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The combination of mammaglobin A and *TWIST-1* increases detection of circulating tumor cells in breast cancer

A combinação de mamaglobina A e TWIST-1 *aumenta a detecção de células tumorais circulantes no câncer de mama*

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ABSTRACT

Objectives: Breast cancer cells that are released into the bloodstream are called circulating tumor cells (CTCs). CTCs can express different genes, like *TWIST-1* and mammaglobin A (*MGA*). The aims of this study were to analyze the expression of *TWIST-1* and *MGA* in the blood of breast cancer patients to detect CTCs and to assess the association between the presence of CTCs and prognostic parameters of breast cancer. **Methods:** Prospective study. Total ribonucleic acid (RNA) from blood mononucleated cells was obtained from breast cancer patients ($n = 36$; age: 51.5 ± 12.5 years) and healthy donors ($n = 14$; age: 49.4 ± 9.4 years). Real-time polymerase chain reaction (RT-PCR) was performed to analyze the expression of *TWIST-1* and *MGA*. **Results:** Patient carcinomas – ductal (86.7%), other types (13.3%). *MGA* gene expression was not detected in the donors' samples, while it was detected in 14% of the patient samples. Overexpression of *TWIST-1* gene was observed in 17% of the patient samples. The combined analysis of both markers allowed the detection of CTCs in 27.8% of the samples, resulting in a significant ($p < 0.05$) sensitivity increase of detection. No significant associations ($p > 0.05$) were found between expression of the analyzed genes and the breast cancer prognostic factors. **Conclusion:** Combined analysis of *TWIST-1* and *MGA* increased the sensitivity of CTCs detection compared to the single analysis of each gene. The detection of CTCs was not associated with known prognostic factors, suggesting that it is able to provide clinical information in addition to routine breast cancer clinicopathological parameters.

Key words: neoplastic cells circulating; breast neoplasms; mammaglobin A; *TWIST-1*; prognosis.

RESUMO

Objetivos: *As células cancerígenas da mama liberadas na corrente sanguínea são chamadas de células tumorais circulantes (CTCs). As CTCs podem expressar diferentes genes, como* TWIST-1 *e mamaglobina A (*MGA). Os objetivos deste estudo foram analisar a expressão de TWIST-1 *e* MGA *no sangue de pacientes com câncer da mama (CM) para detectar CTCs e avaliar a associação entre a presença de CTCs e os parâmetros prognósticos do CM.* ***Métodos:*** *Estudo prospectivo. O ácido ribonucleico (RNA) das células mononucleadas no sangue foi obtido de pacientes com CM (*n = *36, idade: 51,5 ± 12,5 anos) e doadoras saudáveis (*n = *14; idade: 49,4 ± 9,4 anos). Reação da cadeia da polimerase em tempo real (RT-PCR) foi realizada para analisar a expressão de* TWIST-1 *e* MGA. ***Resultados:*** Carcinoma ductal (86,7%), outros tipos (13,3%). A expressão do gene MGA *não foi detectada nas amostras das doadoras, mas foi observada em 14% das amostras das pacientes. Superexpressão de* TWIST-1 *foi observada em 17% das amostras dos indivíduos com CM. A análise combinada de ambos os marcadores permitiu a detecção de CTCs em 27,8% das amostras, resultando em um aumento significativo (*p < *0,05) na sensibilidade da detecção. Associações significativas (*p > *0,05) entre a expressão dos genes e os fatores prognósticos não foram encontradas.* ***Conclusão:*** *A análise combinada de* TWIST-1 *e* MGA *aumentou a sensibilidade da detecção de CTCs em comparação com a análise de cada gene. A detecção de CTCs não foi associada a fatores prognósticos conhecidos, sugerindo que ela pode fornecer informações clínicas adicionais aos parâmetros clinicopatológicos de rotina do CM.*

Unitermos: *células tumorais circulantes; câncer de mama; mamaglobina A; TWIST-1; prognóstico.*

