Cancer stem-like cells with increased expression of NY-ESO-1 initiate breast cancer metastasis

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Abstract. Breast cancer stem-like cells (BCSLCs) with a CD44$^+$/CD24$^{-/low}$ phenotype initiate the invasion and metastasis of breast cancer. The expression of New York oesophageal squamous cell carcinoma 1 (NY-ESO-1), one of the most immunogenic cancer-testicular antigens, is largely restricted to cancer and germ cells/placental trophoblasts, with little to no expression in normal adult somatic cells. Currently, few studies have reported the expression or function of NY-ESO-1 in BCSLCs. In the present study, immunohistochemistry indicated enhanced expression levels of NY-ESO-1/CD44 ($P<0.01$) and decreased expression levels of CD24 ($P<0.01$) in metastatic breast cancer tissues (MBCT) compared with non-MBCT. Additionally, the co-localization of CD44, CD24 and NY-ESO-1 in tissue samples was determined using immunofluorescence analysis. The results revealed that the expression of NY-ESO-1/CD44/CD24 was associated with breast cancer metastasis. Moreover, Spearman's rank correlation analysis indicated that CD44/CD24 expression was significantly correlated with that of NY-ESO-1. In the present study, mammosphere culture, a valuable method of BCSLC enrichment, was used to enrich MCF-7 and SK-BR-3 BCSLCs; immunofluorescence, western blotting and flow cytometry demonstrated increased expression levels of NY-ESO-1 and CD44, and low expression levels of CD24 in BCSLCs. Furthermore, the cell migration and invasion assays verified that BCSLCs with an increased NY-ESO-1 expression level exhibited greater invasive and migratory capacity compared with parental breast cancer cells. In addition to previously reported findings from the Oncomine database, it was ascertained that CD44$^+$/CD24$^{-/low}$ BCSLCs with an increased level of NY-ESO-1 expression initiated the invasion and metastasis of breast cancer; therefore, NY-ESO-1 may serve as a novel target for metastatic breast cancer immunotherapy.

Introduction

Tumour metastasis is the primary cause of mortality in patients with breast cancer (1), and reports have indicated that mortality rates rise sharply from 10% for localized carcinoma, to 80% for metastatic breast cancer (2,3). Previous studies have demonstrated that metastasis involves tumour cells (the 'seed') detaching from the primary tumour, migrating through lymphatic and blood vessels, adhering to the target organ (the 'soil'), forming a micro-metastasis and ultimately developing into clinically detectable metastases (4,5). However, due to the complexity of metastasis and the heterogeneity of solid tumours, only a small subset of cells can successfully navigate the entire metastatic course and re-initiate tumour growth (6). Accumulating studies have reported that metastatic cancer stem-like cells may represent the small subset of cells that are responsible for tumour metastasis (7,8). As a focus of current cancer research, breast cancer stem-like cells (BCSLCs), or breast tumour initiating cells, are comparable to dormant adult stem cells; they not only have the ability to self-renew, maintain stemness and differentiate into tumour cells, but are also able to transform into metastatic BCSLCs and promote tumour metastasis when their epigenetics or resident microenvironments are altered (9,10).

Cancer-testicular antigen (CTA) is a type of embryonic antigen whose expression is limited to the testes, ovaries and endometrium, and is notably re-expressed in metastatic tumours (11). To date, New York oesophageal squamous cell carcinoma-1 (NY-ESO-1) has been the most promising and attractive CTA for immune-based therapy, with a clear correlation between its expression in tumours and the induction of an immune response in the majority of malignancies (12), in addition to limited off-target toxicity (13). NY-ESO-1 is also known as cancer-testis antigen 1B, which was cloned from a cDNA library of oesophageal carcinoma using recombinant cDNA library serological analysis technology in 1997 (14). NY-ESO-1 expression has been demonstrated in a variety of tumour types, including bladder, oesophageal, ovarian, prostate, gastrointestinal and triple-negative breast cancers

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(TNBC) (15-20). A number of studies have confirmed a greater prevalence of NY-ESO-1 in metastatic melanoma compared with primary lesions (19,21). Furthermore, NY-ESO-1 possesses a putative role in the proliferation of stem cells and cancer cells; as mesenchymal stem cells differentiate, NY-ESO-1 expression is downregulated (22). Another report also verified that the expression level of NY-ESO-1 was increased in glioma cancer stem cells compared with differentiated cells (23). However, the protein expression level and function of NY-ESO-1 in BCSLCs remain unclear, although studies have reported the expression of NY-ESO-1 mRNA in 10-30% of patients with breast cancer (19,24).

Considerable progress has been made in the early diagnosis and treatment of breast cancer, but metastasis remains an inevitable outcome for the majority of patients. Previous studies have primarily focused on cancer stem-like cells (CSLCs) *or* NY-ESO-1; the association between BCSLCs, NY-ESO-1 and metastasis has seldom been described. Thus, the present study aimed to investigate the correlation between NY-ESO-1$^+$ and CD44$^+$/CD24$^{-/low}$ cells, and to analyse the association between BCSLCs with increased expression of NY-ESO-1 and metastasis to provide a potential target for the treatment of breast cancer.

