S100B expression in breast cancer as a predictive marker for cancer metastasis

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Received September 12, 2017; Accepted December 5, 2017

DOI: 10.3892/ijo.2017.4226

Abstract. In the tumor microenvironment, soluble molecules play important role in the establishment of a pre-metastatic niche. The S100 calcium-binding protein family are inflammatory molecules that contribute to the development of a pro-inflammatory tumor microenvironment. S100B belongs to the S100 family and serum S100B (also known as S100beta) serves as a marker for metastasis in lung cancer, ovarian cancer and melanoma. However, the association between S100B and the metastasis of breast cancer is not yet well understood. In the present study, a relatively low S100B expression was observed in the tumor samples compared to normal breast tissue among online microarray datasets. When the estrogen receptor (ER)-negative breast cancer cell lines, MDA-MB-231 and Hs578T, were treated with recombinant human S100B, cell migration was significantly inhibited and epithelial cadherin expression was increased. Our results revealed that a high S100B expression predicted a good overall survival in patients with ER-negative breast cancer, and good distant metastases-free survival in all patients with breast cancer via the analysis of the KM plotter and SurvExpress databases. Although previous studies have indicated that the interaction of S100B with wild-type p53 inhibits p53 function, a high S100B expression is associated with a good prognosis in patients with p53 mutant and p53 wild-type breast cancers. On the whole, our findings demonstrate that S100B treatment suppresses the migratory capacity of ER-negative breast cancer and that S100B expression may serve a predictive marker for metastasis in breast cancer.

Introduction

Breast cancer remains a leading cause of cancer-related mortality in women worldwide (1). Distant metastasis is a major cause of cancer-related mortality (2). Clinical studies have shown that breast cancer frequently metastasizes to the brain, lungs, bone, liver and lymph nodes (3). Compared to the cancer cells in the primary site, metastatic cells are less sensitive to breast cancer treatments (4). Therefore, the investigation of the factors which are involved in the development of a pre-metastatic niche is of utmost importance.

Inflammatory molecules, including chemokines, cytokines and other secreted molecules are a driving force that affects the microenvironment of extracellular matrix, and extracellular matrix remodeling is an important process for the establishment of a pre-metastatic niche (5). The S100 calcium-binding protein family are inflammatory molecules and contribute to develop an inflammatory tumor microenvironment (6). Although previous studies have indicated that the interaction of S100B with wild-type p53 inhibits p53 function, a high S100B expression is associated with a good prognosis in patients with p53 mutant and p53 wild-type breast cancers. On the whole, our findings demonstrate that S100B treatment suppresses the migratory capacity of ER-negative breast cancer and that S100B expression may serve a predictive marker for metastasis in breast cancer.

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Key words: S100B, migration, epithelial-mesenchymal transition, p53 mutation, breast cancer
However, serum concentrations of S100B and HER2 may not be biomarkers of brain metastasis in breast cancer (16). By contrast, a recent study revealed that the serum levels of S100B were an independent prognostic determinant for patients with brain metastasis (17). In the serum of breast cancer patients subjected to endocrine therapy treatment, elevated levels of S100B are associated with a poor disease-free survival (18). Currently, the role of S100B in breast cancer is not well known and the association between the cell metastatic capacity and S100B has not yet been determined. Thus, in the present study, we aimed to investigate whether S100B directly regulates in vitro migration by performing in vitro experiments and using bioinformatics databases.

