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Research Article

XAV-939 Suppress Metastasis By Inhibiting Breast Cancer Stem Cells

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Abstract

Dysfunctions of Wnt, Hedgehog and Notch pathways are evident in multiple tumor types and malignancies. A number of studies have suggested that dysregulation of Wnt/β-catenin signaling occurs in human breast cancer. Specifically, inhibition of Wnt/ β-catenin pathway is implicated in arresting of cancer stem cells (CSCs), a small subset of cancer cells capable of self-renewal and differentiation into heterogeneous tumor cells. Here, we investigated tumor initiating property of breast cancer stem cell *in-vitro* with XAV-939 an inhibitor of Wnt/β-catenin signaling pathway. Targeting Wnt/β-catenin signaling with this inhibitor represents a promising strategy to suppress metastasis.

Keywords: Cancer Stem Cell, 3D Mammosphere, Wnt/β-catenin, metastasis, CD44+/CD24

INTRODUCTION

Breast cancer is the leading cause of cancer death in women globally. Advancement has been made by the combination of better screening and treatments that moderately improved the survival however much to be done for the women who are refractory to the current therapies. The breast, like many other organs, is a hierarchically-organized tissue maintained by a series of stem and progenitor cells that have decreasing potency as they differentiate toward terminally-committed epithelial cells.

The conventional treatments like chemotherapy and radiotherapy fails to cure the disease most of the time and this may be due to the presence of cancer stem cells (CSCs). Cancer metastasis, resistance to therapies and disease recurrence are significant hurdles to successful treatment of breast cancer. Cancer stem cells (CSCs) are a small subset of cancer cells with the capability of self-renewal and differentiation into heterogeneous tumor cells, and they have been believed to be responsible for tumor initiation, growth, and recurrence [(1)]. First, CSCs possess a high tumor-initiating capacity, which is an essential characteristic that enables the formation of new tumors[(2)]. Moreover, CSCs also associated epithelial-mesenchymal transition (EMT) markers which helps the tumor cells to migrate into other organs and tissues [(3)].

Wnt signaling is essential for normal breast stem cell function and mammary gland development. Numbers of studies have shown that aberrant Wnt signaling in breast cancer stem cells is a crucial event in breast tumorogenesis [(4)]. Abnormal activation of Wnt signaling has been implicated in the regulation of a plethora of CSC types including colorectal cancer, breast cancer, hematologic can-

cer, skin cancer, liver cancer and lung cancer [5-10].

Recent study also demonstrated that Wnt/β-catenin signaling activity is higher in breast CSCs than the bulk tumor population[11]. Markers like $CD44^+/CD24^-$ have been proposed to exhibit enhanced tumorigenic and metastatic properties in tumor xenograft models[12,13].

Here, we investigated the effects of inhibiting Wnt/β-catenin signaling on breast cancer stem cell to link its tumor initiating ability through 3D mammosphere formation. Targeting Wnt/β-catenin signaling with this XAV-939 inhibitor represents a promising strategy to suppress metastasis.

Materials and Methods

Cell lines and Culture Conditions:

TMD-231 breast carcinoma cell line was obtained directly from the ATCC (Manassas, VA, USA), and grown in DMEM (Thermo Fisher Scientific, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) at 37°C in a 5% humidified CO_2incubator. Characterization of cell line was done according to their surface expression phenotype.TMD-231 cell lines were grown for 48 hours with 80% confluency. After that the same cell lines were treated with XAV-939 inhibitor for 72 hours. These cells were used to form 3D mammosphere along with controls.XAV-939 Inhibitor was purchased from selleckchem (USA).

3D Mammosphere formation

Single cell suspensions were plated in 6-well tissue culture plates covered with poly-2-hydroxyethyl-methacrylate (Sigma, St. Louis, MO) to prevent cell attachment, at a density of 1,000 cells/ml in serum-free DMEM supplemented with 1% L-glutamine, 1% penicillin/streptomycin, 30% F12 (Sigma), 2% B27 (Thermo Fisher Scientific, Carlsbad, CA), 20 ng/ml EGF (Sigma, St. Louis, MO) and 20 ng/ml FGFb (Thermo Fisher Scientific, Carlsbad, CA). The medium was made semi-solid by the addition of 0.5% Methylcellulose (R&D Systems, Minneapolis, MN) to prevent cell aggregation. After 7 days in culture, mammospheres were collected by gentle centrifugation (200 x g) and dissociated enzymatically (5 min in 1:1 trypsin/DMEM solution at 37°C) and mechanically by passing through a 25G needle (6 strokes). Single cells were re-plated at a density of 1,000 cells/ml for subsequent passages.

Viability Assay:

TMD-231 cells were grown in a 96-well tissue culture plate with drugs and appropriate controls. Subsequently they were incubated with the WST-1 reagent (Dojindo Inc, Japan) for 4 hours. After this incubation period, the formazan dye formed was quantitated with a multi-well spectrophotometer (ELISA reader). The measured absorbance directly correlates to the number of viable cells.