Materials and methods

Patients and tumour samples. Formalin fixed paraffin-embedded (FFPE) human breast cancer samples were obtained from 30 female patients (median age, 48.75 years; range, 20-81 years; average weight, 44 kg; range, 42-60 kg) attending The Second Affiliated Hospital of Guangzhou Medical University (Guangzhou, China) between June 2016 and June 2017. The stage and grade of each sample were confirmed based on the 2002 American Joint Committee on Cancer Tumour-Node-Metastasis and World Health Organisation classifications by two independent pathologists in a blinded manner. A total of 12 metastatic and 18 non-metastatic tissues were ultimately utilized. In addition, the breast cancer type differed between the patients, with 7 cases of luminal A, 14 of luminal B, 5 with human epidermal growth factor receptor 2 overexpression and 4 cases of TNBC. All samples were collected with informed patient consent, and the use of patient tissues was approved by the Clinical Research and Application Institutional Review Board of The Second Affiliated Hospital of Guangzhou Medical University.

Reagents. Mouse monoclonal anti-CD44 (cat. no. 3570), rabbit monoclonal anti-NY-ESO-1 (cat. no. 45437) and rabbit monoclonal anti-GAPDH (cat. no. 2118) primary antibodies, horseradish peroxidase (HRP)-conjugated goat anti-rabbit and horse anti-mouse (cat. no. 7074 and 7076) IgG secondary antibodies, donkey anti-mouse IgG H&L (Alexa Fluor® 488; cat. no. 4408) and donkey anti-rabbit IgG H&L (Alexa Fluor® 555; cat. no. 4413) were purchased from Cell Signaling Technology, Inc., and anti-mouse IgG H&L (Alexa Fluor® 594; cat. no. 987237) from Invitrogen; Thermo Fisher Scientific Inc. Rabbit monoclonal anti-CD24 (cat. no. ab110448) and mouse monoclonal anti-NY-ESO-1 (cat. no. ab139339) antibodies were purchased from Abcam. The antibodies used for flow cytometry are described in the corresponding methods

section. Histostain UltraSensitive™-plus kits (cat. no. 9730) were obtained from Fujian Maixin Biotechnology, Inc. Other chemicals were purchased from Sigma-Aldrich; Merck KGaA, unless otherwise stated.

Oncomine database and The Cancer Genome Atlas (TCGA) analysis. In order to identify potential molecular markers and therapeutic targets based on known gene-drug analysis, a cancer microarray database and web-based data mining platform aimed to analyse and compare the transcriptome data of target genes in prominent tumour types, as well as their corresponding normal tissues or subtypes (http://tcga-data.nci.nih.gov/tcga) (25). The individual gene expression levels of CD44 and NY-ESO-1 were analysed using the Oncomine database (https://www.oncomine.org/resource/main). The mRNA levels of samples within invasive ductal breast carcinoma (IDBC) and ductal breast carcinoma in situ (DBCS) datasets were compared. 'ALL' fold change and P=0.05 were selected, and the top 10% gene rank was selected as the threshold. The median intensity and the 10th and 90th percentile data of the CD44 and NY-ESO-1 genes from the Oncomine database were plotted using GraphPad Prism (version 5.0; GraphPad Software, Inc.).

Immunohistochemistry (IHC) and immunofluorescence co-localisation staining. The procedure was performed using FFPE samples as previously described (26). The tissue sections were deparaffinised and rehydrated using an alcohol gradient. After incubation with hydrogenase inhibitors (3%) for 15 min at room temperature, and the sections were blocked for 2 h in washing buffer containing 5% normal goat serum (Sigma-Aldrich; Merck KGaA) at room temperature. The primary monoclonal antibodies (CD44, CD24 and NY-ESO-1) were diluted to the appropriate concentration for IHC, according to the manufacturer's protocol. Bound primary antibodies were detected using secondary, biotinylated goat anti-mouse/rabbit antibodies and HRP-conjugated streptavidin. The tissues were subsequently stained using diaminobenzidine and the nuclei were counterstained with haematoxylin. For the negative controls, an isotype mouse/rabbit immunoglobulin was substituted for the primary antibody. The stained slides were examined using an Olympus DP70 light microscope (Olympus Corporation) at x200 magnification by two experienced pathologists from The Second Affiliated Hospital of Guangzhou Medical University who were blinded to the clinical data.

The degree of staining was determined, of which CD44 was primarily expressed at the cytoplasmic membrane, and CD24 and NY-ESO-1 in the cytoplasm (27-29); the staining intensity was scored from 0-3 corresponding to negative, weak, intermediate and strong staining, respectively. The number of cells stained at each intensity was evaluated and the H-score determined using the following formula: 1x (% of cells exhibiting weak staining) + 2x (% of cells exhibiting moderate staining) + 3x (% of cells exhibiting strong staining).

For immunofluorescence co-localization staining of breast cancer tissues, the slides were incubated with primary CD44-mouse and CD24-rabbit monoclonal antibodies (1:200) overnight at 4°C. Subsequently, anti-mouse IgG H&L (Alexa Fluor® 594) and anti-rabbit IgG H&L (Alexa Fluor® 555) were

added for 1 h and the slides were washed ≥5 times. Mouse monoclonal anti- NY-ESO-1 (1:200) was then added for 2 h at room temperature, prior to incubation with the corresponding anti-mouse IgG H&L (Alexa Fluor® 488) for 1 h in the dark. Cells were observed with an inverted fluorescence microscope at x1,000 magnification (Zeiss GmbH).