Materials and methods

Cells and cell culture. The human breast cancer cell lines, MCF-7 (ATCC® HTB-22™), Hs578T (ATCC® HTB-126™) and MDA-MB-231 (ATCC® HTB-26™), were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The MCF-7, Hs578T and MDA-MB-231 cells were respectively cultured in Eagle's minimum Essential medium (EMEM), Dulbecco's modified Eagle's medium (DMEM) and L-15 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The MCF-7 and Hs578T cells were cultured at 37°C with 5% CO2, and the MDA-MB-231 cells were cultured at 37°C in a CO2-free incubator.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the MCF-7, MDA-MB-231 and Hs578T cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The PrimeScript RT reagent kit (Clontech Laboratories, Inc., Kusatsu, Japan) was used for the reverse transcription of 500 ng of total RNA to complementary DNA. The relative expression of S100B was determined using a Real-Time PCR system (StepOnePlus Real-Time PCT system; Applied Biosystems, Foster City, CA, USA) using Fast SYBR-Green master mix (Applied Biosystems) with the specific primer: forward, 5'-GAGTCAACGGCTTCACTC-3' and reverse, 5'-TTGGGTTATTGAGGA-3'. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected using the primers: forward, 5'-GAGGGACAGCAAGCAGCTG-3' and reverse, 5'-CGTGCCAGCAGTGAACCCG-3'. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected using the primers: forward, 5'-GAGGTCACCGGATTGGTCTCG-3' and reverse, 5'-TTGATTCTTGGAGGGA-3'. Relative mRNA expression was normalized to the expression level of GAPDH and calculated using the 2-∆∆Ct method (19).

Cell migration assay. The migration ability was analyzed by wound healing assay and Transwell migration assay. For wound healing assay, 2.4x10^5 MDA-MB-231 cells were seeded into 24-well plates. At 24 h after seeding, a scratch was made using a 200-ml pipette tip. After scratching, cell debris was removed by washing twice with phosphate-buffered saline (PBS) and the cells were then incubated in serum-free L-15 medium containing 0.01 and 1 nM recombinant human S100B protein (R&D Systems, Minneapolis, MN, USA). Images were acquired at 0 and 12 h following treatment with S100B. Quantification was performed using AxioVs40 V4.8.2.0 microimaging software (MicroImaging GmbH, Gottingen, Germany). QCM™ 24-well Cell Migration assay and Invasion System uncoated 8-µm pore size polycarbonate membranes (Millipore, Billerica, MA, USA) were used for Transwell migration assay according to the manufacturer's instructions. A total of 4x10^4 MDA-MB-231 or Hs578T cells were seeded in 300 ml of serum-free medium in 24-well upper inserts and 500 ml medium with 10% FBS was placed in the lower chamber. After 12 h, the bottom surface of the membrane was fixed in 5% formaldehyde solution, followed by 0.4 g/l crystal violet (Sigma-Aldrich, St. Louis, MO, USA) staining for 2 h. The cells on the upper surface were removed using a cotton swab after washing the crystal violet-stained membrane. The bottom of the membrane was then visualized using an inverted light microscope (Carl Zeiss Primo Vert Microscope; Carl Zeiss, Munich, Germany) at x100 magnification. In total, 4 random fields of view were counted and the relative fold of migration in each group was compared to the 0 nM S100B treatment group.

Western blot analysis. The cells were lysed on ice for 30 min in radioimmunoprecipitation lysis buffer (RIPA) buffer (Millipore) which was supplemented with protease inhibitor cocktail (Millipore) at a 100:1 ratio. The total cell lysate was collected following centrifugation at 4°C, 12,000 x g for 15 min. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of protein were loaded and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred onto polyvinylidene difluoride membranes (Millipore). After 1 h of blocking [5% skim milk in Tris-buffered saline with Tween-20 (TBST) buffer], the membranes were incubated with the following primary antibodies: N-cadherin (1:1,000, cat. no. 2167184; Millipore), E-cadherin (1:1,000, cat. no. 610182; BD Biosciences, San Jose, CA, USA), vimentin (1:1,000, cat. no. MAB3400; Millipore) and GAPDH (1:5,000, cat. no. MAB374; Millipore) at 4°C overnight. After washing with TBST, the membranes were incubated with secondary antibodies, including peroxidase conjugated goat anti-rabbit IgG (1:5,000; cat. no. AP132P; Millipore) and peroxidase conjugated goat anti-mouse IgG (1:5,000; cat. no. AP124P; Millipore) for 1 h at room temperature. The results were acquired on Alpha Innotech FluorChem FC2 Imaging system (ProteinSimple; Bio-Technie, Minneapolis, MN, USA).