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RESUMEN

Objetivos: *Las células de cáncer de mama liberadas al torrente sanguíneo se llaman células tumorales circulantes (CTCs). Las CTCs pueden expresar diferentes genes, como* TWIST-1 *y mamaglobina A (*MGA*). Los objetivos de este estudio fueron analizar la expresión de* TWIST-1 *y* MGA *en la sangre de pacientes con cáncer de mama (CM) para detectar CTCs y evaluar la asociación entre la presencia de CTCs y los parámetros pronósticos del CM.* ***Métodos:*** *Estudio prospectivo. Se obtuvo el ácido ribonucleico (ARN) de las células mononucleadas en la sangre de pacientes con CM (*n = *36, edad: 51,5 ± 12,5 años) y donantes sanas (*n = *14; edad: 49,4 ± 9,4 años). Se realizó reacción en cadena de la polimerasa en tiempo real (RT-PCR) para analizar la expresión de* TWIST-1 *y* MGA*.* ***Resultados:*** *Carcinoma ductal (86,7%), otros tipos (13,3%). No se detectó la expresión del gen* MGA *en las muestras de las donantes, pero en el 14% de las muestras de las pacientes. Se observó elevada expresión de* TWIST-1 *en el 17% de las muestras de pacientes con CM. El análisis combinado de ambos marcadores permitió detección de CTCs en el 27,8% de las muestras, resultando en un aumento significativo (*p < *0,05) en la sensibilidad de detección. No se encontraron asociaciones significativas (*p > *0,05) entre la expresión de los genes y los factores pronósticos.* ***Conclusión:*** *El análisis combinado de* TWIST-1 *y* MGA *aumentó la sensibilidad de detección de CTCs en comparación con el análisis de cada gen. La detección de CTCs no se asoció a factores pronósticos conocidos, sugiriendo que podría ofrecer informaciones clínicas adicionales a los parámetros clínico-patológicos de rutina del CM.*

Palabras clave: *células tumorales circulantes; cáncer de mama; mamaglobina A;* TWIST-1*; pronóstico.*

INTRODUCTION

Breast cancer is the most frequently diagnosed type of female cancer, with 450 thousand annual deaths worldwide[1]. Metastasis is the main cause of death related to this type of cancer[2]. The process of metastasis requires a complex sequence of biological events, collectively called invasion-metastasis cascade. One of the initial steps in this process is the intravasation of primary tumor cells into blood vessels, where they are named circulating tumor cells (CTCs) and are usually not detected by common diagnostic methods[3]. CTCs play a necessary role in metastatic cascade, tumor spreading and progression of the disease. This can be explained by the fact that these cells may experience mechanisms such as the epithelial-mesenchymal transition (EMT)[4]. During the EMT course, epithelial cells lose cell-cell adhesion and apical-basal polarity, reorganize their cytoskeleton, undergo changes in signaling programs and reprogram gene expression, increasing cell mobility and allowing the development of an invasive phenotype[5]. Also, CTCs can colonize their original tumor, accelerating tumor growth, angiogenesis, and stromal recruitment[6]. This action, known as self-seeding, could contribute to cancer recurrence after surgical removal of the tumor[7].

Most cancer diagnostic methods are based on histological examination of the tumor tissue. However, alternative techniques that allow detection of tumor cells with metastatic ability are less utilized[8]. As metastasis is caused by tumor-cell spreading through the bloodstream, one of the approaches to prevent metastatic breast cancer would require monitoring of CTCs to assess the response to different therapies, raising the need of reliable biomarkers for these cells[9]. In addition, analyses of CTCs, circulating tumor deoxyribonucleic acid (DNA) and tumor-derived exosomes by the evaluation of blood samples, which have been called "liquid biopsies", could allow molecular tumor characterization using minimally invasive procedures[10]. It has been suggested that the detection of CTCs may potentially be used for an early assessment of the recurrence risk and provide a more accurate risk stratification for metastasis, contributing to subsequent therapeutic decisions, or even helping to assess additional therapies – conventional or targeted –, aimed to eradicate these cells before metastasis development[11, 12]. Therefore, ongoing studies are directed toward the search for biomarkers to identify these CTCs in the bloodstream[13, 14].

