The role of CD44^{+}/CD24$^{-/low}$ biomarker for screening, diagnosis and monitoring of breast cancer

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Received September 4, 2013; Accepted September 30, 2013

DOI: 10.3892/or.2013.2943

Abstract. Cancer stem cells (CSCs) have been defined as 'a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor'. The CSC hypothesis postulates that a small subpopulation of cancer cells drives tumor initiation, growth and metastasis. CSCs have been isolated from breast cancer using CD44^{+}/CD24$^{-/low}$ phenotype. The purpose of the present study was to evaluate the expression of CD44^{+}/CD24$^{-/low}$ in two diverse breast carcinomas (ductal and lobular), and to determine the correlation between expression of CD44^{+}/CD24$^{-/low}$, and clinicopathological characteristics starting from human fresh breast cancer specimens. We analyzed specimens from 57 patients using CD44 and CD24 markers by flow cytometry and immunohistochemistry and correlated the CD44^{+}/CD24$^{-/low}$ phenotype with clinicopathological characteristics. Moreover, mammosphere formation was tested. In all specimens tested, CD44^{+}/CD24$^{-/low}$ phenotype was detectable with mean percentage of 4.73% as confirmed also by immunohistochemical analyses. A significant statistical association was found among these phenotypic groups and age, grade G3, estrogen and progesterone receptor, Ki-67 as well as lymph node metastasis. No correlation was found for histological type. In conclusion, our data showed that CD44^{+}/CD24$^{-/low}$ phenotype was found at a high frequency in tumors pT2, G3, pN3, positive for Ki-67, and negative for estrogen and progesterone receptors highlighting the hypothesis that CD44^{+}/CD24$^{-/low}$ profile correlates with the more aggressive clinical-pathological features of the disease.

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Key words: CD44^{+}/CD24$^{-/low}$, biomarkers, breast cancer, early diagnosis

Introduction

Breast cancer is the most common malignant disease affecting women, and a principal cause of cancer morbidity and mortality in the industrialized world (1). Breast cancer still represents a major public health problem, with more than 400,000 new cases and 200,000 deaths per years in Europe (2). Breast cancer is currently regarded as a heterogeneous group of tumors with diverse morphologic and molecular behavior, outcome and response to therapy (3). The prognosis is generally related to the stage of the disease and our currently applied prognostic parameters include mainly the following histopathological characteristics: tumor size, lymph node status and the presence of distant metastases (4). Despite combined treatment with surgery, radiotherapy and chemotherapy, a great percentage of breast cancer patients will eventually develop recurrent and metastatic disease (1). Breast tumors are well known to be composed of phenotypically diverse groups of cells; however, it is unclear which of these cell types contribute to tumor development. In contrast to the hypothesis that all cell populations have the capacity to become tumorigenic through accumulation of mutations, another hypothesis limits this ability to an elite group of cells that share classic features of stem cells such as the ability to self-renew and to differentiate (5-7). Evidence suggests that many malignant tumors contain heterogeneous cell population with diverse biological properties and within these there is small proportion of tumor cells termed cancer stem cells (CSCs) (8-13). The CSCs are thought to have the characteristics of self-renewal and, therefore, could be responsible for tumor formation and progression (14). Furthermore, they are thought to have features, which enable resistance to chemotherapy and represent the source of recurrence (15,16). The maintenance of the heterogeneity of cells within a tumor is not fully understood. The cancer stem cell hypothesis was proposed to explore breast cancer heterogeneity and the risk

of breast cancer recurrence, and these cell subpopulations may contribute to drug resistance that drives tumor recurrence or metastasis (17).