Evaluation of S100B expression from public microarray datasets. The mRNA expression of S100B in breast cancer samples was obtained from the Oncomine Research Edition (Thermo Fisher Scientific; http://www.oncomine.org, v4.5) which includes the TCGA breast dataset of Ma et al (20). The clinical data of TP53 mutation and the expression levels of S100B in the breast cancer samples were downloaded as z-scores from the cbioPortal (http://www.cbioportal.org, Breast cancer, TCGA, Cell 2015) (21). The expression of S100B in different subtypes (PAM50) of breast cancer was evaluated using the GOBO database (http://co.bmc.lu.se/gobo) (22).

Assessment of the patient survival rate. The distant metastasis-free survival (DMFS) analysis of the patients with breast cancer...
with different expression levels of S100B was performed using Kaplan-Meier plotter (KM Plotter) database (http://kmplot.com/analysis/) (23). Briefly, the prognostic value of each gene was analyzed by splitting the patient samples into 2 groups according to the median value. To investigate the association between S100B expression and DMFS in different subtypes of breast cancer patients, the DMFS was determined in the subtype of estrogen receptor (ER)-positive (n=664) patients, ER-negative (n=218) patients, or patients subjected to endocrine therapy (n=645). The hazard ratio with 95% confidence intervals and log-rank P-value are shown according to the KM plotter database. To further examine whether the expression of S100B is associated with the TP53 status, the subtype of breast cancer was restricted to TP53 status ‘mutated’ (n=188) or ‘wild-type’ (n=273) and the DMFS of the patients was analyzed. The overall survival was accessed in the dataset ‘Breast Invasive Carcinoma TCGA’ from the SurvExpress database (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp, Interface v2.0, Database Update: November 5, 2017) (24,25). The censor was set to ‘Survival_months’, groups were divided by ‘maximize risk groups’ and stratification was set to ‘class: ER’. To determine the distant recurrence rate, the dataset ‘Vincent Darbon Breast’ (Accession no. GSE9893 in the Gene Expression Omnibus database) in the SurvExpress database. The censor was set ‘Distant recurrence months’, and the raw data of the distant recurrence-free survival curve and mRNA expression levels were sequentially obtained.

The low- and high-risk groups were divided according to the median risk index on the SurvExpress website. The survival curve and mRNA expression were re-drawn using GraphPad Prism 7 software (GraphPad Software, Inc., San Diego, CA, USA).

**Results**

Relatively low S100B expression was observed in breast cancer. To investigate the expression levels of S100B in the breast cancer samples, the Oncomine database (http://www.oncomine.org) was used. Compared to the normal breast tissue, the expression of S100B in various types of breast cancer tissue was significantly low in 2 independent datasets (Fig. 1A and B). To further examine S100B expression in different subtypes of breast cancer according to PAM50 subtypes, S100B expression was evaluated using the GOBO database. As shown in Fig. 1C, a relatively high S100B expression level was observed in the...
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basal-like type when compared to the luminal A (LumA), LumB and HER2-enriched groups. Moreover, S100B expression in the ER-negative group was higher than that in the ER-positive group (Fig. 1D). Based on these results, we hypothesized that S100B may directly regulate the behaviors of the breast cancer cells, particularly in basal-like or ER-negative breast cancer. mRNA expression levels in 3 cancer cell lines, including MCF-7 (luminal A, ER-positive), MDA-MB-231 (basal-like, ER-negative) and Hs578T (basal like, ER-negative) were then determined. As shown in Fig. 1E, the mean S100B expression in the MDA-MB-231 and Hs578T cells was higher than that in the MCF-7 cells; however, there was no statistical difference among these 3 cell lines.

**Treatment with recombinant S100B inhibits cell migration and promotes the epithelial-phenotype.** The effects of extracellular S100B were further examined in the MCF-7, MDA-MB-231 and Hs578T cells. The cells were treated with various concentrations of human recombinant S100B protein. In the Transwell migration assay, S100B treatment significantly inhibited the migration of the two basal-like cell lines (Fig. 2A and B); however, it did not significantly affect the migration of the MCF-7 cells (Fig. 2C). In addition, the inhibitory effects of S100B treatment were observed in the MDA-MB-231 cells (Fig. 2D), but not in the MCF-7 (Fig. 2E) cells, in wound healing assay. The process of epithelial-mesenchymal transition (EMT) in cancer cells is associated with metastatic potential in breast cancer and EMT is related to the basal-like type of breast cancer (26). The MDA-MB-231 and Hs578T cells are classified as the basal B type (27). In order to determine whether S100B affects the mesenchymal or epithelial phenotype transition, the markers of the mesenchymal phenotype, N-cadherin and vimentin, and the marker of the epithelial phenotype, E-cadherin (28), were determined in the MDA-MB-231 cells. The results of western blot analysis revealed that S100B treatment induced the expression of E-cadherin. Thus, S100B treatment led to the acquisition of the epithelial phenotype (Fig. 3).