Cell and mammosphere suspension culture. MCF-7 and SK-BR-3 breast cancer cells were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) according to standard protocols. Mammospheres are one of the most commonly used breast cancer spherical models and are able to enrich $CD44^+/CD24^{-/low}$ BCSLCs. MCF-7 ($1x10^3$ cells/ml) and SK-BR-3 cells ($1x10^4$ cells/ml) were cultured in suspension in serum-free DMEM-F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with B27 (1:50; Gibco; Thermo Fisher Scientific, Inc.; cat. no. 17504-044), 20 ng/ml EGF recombinant human protein (Gibco; Thermo Fisher Scientific, Inc.; cat. no. PHG0311), 0.4% BSA (Sigma-Aldrich; Merck KGaA), 4 mg/ml insulin (Sigma-Aldrich; Merck KGaA) and penicillin-streptomycin (100X; Invitrogen; Thermo Fisher Scientific, Inc.). The cells were shaken twice a day, 20 times each time, to prevent adhesion to the flask walls. Mammospheres was apparent after 7-10 days (30,31).

Flow cytometric analysis. Stem-like mammosphere and parental cells were collected by centrifugation, trypsinized, and resuspended in serum-free medium to obtain a single-cell suspension. Anti-CD44-PE (1:100; cat. no. 338808) and anti-CD24-Percp/cy5.5 (1:100; cat. no. 311116), in addition to the isotype control antibodies for CD44 (1:100; cat. no. 400114) and CD24 (1:100; cat. no. 400252) (all from BioLegend, Inc.), were added to the appropriate sample tubes, mixed and incubated at room temperature in the dark for 30 min. The samples were washed and centrifuged at 400 x g for 5 min at room temperature. For the intracellular detection of NY-ESO-1, the cells were permeabilised using the FIX & PERM™ Cell Permeabilization kit (Invitrogen; Thermo Fisher Scientific Inc.), and stained with the NY-ESO-1 rabbit mAb (1:50) for 1 h at room temperature. The samples were washed, and anti-rabbit IgG H&L (Alexa Fluor® 488; 1:200; cat. no. 1583138; Thermo Fisher Scientific Inc.,) was added for 30 min in the dark. Finally, 500 μl PBS was added and the cells were analysed using a BD FACScalibur™ flow cytometer with CellQuest software (version 5.1; Beckman Coulter, Inc.).

Immunofluorescence. The procedure was conducted as previously described (32). Stem-like mammosphere and parental cells ($5.0x10^4$ cells/glass slip) were fixed with 0.4% polyoxymethylene, and blocked with 1% BSA (Guangzhou Tianjun Biotechnology Co., Ltd.) for 2 h at room temperature. Following incubation with primary antibody against NY-ESO-1-rabbit (1:100) overnight at 4°C, the cells were incubated with anti-rabbit IgG H&L (Alexa Fluor® 488; 1:200) in the dark for 1 h at room temperature. The cells were then counterstained with DAPI and observed using an inverted fluorescence microscope (Olympus Corporation) at x400

magnification, with a core data acquisition system (model no. IX71-A21PH; Applied Precision, Ltd.).

Western blotting. The procedure was conducted as previously described (32). Stem-like mammosphere and parental cells ($2x10^6$ cells/ml) were harvested and washed in triplicate with cold PBS. The cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl; pH 7.4; 250 mM NaCl; 50 mM NaF; 5 mM EDTA; 0.1% Triton X-100; and 0.1 mM Na3VO4). Following centrifugation at 12,000 x g for 15 min at 4°C to remove all organelles, the supernatants were removed, and the protein levels were estimated using a Super-Bradford Protein Assay kit (CoWin Biosciences Co., Ltd.), according to the manufacturer's protocol. Equal amounts of proteins (25 μg/lane) were denatured in SDS-PAGE buffer, subjected to SDS-PAGE on a 20% Tris-glycine gel and transferred onto polyvinylidene fluoride membranes (EMD Millipore). Membranes were blocked with 5% skimmed milk in TBS containing 0.1% Tween-20 for 1 h at room temperature. After washing with TBST one times, membranes were co-incubated with the primary antibodies against NY-ESO-1-rabbit (1:1,000), CD44-mouse (1:1,000), CD24-rabbit (1:500) and GAPDH-rabbit (1:1,000) overnight at 4°C in TBST. Following incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:5,000; cat. no. 7074) and horse anti-mouse (1:5,000; cat. no. 7076) IgG secondary antibodies in TBST at room temperature for 60 min, bands were detected using enhanced chemiluminescence reagents (Thermo Fisher Scientific, Inc.).

Cell migration and invasion assays. The migration and invasion capacities of stem-like mammosphere cells and parental cells were determined using 24-well chambers with 8-μM inserts (Corning Inc.); $2x10^5$ cells/well with 5% (MCF-7) or 1% (SK-BR-3) FBS were placed into the upper bovine fibronectin (2:50; cat. no. 03-090-1-01; Biological Industries) and Matrigel-coated wells (300 μg/ml; Corning Inc.,; cat. no. 356234; invasion only), respectively. DMEM/F12 (BCSLCs) or DMEM (BC) containing 20% FBS was added to the lower chambers. After 12 h (MCF-7) or 24 h (SK-BR-3) for migration, and 24 h (MCF-7) or 36 h (SK-BR-3) for invasion at 37°C, the migratory and invasive cells were fixed for 30 min with 4% paraformaldehyde and stained for 10 min with crystal violet (0.005%; Sigma-Aldrich; Merck KGaA) at room temperature. Images were captured with a light microscope (Olympus Corporation) at x200 magnification, and the cells were counted as previously described (32).