Al-Hajj and colleagues (8) observed that human breast cancer cells that have a strong expression of the adhesion molecule CD44 together with very low levels or no expression of the adhesion molecule CD24 ($CD44^+/CD24^{-/low}$ phenotype) could efficiently form tumors containing an array of cell types similar to those found in the original carcinoma samples when injected into immunocompromised mice. Moreover, the cells showing this antigenic profile, were able to form mammospheres, and were resistant to drug administration. Therefore, this $CD44^+/CD24^{-/low}$ phenotype has been associated with stem cell-like characteristics, with enhanced invasive properties, with radiation resistance and with distinct genetic profiles suggesting correlation to adverse prognosis (18-23). The prevalence of $CD44^+/CD24^{-/low}$ cells within breast tumors, however, has not been significantly associated with clinical characteristics, although tumors with a higher fraction of $CD44^+/CD24^{-/low}$ cells were more commonly found in patients diagnosed with distant metastases. In breast cancer patients, $CD44^+/CD24^{-/low}$ cells were readily detectable in metastatic pleural effusions, moreover, the presence of these cells, in breast cancer, may not be associated with clinical outcome but may favor distant metastasis, related to angiogenic properties (24). However, progress in screening programs, understanding of cancer biology, and the development of new treatments, have reduced the likelihood of dying from breast cancer. Despite this progress and the availability of adjuvant therapies that have significantly reduced recurrence, breast cancer recurrence still occurs in a substantial proportion of women after treatment and mortality rates remain unacceptably high (1). Thus, a more effective treatment of breast cancer is urgently needed. In particular, it is important to identify new prognostic markers to accurately establish innovative therapies to eradicate the metastatic breast cancer cells at the stage of the primary tumor. In this regard, much effort has been expended in recent years to delineate biomarkers which enable identification of CSCs. Biomarkers have the potential to increase the safety and effectiveness of new therapeutics. The development of novel markers is therefore urgently needed to better evaluate prognosis and to designate an appropriate treatment for each individual case. Breast cancers have been classified based on their histopathological profile (invasive ductal and lobular carcinoma) and based on gene expression profiles into luminal A and B, basal-like, $HER2^+$ and normal breast-like subtypes (25). Each subtype is associated with a special natural history and treatment responsiveness. The luminal subtypes are associated with expression of the estrogen receptor (ER), while basal-like and normal-like tumors are essentially all ER-negative, as are the majority of $HER2^+$ tumors. Multiple studies have demonstrated that basal-like tumors had a particularly poor prognosis (26), although it is unclear whether basal-like tumors have a significantly worse clinical outcome than other ER-negative tumors (27). Immunohistochemical features have been used to characterize basal-like tumors as typically negative for ER, for the progesterone receptor (PgR) and for HER2 but positive for basal cytokeratin (CK5/6/14/17), for epidermal growth factor receptor (EGFR) and/or for c-kit (28). In addition to inter-tumor heterogeneity, there is also a high degree of intra-tumor diversity. Specifically, a single tumor can contain tumor cell population with distinct molecular profiles and biological properties. A correlation of the $CD44^+/CD24^{-/low}$ phenotype to specific breast cancer subtypes has not yet been reported in human breast tumors. In the present study, we have determined the expression of CD44 and CD24 in human breast tumors using double-staining flow cytometry and have correlated the presence of $CD44^+/CD24^{-/low}$ cells to subgroups of breast cancer, classified using the expression of ER, PgR and Ki-67, as well as by following the histopathological characteristics tumor size, grade and lymph node status.

Materials and methods

Collection of human tissue specimens and cell cultures. Breast tumor and adjacent non-tumor tissue specimens were obtained by surgical procedures, after informed consent, from 57 consecutive patients who underwent radical mastectomy for primary breast cancer without neo-adjuvant radiotherapy or chemotherapy at the National Cancer Institute of Naples. Diagnosis was based on clinical and histological parameters [46 invasive ductal carcinoma (IDC) and 11 invasive lobular carcinoma (ILC)]. The patients ranged in age from 27 to 85 years. Tumor specimens were subjected to enzymatic dissociation by type IV collagenase (1 mg/ml) in phosphate-buffered saline (PBS) at 37˚C in agitation for 60 min. The digestive solution was filtered through 70 μm filters (Becton-Dickinson, Sunnyvale, CA, USA). After filtration and washing, the cell suspension was centrifuged at 1,300 rpm for 7 min and the pellet, in part, was cultured in 5 ml Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), and another part was used for cytometric analyses. Flasks were incubated at 37˚C under 5% CO_2 and the medium changed twice a week. All of the tumors capable of growing after 9-15 culture passages were considered to be breast-stabilized cell lines, whereas all tumor samples capable of growing only after 1-8 culture passages were considered to be primary cell lines.

