Circulating Levels of Transforming Growth Factor-βeta (TGF-β) and Chemokine (C-X-C Motif) Ligand-1 (CXCL1) as Predictors of Distant Seeding of Circulating Tumor Cells in Patients with Metastatic Breast Cancer

ROSA DIVELLA[1], ANTONELLA DANIELE[1], EUFEMIA SAVINO[1], FABIOLA PALMA[2], ANTONIA BELLIZZI[3], FRANCESCO GIOTTA[4], GIOVANNI SIMONE[2], MARCO LIOCE[5], MICHELE QUARANTA[1], ANGELO PARADISO[6] and ANTONIO MAZZOCCA[7]

[1]Clinical Pathology Laboratory, Department of Experimental Oncology, [2]Department of Pathology, [3]Experimental Oncology Laboratory, Department of Experimental Oncology, [4]Clinical Experimental Oncology Unit, [5]U.O.C. Radiotherapy, [6]Scientific Directorate National Cancer Institute Giovanni Paolo II, Bari, Italy; [7]Department of Emergency and Organ Transplantation, Section of Internal Medicine, Allergology and Clinical Immunology, University of Bari Medical School, Bari, Italy

Abstract. *Background: The presence of circulating tumor cells (CTCs) in the peripheral blood is a prerequisite for the formation of distant metastases. Transforming growth factor-βeta (TGF-β) and Chemokine (C-X-C Motif) Ligand-1 (CXCL1) are cytokines involved in the colonization of distant sites by CTCs in several pre-clinical animal models. However, their role is poorly-investigated in patients with metastatic cancer. Here, we investigated whether circulating levels of TGF-β and CXCL1 are predictors of CTC seeding in preferential distant sites in patients with metastatic breast cancer. Materials and Methods: CTCs were isolated from the peripheral blood of 61 patients with metastatic breast cancer by immunomagnetic separation. Plasma samples were collected from the same patients and assayed for TGF-β and CXCL1 by enzyme-linked immunoassay. Results: Patients were grouped in $CK1_{+/-}$ (N<10), $CK2_{+}$ (N≥10<50) and $CK3_{+}$ (N≥50), according to the number (N) of cytokeratin 7/8-positive CTCs: the highest number of CK7/8-positive CTCs was detected in patients with negative Human epidermal growth factor receptor-2 (HER-2/NEU) status (p<0.0001) antigen, identified by the monoclonal antibody Ki-67 (Ki-67) ≥15% (p=0.003), Carcinoma antigen 15-3 (CA-15.3) ≥40 U/ml (p=0.004) and those with lung metastases (p=0.01). We found that elevated plasma concentrations of TGF-β and CXCL1 are predictive for the detection of CTCs. In particular, patients with $CK3_{+}$ CTCs and plasma concentrations of TGF-β and CXCL1 higher than the median value had a poor prognosis in comparison to patients with $CK1_{+/-}$ CTCs and TGF-β and CXCL1 concentrations below the median value. Conclusion: Our study shows that elevated circulating levels of TGF-β and CXCL1 are associated with a poor prognosis, and higher detection of CTCs and propensity of these cells to seed lung metastases in patients with breast cancer.*

Correspondence to: Rosa Divella, Department of Experimantal Oncology, Clinical Pathology Laboratory National Cancer Institute Giovanni Paolo II, Viale Orazio Flacco, 65, 70100 – Bari, Italy. E-mail: rosadive@inwind.it and Antonio Mazzocca, Department of Emergency and Organ Transplantation, Section of Internal Medicine, Allergology and Clinical Immunology, University of Bari Medical School, Bari, Italy. Piazza G. Cesare, 11, 70124 – Bari, Italy. E-mail: a.mazzocca@intmed.uniba.it