Statistics. All statistical analyses were performed using SPSS for Windows version 16.0 (SPSS, Inc.). A t-test or non-parametric rank test was used to analyse the differences between two variables. Correlation analysis was performed with Spearman's rank correlation. All experiments were performed in triplicate and P<0.05 was considered to indicate a statistically significant difference. Canvas 12 and Prism 5 (GraphPad Software, Inc.) were used for image gathering and processing.

Results

Expression of NY-ESO-1, CD44 and CD24 is correlated with the metastasis of breast cancer. Surface markers such
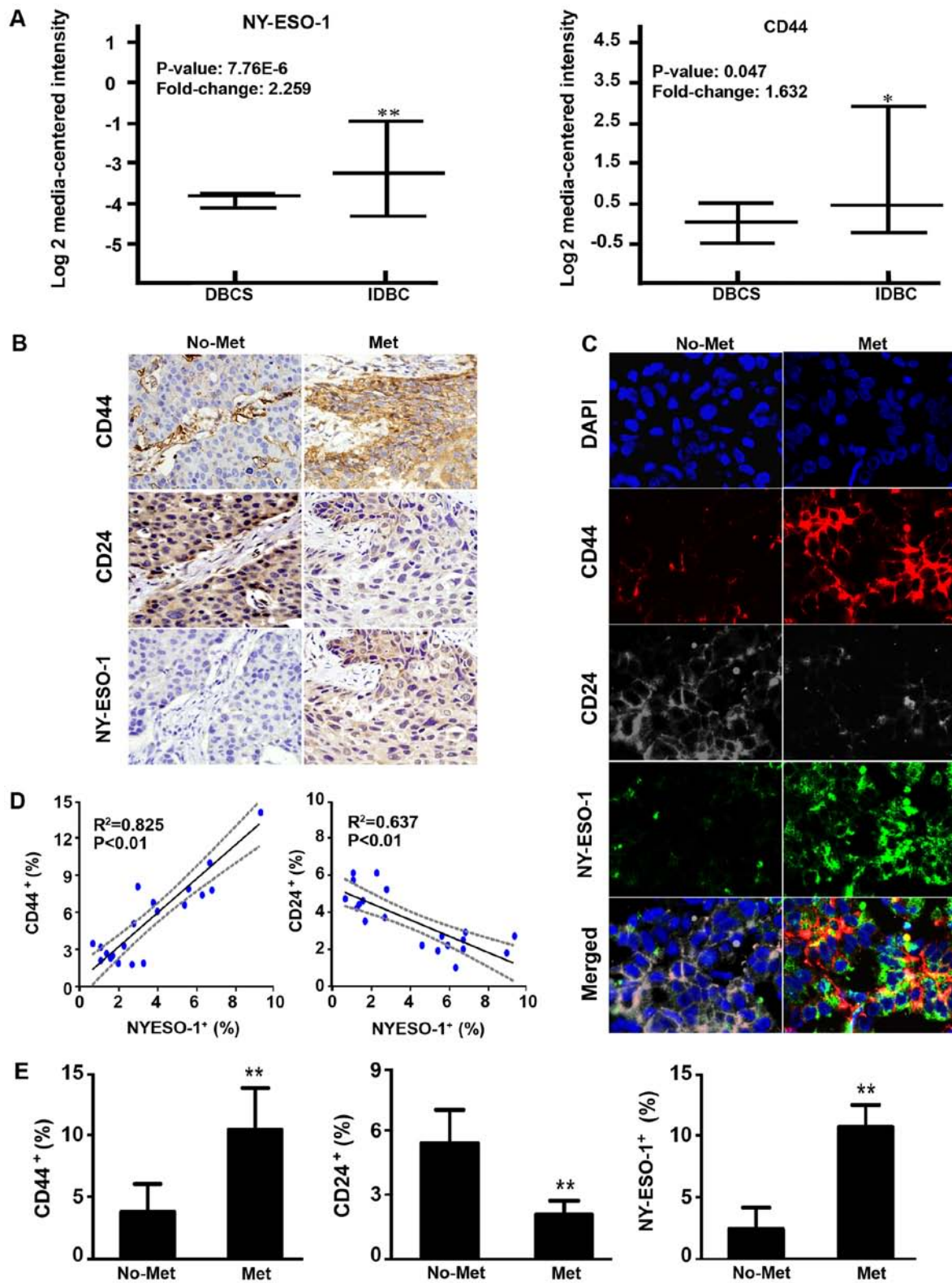
Figure 1. Expression of NY-ESO-1, CD44 and CD24 in MBCT and non-MBCT. (A) NY-ESO-1 and CD44 mRNA expression analysis of IDBC vs. DBCS data from the Oncomine database. (B) IHC of NY-ESO-1, CD44 and CD24 in MBCT and non-MBCT. Magnification, x400. (C) Co-localization of CD44 (red), CD24 (grey) and NY-ESO-1 (green) proteins in breast cancer tissues. Magnification, x1,000. Experiments were performed in triplicate. (D) Correlation between the positive cell rate of NY-ESO-1 and that of CD44/CD24. (E) Error bars correspond to the mean ± standard deviation of IHC. **P<0.01, *P<0.05 compared with non-MBCT or DBCS. NY-ESO-1, New York oesophageal squamous cell carcinoma 1; MBCT, metastatic breast cancer tissue; IDBC, invasive ductal breast carcinoma; DBCS, non-invasive ductal breast carcinoma; IHC, immunohistochemistry; Met: metastatic breast cancer tissues; no-Met, non-metastatic breast cancer tissues.

