Cancer Investigation

ISSN: 0735-7907 (Print) 1532-4192 (Online) Journal homepage: http://www.tandfonline.com/loi/icnv20

Putative Biomarkers of Response to Treatment in Breast Cancer Patients: A Pilot Assay

María J. Rico, Herman A. Perroud, Cintia Herrera, Carlos M. Alasino, Eduardo A. Roggero, Stella M. Pezzotto, Ana Lía Nocito, Viviana R. Rozados & O. Graciela Scharovsky

To cite this article: María J. Rico, Herman A. Perroud, Cintia Herrera, Carlos M. Alasino, Eduardo A. Roggero, Stella M. Pezzotto, Ana Lía Nocito, Viviana R. Rozados & O. Graciela Scharovsky (2017): Putative Biomarkers of Response to Treatment in Breast Cancer Patients: A Pilot Assay, Cancer Investigation, DOI: 10.1080/07357907.2017.1309545

To link to this article: http://dx.doi.org/10.1080/07357907.2017.1309545

CANCER INVESTIGATION
2017, VOL. 0, NO. 0, 1–9
http://dx.doi.org/10.1080/07357907.2017.1309545

Taylor & Francis
Taylor & Francis Group

Putative Biomarkers of Response to Treatment in Breast Cancer Patients: A Pilot Assay

María J. Rico[a,b], Herman A. Perroud[a,b], Cintia Herrera[a], Carlos M. Alasino[c], Eduardo A. Roggero[a], Stella M. Pezzotto[d,e], Ana Lía Nocito[f], Viviana R. Rozados[a], and O. Graciela Scharovsky[a,b,e]

[a]Institute of Experimental Genetics, School of Medicine, National University of Rosario, Rosario, Argentina; [b]National Scientific and Technological Research Council (CONICET), Rosario, Argentina; [c]Institute of Oncology of Rosario, Rosario, Argentina; [d]Institute of Immunology, School of Medical Sciences, National University of Rosario, Rosario, Argentina; [e]National University of Rosario Research Council (CIUNR), Rosario, Argentina; [f]Department of Pathological Anatomy and Physiology, School of Medical Sciences, National University of Rosario, Rosario, Argentina

ABSTRACT

Identifying tumor biomarkers associated with clinical behavior in breast cancer patients may allow higher accuracy in the selection of treatment. Different types of cells were determined in the primary tumors of stage I, II, and III of breast cancer patients, who were assigned to one of the two groups: (1) disease-free or (2) relapsed/progressed, at 5 years after primary treatment. We studied 32 tumor samples. $CD4^+$ lymphocytes and $CD44^+CD24^{-/low}$ cells (cancer stem cells) showed a significant association with clinical outcome at 5 years of primary treatment, while $CD8^+$, $Foxp3^+$, $CD34^+$, and myeloid-derived suppressor cells did not show any association. Coincident with the results of individual analysis, we identified $CD4^+$ cells and $CD44^+CD24^{-/low}$ cells as good predictors of long-term clinical outcome in a logistic regression.

ARTICLE HISTORY
Received 15 February 2016
Revised 19 October 2016
Accepted 18 March 2017

KEYWORDS
Cancer biomarkers; Cancer stem cells; Immune response

Introduction

In Argentina, breast cancer is the leading cause of cancer death in women (1). Its high mortality is mainly due to the development of metastatic disease with the acquisition of invasive characteristics by malignant cells and their ability to avoid antitumor immune responses (2, 3). Breast tumors have developed different mechanisms to evade the immune system and create a tolerogenic microenvironment, allowing it to grow, disseminate, and metastasize (4–8).

Many authors have described the process by which the immune system is able to recognize tumor antigens and eliminate or control tumor growth; however, tumor cells can also suppress the immune response, thus allowing their own growth (9–11). Therefore, manipulation of the immune system, in order to inhibit tumor growth and metastasis development, is a feasible treatment option (12).

Different tumor characteristics, such as inflammatory infiltrate intensity and number of T regulatory (Treg) cells, could be useful to anticipate response to therapy (13, 14). In several studies, the tumor infiltrating lymphocytes (TILs) have been proposed as prognostic marker for a variety of cancers (15). Breast carcinomas are often infiltrated by inflammatory cells, particularly macrophages and T lymphocytes, which may represent a cell-mediated immune response against the tumor (16). However, their importance as biomarkers is not clear yet. Many studies that use Hematoxylin–Eosin (H&E)-stained sections and multivariate analyses have shown that elevated amounts of TILs in breast carcinoma tissues predict the response of patients to neoadjuvant chemotherapy (17).

$CD8^+$ lymphocytes are another putative biomarker known as crucial components of cell-mediated immunity (15). On the other hand, the presence of $Foxp3^+$ TILs has been reported to be associated with poor clinical outcome (CO) in a variety of cancer types, including prostate, lung, hepatic, and renal cell carcinomas, indicating that cancer patients may benefit from blocking the capacity of tumor cells to recruit Tregs (18).

CONTACT Dr. O. Graciela Scharovsky graciela.scharovsky@gmail.com Institute of Experimental Genetics, School of Medical Sciences, National University of Rosario, Santa Fe 3100, Rosario 2000, Argentina.
Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/icnv.
Supplemental files referred to in this article can be accessed on the publisher's website.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells such as macrophages, granulocytes, and dendritic, among others (19). In humans, this population, defined as $CD33^{+}CD11b^{+}$ cells, is increased in patients with advanced cancer (20). The MDSCs have the ability to suppress various functions of immune response, particularly by inhibition of Th1 cytokine production and T cells proliferation (21).