Flow cytometry:

PE-conjugated CD44, FITC-conjugated CD24 monoclonal antibody was purchased from BD Bioscience. After 3 days of drug treatment the TMD-231 cells were dissociated with 0.25 % trypsin-EDTA (1 mM) (Invitrogen) for 3 min and washed with Calcium and magnesium free dulbecco phosphate buffered saline solution by spinning at 400g for 7 minutes. Then these cells were diluted in 100 µl FACS buffer (PBS containing 1 % fetal calf serum) and then incubated for 1 hr at 4 °C in FACS buffer with the corresponding mAb: anti-CD44-PE, CD24-FITC. Flow cytometry analysis was performed with a BD FACSCanto II flow cytometer (BD Biosciences).

Results

Viability of TMD-231-Cell lines in the presence of XAV-939 inhibitor

After 3 days of XAV-939 treatment, TMD-231 cell lines were measured for the viability by WST-1 method. We observed very minimal or negligible difference between control and XAV-939 treated cell lines, and the same was observed in the morphological images under inverted microscope (data not shown here). This result indicates, even though XAV-939 play a major role in suppressing wnt β-catenin signaling pathway it did not affect the viability of the cells.

Expression profiles of breast CSC markers

We examined the expression profiles of breast cancer stem cell markers in TMD-231 cells with or without Wnt1 inhibitor i.e XAV-939. We noticed significant decrease of breast cancer stem cell markers $CD44^+/CD24^-$ when compared to the control. However, in control the expression pattern of breast cancer stem cell was unchanged. These

findings were very evident that wnt/β-catenin signaling plays a vital role in cancer stem cell. So, arresting or blocking the wnt/β -catenin signaling can suppress the breast cancer stem cell which involves in multiple roles like tumorigenesis, chemoresistance and tumor relapse.

Suppression of metastasis

To further confirm the effects of Wnt/β-catenin signaling on tumor cells we formed 3D mammospehres. We used XAV-939 treated cells along with normal TMD-231 cells as a control for spheroid formation assay. Interestingly the data was very much correlated with the flow cytometry analysis. The spheroids were started forming at day 3 itself (data not shown here) in control whereas in XAV-939 treated cells we couldn't find any mammosphere even after 9th day of culture. These results suggest that wnt-β catenin signaling is very much required for tumor initiating capability and metastasis which are conferred by CSCs.

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Discussion

Like in any other cancers, breast cancer also fails to responds to their current chemotherapies. The resistance and recurrence are contributed by cancer stem cells. Hence, the identification of CSCs in breast cancer represents an important milestone in the understanding of chemo-drug resistance and cancer recurrence. Targeting and eradication of these cells represents a potential strategy to improve the clinical outcomes.

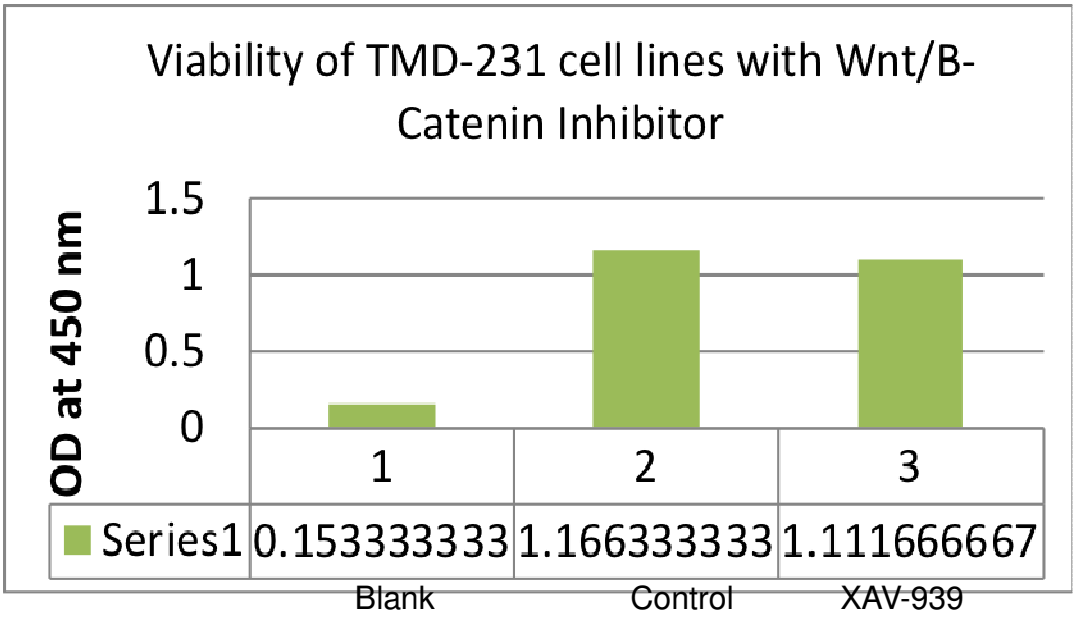
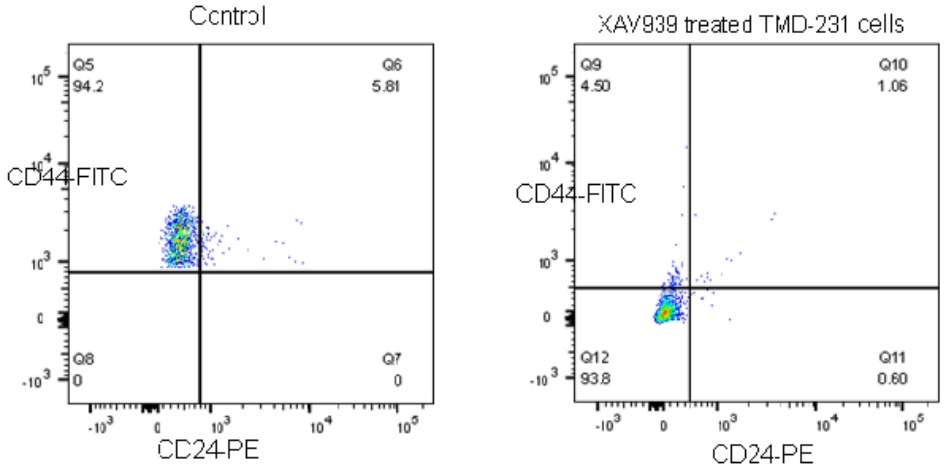