as CD44 and CD24 have been reliably used to identify and isolate BCSLCs. In order to confirm the expression of CD44, CD24 and NY-ESO-1, the mRNA expression levels of NY-ESO-1^{+}/CD44^{+} samples were investigated using Oncomine
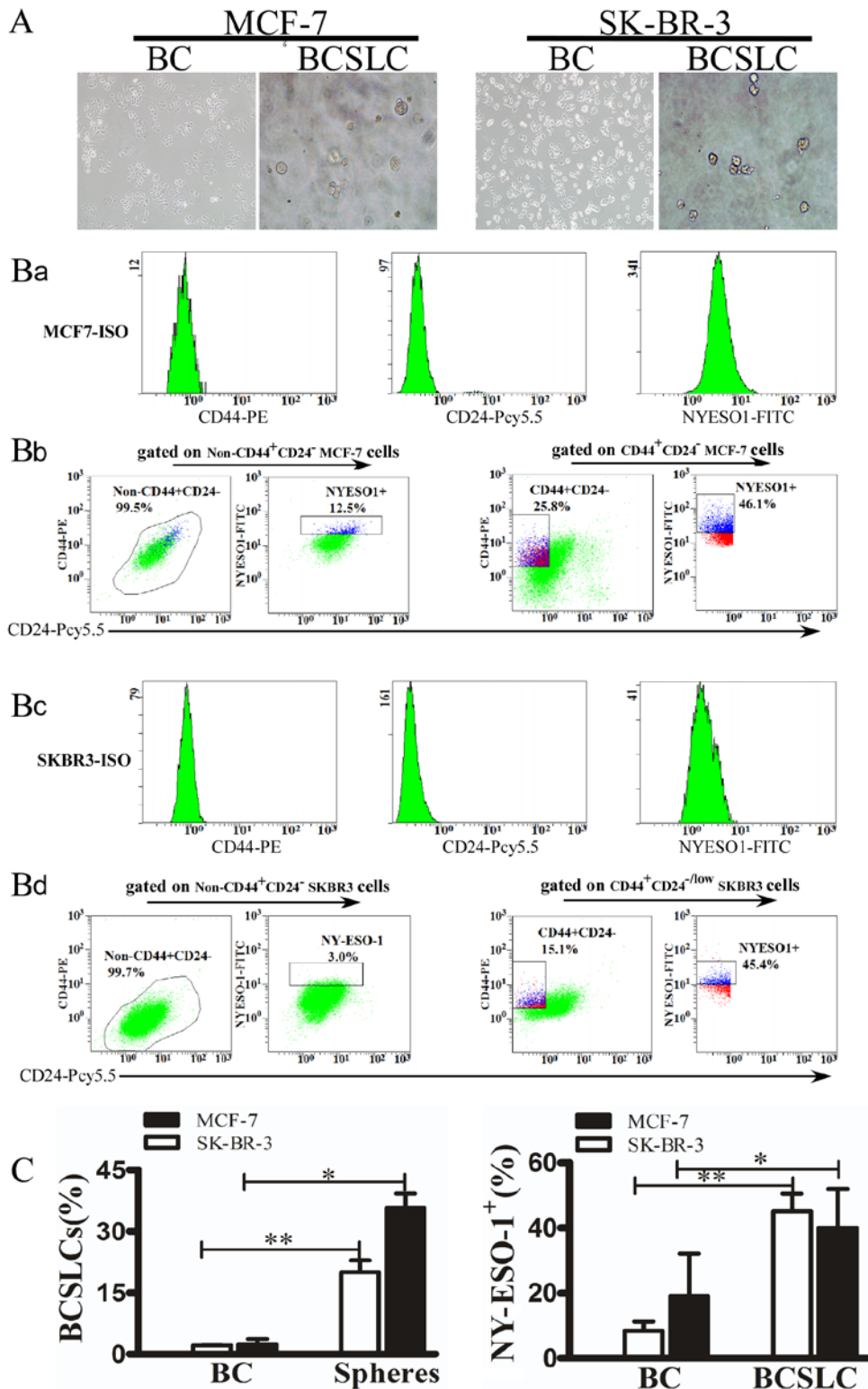
Figure 2. Protein expression of NY-ESO-1 in CD44+/CD24-/low BCSLCs and parental cells. (A) Mammospheres generated from single-cell cultures of SK-BR-3 and MCF-7 cells. Parental breast cancer cells (left; magnification, x100) and mammospheres (right; magnification, x400). (B) Flow cytometric analysis of the expression of NY-ESO-1 in SK-BR-3 and MCF-7 BCSLCs. The isotype controls for CD44, CD24 and NY-ESO-1 gating are presented in Fig. 2Ba and Bc. The non-CD44+CD24- MCF-7 and SK-BR-3 cells were gated to analyse the expression of NY-ESO-1 (left panel), while in the right panel, CD44+/CD24- BCSLCs were gated. The y and x axes represent the mean fluorescence intensity of CD44, CD24 and NY-ESO-1 (Fig. 2Bb and Bd). (C) Error bars correspond to the mean ± standard deviation. Percentage of mammospheres and parental breast cancer cells expressing CD44+/CD24-/low (left) or NY-ESO-1 (right). *P<0.05, **P<0.01 vs. parental SK-BR-3 and MCF-7 cells. All experiments were performed in triplicate. NY-ESO-1, New York oesophageal squamous cell carcinoma 1; BC, breast cancer cells; BCSLC, breast cancer stem-like cells.