Cancer tumors are composed by different heterogeneous cell populations, with different proliferative rates as well as different ability to reconstitute after tumor transplant. Cancer stem cells (CSCs) are one such subpopulation. CSCs are capable of both self-renewal and able to give rise to phenotypically different differentiated cells, specific for a determined organ (22). The epithelial–mesenchymal transition allows CSC to migrate and invade. They would be responsible for recurrence and development of metastases (23). These cells constitute only a small fraction of the tumor cell population, and frequently are resistant to standard anticancer therapies (24). In recent studies, these cells were proposed as a therapeutic target (25, 26).

During the last years, considerable progress has been made in understanding the role of immune system and the processes of angiogenesis and lymphangiogenesis in tumor progression (27, 28).

The importance of identifying tumor biomarkers associated with clinical behavior in breast cancer patients resides in the fact that it may let physicians to make recommendations to be more accurate in the selection of primary treatment. Such data would allow analyzing, individually or in combination with other prognostic factors, their usefulness for classifying patients in different groups according to potential outcome.

The aim of this study was to identify potential predictive or prognostic biomarkers of response to treatment in patients with breast cancer.

Materials and methods

Samples

Paraffin-embedded tissues from primary (mainly stage II but also stages I and III) breast cancer were collected. According to their medical history and long-term clinical outcome at 5 years after the primary treatment, patients were classified in two main groups: disease-free (DF) and relapsed/progressed (R) groups. The evaluation of tumor relapse was made with CT scan or other imaging methods. The patients who presented new lesions, either local or metastatic, were considered relapsed/progressed. Those who presented a contralateral new tumor were not included in this group.

Biomarkers

Histological 5-µm thick sections were obtained from the tumor samples, de-paraffinized and utilized for several determinations. The cells were quantified with a semi-quantitative scale ranging from 0 to +++ (0: null; +: low; ++: moderate; +++: high).

Lymphocyte infiltration

The histological sections were stained with Hematoxylin–Eosin (H&E). The intensity of lymphocyte infiltration was calculated in 20 fields of hotspot areas at 400×. The intensity of lymphocytes infiltration for each sample was assigned to one of the two groups: low 0/+, or high ++/+++.

Immunohistochemistry for CD4, CD8, CD34, and Foxp3

The histological sections of breast tumors were incubated overnight at 4°C with anti-CD4 (Leica Microsystems, Wetzlar, Germany) 1:50, anti-CD8 (Leica Microsystems) 1:50, anti-CD34 (BD Pharmingen) 1:40, or anti-Foxp3 (eBioscience, Waltham, MA, USA) 1:25 and then with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Sections were visualized with 3,3′-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) as chromogen, and counterstained with methyl green. The number of positive cells was calculated in 20 fields of hotspot areas at 400×. The quantity of positive cells for each molecule in the sample was assigned to one of the two groups: Low 0/+ number of positive cells or high ++/+++ number of positive cells.

Quantification of $CD44^{+}CD24^{-/low}$ Cells (CSCs) and $CD33^{+}CD11b^{+}$ Cells (MDSC)

The histological sections were incubated overnight at 4°C with anti-CD24 (Abcam, Cambridge, UK) 1:50 for CSCs or CD33 (eBioscience) 1:40 for MDSC. Next,

sections were incubated with biotinilated antibody and subsequently with PE-Streptavidin (eBioscience). Afterwards, the samples were incubated overnight at 4°C with anti-CD44 (BD Pharmingen, Franklin Lakes, NJ, USA) 1:100 for CSC or anti-CD11b (R&D Systems, Minneapolis, MN, USA) 1:40 for MDSC. Finally, sections were incubated with biotinilated antibody and Alexa Fluor Streptavidine (Invitrogen, Carlsbad, CA, USA). CSCs are described as $CD44^{+}CD24^{-/low}$ and MDSC as $CD33^{+}CD11b^{+}$. Cells were visualized and counted in a fluorescent microscope in 20 fields of hotspot areas at 400×.

Ethical considerations

The confidentiality of these data (Data Protection Law No. 25326, Argentine Republic) is ensured. This project was approved by the Bioethics Committee of the School of Medical Sciences, National University of Rosario (#2015-44405003).

Design and statistical analysis

This is an observational, retrospective study based on tissue samples of primary breast cancer to determine putative biomarkers that could predict clinical outcomes in breast cancer patients. According to their medical history and clinical outcome at 5 years of primary treatment, each patient was assigned to one of the two main groups: DF or R.

Logistic regression

In the subsequent analysis, different studied variables, infiltrating lymphocytes, $CD4^{+}$, $CD8^{+}$, $Foxp3^{+}$, $CD34^{+}$, CSCs, and MDSCs, plus those obtained from the medical history such as estrogen receptor (ER), progesterone Receptor (PR), and Her-2/neu expression, were simultaneously considered in a multiple logistic regression analysis using forward and backward stepwise elimination algorithms to screen for independent significant predictors, analyzing the effect of each individual measurement on the risk of recurrence, and adjusting for the potential confounding effect of other variables. The significance level (p value) to stop the selection process was set at .10 for arriving to the most robust model.

Association between response to treatment and different variables is summarized using odd ratios (OR) with their corresponding 95% confidence interval (95% CI), and associated p values. These calculations were done using STATA statistical software.

Results

After identifying 36 patients with breast carcinomas (Stage I, II, and III) through their medical records, their archived formalin-fixed paraffin-embedded tumor samples were studied. Only 32 of the 36 samples were in good condition to be processed histologically.

