

Evaluation of morphological differences of breast cancer cells using various biological markers and the Feulgen method

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Abstract

Cancer cell nuclei have undergone chromatin structure condensation and changes in shape, nucleolus volume and staining properties. Nevertheless, it has been suggested that cancer stem cells are responsible for resistance to anticancer treatment and tumour recurrence. The aim of the study was to evaluate morphological differences and distribution of DNA amount in luminal and triple negative breast cancer cell populations using various biological markers and the Feulgen method. Multiple cell surface antigens like CD44 (the cluster of differentiation 44), CD24 (the cluster of differentiation 24), aldehyde dehydrogenase (ALDH) and nucleus antigens like embryonic stem cell marker (Oct3/4) can be used to identify cancer stem cells. Strong expression of antibody CD44 and ALDH and positive expression of CD24 and Oct3/4 were seen in both breast cancer groups among morphologically different cells. Amount of DNA indicated variability of cells. The study showed similarity between development and behaviour of microcells and polyploid cells, and we suggest that polyploid cells are in a later phase of development after anticancer treatment.

Key words: biological markers, breast cancer, Feulgen method, microcells.

Abbreviations: ALDH, aldehyde dehydrogenase; CD24, the cluster of differentiation 24; CD44, the cluster of differentiation 44; ER, oestrogen receptor; HER2, human epidermal growth factor receptor; IOD, integrated optical density; Ki67, cell proliferation marker; Oct3/4, homeobox domain transcription factor of the POU family, expressed in embryonic stem cells and germ cells; OD, optical density; PgR, progesterone receptor.

Introduction

Breast cancer is the most common oncological disease among women. Positive oestrogen, progesterone and HER2 (human epithelial growth factor) receptors are markers of luminal breast cancer (Nishimura et al. 2011). Patients with luminal breast cancer have a relatively good treatment prognosis, because of high survival and low relapse (Reddy 2011). On the other hand, triple negative breast cancer is associated with no oestrogen, progesterone and HER2 receptors (Nawloga et al. 2010). Women with triple negative breast cancer have poor prognosis and do not respond to chemotherapy (William et al. 2010). Improvement in treatment efficiency is a major priority. It is known that after surgical resection cancer relapses and become more aggressive. Cancer stem cells could be the reason for these phenomena, due to self-renewal, non-controlled division and resistance against traditional anticancer treatment. There are three hypotheses of how a cancer stem cell may arise: (1) a stem cell undergoes a mutation, (2) a progenitor

cell undergoes two or more mutations, or (3) a fully differentiated cell undergoes several mutations that drive it back to a stem-like state. In all three scenarios, the resultant cancer stem cell has lost the ability to regulate its own cell division (Goldthwaite 2006).

Cancer stem cells constitute a small part of the cell population and their identification and marker options are currently limited. Cancer stem cells express specific antigens on their cell surface. For example, cell surface CD44⁺/CD24^{-low} are thought to be markers for breast cancer stem cells (Colabeda et al. 2008). The biological marker CD44 is a protein, adhesion molecule of cell surface and it is involved in cell-cell and cell-cell matrix interaction (Goodison et al. 1999). There is a known connection between CD44 and metastasis, but CD44 alone could not enable the metastasis (Goodison et al. 1999). CD24 is a small glycosylated mucine-like glycosyl-phosphatidylinositol-associated cell-surface protein (Crispe, Bevan 1987), and most studies have shown that CD24 may be involved in signal transduction (Magnaldo, Barrandon 1996). Interestingly,

some studies revealed enrichment of the CD44⁺/CD24^{-/low} and CD44⁻/CD24⁺ cell populations in basal-like and luminal breast cancer cell lines, respectively, CD44 being positively associated with stem cell-like characteristics and CD24 expression related to differentiated epithelial features (Cobaleda et al. 2008; Ricardo et al. 2011).

Aldehyde dehydrogenase (ALDH) activity is an important parameter, being responsible for oxidation of aldehyde within cells (Ricardo et al. 2011). The detoxification enzyme ALDH is responsible for intracellular ALDH oxidation; thereby it acts as a mediator to self-defence and resistance to the alkylating agent and is used in anti-cancer therapy (Prasmickaite et al. 2010). A positive ALDH is significantly correlated with poor prognosis in mammalian gland and lung cancers. ALDH and its isoform ALDH-1 are being used as functional markers to identify high tumourigenesis and metastatic potential, and also to detect resistance in various epithelial tumours (Prasmickaite et al. 2010).

