Original Article

Expression of *SOX11* and *HER2* and their association with recurrent breast cancer

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Background: Recurrent breast cancer occurs as a result of divergent gene expression in response to therapeutic intervention. A recent report showed that *SOX11*, an embryogenic mammary transcription factor, is overexpressed in breast cancer. *HER2* is also dysregulated in breast cancer stem cells; however, the relative expression of these two genes in recurrent breast cancer has not been investigated.

Methods: Mouse models of mild and advanced stage recurrent breast cancer were developed via implantation of different doses of 4T1 Luc2GFP cells. The cellular morphology of normal and recurrent breast cancer tissues was analyzed using standard histological methods. *SOX11*, *HER2*, and *ALDH1* expression levels were analyzed via immunohistochemistry and western blotting.

Results: Histological analyses revealed that treatment with doxorubicin limited mild recurrent cancer but was ineffective against advanced stage recurrent cancers, as evidenced by increased cell proliferation. *SOX11* was consistently overexpressed in mild and advanced stage breast cancers treated with doxorubicin, relative to *HER2*, which exhibited reduced expression in response to doxorubicin treatment in both mild and advanced stage recurrent breast cancer. In advanced stage recurrent breast cancer, *SOX11* expression was more readily observed across the cell surface and was correlated with the overexpression of the breast cancer stem cell marker *ALDH1*.

Conclusions: These results show that *SOX11* expression was directly associated with breast cancer stem cell populations. In contrast, *HER2* expression was strongly associated with drug treatment effects, but was not correlated with breast cancer stem cell survival in recurrent breast cancer.

Keywords: Recurrent breast cancer; 4T1 Luc2GFP cells; *SOX11*; *HER2*; *ALDH1*

Submitted Sep 12, 2018. Accepted for publication Jan 14, 2019.
doi: 10.21037/tcr.2019.01.27
View this article at: http://dx.doi.org/10.21037/tcr.2019.01.27

Introduction

Breast cancer is the most common form of cancer in women and the second leading cause of cancer-related deaths worldwide (1). Despite significant improvements in both early diagnosis and therapeutic interventions, clinical outcomes remain poor (2) due to significant heterogeneity in clinical, histological, and biological presentation (3,4). According to recent reports, 20% of patients who initially respond to therapeutic intervention will develop recurrent breast cancer within 10 years (5). Cancer cells that detach from primary breast cancer tissues can act as seed cells, resulting in metastatic cancer (6,7). These circulating tumor cells possess a variety of phenotypes similar to those of stem cells (8).

In most cases, breast cancer occurs as a result of mutations or dysregulation of important oncogenes,

such as *HER2*, *BRCA1*, *BRCA2*, and *PIK3CA* (9-12). Sex determining region Y-box 11 (*SOX11*), initially discovered in 1995 (13), is 1 of 20 *SOX* genes identified to date that play vital roles in tissue remodeling, organ development, and neurogenesis (14,15). Overexpression of *SOX11* has been reported in ovarian, brain, and mantle cell lymphoma (16-18), with *SOX11* mutations resulting in significant dysregulation of downstream genes (19).

Human epidermal growth factor receptor 2 (*HER2*) is overexpressed in 25% of breast cancers, and is associated with high mortality and disease recurrence (20). Furthermore, *HER2* overexpression is associated with cancer stem cell self-renewal, proliferation, and invasion, highlighting the importance of this gene in disease pathology (21). Here, we analyzed the expression of *SOX11* and *HER2* in recurrent breast cancer to better understand the association of these genes with disease outcomes.

Methods

Experimental animals with recurrent breast cancer

Mouse experiments were performed using 6-month-old BALB/CJ female mice. All animals were carefully maintained under standard laboratory conditions, with all study-related protocols approved by our institution's scientific review board. All mice were maintained in standard cages and provided with food and water *ad libitum*. For recurrent breast cancer, mice were injected with trypsinized 4T1 Luc2GFP cells (22) at two different dose ranges (~5,000 and 10,000 cells). After 1 week, mice were treated with doxorubicin (1 mg/kg weekly) and monitored for breast cancer recurrence. All animals subjected to experimental handling were observed regularly twice per day.