Demographic characteristics

We analyzed the records of the patients whose tumors were included in this work. Those patients were followed for at least 5 years since the surgery. The most common histology of tumors was ductal breast adenocarcinoma stage II. Nevertheless, the expression of hormonal receptors and Her-2/neu was heterogeneous, corresponding three samples to triple negative tumors. Data related to demographics, tumor histology, clinical stage, ER, PR, and Her-2/neu status, adjuvant treatment and long-term clinical outcome are summarized in Table 1.

Infiltrating lymphocytes

The evaluation of the intensity of lymphocytes infiltrate showed that the proportion of low and high samples in each group of patients (DF and R at 5 years after the primary treatment) did not evince statistical differences (Figure 1).

$CD4^{+}$, $CD8^{+}$, and $Foxp3^{+}$ lymphocytes

A significant association was found between the quantity of $CD4^{+}$ lymphocytes and clinical outcome after 5 years of primary treatment. Thus, a higher number of primary tumors from R patients showed high quantity of $CD4^{+}$ lymphocytes compared with those of DF patients. Conversely, the number of tumors with low density of $CD4^{+}$ lymphocytes was higher in the DF group ($p < .05$, Fisher's exact test; Figure 2A). On the contrary, no differences between groups were found for CD8 (Figure 2B) and Foxp3 molecules (Figure 2C). Hotspot areas for $CD4^{+}$ lymphocytes with low and high staining are shown in Supplementary Materials 1A and 1B, respectively.

Table 1. Clinical pathological data of the patients.

S. No.	Histology	Stage	ER	PR	Her2/neu	Adyuvant treatment	Clinical outcome
1.	Ductal carcinoma	II	—	—	+	AC x6 + RT + Tzb	R
2.	Ductal carcinoma	II	+	—	—	AC x4 + T x4 +Tam (5 years)	DF
3.	Ductal infiltrating carcinoma	I	+	+	—	Tam (5 years)	R
4.	Ductal carcinoma	II	+	+	—	AC x6 + Tam (5 years)	DF
5.	Ductal carcinoma	II	+	+	—	AC x4 + T x4 + Tam (5 years)	DF
6.	Ductal carcinoma	II	+	+	+	Tam (5 years), LHRH analog + AI + Tzb	DF
7.	Ductal infiltrating carcinoma	II	+	+	—	Tam (5 years)	DF
8.	Ductal infiltrating carcinoma	I	+	+	—	Tam (5 years)	DF
9.	Ductal infiltrating carcinoma	II	—	—	—	AC x6	R
10.	Ductal carcinoma	II	+	+	—	AI	DF
11.	Ductal infiltrating carcinoma	II	+	—	—	Tam (5 years) + LHRH analog, + AI	DF
12.	Ductal infiltrating carcinoma	I	+	+	+	AC x4 + Tzb	DF
13.	Ductal carcinoma	II	—	—	+	AC x6 + AI + Tzb	DF
14.	Ductal carcinoma	II	+	+	—	FAC + T x4 + Tmx	DF
15.	Ductal *in situ* carcinoma.	II	+	+	+	AC x4 + Tmx + Tzb	DF
16.	Lobular infiltrating carcinoma	II	—	—	—	AC x4, T x4	DF
17	Ductal Infiltrating carcinoma	II	—	—	+	AC x4, T x4 + Tzb	DF
18.	Ductal infiltrating carcinoma	II	—	—	—	AC x4, T x4	DF
19.	Ductal infiltrating carcinoma	II	+	—	+	AC x4, T x4 + Tmx + Tzb	DF
20.	Ductal Infiltrating carcinoma	II	+	—	+	AC x4 + AI + Tzb	DF
21.	Ductal carcinoma	II	+	—	—	AC x4, T x4	DF
22.	Ductal infiltrating carcinoma	II	+	+	+	AC x4 + Tmx + Tzb	DF
23.	Ductal carcinoma	II	+	—	—	AC x4 + Tmx+ AI	DF
24.	Ductal carcinoma	II	+	+	+	AC x4 + Tx4 + Tam (5 years) + Tzb	R
25.	Ductal carcinoma	II	—	—	+	AC x4 + Tx4 + Tam (5 years) + Tzb	R
26.	Ductal carcinoma	II	—	—	+	AC x4, Tx4 + Tzb	R
27.	Ductal infiltrating carcinoma	II	+	+	—	AC x4 + Tmx	R
28.	Ductal carcinoma	II	—	—	+	ACx4 + Tzb	R
29.	Lobular infiltrating carcinoma	II	—	+	—	T	R
30.	Ductal infiltrating carcinoma	II	+	+	—	FAC x6, Tam (5 years)	R
31.	Ductal infiltrating carcinoma	III	+	+	+	AC x4 + Tmx + Tzb	R
32.	Lobular infiltrating carcinoma	III	+	+	—	Tam (5 years)	R

R: relapsed; DF: disease-free; AC: adriamycin + cyclophosphamide; RT: radiotherapy; T: taxanes; Tam: tamoxifen; LHRH analog: chemical castration; FAC: fluorouracil + adriamycin + cyclophosphamide; AI: aromatase inhibitors; Tzb: trastuzumab.

CD34$^+$ cells

The density of blood vessels in the primary tumors was estimated with the endothelial marker CD34. No association was found between the quantities of CD34$^+$ cells and the clinical outcome after treatment (Figure 2D).