There are some studies in which the embryonic stem cell marker Oct3/4 has been associated with pluripotency of a cell. Nevertheless, a low level of Oct3/4 characterizes decrease of pluripotency (De Jong, Looijenga 2006). Pluripotency is considered a sign of embryonic stem cells (De Jong, Looijenga 2006).

The Feulgen method has been used for qualitative DNA detection in the cell nucleus. This staining is directly proportional to the amount of DNA in a sample (Chieco et al. 1999). Differences in the amount of DNA shows cell variability and differences between cells with diploidic or haploidic DNA. In our research we studied histological samples of two types of breast cancer. The aim of the study was to evaluate morphological differences of the cell population and the distribution of DNA amounts in luminal and triple negative breast cancer cell population using various biological markers and the Feulgen method. Amount of DNA was determined using the Feulgen method.

Materials and methods

Subjects

Permission for this study was received from the Research Ethics Commission of the Riga East University Hospital Oncology Centre and Experimental and Clinical Medicine Institute, University of Latvia. Primary surgical material of women's breast cancer was collected from September 2011 till February 2012. There were two groups of breast cancers – luminal (32 histological samples) and triple negative breast cancer (11 histological samples). Mean patient age in the luminal breast cancer group was 59 years, and in the triple negative breast cancer group – 52 years. The material was prepared for sectioning in paraffin blocs. For each sample, six cuts (1.5 µm) of the paraffin section were performed using a microtome, at the Pathology Centre of the Riga East University hospital.

Immunohistochemical staining

Nuclei were counterstained with haematoxylin. For both groups of breast cancer, slide samples were stained with haematoxylin-eosin.

The primary antibodies against CD44 (the cluster of differentiation 44, clone: DF1485, mouse monoclonal antibody, Novocastra™, Leica Biosystems Newcastle, United Kingdom), CD24 (the cluster of differentiation 24, clone: ML5, mouse monoclonal antibody, BioSite, Finland), Oct3/4 (homodomain transcription factor of the POU family, expressed in embryonic stem cells and germ cells, clone: N1NK, mouse monoclonal antibody, Novocastra™, Leica Biosystems Newcastle, United Kingdom) and polyclonal antibody against ALDH (aldehyde dehydrogenase, ALDH1A1, clone: 5A11, mouse monoclonal antibody, BioSite, Finland) were used to differentiate cancer cell populations. The slides were immunohistochemically stained following the manufacturer's recommendations.

For each biological marker, one slide was analysed under a microscope and 16 images were taken for each sample.

Feulgen method

The Feulgen method was used to determine breast cancer cell population heterogeneity, integrated optical density (IOD), mean optical density of cell nuclei, and size of cell nuclear area. The breast cancer histological sections were subjected to hydrolysis for 50 min at 37 °C in 3N HCl and rinsed in 1N HCl. Then, samples were transferred to Schiff's reagent (pH 3.5 to 4.0) for 1h at 37 °C in a hermetically sealed container.

Microscopy and image capture

A Leica DM1000B (Leica Microsystems) microscope with 63 × objective (apochromatic, aperture 1.40) was used for sample visualization and a Leica DFC400 (Leica Microsystems) digital camera was used for image capture.

Data evaluation

Biological marker (CD44, CD24, ALDH, Oct3/4) expression was detected by semi-quantitative method, where 0, expression was not detected; 1, low expression; 2, expression was detected in average level; 3, high expression, but not in a whole sample; 4, strong expression in the entire sample.

In addition, both types of cancer histological samples were stained by the Feulgen method to estimate DNA amount in the nuclei of cancer cells. The integrated optical density, proportional to the amount of DNA, and mean optical density, proportional to the concentration of DNA of nucleus, were measured using ImagePro Plus 6.0 software. Optical density (OD) detection and analyses allows for a variety of measurements. OD detection is a method using light absorption to detect amount of a substance in a sample (Farrel 2009).