Histological imaging

Breast tissues were dissected and fixed in 10% neutral formalin solution. After 24 h, the tissues were washed thoroughly with distilled H_2O and dried using a series of increasing concentrations of isopropyl alcohol (70% to 100% concentration). Finally, the tissues were rinsed with xylene and embedded in paraffin. Tissues were cut into thin 7-µm sections using a microtome and placed on glass slides. Tissue sections were then dewaxed and processed stepwise with xylene, isopropyl alcohol, and dH_2O, and stained with hematoxylin and eosin.

Immunohistochemistry

After sectioning, the tissues were incubated with 3% H_2O_2 solution for 10 min, washed, and trypsinized for 5 min to unmask target antigens. Slides were then incubated with blocking solution [4% bovine serum albumin (BSA)] for 2 h at room temperature and incubated with primary antibodies (anti-*SOX11*, anti-*HER2*, or anti-ALDH1 antibodies; Abcam) at 4 ℃ for 8 h. Slides were then washed three times in 1× phosphate buffered saline (PBS) (2 min each), followed by treatment with secondary antibody at room temperature for 45 min. After washing, the slides were overlaid with a freshly prepared DAB solution, incubated at room temperature for 5 min, washed once in 1× PBS, and counterstained with hematoxylin.

Western blotting

Dissected tissue samples were mechanically lysed in ice-cold sample buffer. Cell lysates were then boiled for 10 min and stored at –80 ℃ until needed. Samples were loaded in equal concentrations (60 µg), resolved on 12% SDS-PAGE gel run at 50 V for 4 h, transferred to a PVDF membrane, and blocked in 5% non-fat milk for 1 h. The membrane was then incubated in primary antibody solution (anti-*SOX11*, anti-*HER2*, or anti-*ALDH1* antibody; Abcam) at 4 ℃ overnight with gentle agitation. Blots were then washed, incubated with secondary antibody, and visualized with DAB solution.

Statistical analysis

All experiments were performed three or more times. Statistical analyses were performed using SPSS version 21.0, and the results expressed as the mean ± standard error. Comparisons between groups were performed using an ANOVA with Tukey's *post hoc* test for multiple data comparison. P values <0.01 were considered statistically significant.

Results

Murine model of recurrent breast cancer

A murine model of mild and advanced stage recurrent breast cancer was developed using two groups of mice (n=5 per group) implanted with either low (5,000) or high