Flow cytometric analysis. At least 200,000 cells were tested for a panel of fluorescent labeled monoclonal antibodies and respective isotype controls. The antibodies were incubated for 30 min at 4˚C. After washing, the labeled cells were analyzed by flow cytometry using a FACSAria II cell sorter (Becton-Dickinson, Mountain View, CA, USA). The antibodies used were: anti-human CD44 FITC (BD Pharmingen), anti-human CD24 PE (BD Pharmingen) and anti-human CD45 APC (BD Pharmingen). All data were analyzed using Diva 6.1.1 software.

Mammosphere formation. Single cells are plated at 1,000 cells/ml in ultra-low attachment plates (Corning) in serum-free DMEM-F12 supplemented with 10 ng/ml bFGF, 20 ng/ml EGF, 5 μg/ml insulin and 0.4% BSA. Cells grew in these conditions as non-adherent spherical clusters of cells (usually named spheres or mammospheres) and were enzymatically dissociated by incubation in a trypsin-EDTA solution or mechanically disaggregated every 3 days for 2 min at 37˚C. Fresh aliquots of EGF and bFGF were added every day. After

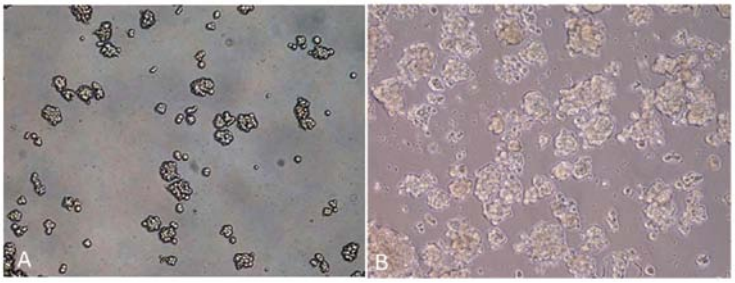

Figure 1. CSC characterization by sphere generation. Images show examples of floating spheres in serum-free medium, obtained from primary breast cancer cell line at (A) second passage and (B) at third passage of culture using serum-free medium in ultra-low attachment plates. With this method, it is possible to select and characterize CSCs. Original magnification, x100 in A and x200 in B.

culture for 48-72 h, mammospheres were visible with an inverted phase-contrast microscope (Carl Zeiss, Milan, Italy).

Histopathological diagnosis. The histopathological diagnoses were made according to the criteria of the World Health Organization and were recorded as invasive ductal or invasive lobular, using standard tissue sections and appropriate immunohistochemical slides. Clinicopathological parameters were evaluated for each tumor and included patient age at initial diagnosis; tumor size, histologic subtype, histologic grade, nuclear grade, nodal status, number of positive lymph nodes, tumor stage and type of surgery. In addition, estrogen receptor (ER), progesterone receptor (PgR), and Ki-67 were considered. Immunohistochemical analyses for CD44 (Dako Cytomation), E-cadherin (Abcam) on paraffin-embedded tissue sections were performed with the Dako AEC kit, according to the manufacturer's instructions. The nuclei were stained with hematoxylin, and the cells were observed under an inverted light microscope (Carl Zeiss).