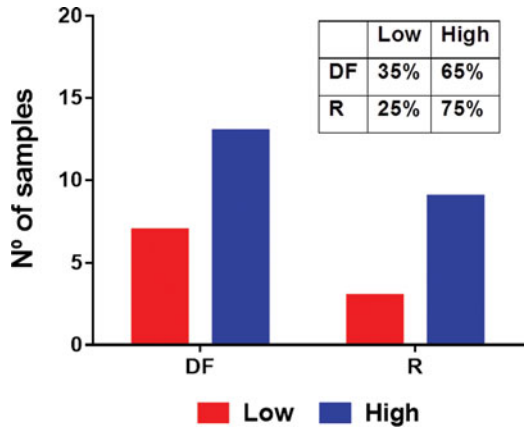

Figure 1. Quantification of lymphocytes infiltration. Low: samples with 0/+ lymphocytes infiltrate; High: samples with ++/+++ lymphocytes infiltrate. Disease-free (DF) patients vs. relapsed (R) patients, NS. The insert shows the percentage of low and high samples with respect to the total number of samples of each group of patients (DF and R); NS: Fisher's exact test.

CD44$^+$CD24$^{-/low}$ Cells (CSCs)

The presence of CD44$^+$CD24$^{-/low}$ cells showed a strong association with clinical outcome after treatment. A higher number of primary tumors with high density of CSCs were found among R patients than in the group of DF patients. Conversely, the number of tumors with low density of CSC was higher in the DF group ($p < .01$; Fisher's Exact test; Figure 2E). CD44$^+$ staining was found along the membrane and in the nucleus. Hotspot areas for CSC with low/negative and high staining are shown in Supplementary Material 1 (C, D, E, and F).

CD33$^+$CD11b$^+$ Cells (MDSC)

The number of CD33$^+$CD11b$^+$ cells was lower in R patients than in DF ones, contrary to the expected results. Nevertheless, no statistical differences between the groups of patients were found for these types of cells (Figure 2F).

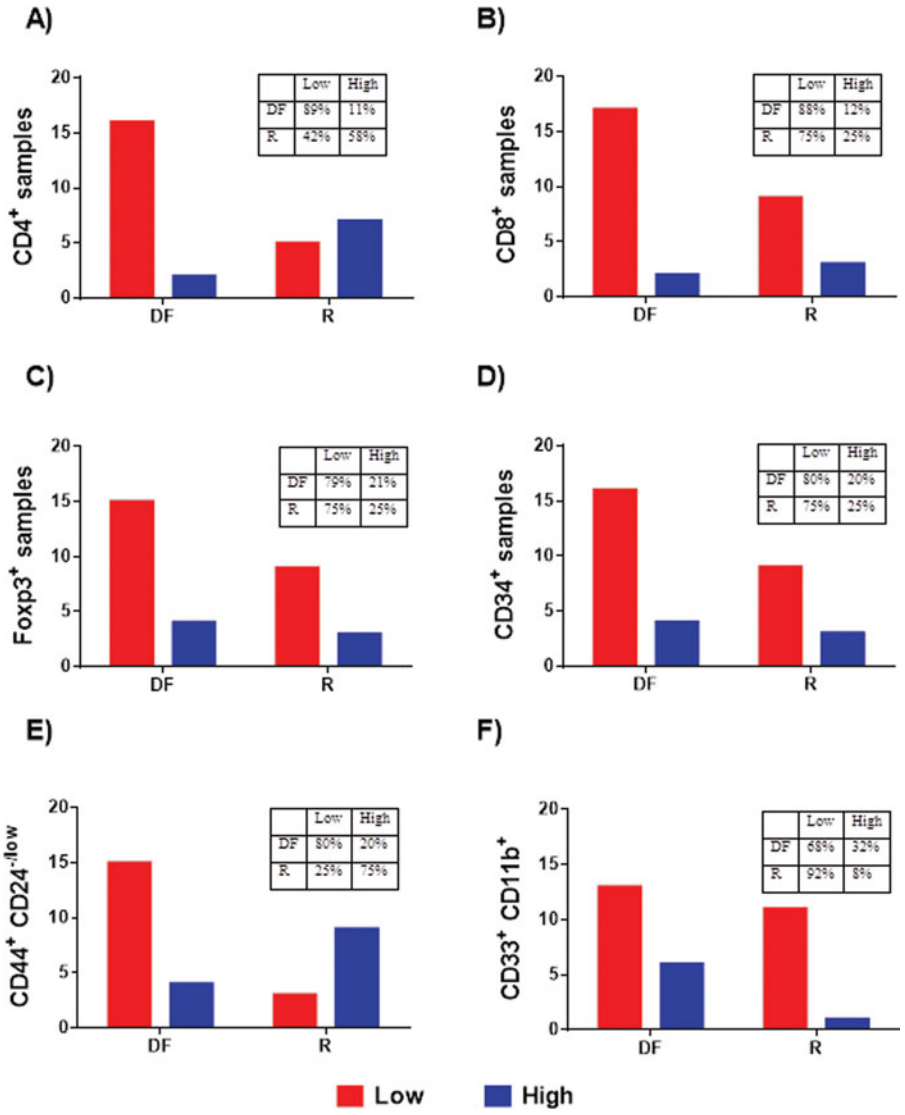

Figure 2. Quantification of $CD4^+$, $CD8^+$, and $Foxp3^+$ lymphocytes, $CD34^+$ cells, CSC, and MDSC in disease-free (DF) and relapsed (R) patients' tumors. Low: samples with 0/+ number of positive cells; High: samples with ++/+++ number of positive cells. (A) $CD4^+$ cells: DF vs. R, $p < .05$; (B) $CD8^+$ cells: DF vs. R, NS; (C) $Foxp3^+$ cells: DF vs. R, NS; (D) $CD34^+$ cells: DF vs. R, NS; (E) CSC: DF vs. R, $p < .01$; (F) MDSC: DF vs. R, NS: Fisher's exact test. The inserts show the percentage of low and high samples with respect to the total number of samples of each group of patients (DF and R) for each putative marker.