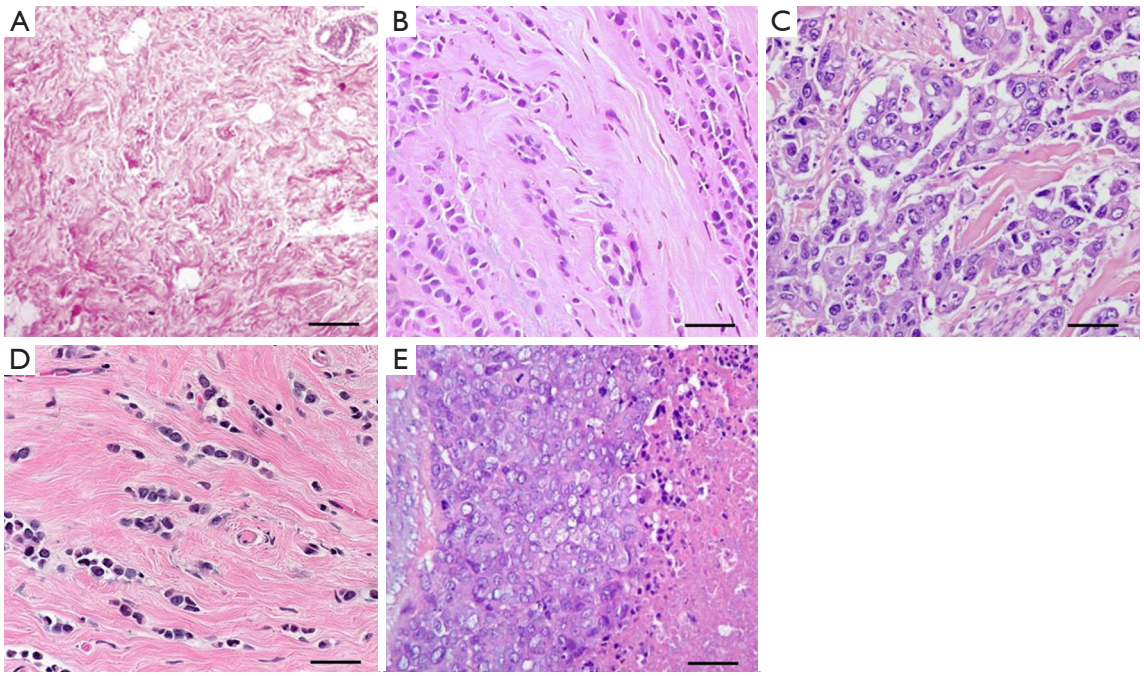

Figure 1 Murine models of mild and advanced stage recurrent breast cancer. (A) Histopathology of normal breast tissue from BALB/CJ control mice. (B) Primary breast tumors developed in response to low-dose 4T1 Luc2GFP cell injection (5,000 cells). (C) Primary breast tumors developed in response to high-dose 4T1 Luc2GFP cell injection (10,000 cells). (D) Mild recurrent breast cancer developed after 20 days of low-dose 4T1 Luc2GFP cell injection. (E) Advanced stage recurrent breast cancer developed after 20 days of high-dose 4T1 Luc2GFP cell injection. Scale bar =100 µm; haematoxylin and eosin stained.

(10,000) inocula of 4T1 Luc2GFP cells. After 1 week, mice were injected with 1 mg/kg doxorubicin weekly, with primary tumors excised on day 10. At 20 days post-implantation, mice inoculated with 5,000 cells exhibited signs of mild recurrent breast cancer, whereas the high inoculum group (10,000 cells) exhibited signs of advanced stage recurrent breast cancer. To better characterize tumor development, the primary tumors were subjected to histological analysis (*Figure 1A*,*B*,*C*,*D*,*E*). Both the high and low inoculum groups developed primary tumors with similar morphological features (*Figure 1B*,*D*); however, more divergent phenotypes were evident by day 20 (*Figure 1C*,*E*). The low inoculum group exhibited isolated clusters of cells with mild recurrent breast cancer (*Figure 1C*), whereas the high inoculum group harbored a larger number of proliferative cell clusters, consistent with that seen in advanced stage recurrent breast cancer (*Figure 1E*).

SOX11 expression in different stages of recurrent breast cancer

Expression of the transcription factor *SOX11* is associated with tumor growth, progression, and invasion, with overexpression seen in a variety of cancers including basal-like breast cancer and ductal carcinoma (23,24). Here, we analyzed the expression of *SOX11* in recurrent breast cancer induced by 4T1 Luc2GFP cell injection, along with their respective controls (*Figure 2A*,*B*,*C*,*D*,*E*). *SOX11* was minimally expressed in normal breast tissue (*Figure 2A*), with modest increases seen in the primary tumors resected from the low inoculum group (*Figure 2B*). Following doxorubicin treatment, recurrent tumors exhibited a controlled pattern of *SOX11* expression in the low inoculum group (*Figure 2D*). In contrast, mice in the high inoculum group exhibited stable overexpression of *SOX11* (*Figure 2C*,*E*). Primary tumors excised on day 10 showed consistent overexpression of *SOX11* around the