Correlation between CD44$^+$/CD24$^{-/low}$, clinical and histopathological parameters. To correlate the presence of CD44$^+$/CD24$^{-/low}$ cells with the outcomes of disease, the following clinical parameters were analyzed: age, histotype, tumor size, grading, lymph node status and clinical stage. Correlation between presence of CD44$^+$/CD24$^{-/low}$ cells and clinicopathological parameters was analyzed by Fisher's exact test. Levels of significance were set at $P<0.05$ and $P<0.005$.

Results

Collection of human tissue specimen. The clinical and histological characteristics of the breast cancer patients enrolled in the study are summarized in Table I. There were 57 females, with mean age of 56.5 years. Breast cancer histotype was invasive in all cases (46 invasive ductal carcinoma and 11 invasive lobular carcinoma).

Primary cell culture and mammosphere formation. Primary cell lines obtained were maintained only for few passages (from 3 to 5 passages), after which the cells displayed morphological findings of senescence, namely, enlargement and flattening. Although mammospheres were obtained for all primary cell lines, in this case the spheres became senescent between the second and third culture passage (Fig. 1).

Table I. CD44$^+$/CD24$^{-/low}$ expression and clinicopathological characteristics in primary breast cancer.

Variables	No. of patients	Mean CD44$^+$/CD24$^{-/low}$ (%)	P-value
Total	57	4.86	
Age (years)			
>50	34	5.68	3.19E-04
≤50	23	3.65	
Tumor size (cm)			
pT1 (≤2.0)	27	4.43	0.004925
pT2 (>2.0)	30	4.86	
Lymph node metastasis			
pN0	30	4.51	
pN1	9	4.15	0.415821
pN2	10	10.00	0.517209
pN3	8	7.27	0.000209
Histological type			
IDC	46	5.06	0.395637
ILC	11	4.06	
Histological grade			
G2	18	3.08	4.90E-05
G3	39	5.69	
Estrogen receptor			
Negative	12	6.25	0.037009
Positive	45	4.50	
Progesterone receptor			
Negative	16	5.42	0.016659
Positive	41	4.65	
Ki-67			
Negative (<20%)	16	4.44	0.002914
Positive (≥20%)	41	5.03	

Flow cytometric analysis. Surgical biopsies of IDC and ILC obtained from 57 patients undergoing radical mastectomy were enzymatically disaggregated and the resulting cell suspensions were analyzed by flow cytometry to identify the stem phenotypic characteristics of different cell populations. In order to investigate the possible presence of a tumor

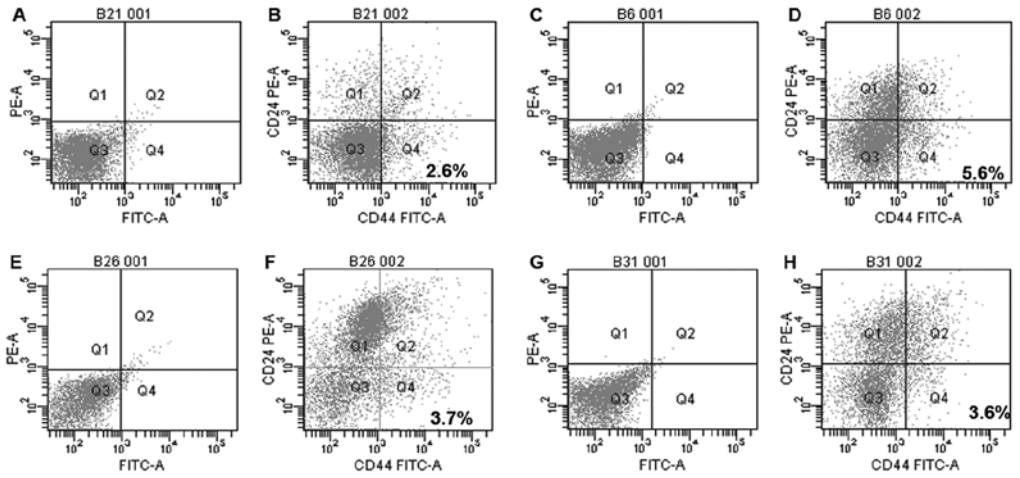

Figure 2. Flow cytometry detection: (A, C, E and G) Negative isotype control; (B, F, D and H) expression of CD44 and CD24.