Integrated optical density (IOD) is used in microscopy and cell research for DNA quantification in cells. IOD is

obtained by deducting background pixel value of a certain field of interest from the value of a single pixel. IOD is produced from a grayscale image (Farrel 2009).

Results

Luminal and triple negative breast cancer

Histochemical samples stained with haematoxylin-eosin were used to evaluate cancer histology. Luminal breast cancer samples were relatively similar to normal breast histological samples. Luminal breast cancer sections had similar structure to that of triple negative breast cancer.

In triple negative breast cancer samples stained with haematoxylin-eosin histological structure was changed in comparison to normal samples. Large cells with intensive stained nucleus and clearly detectable nucleoli, large size cells with modified nucleus shape, and small cells (microcells) with condensed chromatin were identified in the samples. Structures (*alveoli, ductus lactiferi* etc.) with normal appearance were rare in triple negative breast cancer.

Luminal and triple negative breast cancer and biological markers

In both breast cancer groups, expression of antigens CD44 and ALDH was observed in large cells with rounded nucleus, in large cells with non-symmetrical nucleus and in small rounded cells. Expression intensity of CD24 was lower than that of CD44 and ALDH. Antigen CD24 expression was observed in cells of specific morphology, small rounded cells and large, oval or rounded cells. Cancer stem cell antigen CD44 and ALDH expression was strong in cells of variable morphology and in mammary gland *alveoli* (photographs not shown). Biological marker CD24 (Fig. 1) was seen mostly in two types of cells: large size cells (Fig. 1, B) and microcells (Fig. 1, A.), and the expression of CD24 (Fig. 1, D, F) was seen in different cells within both breast cancer groups.

Expression of embryonic stem cell antigen Oct3/4 and cell surface antigen CD24 (Fig. 1) was pronounced in microcells (Fig. 1 D) and in large size cells (Fig. 1 F). Additionally, we observed microcells without CD24 (Fig. 1