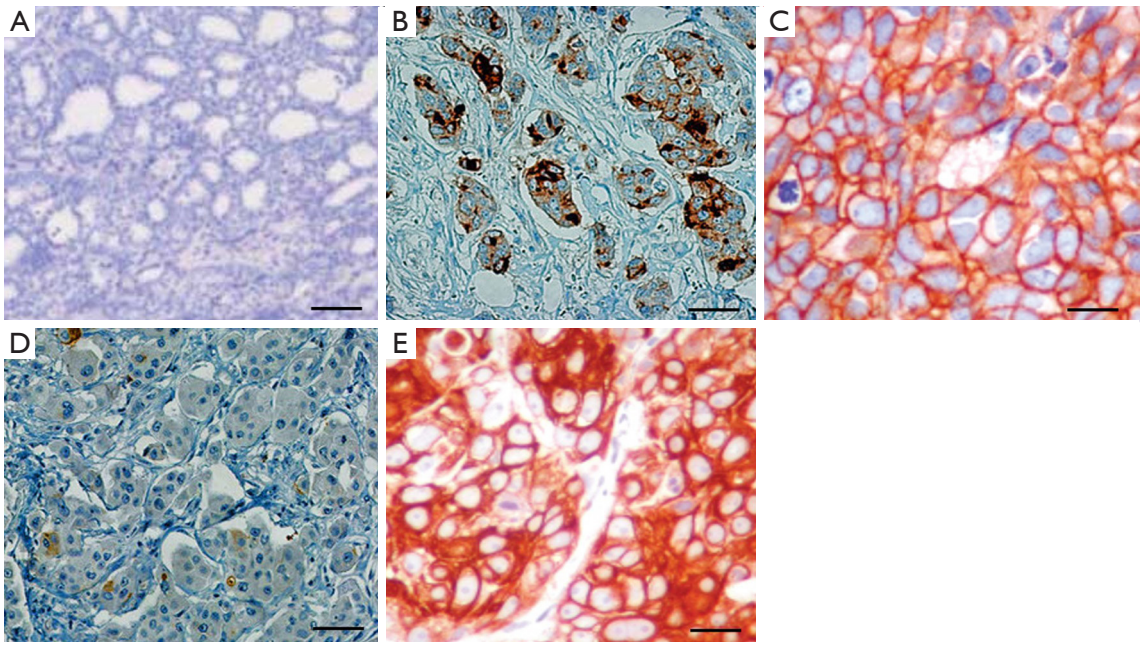

Figure 2 *SOX11* expression at different stages of recurrent breast cancer. (A) Immunohistochemical analysis of *SOX11* expression shows very low expression in control breast tissue. Primary breast tumors from low (B) and high (C) inoculum-treated mice exhibit prominent expression of *SOX11*. (D) Mild recurrent breast cancer developed after 20 days in low inoculum animals and exhibits prominent expression of *SOX11*. (E) Advanced stage recurrent breast cancer exhibits pervasive *SOX11* expression across the cell surface. Scale bar =100 µm; haematoxylin stained.

cytoplasm (*Figure 2C*), whereas advanced stage recurrent breast cancers exhibited more pervasive expression of SOX11 that was evident throughout the cell (*Figure 2E*).

Controlled expression of HER2 upon treatment with doxorubicin

Overexpression of *HER2* is often seen in recurrent breast cancer and is associated with more aggressive disease with shorter survival (20,25). Expression of *HER2* was upregulated in a dose-dependent manner, with higher 4T1 Luc2GFP inocula exhibiting higher overall expression (*Figure 3A,B,C*). Upon treatment with doxorubicin, both mild (*Figure 3D*) and advanced stage (*Figure 3E*) recurrent cancers exhibited reduced *HER2* expression. *HER2* expression in recurrent cancer was consistent with that seen in primary tumors; however, the overall expression of *HER2* was downregulated in these cells (*Figure 3D*).

Comparative analysis of SOX11, HER2, and ALDH1 expression

Western blotting was used to assess the relative expression of *SOX11*, *HER2*, and *ALDH1*, *SOX11* and *HER2* proteins (*Figure 4*) were expressed at levels similar to those observed by immunohistochemistry (*Figures 2,3*), with *SOX11* expression consistently upregulated in aggressive recurrent breast cancer, even after treatment with doxorubicin (*Figure 2E*). To contextualize these findings, we next examined *ALDH1* expression, a breast cancer stem cell marker (26), at different stages of recurrent breast cancer tissues. *ALDH1* expression was strongly associated with *SOX11* expression patterns, but not *HER2* expression (*Figure 4*). Despite these differences in relative expression, elevated levels of *SOX11*, *HER2*, and *ALDH1* were observed across breast cancer samples (*Figure 5*).