analysis, comparing IDBC with DBCS sample data. The GSE14548 dataset obtained by Ma *et al* (33) (http://www. ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14548) demonstrated that the mRNA expression level of CD44 in IDBC
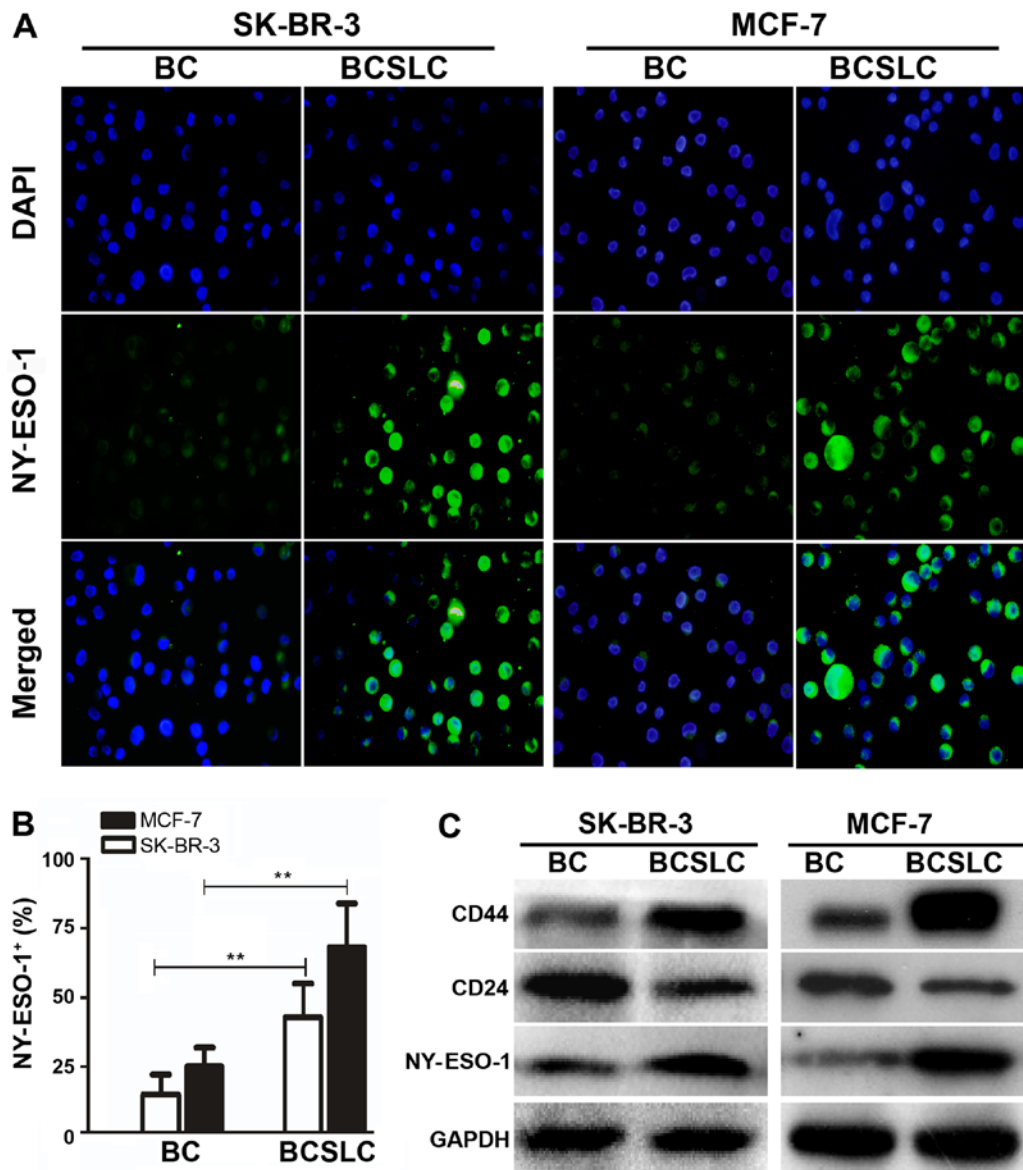
Figure 3. Protein expression of NY-ESO-1, CD44 and CD24 in BCSLCs and parental cancer cells. (A) The expression of NY-ESO-1 in BCSLCs and parental cells using immunofluorescence (magnification, x640). (B) Percentage of NY-ESO-1⁺ BC and BCSLC cells. Error bars correspond to the mean ± standard deviation. **P<0.01 vs. parental cells. All experiments were performed in triplicate. (C) Western blotting for the expression of NY-ESO-1, CD44 and CD24 in BCSLCs and BCs. NY-ESO-1, New York oesophageal squamous cell carcinoma 1; BC, breast cancer cells; BCSLCs, beast cancer stem-like cells.

was increased by 1.632-fold (P=0.047). The TCGA database demonstrated that the NY-ESO-1 mRNA expression level in IDBC samples was 2.259-fold greater than that of DBCS samples (P=7.76x10⁻⁶; Fig. 1A).

Subsequently, IHC was performed on 12 metastatic and 18 non-metastatic tissue samples. Compared with the non-metastatic tissues, the positive expression levels of NY-ESO-1 and CD44 increased 4.14- and 2.67-fold, respectively, while that of CD24 decreased 2.54-fold in metastatic tissues (P<0.01; Fig. 1B and E). Four-color immunofluorescent staining illustrated the co-localization of CD44, CD24 and NY-ESO-1 proteins; this revealed that the expression level of CD44 and NY-ESO-1 in metastatic tissues was greater than in non-metastatic tissues, while that of CD24 was reduced (Fig. 1C). Spearman's rank correlation indicated that the expression level of NY-ESO-1 significantly correlated with that of CD44 (R^2=0.825; P<0.01) and CD24 (R^2=0.637; P<0.01; Fig. 1D).