Logistic regression

Coincident with the results of individual analysis, we identified $CD4^+$ and CSCs as good predictors of recurrence ($R^2 = 0.3230$). For patients with similar CSC levels, the risk of recurrence increases 6.28 times in those with high levels of $CD4^+$ cells (OR = 9.00; 95% CI: 1.10 to 73.46; $p = .04$). In turn, for subjects with similar quantity of $CD4^+$ cells, patients with high levels of CSC showed that a risk of recurrence increased by 8.49 times (OR = 8.49; 95% CI: 1.24 to 57.94; $p = .029$). In addition, for the sake of homogeneity, the same analysis was performed excluding stage I and III patients. In the first and the second analysis, both methods (backward and forward) resulted in the same model, so we can consider that the model is robust. We found that $CD4^+$ cells and CSCs are also good predictors of recurrence in stage II invasive ductal carcinoma patients ($R^2 = 0.336$). For patients with similar CSC levels, the risk of recurrence increases by 7.93 times in those with high levels of $CD4^+$ cells (OR = 7.93; 95% CI: 0.72 to 86.49; $p = .08$). Likewise, for subjects with similar quantity of $CD4^+$ cells, patients with high levels of CSC showed that a risk of recurrence increased by 18.82 times (OR = 18.82; 95% CI: 1.77 to 199.40; $p =$

.015). Hence, both types of cells are good predictors of recurrence when analyzed for stage II patients or for patients in stage I, II, and III.

Discussion

Cancer is a global health problem. While many infectious diseases are eliminated, neoplastic diseases remain the leading cause of death and disability in the world due to the aging population. It is estimated that around 1,200,000 new cases of breast cancer occur annually in the world, which involves more than 500,000 deaths (29). Argentina has the second highest death rate by cancer in Latin America (1). In 2009, 60,117 people died of cancer, with 95.79% of the cases due to malignant tumors and 4.2% due to in situ tumors, benign tumors of uncertain or unknown carcinomas. Of all cancer deaths, 9.09% of the cases were due to breast cancer.

In daily practice, breast cancer treatment decision is based in clinical and pathological prognostic and predictive factors such as tumor size, regional lymph node metastasis, expression of ER, PR, Her-2, and Ki-67, and lymphovascular invasion, which are well established prognostic markers. In addition, the use of baseline 21-gene Recurrence Score (Oncotype DX; Genomic Health Inc, Redwood City, CA, USA) can be useful to predict the benefits of adjuvant chemotherapy (30, 31). However, this technology is not affordable in low- and middle-income countries. Nowadays, research is focused on the investigation of new predictive markers of response in order to select better treatments for patients and to get better clinical outcome. Thus, different biomarkers are analyzed in tumor biopsies trying to identify possible predictors of response to tumor therapy.

Tumor infiltrating lymphocytes are the main players in response against tumor cells, and they may constitute markers of immune balance between the host and the tumor. Different authors have shown that TILs have an important role in breast cancer outcome (32). Studies addressing the issue of tumor immune cell infiltration have consistently demonstrated that a high lymphocytic infiltration predicts a better prognosis and a better response to neoadjuvant chemotherapy in almost all breast cancer subtypes, except for the hormone receptor negative subtype (33). The relationship between certain subtypes of TIL and breast cancer survival is supported by some studies (32). However, conflicting results exist regarding the exact prognostic or predictive value of immune cell infiltrates in the adjuvant setting (33). In our pilot study, we did not find association between TILs and clinical outcome at 5 years after the primary treatment. The same happened with $CD8^+$ cells. Nevertheless, we demonstrated a significant association between $CD4^+$ lymphocytes and clinical outcome, the DF group being the one that showed lowest values in their primary tumors. Moreover, other authors found that intra-tumoral $T\gamma\delta$ cells act as prognostic biomarkers for human breast cancer (34). Mahmoud et al. found that tumor-infiltrating $CD8^+$ T lymphocytes have antitumor activity and could potentially be utilized in the treatment of breast cancer (16). Moreover, some authors have found that $CD8^+$ T cells were the key effector cell population mediating effective antitumor immunity that resulted in better clinical outcome (35). On the other hand, intra-tumoral $CD4^+$ T cells have negative prognostic effects on breast cancer patient (36). These findings are in accordance with our results.

Tregs are commonly identified by expression of the transcription factor Foxp3 and are conventionally thought to promote cancer progression by suppressing antitumor immune responses (37) and facilitating tumor growth (38). However, the prognostic value of Tregs in breast cancer remains controversial. A meta-analysis conducted by Shang et al. concluded that a high Treg infiltration was significantly associated with a shorter overall survival in several tumors, including breast cancer (18). On the contrary, in our group of patients, we found no association between $Foxp3^+$ cells and patients' evolution.