Over the past few years, many techniques have been developed to detect peripheral blood CTCs in patients with solid epithelial tumors. However, molecular detection of CTCs remains challenging, requiring extremely sensitive and specific analytical methods, which are often a combination of tumor-cell enrichment and detection procedures. Different strategies have been designed to detect CTCs grounded in the identification of proteins or mRNAs, which are expressed in tumor cells[15-17].

Detection techniques based on nucleic acid analysis, such as quantitative reverse transcription assays followed by real-time polymerase chain reaction (RT-PCR), are highly sensitive and consist in differential gene expression analysis between tumor cells and normal blood cells[18]. Since CTCs do not always express all tumor-associated genes, a simultaneous analysis of more than one gene may improve the detection of these cells[19].

The expression of mammaglobin A (*MGA*) is mainly restricted to the mammary tissue and increases in most breast tumors[20, 21]. Thus, considering that *MGA* expression is highly specific for breast tissue, it has been used to detect epithelial CTCs from breast origin[22-24].

TWIST-1 gene codes for a transcription factor involved in the EMT process in tumor cells and metastasis[25]. Consequently, the study of *TWIST-1* expression has been proposed to assess CTCs with mesenchymal features, which would possess high expression levels of the gene[26-28].

Based on the above mentioned, the aim of the present study was to detect CTCs in blood samples of patients diagnosed with breast cancer by assessing the expression of *TWIST-1* and *MGA* genes. In addition, association studies between the expression of the analyzed genes and prognostic parameters of breast cancer were performed.

METHODS

This study was performed in accordance with the Declaration of Helsinki ethical guidelines, as approved by the Ethical Board of Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (Res. 1068/2014). Written consents were obtained from all patients and donors.

Chemicals and reagents

Unless otherwise indicated, all the chemicals and reagents were obtained from Sigma-Aldrich Inc. or Merck SAIC, and were of the highest purity available.

Patients and donor samples

Five milliliters of blood were obtained by venous puncture from patients or from female healthy donors and collected in tubes with heparin.

The patients were diagnosed with breast cancer (excluding those with a family history of breast cancer) by the Mastology Service of Hospital del Centenario, and samples were obtained before any surgical intervention or antitumor treatment.

Nucleated blood cells were isolated by Ficoll 400 (Fluka, Steinheim, Germany) density gradient. Briefly, blood samples were diluted at a ratio 1:1 with NaCl 0.9% p/v and 5 ml were added gently onto 3 ml of Ficoll solution (density = 1.077 g/ml) and centrifuged for 20 min at 350 × g. Then, cells were collected from the Ficoll-blood interphase by aspiration, placed in clean tubes and washed twice with phosphate-buffered saline (PBS, pH 7.4) for 10 min at 200 × g. Finally, total RNA was extracted from the cell pellet.

Small breast tissue fragments were obtained from patients undergoing reduction mammoplasty procedures at Hospital del Centenario. Total RNA was extracted from blood nucleated cells or breast tissue using Trizol reagent (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. In the end, the RNA pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water by gentle pipetting, and the concentration was estimated by measuring absorbance at 260 nm in a spectrophotometer Ultrospec 2000 (Amersham Pharmacia Biotech, Uppsala, Sweden).

The integrity of RNA samples was analyzed by electrophoresis in 1% agarose (Biorad, Hercules, CA, USA) gels, further stained with ethidium bromide, and bands were visualized using a UV transilluminator (UVI complexion, Cambridge, MA, USA). The appearance of two ribosomal RNA bands (28S and 18S) indicated that the RNA was not damaged. The samples with damaged RNA were discarded. In all cases, nucleic acid samples were stored at -70°C until analyzed.