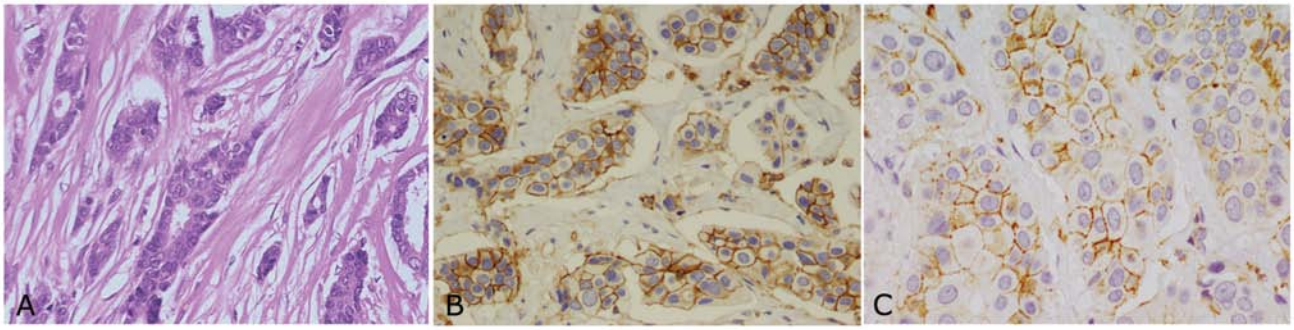

Figure 3. $CD44^+/CD24^{-/low}$ stem phenotype. (A) H&E morphology (IDC) (original magnification, x200); (B) immunopositivity for CD44 (original magnification, x100); (C) immunopositivity for E-cadherin (original magnification, x200).

initiating stem cells, we analyzed the expression of CD44 and CD24 antigens on cell suspensions. All samples tested, showed $CD44^+/CD24^{-/low}$ stem phenotype with mean percentage of 4.86% of total cell population (Fig. 2) as confirmed also by immunohistochemical staining (Fig. 3).

Correlation between $CD44^+/CD24^{-/low}$, clinical and histopathological parameters. It has been hypothesized that the CSC content in tumors may correlate with the more aggressive clinicopathological features of the disease and clinical parameters and outcome of the disease. Of the 57 specimens of breast cancer, 57 (100%) were found to be $CD44^+/CD24^{-/low}$, and these breast cancers were significantly more likely to be ER^+ (P=0.037009), PR^+ (P=0.016659), Ki-67^+ (P=0.002914) with invasion to lymph nodes (P=0.000209). Statistical analyses for correlation of $CD44^+/CD24^{-/low}$ expression and clinicopathological parameters in breast cancers selected for the present study showed that $CD44^+/CD24^{-/low}$ profile was significantly associated to age (P=3.19E-04). Moreover, significant statistical association was detectable for tumor size [P=0.004925, tumor grade (P=4.90E-05) and pN3 (P=0.000209] as showed in Table I and Fig. 4. No correlation was found for histological type.

$CD44^+/CD24^{-/low}$ profile was found to be strongly expressed in poorly differentiated tumors, negative for hormone receptors, positive for Ki-67.