Myeloid-derived suppressor cells are a heterogeneous population of immature myeloid cells inhibiting innate and adaptive immunity through multiple mechanisms, including depletion of arginine, production of reactive nitrogen and oxygen species, and secretion of inhibitory cytokines (39). De Sanctis and colleagues provided support for the hypothesis that the levels of MDSC could have a value to predict prognosis in several types of tumors (40). Conversely, the tumors herein studied did not show association between levels of MDSC and clinical outcome in spite of showing unexpected, nonsignificant, and slightly lower levels of MDSCs in R patients. Other authors found that Tregs and MDSC were associated with a tolerogenic cytokine milieu and impaired clinical efficacy of vaccine responses in patients with lung, pancreatic, esophageal, and gastric cancers (41, 42).

Cancer stem cells are heterogeneous cancer cells with different implications in tumorigenesis, progression, metastatic process and clinical outcome. We have described CSC as $CD44^+CD24^{-/low}$, however, very recently, other authors described breast CSCs as $CD44^+/CD24^-/ALDH1^+$ (43). Nevertheless, they showed that in HER-2^+ non-metastatic patients, the expression of $CD44^+/CD24^-$ but not $ALDH1^+$ was an independent factor related to DF survival and overall survival. Moreover, presently we are devoid of treatments that specifically target this type of cells. In addition, it is scarce the number of studies conducted to validate or associate CSCs with treatment response (44). Recently, breast CSCs where suggested as a prognostic biomarker. Seo et al. proposed that cells $CD44^+/CD24^{low}$ can be used as a prognostic factor for clinical outcome and a predictive factor of trastuzumab treatment in HER2-positive breast cancer patients (43) Interestingly, we were able to show a strong and significant association between the presence of CSCs in primary tumors and clinical outcome. As expected, the higher number of CSCs was found in R patients. As far as we know, this is the first time that such a putative biomarker was found to have predictive value in breast cancer.

Interestingly, the CD44 molecule, along with the membrane staining, was also found in the nucleus. The expected localization of CD44 was the cell membrane, as several authors have shown. Park et al. found that in normal breast cancer tissue, CD44 was localized in the cell membrane of basal/myoepithelial and a subset of luminal epithelial cells; however, some cells could show an incomplete membrane-staining pattern (45). In addition, Ali et al. showed membrane CD44 expression in breast cancer (46). However, other authors demonstrated that CD44 can be translocated to the nucleus (47, 48), a result in line with our findings. The future studies may give information about the biological significance of such an event.

It is well known that angiogenesis is critical for tumor growth, invasion, and metastasis. Extensive neovascularization and tumor thrombus in vessels have been reported to be the signs of poor prognosis in breast cancer (49, 50). The assessment of microvascular density with CD34 in breast tumors led to the conclusion that microvascular density correlated positively with Her-2 expression but negatively with hormone receptor expression (49). However, we did not find, in our samples, association with clinical outcome. Other authors found that expression of VEGF-A and VEGF-C in breast cancer might be beneficial for the identification of tumors that have a higher probability of recurrence and metastatic spread (51).

It is noteworthy that in this work we found that $CD4^+$ cells and CSC are good predictors of recurrence. The importance of this finding, if confirmed with a higher number of samples, is that it will help to choose a suitable treatment for each individual patient.

Conclusions

Tumor biomarkers are a hard field to study and in spite of the fact that several molecules are being proposed as biomarkers, not many of them will be able to achieve that proposal. Our findings in this pilot assay showed the potential role of $CD4^+$ cells, already known putative biomarker, as a predictor of relapse during the first 5 years after the primary treatment. Moreover, the elevated number of CSCs, a novel biomarker for breast cancer, was strongly associated with poor clinical outcome. It is noteworthy that the logistic regression analysis identified both types of cells, $CD4^+$ and CSC, as good predictors of recurrence. Nonetheless, the obvious limitation of this study is the low number of samples analyzed. Hence, a higher number of samples will allow arriving to stronger conclusions. The door is open for future prospective studies.

Executive summary

The identification of several types of cells as putative tumor biomarkers associated with certain clinical behavior in breast cancer patients yielded the following results:

- $CD4^+$ lymphocytes and CSCs showed a significant association with clinical outcome after 5 years of primary treatment.
- These types of cells could be useful as biomarkers of clinical outcome, but a study with higher number of tumor samples is needed for its confirmation.

Acknowledgments

We would like to thank Institute of Experimental Physiology (IFISE) for allowing the use of the fluorescence microscope, and to Dr. José Pellegrino for his microscope technical advice.

Conflict of interest

The authors declare no potential conflicts of interest. The authors alone are responsible for the content and writing of the article.

Funding

This work was supported by Roemmers Foundation and the Ministry of Science, Technology and Innovation of Santa Fe, Argentine. María J. Rico, Herman A. Perroud and O. Graciela Scharovsky are the fellows of National Scientific and Technologic Research Council (CONICET). Stella M. Pezzotto and O. Graciela Scharovsky are the fellows of National University of Rosario Research Council.