MCF-7 cells processing

A breast cancer cell line, Michigan Cancer Foundation-7 (MCF-7) cells, was used as a positive expression control for *TWIST-1*, since it was reported that they express this gene. The MCF-7 cells (generously provided by Dr. Mauricio Menacho) were cultured in DMEM/F12 medium (Gibco; Life Technologies, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Natocor, Argentina), penicillin (100 units/ml), and streptomycin (100 mg/ml) at 37°C with 5% CO_2. After culture, the cells were collected by scraping the culture dishes, washed twice in PBS (Gibco, Invitrogen, Grand Island, NY, USA), and centrifuged for 10 min at 350 × g. Total RNA from MCF-7 cells was obtained using Trizol as described above.

Reverse transcription (RT)

Before converting the RNA to complementary DNA (cDNA), RNA samples were treated with RNase-free DNase RQ1 enzyme (Promega, Madison, USA) to remove remaining genomic DNA. Briefly, 2 µg of total RNA were mixed with two units of RNase-free DNase RQ1 and 2 µl of the reaction buffer 10×, completing with DEPC-treated water until a final volume of 10 µl. After incubation for 30 min at 37°C, 1 µl of the RQ1 DNase stop solution was added to stop the reaction. In the end, the samples were heated at 65°C for 15 min to inactivate DNase activity.

cDNA was synthesized via RT from 2 µg of total RNA (treated as mentioned above) from each sample using the M-MLV enzyme (Invitrogen, Carlsbad, USA) in a final reaction volume of 25 µl. The reaction mixture contained 5× enzyme buffer, 0.5 mM deoxynucleoside triphosphates (dNTPs) (Invitrogen, Carlsbad, USA), oligo-dT (Biodynamics, Buenos Aires, Argentine), and 200 units of M-MLV in nuclease-free water. The mixture was incubated at 42°C for 50 min.

As RT performance control, PCR reactions using beta-actin gene commercial primers (Biodynamics, Buenos Aires, Argentina) were performed with the RT products. The PCR consisted of 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final round at 72°C for 7 min. The PCR products were analyzed by electrophoresis in 2% agarose gels, and the appearance of a 289 bp band confirmed the presence of the beta-actin cDNA. Only the samples that expressed beta-actin were further considered for this study.

Detection of *MGA* expression

MGA mRNA expression in blood samples was detected by RT-PCR. Reactions were carried out with cDNAs obtained by RT and specific primers reported by Tjensvoll *et al*. (2010)[26] using commercial SYBR-Green PCR reaction mixture (Productos Bio-Lógicos, Buenos Aires, Argentina). As a negative control, cDNA was replaced by nuclease-free water. The protocol was set up as follows: cycling conditions consisted of 40 cycles of 95°C for 15 s, 62°C for 30 s, 72°C for 30 s and 76°C for 10 s, and reactions were carried out in a StepOne Real-time PCR thermocycler (Applied Biosystem, USA).

The cDNA obtained from normal breast tissue was used as positive control for *MGA* expression.

Analysis of *TWIST-1* and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expressions

To detect the *TWIST-1* expression, a real-time PCR was performed using previously published primers[26]. The cDNAs from RT were mixed with *TWIST-1* specific primers using a commercial PCR mix containing SYBR-Green (Productos Bio-Lógicos). As a negative control, cDNA was replaced by nuclease-free water. The protocol of the reaction conditions was set up as follows: 40 cycles of 95°C during 15 s, 57°C for 1 min and 72°C for 30 s, and reactions were carried out in a StepOne Real-time PCR thermocycler. The cDNA obtained from MCF-7 cells was used as positive control for *TWIST-1* expression.

The $2^{-\Delta\Delta Ct}$ comparative method was used to measure the *TWIST-1* expression level[29]. This method compares the expression of the interest gene with the expression of a control reference gene, considering cycle threshold (Ct) as the minimum number of cycles required for the fluorescence signal to pass the threshold line, above the background fluorescence. The expression of GAPDH was chosen as the reference gene. Briefly, RT-PCR for GAPDH with previously reported primers[30] was carried out under conditions similar to those described above to amplify *TWIST-1*.