**High S100B expression is associated with a good prognosis.** As S100B treatment inhibited cell metastasis and the acquisition of the epithelial phenotype, we further investigated whether S100B expression could be a prognostic marker for metastasis in patients with breast cancer, particularly in patients with metastatic breast cancer via the online databases, SurvExpress (http://bioinformatica.mty.itesm.mx:8080/Biomatec/Survivax.jsp) and KM plotter (http://kmplot.com/analysis/). In a dataset from the SurvExpress database, patients...
with a low S100B expression (high-risk group) had a good survival rate in a distant recurrence-free survival analysis (Fig. 4A and B). Furthermore, we examined whether the ER status was an important factor in the S100B-induced inhibitory effect on cell migration. In breast invasive carcinoma, the TCGA dataset from the SurvExpress database, a high S100B expression was associated with a good overall survival rate in only ER-negative breast cancer patients, but not in ER-positive breast cancer patients and in breast cancer patients as a whole (Fig. 4C-E). As S100B treatment resulted in the inhibition of the migration of ER-negative breast cancer cell lines, we further investigated the DMFS using the KM plotter database. S100B expression was not associated with DMFS in all breast cancer patients as a whole (Fig. 4F). By contrast, S100B
expression was associated with a good DMFS in ER-positive (\(P=0.018\)) and ER-negative (\(P=0.049\)) breast cancer patients (Fig. 4G and H). Of note, S100B expression was a marker of prediction of DMFS in ER-positive breast cancer patients who received endocrine therapy (Fig. 4I).

High S100B expression is associated with good prognosis. In melanoma, S100B decreases p53 activity and expression (14,15). In this study, the association between S100B and p53 expression was examined using the TCGA breast dataset from cBioPortal (http://www.cbioportal.org). Compared to the p53 wild-type group, a significantly higher p53 expression was observed in the p53 mutant group (Fig. 5A). Both the MDA-MB-231 and Hs578T cells exhibited p53 mutation (27). However, in this study, S100B treatment resulted in the inhibition of cell migration. Therefore, the association between the p53 status, S100B expression and the prognosis of breast cancer patients was evaluated. The DMFS in breast cancer patients with mutant p53 and wild-type p53 was respectively accessed in the KM plotter database. The results revealed that a high S100B expression was associated with a good outcome (Fig. 5B and C). These findings suggest that a low S100B expression is associated with a high metastatic rate irrespective of the p53 status in breast cancer.

**Discussion**

Inflammation is a critical to create a pre-metastatic niche in cancer (5). The interaction of inflammatory molecules which are secreted from tumor cells, adipocytes and tumor-infiltrating macrophages, neutrophils, myeloid-derived suppressor cells and other types of immune cells regulate the behaviors of tumor cells (29). Metastasis-related pathways, such as the transition between the epithelial and mesenchymal phenotype, were altered by inflammatory molecules (29). Tumor cells secrete interleukin (IL)-6 and IL-8, which are important mediators of EMT in breast cancer (30). In addition, tumor cells secrete IL-6 and IL-8, which affect the phenotypes in an autocrine manner and IL-8 promotes the mesenchymal phenotype (31,32). S100B is an inflammatory mediator and secreted from several types of cells, such as cancer cells and neuronal cells. Previous studies have suggested that S100B-expressing neuronal cells...
exert an autocrine/paracrine effect under physiological conditions (32,33). We thus hypothesized that S100B may directly regulate the phenotype of breast cancer in an autocrine or paracrine manner. Therefore, this study focused on S100B expression in breast cancer cells and on the effects of S100B treatment. Of note, a previous study suggested that S100B was associated with brain metastasis in lung cancer (10). Thus, the tumor microenvironment may be a key factor which results in controversial effects in breast cancer. Compared to lung cancer, breast cancer grows near adipose tissue. Adipocytes secrete many molecules which regulate tumorigenesis and metastasis (34). In addition, adipocytes are an important source of serum S100B (35). The interaction of soluble factors and S100B in the tumor microenvironment exert differential effects on lung cancer cells and breast cancer cells.