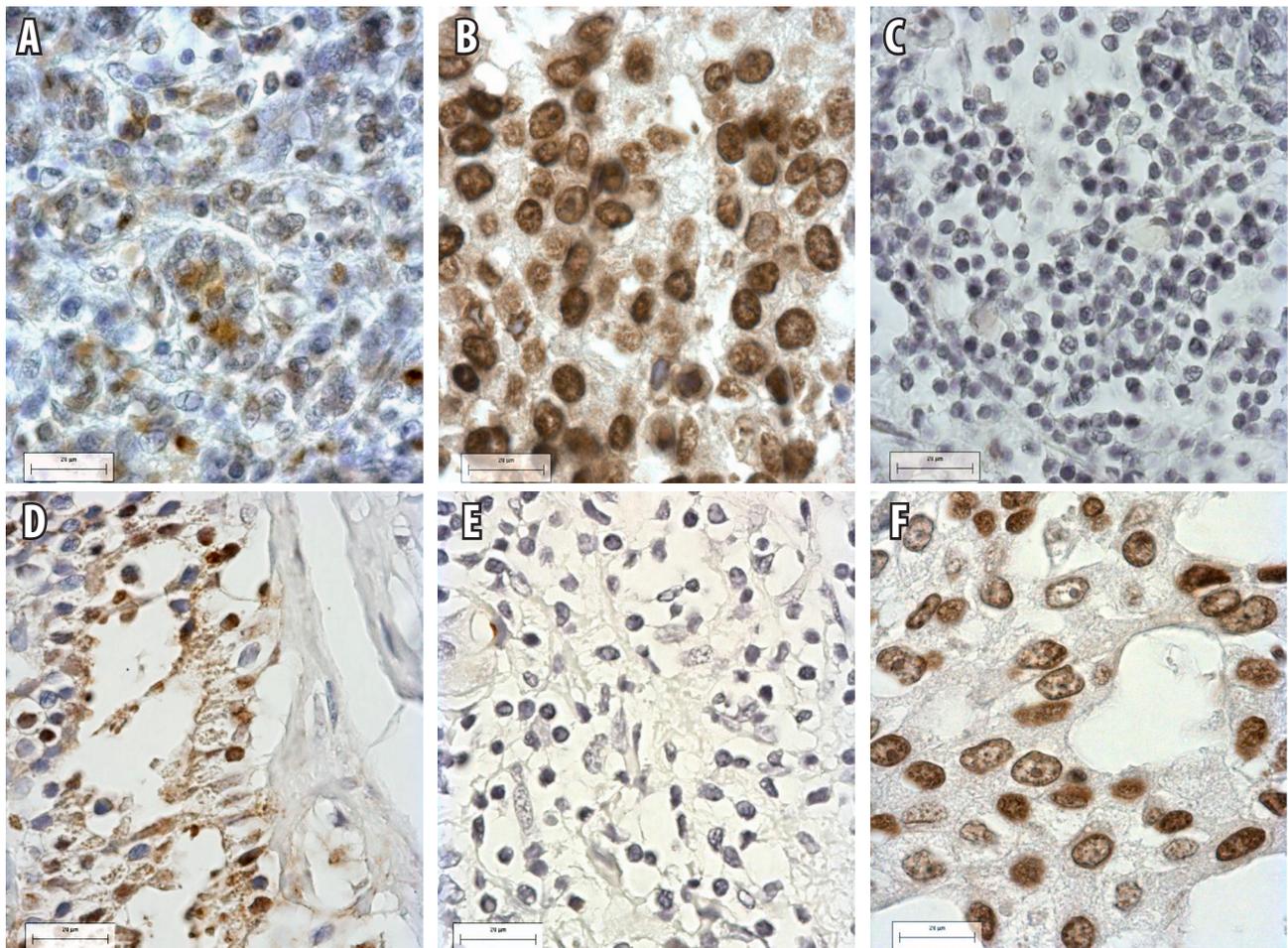


Fig. 1. Antigen CD 24 expression in luminal and triple negative breast cancer histological samples. Triple negative breast cancer histological samples, immunocytochemically stained against CD24, counterstained with haematoxylin: A, small size cells with CD24 expression; B, large size cells with CD24 expression; C, small size cells, where CD24 expression not detected. Luminal breast cancer histological samples, stained against CD24, counterstained with haematoxylin: D, CD24 expressing in small size cells; E, small cells without CD24 expression; F, CD24 expression in large size cells.

E) and Oct3/4 expression.

Biological marker (CD44, CD24, ALDH and Oct3/4) expression in triple negative breast cancer was observed in morphologically variable cells. For example, CD44 and ALDH had very strong expression throughout samples and in morphologically different cells, whereas semi-quantitative histological section evaluation showed that expression of CD44, ALDH (Fig. 2 A and B), CD24 and Oct3/4 (Fig. 2 C and D) antigens was stronger in the triple negative breast cancer group, compared to the luminal breast cancer group. Both luminal and triple negative breast cancer group histological samples that were immunohistochemically stained against CD44 and ALDH showed strong expression, whereas CD24 expression was low. We determined that CD44, CD24, Oct3/4 and ALDH expression in triple negative breast cancer was stronger than in luminal breast cancer ($p < 0.05$; Fig. 2).

Amount of DNA

The microcell population of the luminal breast cancer contained cells with low and high DNA concentration while large cells exhibited only low DNA concentration (Fig. 3 B). In the triple negative breast cancer cell population, polyploid cells and microcells contained high and low concentration of DNA, respectively (Fig. 3 A). Cell population with a larger nucleus area and lower average optical density was dominant in luminal breast cancer samples. In contrast, the triple negative breast cancer cell population showed

cells with smaller nucleus area and higher average optical density (Fig. 3 A and B).

Discussion

As a rule, relation between CD44 and CD24 expression is used to detect breast cancer stem cells. Generally, the proportion of cells having CD44⁺/CD24⁻ phenotype (expression of CD44 and no CD24 expression) increased after chemotherapy (Lu et al. 2009; Freivalds et al. 2011). As described in literature, to identify breast cancer stem cells researchers have used mainly three biological markers: CD44, CD24 and ALDH. In this study, breast cancer histological samples were used to identify different cell populations: large size cells with phenotype CD44⁺, ALDH⁺, CD24⁺ and CD44⁺, ALDH⁺, CD24⁻; and microcells with phenotype CD44⁺, ALDH⁺, CD24⁺ and CD44⁺, ALDH⁺, CD24⁻. We suggest that the cell phenotype with high CD44⁺, CD24⁻ and ALDH⁺ expression levels might represent cancer stem cells. Also, Ricardo (2011) found that in breast cancer cell lines, CD44⁺/CD24^{-low} and ALDH positive (ALDH⁺) expression could be used to identify cancer stem cells with various differentiation levels.