The patient samples presenting overexpression of *TWIST-1* were identified considering as a threshold the highest value of the estimated reference range for the expression of the gene in samples from healthy donors. The reference range was set up as the mean expression level of the gene in blood from healthy donors ± two standard deviations (SD)[31].

In summary, $2^{-\Delta\Delta Ct}$ shows the fold difference between *TWIST-1* expression in each patient sample and the expression in healthy donors, considering:

$\Delta\Delta Ct = (\Delta Ct_{\text{healthy controls}} - \Delta Ct_{\text{patients}})$, where $\Delta Ct_{\text{healthy controls}} = (Ct_{\text{TWIST-1}} - Ct_{\text{GAPDH}})$, and $\Delta Ct_{\text{patients}} = (Ct_{\text{TWIST-1}} - Ct_{\text{GADPH}})$.

Characteristics of patient tumors

Data on tumor size, histology, nuclear grade and presence of lymph node metastases were obtained from the pathology reports. Tumors had been diagnosed by senior pathologists using standard criteria for histology and graded by Scarff-Bloom-Richardson criteria[32]. The immunostaining for estrogen receptor (ER) and progesterone receptors (PR) was performed in fixed sections from tumor tissue using a standard three-layered streptavidin-avidin-biotin horseradish peroxidase method with a mouse anti-human ER primary antibody (1:100 dilution; M7047, Dako, Ely, Cambridgeshire, UK) or anti-PR antibody 636 (1:100 dilution; M3569, Dako), respectively, and a biotinylated rabbit anti-mouse secondary antibody (1:350 dilution; E354, Dako). ER or PR expressions were classified as positive when the staining was observed in more than 10% of cancer cell nuclei. The absence of staining was considered a negative result[33].

Statistical analysis

Association studies between detection of *TWIST-1* or *MGA* gene expressions and different prognostic factors were performed using contingency tables based on the Fisher's exact test (Graph-Pad Instat, CA, USA). The sensitivity of CTC detection was analyzed with the Q-Cochran test. A p-value < 0.05 was considered statistically significant. The following patient data and tumor characteristics were considered for the analysis: patient age (older or younger than 50 years old), tumor size (≤ 2 cm vs. > 2 cm), histologic grade

(1 or 2 vs. 3), nuclear grade (1 or 2 vs. 3), presence or absence of Her2-neu overexpression, presence or absence of ER, presence or absence of PR, and presence or absence of lymph node metastasis.

RESULTS

Thirty-six breast cancer patients (average age: 51.5 ± 12.5 years) and 14 healthy donors (average age: 49.4 ± 9.4 years) were recruited for the study. **Table 1** provides clinical information about patients.

According to the anatomopathological assessment, the breast carcinomas were classified as ductal (86.7%), lobular (6.7%), mucinous (3.3%), and mixed (3.3%). Ductal carcinomas correspond to the most frequent clinical form of breast cancer observed in our hospital (Table 1).

TABLE 1 – Clinical features of tumors from breast cancer patients who participated in the study

	n	%
Age (years)		
< 50	16	44.4
> 50	20	55.6
ER		
Positive	26	78.8
Negative	7	21.2
PR		
Positive	27	81.8
Negative	6	18.2
Her2-neu		
Positive	10	30.3
Negative	23	69.7
Tumor size		
< 2 cm	14	50
> 2 cm	14	50
Nuclear grade		
1-2	24	80
3	6	20
Histologic grade		
1-2	14	58.3
3	10	41.7
Metastatic node		
Positive	14	56
Negative	11	44

ER: estrogen receptors; PR: progesterone receptors; n *and %: number and percentage of patients with a given characteristic. Some data were not available for all the tumors in the patient medical record.*

Analysis of the CTC biomarkers

No *MGA* expression was detected in samples from healthy donors, confirming that its expression is absent in peripheral blood and it is mainly restricted to the breast tissue, as it was assessed with cDNA obtained from breast tissue. The analysis allowed the detection of *MGA* expression in about 14% (5/36) of patient samples (**Figure 1**).