Discussion

The hypothesis that the tumors originate from a small cell population with stem cell characteristics has been demonstrate by different studies in breast, brain and other solid tumors (8-13). Studies supporting this theory are based on xenotransplantation experiments, where human cancer cells are grown in immunocompromised mice and only CSC generate tumors and different phenotypes of these cells play a different goal in the behavior of tumors (7-13). In the present study, we investigate whether breast cancer could contain CSCs correlating them with clinicopathological parameters. The recent study of Al-Hajj *et al* (8) is believed to have provided evidence supporting the existence of stem cells in breast cancer. We have analyzed the presence of CD44 and CD24 antigens in fresh human breast cancer specimens. In the present study, as confirmed previously (29), $CD44^+/CD24^{-/low}$ cell subsets were isolated from the tumor specimens collected using flow cytometry. In accordance with the concept that CSCs are only a minimal part of the total tumor cell population (30), we found that the $CD44^+/CD24^{-/low}$ was expressed in a very small percentage of the total cell population of breast cancer supporting the hypothesis of the existence of CSCs in breast cancer. CD44 and CD24 have been shown to regulate invasion and metastasis of breast cancer cells (23). However, others have shown inhibition of breast cancer metastasis by

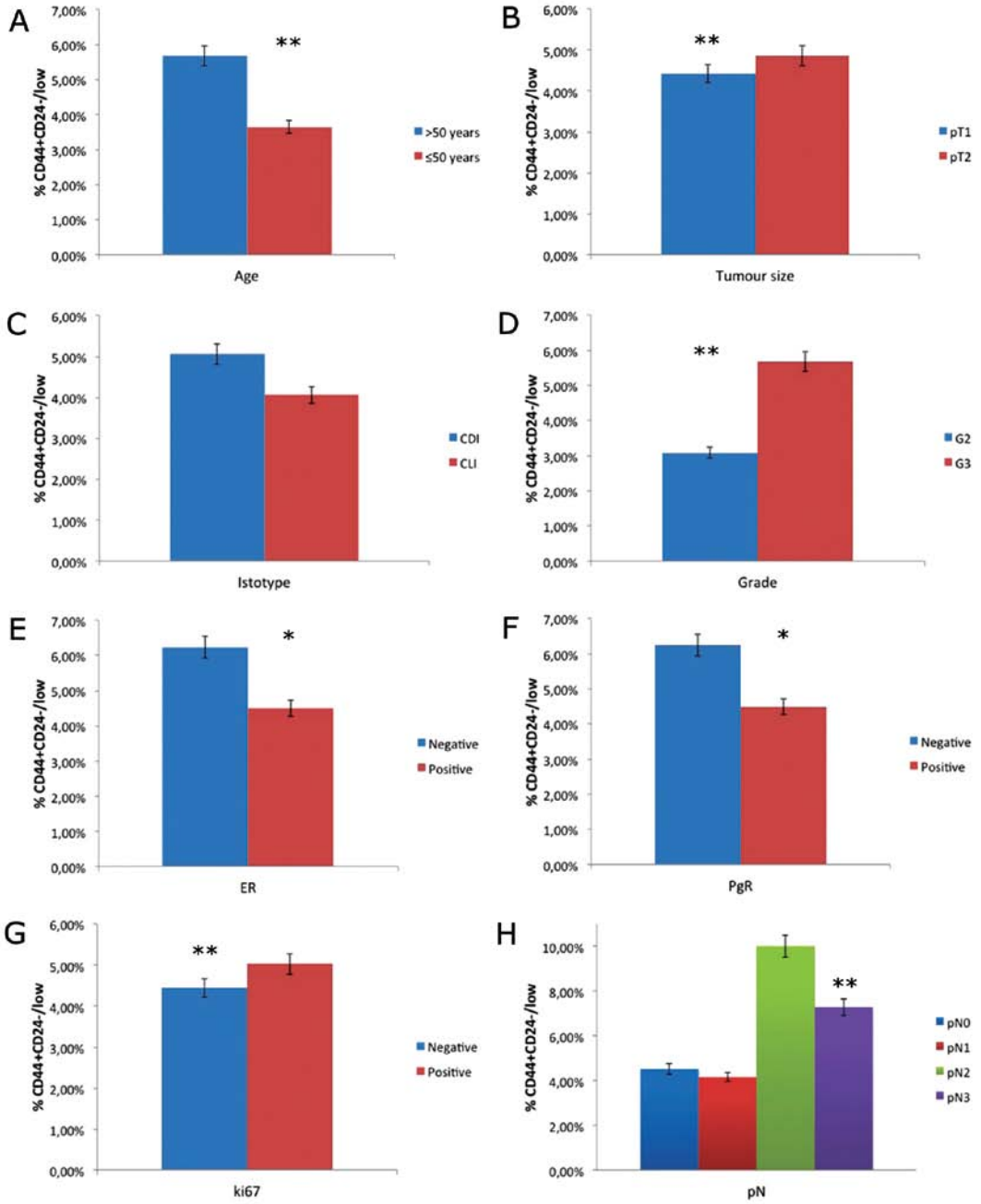

Figure 4. Correlation between $CD44^+/CD24^{-/low}$, clinical and histopathological parameters. Of the 57 specimens of breast cancer, 57 were found to be $CD44^+/CD24^{-/low}$, and these breast cancers were significantly more likely to be ER^+ (P=0.037009), PR^+ (P=0.016659), Ki-67^+ (P=0.002914) and pN3 (P=0.000209). Significant statistical association was detectable for tumor size (P=0.004925) tumor grade (P=4.90E-05) and age (P=3.19E-04). No correlation was found for histological type. *P<0.05; **P<0.005.

these molecules (31). Association of $CD44^+/CD24^{-/low}$ phenotype with invasion is correlated with invasive properties of other clinical parameters such as ER, PR and Ki-67 status.