Comparing the two breast cancer groups, literature indicates that the expression of CD44 and ALDH is higher in the triple negative breast cancer group (prognostic adverse case with higher cancer stem cell number). In concordance with Balic et al. (2013), in breast cancer cell

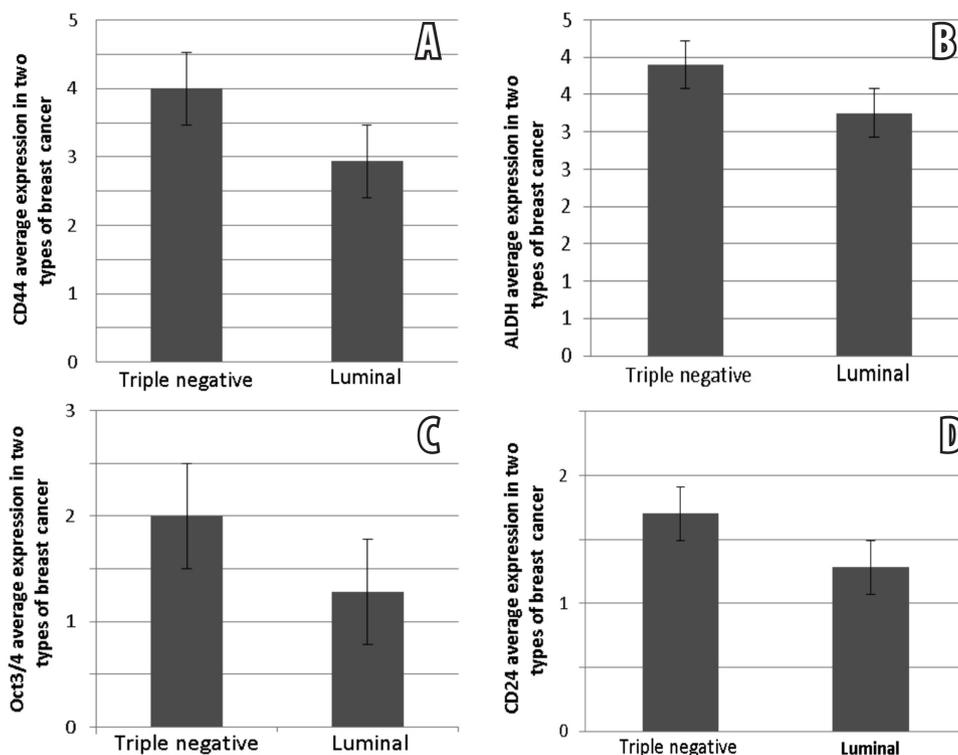


Fig. 2. Average antigen expression in luminal and triple negative breast cancer histological samples. CD44 (A), ALDH (B), Oct3/4 (C) and CD24 (D).

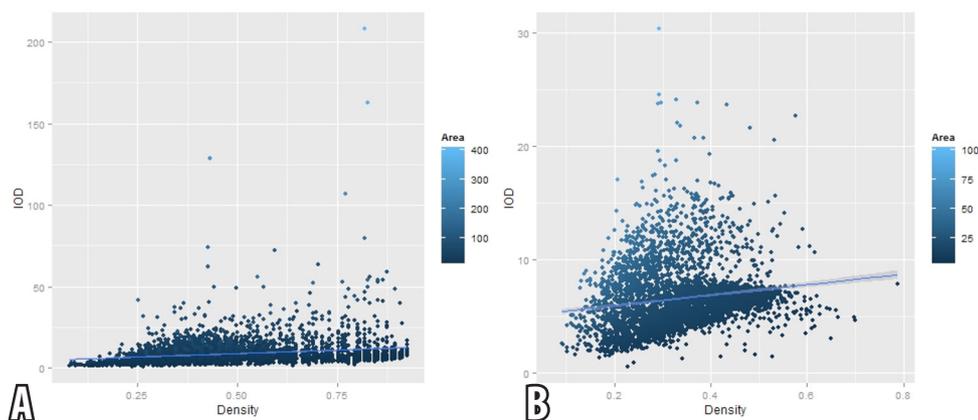


Fig. 2. Integrated optical density versus nucleus mean optical density and nucleus area in cancer cell population. A, in triple negative breast cancer histological samples; B, in luminal breast cancer histological samples.