The association analysis between the results of *MGA* expression and the prognostic factors for breast cancer did not reveal significant associations ($p > 0.05$, **Table 2**).

TWIST-1 overexpression in patient samples was reported when the gene expression was higher than the estimated maximal expression in the blood of healthy donors. The results indicated that around 17% of patient samples showed expression of *TWIST-1* higher than that observed in healthy donors, suggesting the presence of CTCs (Figure 1). Additionally, association studies between the overexpression of *TWIST-1* and prognostic factors also indicated no significant results (Table 2).

Interestingly, the three patients who presented the highest levels of *TWIST-1* expression were further diagnosed with metastasis in distant organs (**Figure 2**).

Combined analysis of the CTC biomarkers

Considering the detection of the expression of at least one of the investigated genes, the results indicated that the combination of both genetic markers allowed detection of CTCs in 27.8% (10/36) of patient samples (Figure 1), which almost doubles the sensitivity ($p < 0.05$) of CTC detection in relation to the use of only one of them, as assessed with Cochran's Q test.

It is important to note that only one of the patient samples showed *MGA* expression together with a high *TWIST-1* expression.

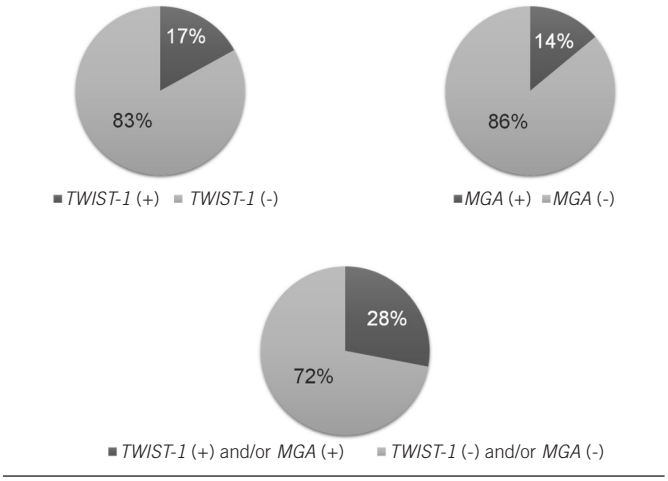

FIGURE 1 – *Pie charts showing percentages of patients with CTCs detected by analyses of the expression of* TWIST-1, MGA *and the combination of expression of both genes*

CTCs: circulating tumor cells.

TABLE 2 – Association analysis (Fisher's exact test) between the expression of *MGA* and *TWIST-1* in peripheral blood and clinicopathological parameters for breast cancer

	MGA (+)	*MGA* (-)	*p*	*TWIST-1* (+)	*TWIST-1* (-)	*p*	*MGA* and/or *TWIST-1* (+)	*MGA* (-) and *TWIST-1* (-)	*p*
Patients age (years)									
< 50	2	14	1	4	12	0.37	6	16	1
> 50	3	17		2	18		4	10	
ER									
Positive	2	25	0.18	5	21	1	6	21	0.35
Negative	2	5		1	6		3	4	
PR									
Positive	2	26	0.13	5	22	1	6	22	0.3
Negative	2	4		1	5		3	3	
Her2-neu									
Positive	1	9	1	1	9	0.64	3	7	1
Negative	3	21		5	18		6	18	
Tumor size									
< 2 cm	4	12	0.33	3	11	1	6	9	0.21
> 2 cm	1	13		3	11		2	12	
Nuclear grade									
1-2	2	22	0.17	5	19	0.55	6	18	0.64
3	2	4		0	6		2	4	
Histologic grade									
1-2	3	11	0.61	5	11	0.34	6	8	0.23
3	1	9		1	9		2	8	
Metastatic node									
Positive	4	11	0.34	3	13	0.64	3	9	0.69
Negative	1	11		3	7		5	10	

