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BREAST CANCER STEM CELLS – DIAGNOSTIC AND PROGNOSTIC SIGNIFICANCE

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Abstract

The aim of this study was to investigate the diagnostic and prognostic significance of CSCs cell population in human breast cancer. Tissue samples from primary and recurrent invasive ductal breast carcinomas, regional lymph nodes (metastatic and non-metastatic), distant bone metastases, as well as cells from permanent breast carcinoma cell lines (MCF7 and MDA-MB-231) and a normal mammary tissue cell line (MCF10) were included in the study. Tumour stem cells were identified immunohistochemically by the markers CD24, CD44 and ALDH1. The results of this study showed an increased population of tumour stem cells with high expression of CD44 and ALDH1 in invasive ductal carcinomas with metastatic regional lymph node and distant metastasis. Determination of the expression of markers characterizing the stem cell phenotype in breast cancer – CD44, CD24 and ALDH1 early in the diagnostic process would be of high informative value regarding the biological behaviour of the tumour, in particular to assess its metastatic potential.

Key words: cancer stem cells, breast cancer, CD44, ALDH1, multiple drug resistance

Introduction. Cancer stem cells (CSCs) are a small cell population, representing 0.1–1% of the composition of tumours. They are characterized by the potential for self-renewal and differentiation into specialized mature cell types and possess tumourigenic potential [1]. The hypothesis that a small fraction of

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tumour cells described as cancer stem cells underlie most tumours was first proposed in 1994 [2]. Initially, such a cell subpopulation was identified in acute myeloid leukemia. Subsequent studies have demonstrated CSCs activity in numerous solid tumours such as carcinomas, mesenchymal tumours, and also in malignant melanomas. A variety of cell surface markers have been used to identify CSCs in cell lines and tumours with different histogenesis (Cluster of differentiation – CD) in particular CD133, CD44, CD24, and CD166 [3]. None of these studies clearly define CSCs as a single universal entity, suggesting that their phenotype may vary considerably between tumours [4]. Experimental confirmation of the presence of a subpopulation of cancer stem cells in breast carcinoma was reported by Al-Hajj et al. [5]. The study showed that the cell population with CD44+CD24−/low phenotype has 10 to 50 times higher ability to induce tumours in NOD SCID mice with respect to unfractionated tumour cells. Breast cancer stem cells (BCSCs) are responsible for resistance to chemo- and radiotherapy and the occurrence of tumour recurrence. They express drug efflux proteins associated with multidrug resistance (MDR) [6] and exhibit resistance to radiotherapy through altered/higher expression of DNA repair enzymes and activation of the free radical neutralization system [7]. BCSCs show expression of specific molecular markers such as CD44+/CD24−/low, Aldehyde dehydrogenase1 high (ALDH1 high), CD133+, Ganglioside 2+ (GD2+), etc. [8]. **CD44** is a membrane glycoprotein involved in cell signalling, adhesion and migration [9]. It is a receptor for hyaluron and other extracellular matrix (ECM) components and regulates tumour cell proliferation, angiogenesis, invasion and metastasis [10]. **CD24** is an adhesion glycoprotein that is expressed on the surface of many cell types and is a ligand for P-selectin. CD24 expression is found in highly differentiated cells [11]. Higher expression of CD44 combined with low expression of CD24 mark the cancer stem cell population [8]. Breast carcinomas that contain a high proportion of CD44+/CD24− cells are associated with the formation of distant metastases [12].

Aldehyde dehydrogenase 1 (ALDH1) is also a marker of BCSCs and belongs to the family of cytosolic enzymes, has a detoxifying function and catalyzes the oxidation of intracellular aldehydes to carboxylic acids [13]. ALDH1 activity in cells is part of the stem phenotype and its expression is associated with poor clinical outcome [14]. In vitro studies have shown that BCSCs are resistant to paclitaxel, doxorubicin, 5-fluorouracil and platinum [15].

The aim of the present study was to investigate the diagnostic and prognostic significance of the tumour stem cell population in breast cancer stem cells (BCSCs) and the feasibility of their identification by routine diagnostic immunophenotyping.

Materials and methods. ***Tissue samples.*** Histological specimens were obtained during routine surgical interventions in specialized cancer clinics in 2021. The examinations were performed after completion of the main treatment and

diagnostic process and were in accordance with current ethical requirements.