In the present study, we found that the CSCs ratio was significantly related to the hormone receptor (HR) status, Ki-67 status, lymph node status, tumor size, age and especially to histological grade. The $CD44^+/CD24^{-/low}$ cell population was found to be strongly expressed in young patients with poorly differentiated tumors, high histological grade, high tumor size, negative to hormone receptors and high proliferation rate. For tumor size, although a correlation, statistically significant is present, no strong difference was detectable. In addition, no correlation was found for histological type, even though $CD44^+/CD24^{-/low}$ phenotype seems to be more frequent in patients with invasive ductal carcinoma. Notably, we found also that $CD44^+/CD24^{-/low}$ tumor cells were associated with the lymph node status pN3. Therefore, we can assume that the $CD44^+/CD24^{-/low}$ population plays a critical role in metastasis. Metastasis is a complex process that involves integrated activity of genes, which function in different steps that include angiogenesis, invasion, intravasion, survival in circulation, extravasion and homing and proliferation at sites of metastasis (32,33). Baumann *et al* (34) showed that $CD44^-/CD24^+$ tumor cells acquired enhanced spreading, motility and invasive properties that facilitated metastasis.

Consequently, this means that the CSCs are not only able to start the tumor from the transformation of multiple stem cells and/or progenitor cells in the normal breast, but also to cause relapse and metastasis. Hence, in this context, CD44$^+$/CD24$^{-/low}$ cell population could reflect the characteristics of breast cancer progress, which may be related to proliferation and invasion of CSCs. Dontu *et al* (35) suggested a model in which the transformation of different subsets of stem and progenitor cells generates the diversity of breast cancer phenotypes, including estrogen receptor positive (ER$^+$) and negative (ER$^-$) breast cancer subtypes.

In conclusion, the present study shows new important insight into breast cancer. In particular, we have confirmed CD44$^+$/CD24$^{-/low}$ cell population as a potential breast cancer stem/initiating-cell profile. In addition, as CD44$^+$/CD24$^{-/low}$ cell subpopulation correlated with a more aggressive tumor phenotype, CD44$^+$/CD24$^{-/low}$ profile could be used in diagnostics, and as a predictive tool indicating poor prognosis.

Acknowledgements

We thank Dr Alessandra Trocino and the librarians of the National Cancer Institute, Naples, for providing excellent bibliographic services and assistance. We thank Dr Virginia Tirino, Researcher of the Second University of Naples, for providing excellent technical and scientific support. The present study was supported by grants from the Current Research of 2011/12 of the Italian Department of Health to G. Pirozzi.

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