MGA *(+): number of patients in whom* MGA *expression was detected;* MGA *(-): number of patients in whom no* MGA *expression was detected;* TWIST-1 *(+): patients in whom increased expression of the* TWIST-1 *gene was detected;* TWIST-1 *(-): patients in whom the expression of* TWIST-1 *was within the values of the control population. Some data were not available in the medical records of all the patients;* MGA *and/or* TWIST-1 *(+): patient samples that were positive either for* MGA *or for* TWIST-1*; MGA: mammaglobin A; PR: progesterone receptors; ER: estrogen receptor;* $p < 0.05$ *was considered significant; no significant associations were found (*$p > 0.05$*).*

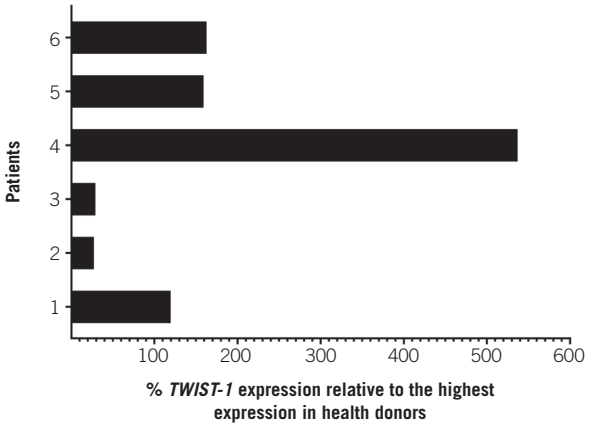

FIGURE 2 – *Overexpression of* TWIST-1 *gene in six of the breast cancer patients studied. The graph shows percentage of increase in the expression over the estimated maximum value of the expression in healthy donors*

The statistical analysis did not indicate significant associations between the combined detection of both genes and the prognostic parameters for breast cancer (Table 2).

DISCUSSION

The main cause of mortality associated with breast cancer is metastasis[34]. The spread of cells from the primary tumor to distant sites through the bloodstream cannot be detected by traditional methods. Thus, CTC detection could reflect the progression of the disease in real time. This information is perhaps particularly useful for the application of systemic therapies, and it would help to guide specific treatments for a given patient in a defined therapeutic window, supporting the concept of personalized medicine[35].

In the current report, the presence of CTCs from breast cancer was assessed through the analysis of expression of *MGA* and *TWIST-1* genes. The detection of *MGA* expression is highly specific for the breast tissue, since it is mainly limited to the mammary gland and is not usually found in the blood[36]. As expected, *MGA* was not detected in blood samples from healthy donors, confirming its expression restricted to breast tissue. Based on the highly specific *MGA* expression in breast tissue, different studies have used the detection of this gene as biomarker for CTCs derived from breast cancer[37-40]. The results indicated that the expression of *MGA* was detected in peripheral blood from about 14% of the patients, indicating the presence of CTCs derived from breast tissue. The reported percentages of *MGA* detection in peripheral blood of breast cancer patients range from 8% to 60%, according to different studies[23, 26, 40, 41]. This variation is probably related to differences in the experimental setup, such as the type of sample obtained, and the method used to assess the expression of *MGA*.

Some authors have reported that detection of *MGA* expression in blood was related to bad prognostic factors and lower rates of disease-free survival in patients with breast cancer[41, 42]. Furthermore, in a previous study in our laboratory, the detection of CTCs by analyzing *MGA* expression in breast cancer patients was associated with the absence of ERs in the primary tumor, which is a negative prognostic factor for the disease[23]. However, the use of *MGA* has not yet been implemented as a reliable tool for CTC detection, and more studies will be necessary to validate its clinical use.