References

1. Instituto Nacional Del Cancer – Argentina. Analysis of the cancer situation in Argentina based on the Globocan database. Argentina: Instituto Nacional Del Cancer; 2012.
2. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 2011;331:1565–1570.
3. Croci DO, Zacarias Fluck MF, Rico MJ, Matar P, Rabinovich GA, Scharovsky OG. Dynamic cross-talk between tumor and immune cells in orchestrating the immunosuppressive network at the tumor microenvironment. Cancer Immunol Immunother 2007;56:1687–1700.
4. Heckel MC, Wolfson A, Slachta CA, Schwarting R, Salgame P, Katsetos CD, et al. Human breast tumor cells express IL-10 and IL-12p40 transcripts and proteins, but do not produce IL-12p70. Cell Immunol 2011;266:143–153.
5. Zhang B, Sun T, Xue L, Han X, Lu N, Shi Y, et al. Functional polymorphisms in FAS and FASL contribute to increased apoptosis of tumor infiltration lymphocytes and risk of breast cancer. Carcinogenesis 2007;28:1067–1073.
6. Coussens LM, Pollard JW. Leukocytes in mammary development and cancer. Cold Spring Harb Perspect Biol 2011;3(3):a003285. doi:10.1101/cshperspect.a003285.
7. Razmkhah M, Jaberipour M, Erfani N, Habibagahi M, Talei AR, Ghaderi A. Adipose-derived stem cells (ASCs) isolated from breast cancer tissue express IL-4, IL-10 and TGF-beta1 and upregulate expression of regulatory molecules on T cells: do they protect breast cancer cells from the immune response? Cell Immunol 2011;266:116–122.
8. Dalotto-Moreno T, Croci DO, Cerliani JP, Martinez-Allo VC, Dergan-Dylon S, Mendez-Huergo SP, et al. Targeting galectin-1 overcomes breast cancer-associated immunosuppression and prevents metastatic disease. Cancer Res 2013;73:1107–1117.
9. Kusmartsev S, Gabrilovich DI. Immature myeloid cells and cancer-associated immune suppression. Cancer Immunol Immunother 2002;51:293–298.
10. Solinas G, Schiarea S, Liguori M, Fabbri M, Pesce S, Zammataro L, et al. Tumor-conditioned macrophages secrete migration-stimulating factor: a new marker for M2-polarization, influencing tumor cell motility. J Immunol 2010;185:642–652.
11. Zamarron BF, Chen W. Dual roles of immune cells and their factors in cancer development and progression. Int J Biol Sci 2011;7:651–658.
12. Dunn GP, Ikeda H, Bruce AT, Koebel C, Uppaluri R, Bui J, et al. Interferon-gamma and cancer immunoediting. Immunol Res 2005;32:231–245.
13. Jensen HK, Donskov F, Nordsmark M, Marcussen N, Von Der Maase H. Increased intratumoral FOXP3-positive regulatory immune cells during interleukin-2 treatment in metastatic renal cell carcinoma. Clin Cancer Res 2009;15:1052–1058.
14. Emerson RO, Sherwood AM, Rieder MJ, Guenthoer J, Williamson DW, Carlson CS, et al. High-throughput sequencing of T-cell receptors reveals a homogeneous repertoire of tumour-infiltrating lymphocytes in ovarian cancer. J Pathol 2013;231:433–440.
15. Seo AN, Lee HJ, Kim EJ, et al. Tumour-infiltrating $CD8^+$ lymphocytes as an independent predictive factor for pathological complete response to primary systemic therapy in breast cancer. Br J Cancer 2013;109:2705–2713.
16. Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, et al. Tumor-infiltrating $CD8^+$ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol 2011;29:1949–1955.
17. Issa-Nummer Y, Loibl S, Von Minckwitz G, Denkert C. Tumor-infiltrating lymphocytes in breast cancer: a new predictor for responses to therapy. Oncoimmunology 2014;3:e27926.
18. Shang B, Liu Y, Jiang SJ, Liu Y. Prognostic value of tumor-infiltrating FoxP3(+) regulatory T cells in cancers: a systematic review and meta-analysis. Sci Rep 2015;5:15179.
19. Youn JI, Gabrilovich DI. The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity. Eur J Immunol 2010;40:2969–2975.
20. Almand B, Clark JI, Nikitina E, Van Beynen J, English NR, Knight SC, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. J Immunol 2001;166:678–689.
21. Markowitz J, Wesolowski R, Papenfuss T, Brooks TR, Carson WE, 3rd. Myeloid-derived suppressor cells in breast cancer. Breast Cancer Res Treat 2013;140:13–21.
22. Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ. Opinion: the origin of the cancer stem cell: current controversies and new insights. Nat Rev Cancer 2005;5:899–904.
23. Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells – an integrated concept of malignant tumour progression. Nat Rev Cancer 2005;5:744–749.
24. Jones RJ. Controversies in cancer stem cells. J Mol Med (Berl) 2009;87:1077–1078.
25. Londono-Joshi AI, Oliver PG, Li Y, Lee CH, Forero-Torres A, Lobuglio AF, et al. Basal-like breast cancer stem cells are