Collectively, the data suggested that CD44, CD24 and NY-ESO-1 expression correlated with the metastasis of breast cancer, and that NY-ESO-1 expression may be increased in CD44⁺/CD24⁻/low BCSLCs.

CD44⁺/CD24⁻/low BCSLCs exhibit increased expression of NY-ESO-1. In order to demonstrate the increased expression level of NY-ESO-1 in CD44⁺/CD24⁻/low BCSLCs, MCF-7 and SK-BR-3 cells were cultured in suspension to generate mammospheres (Fig. 2A). The isotype controls for CD44, CD24 and NY-ESO-1 gating are presented in Figs. 2Ba and B3, the non-CD44⁺ CD24⁻ MCF-7 and SK-BR-3 cells were gated to analyse the expression of NY-ESO-1 (left panel), while in the right panel, CD44⁺/CD24⁻ BCSLCs were gated. Independent experiments were repeated three times. The results revealed that 35.67±3.50% of the MCF-7, and 20.00±2.90% of the SK-BR-3 spheres were CD44⁺/CD24⁻/low,
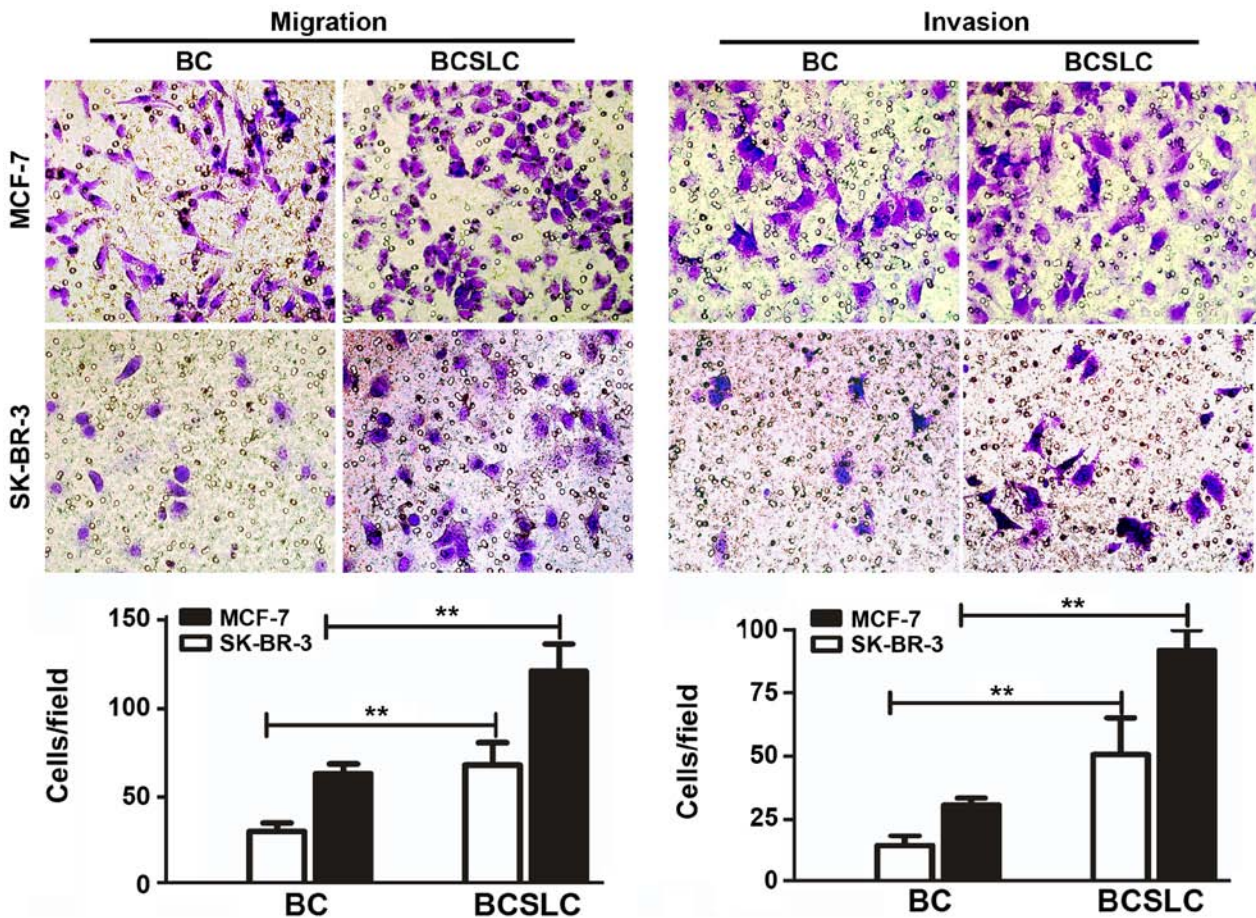
Figure 4. Migration and invasion capacities of BCSLCs and parental cells. Migration (left) and invasion (right) of BCSLCs and parental cells (magnification, x400). Error bars correspond to the mean ± standard deviation. **P<0.01 vs. parental cells. Each experiment was repeated three times. NY-ESO-1, New York oesophageal squamous cell carcinoma 1; BC, breast cancer cells; BCSLCs, beast cancer stem-like cells.