TWIST-1 could be expressed in some blood immune cells in healthy donors[43]. Thus, the highest value of the estimated reference range for the expression of the gene in samples from healthy donors (Methods) was considered as a threshold, to

identify the patient samples with CTCs that overexpressed *TWIST-1*. The results of this study revealed that 17% of patients with breast cancer showed increased expression of *TWIST-1* in the blood compared to the expression in healthy individuals. Other authors have also reported that the expression of *TWIST-1* was detected in about 17%-40% of blood samples analyzed from breast cancer patients[19, 28, 44]. The difference in the reported results among studies can be explained by several aspects, such as the volume of blood samples which were obtained from patients, and the fact that samples could have been drawn before or after the surgical procedure. A higher sample volume could increase the possibility of detecting CTCs, and surgery might result in seeding tumor-derived cells in the bloodstream.

In addition, the different population of patients recruited in distinct studies may also contribute to the difference observed in CTC detection[44,45]. Finally, the fact that *TWIST-1* expression is not completely absent in the blood from healthy individuals requires estimating a suitable normal reference threshold of expression for the gene, in order to detect the genuine overexpression of the gene.

The clinical follow-up of the patients revealed that the three of them who presented the highest overexpression levels of *TWIST-1* had distant organ metastasis. This is possibly correlated with the essential role of *TWIST-1* to increase the ability of tumour cells to undergo the EMT process, leading to an invasive phenotype and the formation of distant metastases[4, 46]. Moreover, a recent systematic review of studies of *TWIST-1* expression in different types of carcinoma has revealed that the overexpression of this gene would be associated with a worse survival prognosis[47].

Since *MGA* detection mainly reflects the presence of CTCs expressing an epithelial phenotype and *TWIST-1* expression is associated with mesenchymal phenotypes and EMT, the combined analysis of both genes would allow the detection of cancer cells that could express epithelial and/or mesenchymal markers. In fact, the combination of two or more genetic markers has been proposed in order to increase the sensitivity in the detection of CTCs[45, 48]. The present results support such idea, because the combined detection of the expression of both investigated genes markedly increased the sensitivity in the detection of CTCs, which showed almost double percentage of detection than the obtained by the single analysis of *MGA* or *TWIST-1* expression. Furthermore, it is interesting to note that *MGA* and *TWIST-1* were simultaneously detected in only one sample, suggesting that both genetic markers were usually complementary in the provided information.

The results of association studies between the detection of CTCs by analysis of *MGA* and/or *TWIST-1* expressions and prognostic factors for breast cancer did not reveal significant associations. Considering that the presence of CTCs is associated with cancer invasiveness and progression, the results could suggest that the detection of CTCs provides additional clinical information about the routine clinicopathological parameters for breast cancer. Other studies have also reported that CTCs detection was not associated with other prognostic factors in breast cancer, suggesting its independent prognostic value[49, 50].

The detection of the presence of CTCs is an indicator of disease spreading from the primary site. Therefore, the finding of CTCs in peripheral blood reflects the potential risk of metastasis progression[50]. As an advantage, this is a minimally invasive procedure and can be performed frequently, achieving real-time monitoring of cells released from the tumor or the response to a given treatment.

The selection of patients who receive different antitumor treatments is mainly based on the statistical risk of tumor recurrence. The screening of CTCs might contribute to predict the risk for cancer metastasis and therapy effectiveness, assessing the response to an anticancer treatment aimed to eradicate these cells before they initiate metastasis. Thus, the clinical implementation of CTCs detection may actually help improve the selection of specific treatments for each patient and, therefore, move towards more efficient and personalized therapies. Over the last years, different clinical trials have been conducted to establish the prognostic value of CTCs in early and advanced stages of breast cancer[50, 51]. However, the standardization of CTCs detection for use in clinical practice is still a great challenge, and further clinical trials should be carried out to validate the methods and the utility of different biomarkers of CTCs.

CONCLUSION

The analysis of *MGA* and *TWIST-1* expression in blood from breast cancer patients can be a suitable method for the detection of CTCs. Since the results indicated that detection of *MGA* expression was usually not associated with *TWIST-1* overexpression, the combined analysis of both genes markedly increased sensitivity for the detection of CTCs in patients with breast cancer.

CONFLICT OF INTEREST

The authors do not have any conflicts of interest to declare.

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