After pathomorphological diagnosis and characterization of the tumours in terms of histologic grade, size, presence of regional metastatic lymph nodes, and distant metastases, presumptive histologic material was selected from 10 primary invasive ductal carcinomas (7 surgically resected and 3 tru-cut biopsies), 2 recurrent invasive ductal carcinomas of the breast, 15 regional lymph nodes (5 metastatic and 10 non-metastatic), 3 distant bone metastases, and 2 benign neoplasms (fibroadenomas). Tissues were routinely processed – fixed in formalin and embedded in paraffin (FFPE). Histological sections were reviewed and diagnoses were reconfirmed by a clinical pathologist with specialty in human mammary pathology.

Cell lines. Cell lines from the IEMPAM-BAS collection with different expression profiles of markers characterizing the biological behaviour of human mammary carcinomas (MCF-7, MDA-MB-231) and a non-tumour mammary cell line (MCF10) were included in the study. Tumour cell lines were cultured in DMEM (Gibco-Invitrogen, UK) supplemented with 10% fetal calf serum (Gibco-Invitrogen, UK) and 1% penicillin/streptomycin solution (Lonza, USA). Human epidermal growth factor (100 ng/ml), hydrocortisone (200 ng/ml), human insulin (10 µg/ml), and cholera toxin (100 ng/ml) were added to the culture medium when MCF-10 was cultured. Culturing was performed in a CO_2 incubator (Thermo Scientific, HEPA class 100) at 37 °C, 5% CO_2 and 95% humidity.

Immunohistochemical phenotyping. Tissue samples were examined after pathomorphological diagnosis. Sections of 3 µm thickness were prepared from paraffin blocks, which were examined immunohistochemically after deparaffinization and heat induced epitope retrieval. Mouse monoclonal antibodies against Estrogen Receptor (ER), Progesterone Receptor (PR), c-erbB-2 Oncoprotein (HER-2), Ki67 (clone MM1) produced by Leica Biosystems, UK and mouse monoclonal antibodies against CD44, CD24 and ALDH1 produced by Quartett Immunodiagnostika, Germany were used. Heat induced epitope retrieval of antigens was achieved by 30 min heating at 98 °C in pH 9 detection buffer (Retrieval buffer pH9.0, Leica Bio Systems, UK) and visualization of antigen-antibody reaction was performed by immunocytochemical peroxidase reaction with polymer labelling and DAB chromogen (Novolink Polymer detection Systems RE7140-K, Leica Biosystems, UK) according to the manufacturer's recommendations. Preparations were dehydrated in ascending alcohol series, blotted in xylene and included in synthetic medium (Bio Maunt Medium, Bio Gnost, Groatia) with coverslips. Reading of the results was performed with a Leica DM5000B light microscope at 100× – 400× magnification.

Results and discussion. ***Characterization of hormone receptors, HER-2 status and proliferative activity of human breast cancer tissues examined.*** The expression of Estrogen Receptor and Progesteron Receptor, as well as Human Epidermal Receptor 2 (HER2) and Ki67, underlies the molecular

classification of breast carcinomas and is closely related to the clinical prognosis of patients. In the 10 invasive ductal carcinomas we studied, 9 tumours showed positive ER status (expression in more than 50% of tumour cells). Positive PR was reported in 7 tumours, with 6 tumours showing 25–50% of tumour cells responding and 1 tumour showing less than 10%. Positive HER2 status was found in 4 carcinomas. One of the immunophenotyped tumours was triple negative – ER (−), PR (−), HER2 (−). The Ki67 proliferative index was high (more than 15%) in all the tumours studied.