lines, primary cancer tissues of triple negative breast cancer had higher frequency of ALDH⁺ and CD44⁺ CD24⁻ cells. In our study, comparing the two breast cancer groups, antigen (CD44, CD24 and ALDH) expression was higher in the triple negative group. Similar results were obtained by Kristiansen et al. (2003) in study of a primary breast carcinoma cell line. They concluded that CD24 expression indicated tumour malignancy. In a previous study, they also concluded that CD24 expression indicates ovary cancer, non-small cell lung cancer and prostate cancer (Ulbricht 2005). Liu et al. (2014) found that each marker (CD24, CD44 and ALDH) identified a different cell sub-population in breast cancer stem cells.

Embryonic stem cell marker Oct3/4 expression in both breast cancer groups was similar to CD24. Oct3/4 expression was present in two cell types: large size cells and microcells. Antibody against Oct3/4 was used as a marker against embryonic carcinoma and germinoma (Ulbricht 2005). In this study we showed that higher expression of Oct3/4 was detected in triple negative breast cancer compared to the luminal breast cancer group. In research with bone marrow cells, Oct3/4 was associated with cell plasticity or a subgroup of passive stem cells, whereas cells with subtype Oct3/4⁺ can make conglomerates and can spontaneously result in differentiated cells *in vitro* outside of bone marrow (Pallante et al. 2007).

There was a tendency that there were more small cells within the luminal breast cancer cell population with low and high concentration of DNA as well as large cells with low DNA concentration. In contrast, the triple negative breast cancer cell population included large and small cells both with high and low concentration of DNA. Also Oct3/4 expression in triple negative breast cancer cell populations occurred both in large and small cells. In addition, the luminal breast cancer cell population was dominated by small cells that expressed Oct3/4. Intra-tumour heterogeneity denotes the coexistence of sub-populations of cancer cells that differ in their genetic, phenotypic or behavioural characteristics within the primary tumour, and

between the primary tumour and its metastasis (Martelotto et al. 2014). Many medical failures can be caused to the outgrowth of clones that have specific resistance mechanisms and were present before the origin of therapy due to intra-tumour genetic heterogeneity (Martelotto et al. 2014).

Conclusions

For identification of cancer stem cells the following biological markers were used: CD44, CD24, ALDH and Oct3/4. However, it is difficult to correctly identify cancer stem cells with these biological markers. Results of this study suggest that microcells and polyploid cells have similar properties, but polyploid cells could become microcells in further development stage. Polyploid cell behaviour seems to be identical with microcells in the conditions defined in this work. Increased presence of microcells and polyploidy cells in cancer cell population could be bad anticancer therapy efficiency indicator.

Consequently, our study suggested that both microcells and polyploid cells with high concentration of DNA could be responsible for resistance. However, it seems that only microcells could be the progenitors of resistant cell population.

It would be necessary to perform research in cell population heterogeneity using flow cytometry for cell sorting, as a continuation of this study.

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References

- Balic M., Schwarzenbacher D., Stanzer S., Heitzer E., Auer M., Geigl J.B., Cote R.J., Datar R.H., Dandach N. 2013. Genetic