Discussion

Breast epithelial cells of embryonic origin are composed of undifferentiated cells which differentiate after birth and give rise to several different breast epithelial cell populations (27). These stem cell-like cells behave as cancer stem cells in many solid tumors and support the growth of cancer cells even after treatment with various chemotherapeutic regimens (28). Here, we developed

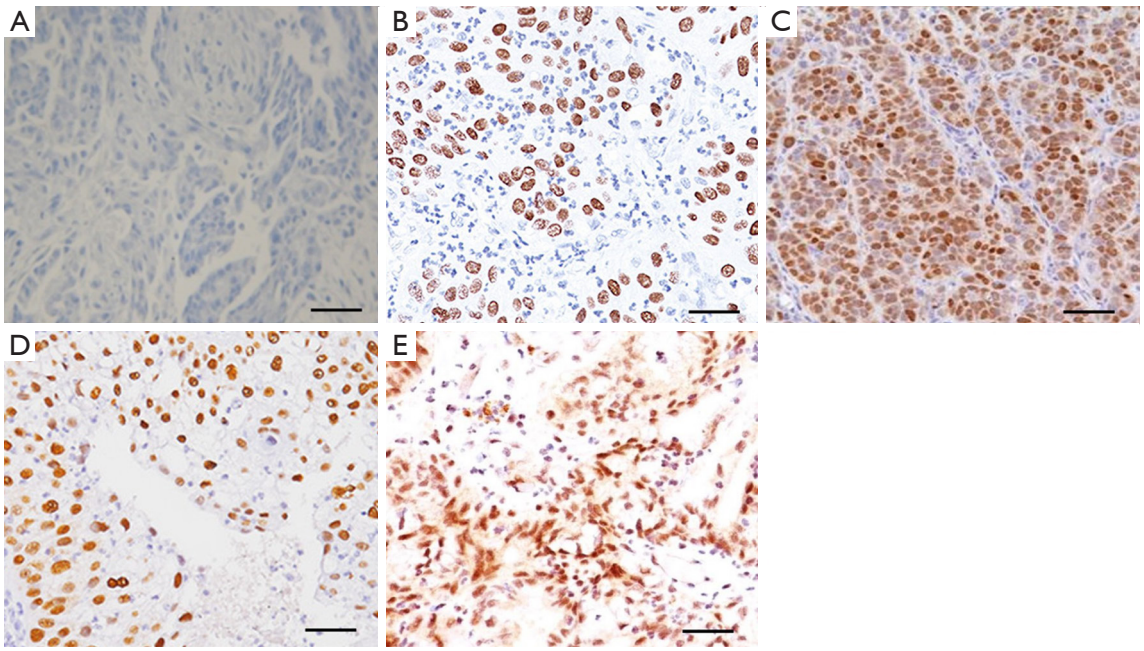

Figure 3 *HER2* expression in mild and advanced stages of recurrent breast cancer. (A) Control breast tissue with mild *HER2* expression. (B) *HER2* is prominently expressed in primary breast tumor tissues isolated from low inoculum-treated mice. (C) Overexpression of *HER2* is also seen in primary breast tumors developed from high inoculum-treated mice. (D) Reduced expression of *HER2* is evident in mild recurrent breast cancers which developed after 20 days in the low inoculum group. (E) Advanced stage recurrent breast cancer with reduced *HER2* expression. Scale bar =100 μm; haematoxylin stained.

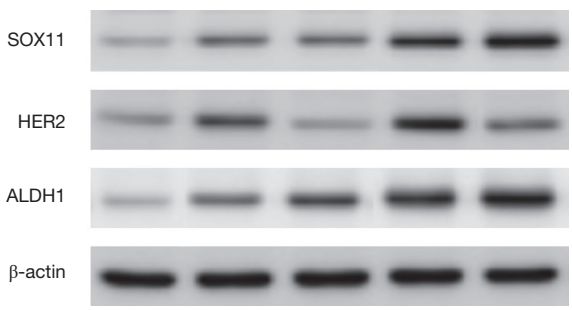

Figure 4 Comparative analysis of *SOX11*, *HER2*, and *ALDH1* expression. Lane 1: *SOX11* expression in control tissue, primary tumors (low dose), mild recurrent breast cancer, primary tumors (high dose), and advanced stage recurrent breast cancer. Lane 2: expression of *HER2* in control tissue, primary tumors (low dose), mild recurrent breast cancer, primary tumors (high dose), and advanced stage recurrent breast cancer. Lane 3: expression of breast cancer stem cell marker *ALDH1* in control tissue, primary tumors (low dose), mild recurrent breast cancer, primary tumors (high dose), and advanced stage recurrent breast cancer. β-actin was used as a loading control.