Expression of markers characterizing tumour stem cell phenotype. Identification of cancer cells with stem cell phenotype in breast carcinoma cell lines (MCF7 and MDA-MB-231) and in tissue samples from invasive ductal carcinoma was performed by immunohistochemical/immunocytochemical monitoring of the expression of membrane markers (CD44 and CD24) and of the cytoplasmic localized enzyme ALDH1. Monoclonal antibodies (Quartett Immunodiagnostika, Germany) were used for this purpose. Brown staining by the chromogen diaminobenzidine (DAB) on the cell membrane surface for the markers CD24 and CD44, and cytoplasmic staining of varying intensity (+), (++), (+ + +) for ALDHA1 was taken as a positive reaction and evidence of expression. Of the mammary carcinoma cell lines examined (MDA-MB-231 and MCF7) and from non-tumour mammary tissue (MCF10), cell populations with a CD44+ CD24−/low ALDH1+ stem cell phenotype were identified in cell line MCF7 derived from pleurally metastatic invasive mammary carcinoma. Metastatic invasive ductal carcinomas exhibited a CD44+/CD24−/ALDH1+ phenotype in more than 10% of tumour cells (Fig. 1). Those mammary carcinomas not accompanied by metastatic axillary lymph nodes at the time of surgical removal have a CD44−/CD24−/ALDH1− profile (Fig. 2). Metastatic regional axillary lymph nodes showed expression of a stem cell phenotype, CD44+/CD24−/ALDH1+, in more than 10% of tumour cells (Fig. 3). In non-metastatic invasive ductal carcinomas, axillary lymph nodes examined did not show CD44 and ALDH1 expression. Distant bone metastases showed CD44 expression and had a CD44+/CD24−/ALDH1+ phenotypic profile (Fig. 4).

The extensive analysis of the results shows the association between the presence of a tumour cell population with CD44+/CD24−/ALDH1+ phenotype and the occurrence of regional and distant metastatic foci, as well as the development of tumour recurrence. The possibility of determining the expression of these markers in the first histological material obtained (tru-cut biopsy, express intraoperative histopathological examination) during the diagnostic process would be of high informative value regarding the biological behaviour of the tumour, in particular for the assessment of its metastatic potential.

Findings and conclusions. BSSCs are emerging as a potential therapeutic target in breast cancer treatment due to their tumourigenic potential, involvement in metastasis and the formation of a drug resistance phenotype. Development of targeted therapy against BCSCs would help to reduce drug resistance and tumour

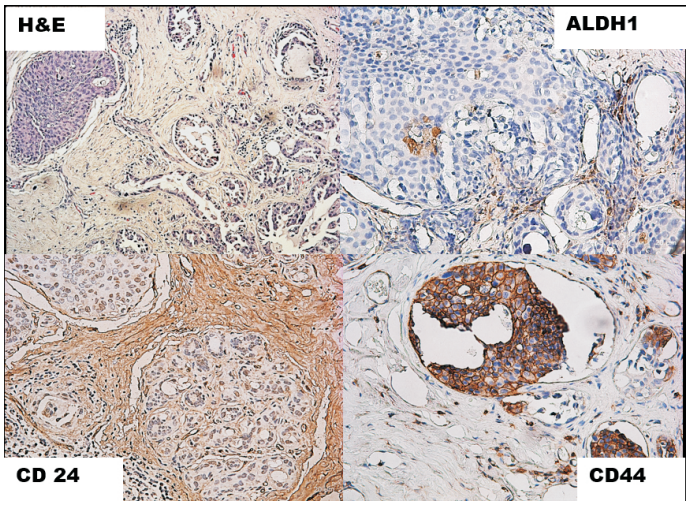


Fig. 1. Invasive ductal carcinoma of the breast with metastatic regional lymph nodes. H&E staining and immunohistochemical reaction with monoclonal antibodies against CD24, CD44 and ALDH1, visualization with chromogen DAB (Leica DM5000B microscope, 200×)

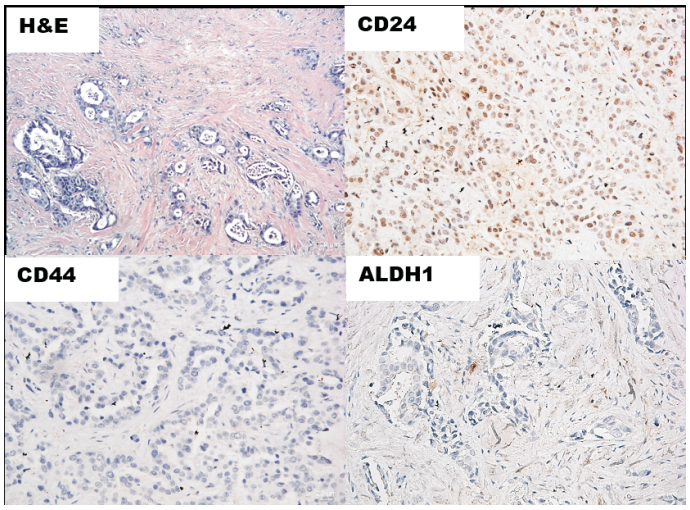


Fig. 2. Non-metastatic invasive ductal breast carcinoma. H&E staining and immunohistochemical reaction with monoclonal antibodies against CD24, CD44 and ALDH1, visualization with chromogen DAB (Leica DM5000B microscope, 200×)