while 97.50±1.22% of parental MCF-7, and 97.90±0.15% of parental SK-BR-3 cells were non-CD44$^+$/CD24$^{-/low}$ (Fig. 2Bb and Bd). In addition, flow cytometric analysis demonstrated that the NY-ESO-1 expression level was 40±12% (MCF-7) and 45.1±5.34% (SK-BR-3) in BCSLCs, while that of the parental MCF-7 and SK-BR-3 cells was 19.1±13 and 8.5±2.76%, respectively (Fig. 2Bb and Bd; P<0.05). These data strongly suggested that mammospheres were able to enrich CD44$^+$/CD24$^{-/low}$ cells. Furthermore, immunofluores-cence indicated a 2.72- (MCF-7) and 2.87-fold (SK-BR-3) increase in NY-ESO-1 expression level in stem-like mammo-sphere cells (Fig. 3A and B; P<0.05). Western blotting also demonstrated that the CD44 and NY-ESO-1 expression level was increased, while that of CD24 was decreased in stem-like mammosphere cells (Fig. 3C; P<0.05). Thus, these data adequately demonstrated that the expression level of NY-ESO-1 was increased in CD44$^+$/CD24$^{-/low}$ BCSLCs.

CD44$^+$/CD24$^{-/low}$ BCSLCs with higher expression levels of NY-ESO-1 promote the invasive and migratory properties of breast cancer cells. To verify whether CD44$^+$/CD24$^{-/low}$ BCSLCs with increased NY-ESO-1 expression levels exhib-ited altered migration and invasion capacities, the migration and invasion assays were performed. The results demonstrated that compared with parental MCF-7 or SK-BR-3 cells, MCF-7 BCSLCs were increased by 1.78-fold (migration) and 1.81-fold

(invasion), and SK-BR-3 BCSLCs by up to 2.05-fold (migra-tion) and 2.09-fold (invasion) (Fig. 4, P<0.05).

Collectively, the presented data indicated that CD44$^+$/CD24$^{-/low}$ BCSLCs with increased expression levels of NY-ESO-1 were able to effectively promote the invasiveness and metastasis of breast cancer cells.

Discussion

CSLCs were first identified in acute myeloid leukaemia, and were termed leukaemia-initiating cells (34,35). Subsequently, in 2003, CD44$^+$/CD24$^-$ BCSLCs were identified and isolated for the first time, and it was verified that these cells possessed metastatic capa-bilities (36). The same conclusion has also been reached by other researchers (37,38). Consistent with previous studies, the present data revealed that CD44$^+$/CD24$^{-/low}$ BCSLCs were involved in cancer metastasis. These genetic signatures in CSLCs support the concept that CSLCs may be metastatic precursors (10).

In addition, the altered expression levels of certain proteins, including KLF49 (10) and phosphatase and tensin homolog (39), are likely to contribute to the maintenance of stem cell-like features (metastatic ability) in breast cancer cells. A previous report suggested that specific CTAs exhibit preferential re-expression in certain tumour types, taking melanoma-associated antigen 1-4 in 70% of metastatic mela-nomas, acrosin-binding protein in 70% of ovarian tumours,

and NY-ESO-1 in 46% of breast tumours as examples (40). Comparably, the present study demonstrated that the expression of the CTA NY-ESO-1 was increased in metastatic breast cancer at the mRNA and protein levels. Additionally, NY-ESO-1 was significantly correlated with the expression of CD44 and CD24. Thus, it was preliminarily verified that NY-ESO-1 may exhibit higher expression levels in CD44^{+}/CD24[-/Low] BCSLCs.

NY-ESO-1 is a representative CTA. Although NY-ESO-1 expression has been reported in a wide range of tumour types, whether NY-ESO-1 was expressed in BCSLCs remained unknown. In the present study, *in vitro* analyses revealed that NY-ESO-1 was abundantly expressed on CD44^{+}/CD24^{-/low} BCSLCs. Furthermore, CD44^{+}/CD24^{-/low} BCSLCs with increased expression levels of NY-ESO-1 exhibited a stronger capacity for invasion and migration compared with parental cells.

In conclusion, NY-ESO-1 was confirmed to be highly expressed in CD44^{+}/CD24^{-/low} BCSLCs, and for the first time, those with increased expression levels of NY-ESO-1 were shown to effectively initiate breast cancer metastasis. NY-ESO-1 may transform BCSLCs from a dormant to a metastatic state, although further research is required to support this hypothesis. NY-ESO-1, with its strong immunogenicity, unique expression pattern and low toxicity, is a promising candidate target for breast cancer immunotherapy, particularly with respect to BCSLCs. Single (NY-ESO-1) or double-target (NY-ESO-1 and CD44) T-cell receptor mimic mAbs may be a potential focus for future research.

Acknowledgments

Availability of data and materials

All data generated or analysed during the present study are included in this published article.

Authors' contributions

Study concept and design, or data acquisition, analysis and interpretation were conducted by MYL, HS, HLH and JQC. Manuscript drafting or revision for important intellectual content, and approval of the final version of the submitted manuscript was also conducted by MYL, HS, HLH and JQC. MYL and HS performed the statistical analysis.

Ethics approval and consent to participate

All patient tissues were collected with written informed consent, and the study was approved by the Clinical Research and Application Institutional Review Board of The Second Affiliated Hospital of Guangzhou Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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