sensitive to anti-DR5 mediated cytotoxicity. Breast Cancer Res Treat 2012;133:437–445.
26. Sigurdsson V, Hilmarsdottir B, Sigmundsdottir H, Fridriksdottir AJ, Ringner M, Villadsen R, et al. Endothelial-induced EMT in breast epithelial cells with stem cell properties. PLoS One 2011;6:e23833.
27. Ni X, Zhao Y, Ma J, Xia T, Liu X, Ding Q, et al. Hypoxia-induced factor-1 alpha upregulates vascular endothelial growth factor C to promote lymphangiogenesis and angiogenesis in breast cancer patients. J Biomed Res 2013;27:478–485.
28. Scharovsky OG, Binda MM, Rozados VR, Bhagat S, Cher ML, Bonfil RD. Angiogenic and anti-angiogenic balance regulates concomitant anti-tumoral resistance. Clin Exp Metastasis 2004;21:177–183.
29. American Cancer Society. Cancer Facts & Figures, 2015. Atlanta, GA: American Cancer Society; 2015.
30. Rutter CE, Yao X, Mancini BR, Aminawung JA, Chagpar AB, Saglam O, et al. Influence of a 21-gene recurrence score assay on chemotherapy delivery in breast cancer. Clin Breast Cancer 2015;30(16):59–62. doi:10.1016/j.clbc.2015.09.008
31. Kelly CM, Krishnamurthy S, Bianchini G, Litton JK, Gonzalez-Angulo AM, Hortobagyi GN, et al. Utility of oncotype DX risk estimates in clinically intermediate risk hormone receptor-positive, HER2-normal, grade II, lymph node-negative breast cancers. Cancer 2010;116:5161–5167.
32. Garcia-Martinez E, Gil GL, Benito AC, Gonzalez-Billalabeitia E, Conesa MA, Garcia Garcia T, et al. Tumor-infiltrating immune cell profiles and their change after neoadjuvant chemotherapy predict response and prognosis of breast cancer. Breast Cancer Res 2014;16:488.
33. Yu X, Zhang Z, Wang Z, Wu P, Qiu F, Huang J. Prognostic and predictive value of tumor-infiltrating lymphocytes in breast cancer: a systematic review and meta-analysis. Clin Transl Oncol 2015;33(18):497–506. doi:10.1007/s12094-015-1391-y
34. Ma C, Zhang Q, Ye J, Wang F, Zhang Y, Wevers E, et al. Tumor-infiltrating gammadelta T lymphocytes predict clinical outcome in human breast cancer. J Immunol 2012;189:5029–5036.
35. Wang K, Xu J, Zhang T, Xue D. Tumor-infiltrating lymphocytes in breast cancer predict the response to chemotherapy and survival outcome: a meta-analysis. Oncotarget 2016;35(7):44288–44298. doi:10.18632/oncotarget.9988
36. Huang Y, Ma C, Zhang Q, Ye J, Wang F, Zhang Y, et al. $CD4^+$ and $CD8^+$ T cells have opposing roles in breast cancer progression and outcome. Oncotarget 2015;6:17462–17478.
37. Takeuchi Y, Nishikawa H. Roles of regulatory T cells in cancer immunity. Int Immunol 2016;37(28):401–409. doi:10.1093/intimm/dxw025
38. Lal A, Chan L, Devries S, Chin K, Scott GK, Benz CC, et al. FOXP3-positive regulatory T lymphocytes and epithelial FOXP3 expression in synchronous normal, ductal carcinoma in situ, and invasive cancer of the breast. Breast Cancer Res Treat 2013;139:381–390.
39. Kao J, Ko EC, Eisenstein S, Sikora AG, Fu S, Chen SH. Targeting immune suppressing myeloid-derived suppressor cells in oncology. Crit Rev Oncol Hematol 2011;77:12–19.
40. De Sanctis F, Solito S, Ugel S, Molon B, Bronte V, Marigo I. MDSCs in cancer: conceiving new prognostic and therapeutic targets. Biochim Biophys Acta 2015;40(1865):35–48. doi:10.1016/j.bbcan.2015.08.001
41. Gabitass RF, Annels NE, Stocken DD, Pandha HA, Middleton GW. Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the Th2 cytokine interleukin-13. Cancer Immunol Immunother 2011;60:1419–1430.
42. Hansen GL, Gaudernack G, Brunsvig PF, Cvancarova M, Kyte JA. Immunological factors influencing clinical outcome in lung cancer patients after telomerase peptide vaccination. Cancer Immunol Immunother 2015;42(64):1609–1621. doi:10.1007/s00262-015-1766-5
43. Seo AN, Lee HJ, Kim EJ, Jang MH, Kim YJ, Kim JH, et al. Expression of breast cancer stem cell markers as predictors of prognosis and response to trastuzumab in HER2-positive breast cancer. Br J Cancer 2016;114:1109–1116.
44. Zhang S, Jing Y, Zhang M, Zhang Z, Ma P, Peng H, et al. Stroma-associated master regulators of molecular subtypes predict patient prognosis in ovarian cancer. Sci Rep 2015;5:16066.
45. Park SY, Lee HE, Li H, Shipitsin M, Gelman R, Polyak K. Heterogeneity for stem cell-related markers according to tumor subtype and histologic stage in breast cancer. Clin Cancer Res 2010;16:876–887.
46. Ali HR, Dawson SJ, Blows FM, Provenzano E, Pharoah PD, Caldas C. Cancer stem cell markers in breast cancer: pathological, clinical and prognostic significance. Breast Cancer Res 2011;13:R118.
47. Lee JL, Wang MJ, Chen JY. Acetylation and activation of STAT3 mediated by nuclear translocation of CD44. J Cell Biol 2009;185:949–957.
48. Cho Y, Lee HW, Kang HG, Kim HY, Kim SJ, Chun KH. Cleaved CD44 intracellular domain supports activation of stemness factors and promotes tumorigenesis of breast cancer. Oncotarget 2015;6:8709–8721.
49. Rau KM, Huang CC, Chiu TJ, Chen YY, Lu CC, Liu CT, et al. Neovascularization evaluated by CD105 correlates well with prognostic factors in breast cancers. Exp Ther Med 2012;4:231–236.
50. Safwat MD, Habib F, Elayat A, Oweiss N, Reffat S, Algaidi S. Morphometric and immunohistochemical study of angiogenic marker expressions in invasive ductal carcinoma of human breast. Folia Morphol (Warsz) 2009;68:144–155.
51. Mohammed RA, Green A, El-Shikh S, Paish EC, Ellis IO, Martin SG. Prognostic significance of vascular endothelial cell growth factors A, C and D in breast cancer and their relationship with angio- and lymph-angiogenesis. Br J Cancer 2007;96:1092–1100.