recurrence and would be beneficial in increasing patient survival. In our study, immunohistochemical method was applied to examine the expression of the biomarkers identifying cancer stem cells in breast cancer – CD44, CD24 and ALDH1 – on standard histological specimens – formalin-fixed and paraffin-embedded (FFPE),

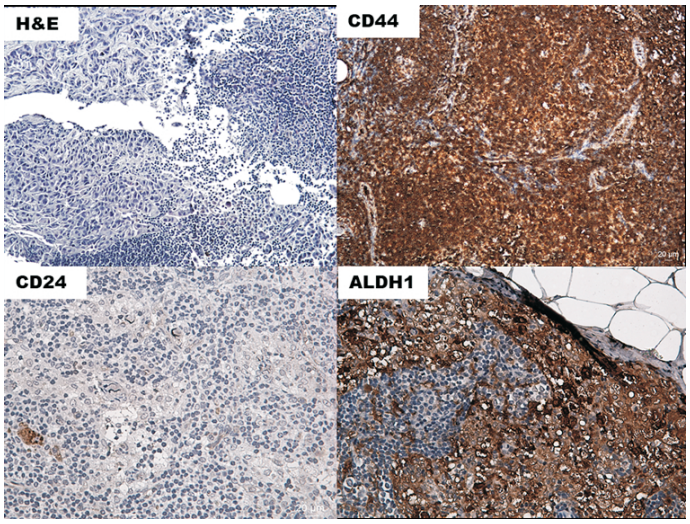


Fig. 3. Metastatic lymph node from invasive ductal carcinoma. H&E staining and immunohistochemical reaction with monoclonal antibodies against CD24, CD44 and ALDH1, visualization with chromogen DAB (Leica DM5000B microscope, 200×)

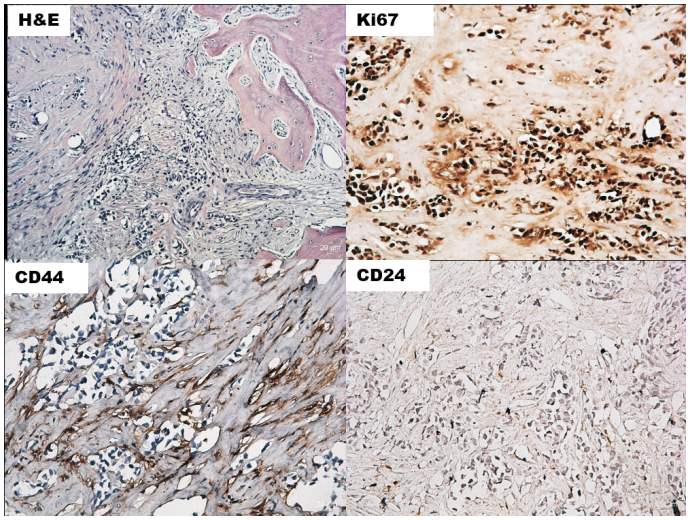


Fig. 4. Distant metastasis (femur) from invasive ductal carcinoma of the breast. H&E staining and immunohistochemical reaction with monoclonal antibodies against CD24, CD44 and Ki67, visualization with chromogen DAB (Leica DM5000B microscope, 200×)

without the need to prepare separate specimens for immunofluorescence study, as reported in current literature [16–18]. The availability and established diagnostic application of the standard methodology we used for immunohistochemical verifi-

cation of tissue biomarker expression will contribute to the application of the CSCs concept in clinical practice, and the establishment of biological characteristics of tumour stem cells may serve prognostic and diagnostic purposes.

REFERENCES

[1] Rycaj K., D. G. Tang (2015) Cell-of-origin of cancer versus cancer stem cells: Assays and interpretations, Cancer Res., **75**, 4003–4011.