- and epigenetic analysis of putative breast cancer stem cell models. *BMC Cancer* 13: 358–373.
- Chieco P., Derenzini M. 1999. The Feulgen reaction 75 years on. *Histochem. Cell Biol.* 111: 345–358.
- Cobaleda C., Cruz J.J., Gonzalez-Sarmiento R., Sanchez-Garcia I., Perez-Losanda J. 2008. The emerging picture of human breast cancer as a stem cell-based disease. *Stem Cell Rev.* 4: 67–79.
- Crispe I.N., Bevan M.J. 1987. Expression and functional significance of J11d marker on mouse thymocytes. *J. Immunol.* 138: 2013–2018.
- De Jong J., Looijenga L.H.J. 2006. Stem cell marker OCT3/4 in tumor biology and germ cell tumor diagnostics: history and future. *Crit. Rev. Oncol.* 12: 171–203.
- Farrell R. 2009. RNA Methodologies, Fourth Edition: Laboratory Guide for Isolation and Characterization. Elsevier, City, pp. 287–288.
- Freivalds T., Simsone Z., Kudaba I., Berzins J. 2011. Prognostic and predictive significance of breast cancer stem cells. *Acta Chir. Lat.* 11: 122–125.
- Goldthwaite C.A. 2006. Are cancer stem cells involved in cancer? *Regen. Med.* 9: 89–95.
- Goodison S., Urquidi V., Tarin D. 1999. CD44: cell adhesion molecules. *J. Clin. Pathol. Mol. Pathol.* 52: 189–196.
- Kristiansen G., Winzer J.K., Mayordomo E., Bellach J., Schlüns K., Denker C., Dahl E., Pilarsky C., Altevoigt P., Guski H., Dietel M. 2003. CD24 expression is a new prognostic marker in breast cancer. *Clin. Cancer Res.* 9: 4906–4913.
- Liu Y., Nenutil R., Appleyard M.V., Boylan M., Thompson A.M., Coates P.J. 2014. Lack of correlation of stem cell markers in breast cancer stem cells. *British J. Cancer* 110: 2063–2071.
- Lu X.Q., Suo Z., Ma C.L., Xu K.J., Liu Y.S., Li H.X. 2009. Quantity and distribution of CD44+/CD24- cells in breast cancer tissue and the cell lines. *Chin. J. Pathol.* 38: 441–444.
- Magnaldo T., Barrandon Y. 1996. CD24 (heat stable antigen, nectadrin), a novel keratinocyte differentiation marker, is preferentially expressed in areas of the hair follicle containing the colony-forming cells. *J. Cell Sci.* 109: 3035–3045.
- Martelotto L.G., Ng K.Y.C., Piscuoglio S., Weigelt B., Reis-Filho J.S. 2014. Breast cancer intra-tumor heterogeneity. *Breast Cancer Res.* 16: R48.
- Nalwoga H., Arnes J.B., Wabinga H., Akslen L.A. 2010. Expression of aldehyde dehydrogenase I (ALDH I) is associated with basal-like markers and features of aggressive tumors in African breast cancer. *British J. Cancer.* 102: 369–375.
- Nishimura R., Osaki T., Okumura Y., Tashima R., Toyozumi Y., Arima N. 2011. Changes in the ER, PgR, HER2, p53 and Ki-67 biological markers between primary and recurrent breast cancer: discordance rates and prognosis. *World J. Surg. Oncol.* 9: 131–136.
- Pallante A.B., Duignan I., Okin D., Chin A., Bressan M.C., Mikawa T., Edelberg J.M. 2007. Bone marrow Oct3/4+ cells differentiate into cardiac myocytes via age-dependent paracrine mechanisms. *J. Am. Heart Assoc.* 100: e1–e11.
- Prasmickaite L., Engesæter B., Skrbo N., Hellenes T., Kristian A., Olover N.K., Suo Z., Mælandsmo G.M. 2010. Aldehyde dehydrogenase (ALDH) activity does not select for cells with enhanced aggressive properties in malignant melanoma. *PlosOne* 5: 1–12.
- Reddy K.B. 2011. Triple-negative breast cancers: an updated review on treatment options. *Curr. Oncol.* 18: 173–179.
- Ricardo S., Vieira A.F., Gerhard R., Leitão D., Pinto R., Cameselle-Teijeiro J.F., Milanezi F., Schmitt F., Paredes J. 2011. Breast cancer stem cell markers CD44, CD24 and ALDH1: expression distribution within intrinsic molecular subtype. *J. Clin. Pathol.* 64: 937–946.
- Ulbright T.M. 2005. Germ cell tumors of the gonads: a selective review emphasizing problems in differential diagnosis, newly appreciated, and controversial issues. *Modern Pathol.* 18: 61–79.
- William D.F., Smith I.E., Reis-Filho J.S. 2010. Triple-negative breast cancer. *New Engl. J. Med.* 363: 1938–1948.