Fig 01: **The graph representing the viability of control and XAV-939 treated TMD-231 cell lines at day 3 culture. The absorbance readings were taken at 450 nm.**

Fig 02: **Flow cytometry analysis representing the expression of breast cancer stem cell markers CD44+/CD24-. A] In control, the expression of CD44/CD24 was 6.05%. B] In XAV-939 treated cells the expression of CD44 was 1.04%.**



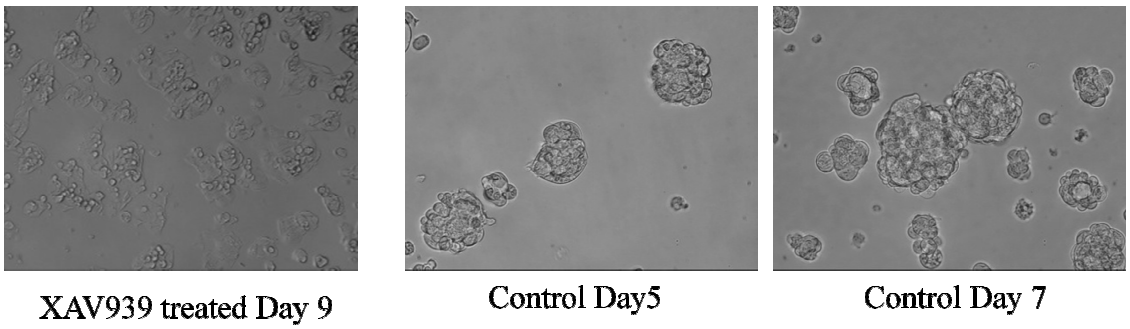


Fig 03: **3D Mammosphere formation assay. The first image is XAV-939 treated cells, no mammosphere was observed even after 9 days of spheroid culture. Second image, TMD-231 cells forming 3D mammosphere at day 5. Third image, TMD-231 cells forming mammosphere at day 7.**

Recent understanding of the biological characteristics of breast cancer stem cells has facilitated the identification of mechanisms underlying the development of malignant breast cancer. One such mechanism is dysregulation of Wnt/β-catenin signaling occurs in breast cancer [14]. In this context, abnormal Wnt/β-catenin signaling activity may be an important clinical and pathologic feature of breast cancer and a predictor of poor overall survival [15]. XAV-939 specifically inhibits tankyrase PARP activity. XAV-939 deregulates the Wnt/β-catenin pathway which has been implicated in many cancers including breast.

In this study, we treated TMD-231 cell lines with XAV-939, an inhibitor of Wnt/β-catenin signaling pathway that resulted in 1) decreased levels of the stem cell markers $CD44+/CD24^-$

2) Reduced 3D mammosphere formation. However, there was not much difference in their cell viability.

Conclusion

Tumor initiating ability is one of the important characteristics of cancer stem cell. In breast cancer presence of these cancer stem cells notably the $CD44^+/CD24^-$ subpopulation is enriched under suspension sphere culture conditions. Abolishing the 3D mammosphere forming capacity

in the TMD-231 cancer cell line is a direct indication of suppression of metastasis with a blockade in Wnt/β-catenin signaling pathway.

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