[2] Lapidot T., C. Sirard, J. Vormoor, B. Murdoch, T. Hoang et al. (1994) A cell initiating human acute myeloid leukaemia after transplantation into SCID mice, Nature, **367**, 645–648.

[3] Walcher L., A. K. Kistenmacher, H. Suo, R. Kitte, S. Dluczek et al. (2020) Cancer Stem Cells – Origins and Biomarkers: Perspectives for Targeted Personalized Therapies, Front. Immunol., **11**, 1280.

[4] Shiozawa Y., B. Nie, K. J. Pienta, T. M. Morgan, R. S. Taichman (2013) Cancer stem cells and their role in metastasis, Pharmacol. Ther., **138**(2), 285–293.

[5] Al-Hajj M., M. S. Wicha, A. Benito-Hernandez, S. J. Morrison, M. F. Clarke (2003) Prospective identification of tumorigenic breast cancer cells, Proc. Natl. Acad. Sci. U S A, **100**(7), 3983–3988.

[6] Vasiliou V., K. Vasiliou, D. W. Nebert (2009) Human ATP-binding cassette (ABC) transporter family, Hum. Genomics, **3**, 281–290.

[7] Wang Q. E. (2015) DNA damage responses in cancer stem cells: implications for cancer therapeutic strategies, World J. Biol. Chem., **6**, 57–64.

[8] Li W., H. Ma, J. Zhang, L. Zhu, C. Wang et al. (2017) Unraveling the roles of CD44/CD24 and ALDH1 as cancer stem cell markers in tumorigenesis and metastasis, Sci. Rep., **7**, 13856.

[9] Aruffo A., I. Stamenkovic, M. Melnick, C. B. Underhill, B. Seed (1990) CD44 is the principal cell surface receptor for hyaluronate, Cell, **61**, 1303–1313.

[10] Senbanjo L. T., M. A. Chellaiah (2017) CD44: a multifunctional cell surface adhesion receptor is a regulator of progression and metastasis of cancer cells, Front. Cell Dev. Biol., **5**, 18.

[11] Kim H. J., M. J. Kim, S. H. Ahn, B. H. Son, S. B. Kim et al. (2011) Different prognostic significance of CD24 and CD44 expression in breast cancer according to hormone receptor status, Breast, **20**, 78–85.

[12] Abraham B. K., P. Fritz, M. McClellan, P. Hauptvogel, M. Athelogou et al. (2005) Prevalence of CD44+/CD24−/low cells in breast cancer may not be associated with clinical outcome but may favor distant metastasis, Clin. Cancer Res., **11**, 1154–1159.

[13] Ginestier C., M. H. Hur, E. Charafe-Jauffret, F. Monville, J. Dutcher et al. (2007) ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome, Cell Stem Cell, **1**(5), 555–567.

[14] Moreb J. S., D. Ucar, S. Han, J. K. Amory, A. S. Goldstein et al. (2012) The enzymatic activity of human aldehyde dehydrogenases 1A2 and 2 (ALDH1A2 and ALDH2) is detected by Aldefluor, inhibited by diethylaminobenzaldehyde and has significant effects on cell proliferation and drug resistance, Chem. Biol. Interact.,

195, 52–60.
[15] Fillmore C. M., C. Kuperwasser (2008) Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy, Breast Cancer Res., **10**(2), R25.
[16] Zhang X. K. Powell, L. Li (2020) Breast Cancer Stem Cells: Biomarkers, Identification and Isolation Methods, Regulating Mechanisms, Cellular Origin, and Beyond, Cancers (Basel), **12**(12), 3765.
[17] Tanei T., K. Morimoto, K. Shimazu, S. J. Kim, Y. Tanji et al. (2009) Association of breast cancer stem cells identified by aldehyde dehydrogenase 1 expression with resistance to sequential Paclitaxel and epirubicin-based chemotherapy for breast cancers, Clin. Cancer Res., **15**(12), 4234–4241.
[18] Johnson B. E., A. L. Creason, J. M. Stommel, J. M. Keck, S. Parmar et al. (2022) An omic and multidimensional spatial atlas from serial biopsies of an evolving metastatic breast cancer, Cell Rep. Med., **3**(2), 100525.

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