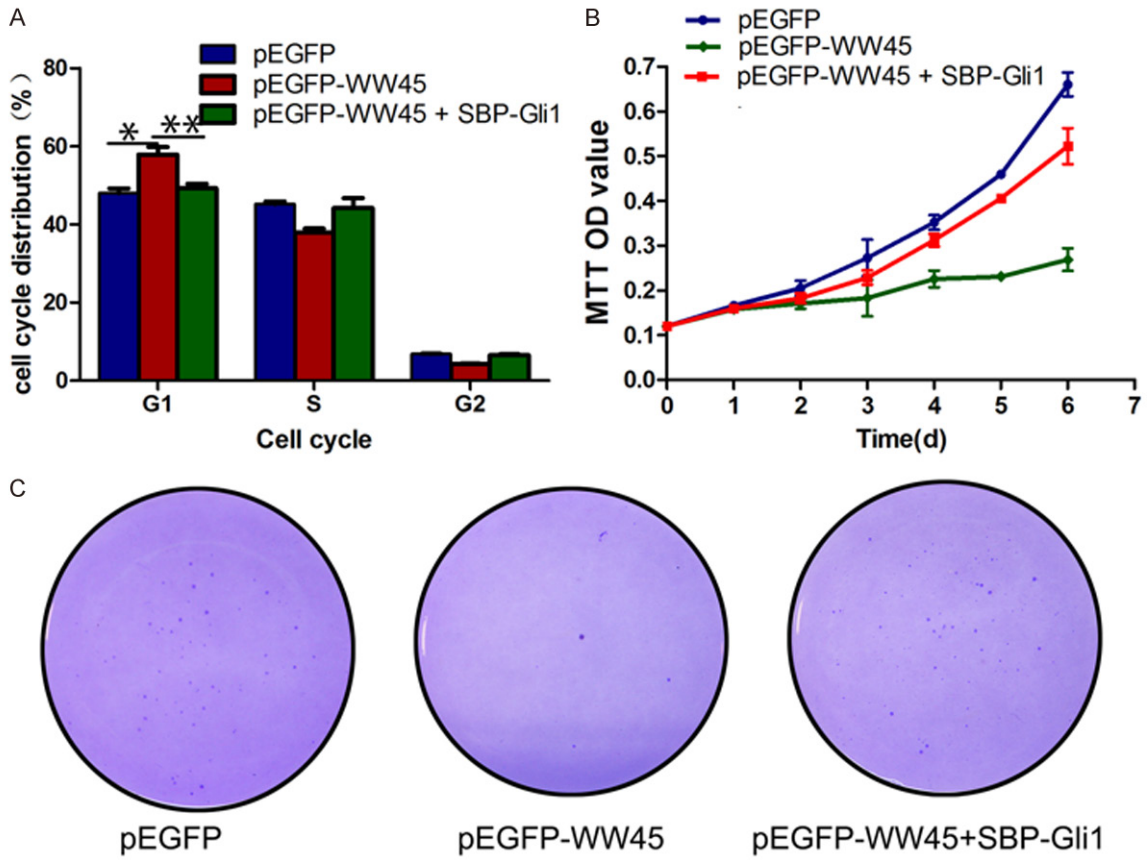

Figure 4. WW45 suppresses Gli1-induced ZR-75-30 cell growth. A. ZR-75-30 cells were transfected with the indicated plasmids, and cells harvested at 72 h and subjected to flow cytometry analysis. Cell cycle distribution is presented as the percentage of cells at each phase. B. Cell proliferation was determined by MTT assay. ZR-75-30 cells (2000 cells per well) were plated in 96-well plates following transfection with indicated plasmids. Medium was changed 6 h later. Cells were incubated with MTT (1 mg/ml) for 2 h at 37°C every 24 h up to 6 days. C. ZR-75-30 cells were transfected with indicated plasmids. Colony formation was determined.

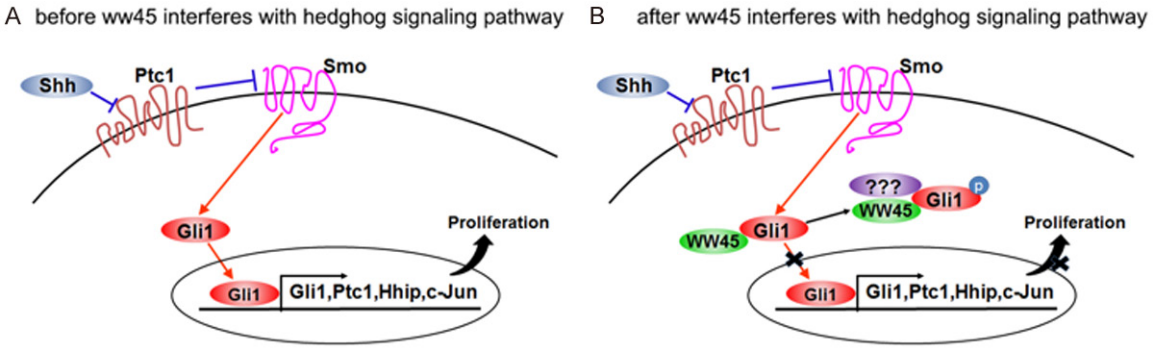

Figure 5. Hypothetical model depicting interaction between WW45 with hedgehog-Gli1 signaling pathway. A. Schematic illustration of WW45 and Gli1 proteins. WW45 contains two WW domains and a short dehydrogenase domain (SDR). Gli1 composed of a combination of two PPxYs and a phospho-serine/proline motif in C-terminal region. B. WW45 interacts with transcription factors Gli1 and inhibits its translocation to the nucleus, hence suppressing its transactivation activity.

Other researchers have shown that WW45 repair can inhibit the growth of colorectal cancer cells [24, 25]. However, research on breast cancer is still very limited. Our research show-

ed significant inhibition of cell growth in pGFP-WW45-transfected cells compared with pGFP-transfected cells, and WW45-induced growth inhibition was reversed by Gli1.

In conclusion, our results strongly suggest that expression of WW45 negatively correlates with Gli1 expression in breast cancer cells. Through co-immunoprecipitation experiments, we found a direct physical interaction between WW45 and Gli1, and WW45 affected Gli1 intracellular localization though WW-PPxY/PsP interaction. Furthermore, Gli1 blocks WW45-induced breast cancer cell growth inhibition.

Conclusions

The function of WW45 in breast cancer is rarely studied. We first discovered that WW45 can inhibit breast cancer cell proliferation. Our study has determined that Gli1 binds to WW45 protein in breast cancer cells, is anchored in the cytoplasm, and cannot be successfully inserted into the nucleus. Further studies have shown that Gli1 binds to WW45 protein through its PPxY/pSP motif.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

Gli1, glioma-associated oncogene homolog 1; Shh, Sonic hedgehog; IP, immunoprecipitation; IB, immunoblotting; BAG3, BCL2-Associated Athanogene 3.

Address correspondence to: Rongcheng Luo, Cancer Center, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou 510310, Guangdong, People's Republic of China; Cancer Institute, Southern Medical University, Guangzhou 510515, Guangdong, People's Republic of China. Tel: +86-15317668176; E-mail: luorongcheng456@126.com

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