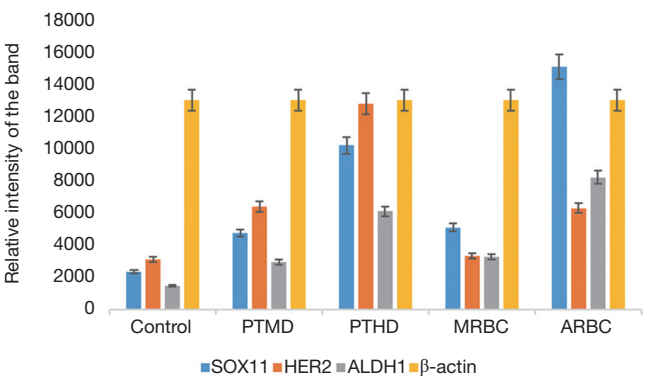

Figure 5 Quantification of *SOX11*, *HER2*, and ALDH1 expression in different breast cancer conditions. Expression of *SOX11*, *HER2*, *ALDH1* and β-actin are plotted based on the band intensity in western blots. Experimental data were collected from three independent experiments and are presented as the mean ± SD. P<0.01. PTMD, primary tumor with a mild dose of 4T1 Luc2GFP cell injection; PTHD, primary tumor with high-dose 4T1 Luc2GFP cell injection; MRBC, mild recurrent breast cancer; ARBC, advanced stage recurrent breast cancer.

a mouse model of recurrent breast cancer using 4T1 Luc2GFP cells. Histological studies showed that mild forms of recurrent breast cancer, in conjunction with the primary tumors excised from these animals, are readily controlled with doxorubicin therapy, whereas more advanced stages of recurrent breast cancer remain resistant to therapeutic intervention. These result suggests that the risk of death in early stage recurrent breast cancer is not correlated with the overall spectrum of breast cancer mortality (29).

Histological analysis of *SOX11* expression in advanced stage recurrent breast cancer revealed consistent overexpression throughout the cell, consistent with the invasive nature and progression of breast cancer (*Figure 1C*,*E*; *Figure 2C*,*E*). *HER2*-positive cells are also a sign of aggressive cancer, representing a significant risk for tumor recurrence. Treatment with doxorubicin was found to reduce *HER2* expression in mild and advanced stage recurrent breast cancer (*Figure 3A*,*B*,*C*,*D*,*E*), whereas both *ALDH1* and *SOX11* were consistently overexpressed in both tumor types (*Figure 4*). These results suggest that cancer progression may be attenuated in response to treatment, as evidenced by the reduction in *HER2* expression, but these effects do not extend to breast cancer stem cells, which continue to exhibit high levels of *SOX11* expression. This comparative analysis showed that *SOX11* and *ALDH1* are better markers for breast cancer stem cells than *HER2*, and may be predictive of cancer recurrence.

Several studies have identified *HER2* as an important regulator of breast cancer stem cells, as inhibition of *HER2* expression reduces cancer stem cell populations (21). However, our data indicated that *HER2* expression levels were not associated with breast cancer stem cell survival in recurrent breast cancers. These data suggest that *HER2* expression is an effective marker for assessing treatment response, but is not appropriate for predicting responses of breast cancer stem cells.

Conclusions

In summary, using 4T1 Luc2GFP cells, we established effective mouse models of mild and advanced stage recurrent breast cancer. *SOX11* expression was associated with breast cancer stem cell survival and was strongly correlated with *ALDH1* expression. In contrast, *HER2* expression was associated with treatment response in recurrent breast cancer but was not predictive of changes associated with breast cancer stem cells.

Acknowledgments

Funding: None.

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.01.27). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animals were carefully maintained under standard laboratory conditions, with all study-related protocols approved by our institution's scientific review board (approval No. TXT24311).

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Cite this article as: Wang FW, Ao X, Fu SM. Expression of *SOX11* and *HER2* and their association with recurrent breast cancer. Transl Cancer Res 2019;8(1):248-254. doi: 10.21037/tcr.2019.01.27