There are >20 molecules in the S100 calcium-binding protein family. Different molecules have specific functions and regulatory mechanisms in different types of cancer. For example, S100A8 and S100A9 are associated with metastasis in breast cancer (36). By contrast, the overexpression of S100A6 inhibits metastasis and invasion in human osteosarcoma (37). Our results revealed a relatively high S100B expression in normal breast tissue, as observed by bioinformatics analysis when compared to breast cancer tissue. Furthermore, recombinant S100B treatment significantly suppressed cell migration and promoted the epithelial phenotype in ER-negative breast cancer cell lines. Our results thus suggest that S100B treatment can trigger the activation of anti-metastatic signaling pathways in ER-negative breast cancer. As the serum S100B level is not correlated with the number of metastatic sites of brain metastases in breast cancer (17), and S100B may affect the behavior of breast cancer cells in an autocrine or paracrine manner, we hypothesized that the expression S100B in breast cancer was not associated with the serum S100B levels. The detailed regulatory mechanisms of S100B expression warrant further investigation in breast cancer.

Our results revealed that S100B expression in ER-negative group was higher than that in the ER-positive group, as shown by bioinformatics analysis (Fig. 1D). RT-qPCR analysis revealed a similar trend in breast cancer cell lines (Fig. 1E). S100B expression was associated with a good overall survival rate in patients with ER-negative breast cancer, and a good DMFS in breast cancer patients as a whole. However, current endocrine therapy-treated patients with a high S100B expression and ER-positive breast cancer reveals poor disease-free survival (18). Based on these findings, it is suggested that ER signaling pathways may affect S100B expression and function. The anti-migratory effect of S100B was not observed in ER-positive MCF-7 cells. In microgliia, extracellular S100B, IL-1β, and tumor necrosis factor-α activate the NF-κB and AP-1 transcriptional activity and then results in the upregulation of cyclo-oxygenase 2 (38). The interaction between S100B-mediated signaling pathways and ER signaling pathways warrants further investigation in breast cancer.

Previous studies have demonstrated that S100B binds to the c-terminal of p53 protein, which is an important tumor suppressor gene (14,15). The interaction of S100B and p53 results in the inhibition of p53-mediated growth suppression and apoptosis in melanoma, lung cancer and ovarian cancer (10,14,39). In this study, S100B treatment inhibited the migration in of 2 p53-mutated breast cancer cells and S100B expression was associated with a good prognosis. Although we did not examine whether S100B binds to mutant p53 in the MDA-MB-231 and Hs578T cells, and did not assess the effects of S100B on breast cancer cell lines carrying wild-type p53, the current results revealed that S100B was associated with an anti-migratory effect. The interaction between p53 and S100B may play a minor role in breast cancer. The association between S100B, wild-type p53, mutant p53 and distant metastasis in breast cancer warrants further investigation.

In conclusion, this study demonstrated a novel role of S100B in the migration of breast cancer cells. S100B expression in breast cancer tissue was lower than that in normal breast tissue, and S100B exerted an inhibitory effect on cell migration and the mesenchymal phenotype. The summarized model is shown in Fig. 6. These results suggest that the regulatory mechanisms of action of S100B in breast cancer differ from those in melanoma, lung cancer and ovarian cancer, and that S100B expression may provide a predictive marker for metastasis in breast cancer.

Acknowledgements

The present study was supported by grants from the Ministry of Science and Technology (MOST 104-2314-B-037-053-MY4; MOST 105-2314-B-037-037-MY3; MOST 106-2314-B-037-046) and the Kaohsiung Medical University Hospital Research Foundation (KMUHS10601; KMUH105-5M23).

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