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In epithelial cancer, invasion of cancer cells through the basal membrane into blood vessels, generating circulating tumor cells (CTCs), is a crucial step for the metastatic spread of cancer cells. The presence of CTCs in the peripheral blood is therefore a prerequisite for the formation of distant metastases (1). Metastases represent a major obstacle for the treatment of epithelial cancer and are responsible for most cancer-related deaths. The influence of the tumor microenvironment on metastatic growth is well-recognized (2). Metastatic spread is influenced by both intrinsic tumor properties and microenvironmental factors that facilitate the formation of the pre-metastatic niche prior to the arrival of disseminated tumor cells (3). The potential of a tumor cell (seed) to become metastatic depends on its interactions with homeostatic factors in a target organ (soil) that promote cell survival, angiogenesis

and tumor growth. According to the 'the seed and soil' theory, postulated by Stephen Paget in 1889, there is a propensity for certain tumors to seed in particular organs (4). In addition, the metastatic process is extremely inefficient and the fate of CTCs can vary according to their molecular profile; fewer than one tumor cell in a million gives rise to metastases (5). CTCs have a low survival rate in the blood circulation. In fact more than 85% of CTCs undergo a rapid phase of intravascular disappearance which is completed in less than 5 min. This process is believed to be due to different events including an augmented susceptibility to anoikis (6). The fate of CTCs is dependent on an active crosstalk within the tumor microenvironment. In this context, inflammatory mediators, such as cytokines and chemokines, play a pathogenic role by stimulating factors that promote angiogenesis, extracellular matrix degradation and motility of tumor cells (7-9). For instance, increased migration and invasion of epithelial cells can be induced in response to Transforming growth factor-beta (TGF-β) (10, 11). The contribution of fibroblasts to cancer initiation and progression has been the subject of recent investigation with respect to TGF-β and cancer progression (12, 13). In fact, in different models of human cancer, TGF-β has shown activity in the regulation of stromal tumor interactions in tumor angiogenesis and progression by acting in an autocrine/paracrine fashion (14-16). For example, carcinoma-derived TGF-β acting on stromal fibroblasts or carcinoma-associated fibroblasts (CAF) remodels the tumor matrix and induces expression of mitogenic signals towards the carcinoma cells, thus promoting tumor progression (17, 18). In addition, acting directly or indirectly (through stimulation of pro-angiogenic factors) on endothelial cells and pericytes, TGF-β regulates angiogenesis (15, 19). Chemokines and their receptors are implicated in many aspects of tumor progression and metastasis (20). Chemokine (C-X-C Motif) Ligand-1 (CXCL1) is a small chemokine belonging to the CXC subfamily of chemokines, previously known as growth-related oncogene-α (GROα), that normally controls leukocyte trafficking from peripheral blood into tissues during inflammation and hematopoiesis (21). Many chemokines, including CXCL1 are strong inducers of chemotaxis and there are several lines of evidence showing that they play a role in tumor progression, for example, by increasing metastasis formation in preferential target organs (22). Targeting chemokines or their receptors may pave the way for therapeutic intervention in cancer metastasis (23). CXCL1 promotes growth of tumor cells and is involved in angiogenesis of several tumors in animal models but its role in human cancer remains to be elucidated (24, 25). In this study, we investigated whether levels of circulating TGF-β and CXCL1 are associated with a higher detection rate of CTCs in metastatic breast cancer and also if these mediators have a role in favoring the colonization of metastatic cells to preferential sites.

Materials and Methods

Patients. A total of 61 patients with metastatic breast cancer median aged 59 (range 37-82) years were enrolled in this prospective study at Giovanni Paolo II National Cancer Institute (NCI) of Bari, Italy. The criteria for inclusion of patients were the following: informed consent; age ≥18 years <85 years; histologically-proven diagnosis of breast cancer; non-treated stage III-IV disease (TNM staging); distant metastatic disease (M1: TNM staging); no double cancer; no major pathologies; at least three months of life expectancy. The clinical characteristics of patients (age, sex, therapeutic interventions, *etc*.) were obtained from medical records. For detection of CTCs 15 ml of peripheral blood were collected from each participant in a vacutainer system with EDTA. For analysis of cytokine, 5 ml of peripheral blood were collected in a vacutainer system with EDTA and plasma was immediately separated from the cellular fraction by centrifugation at 1,500 ×*g* for 10 min and frozen at –20˚C. Blood samples from 20 healthy individuals were used as negative control. Samples were collected from each participant before any invasive procedures or therapy. In according to national and institutional standard procedures, all patients received systemic therapy. Patient evaluation consisted of imaging studies (Computed Tomography, Positron Emission Tomography - Computed Tomography, chest X-ray, abdominal ultrasound) and biochemical analyses and was performed at the beginning, at mid-therapy and at the end of treatment (at different intervals, depending on treatment and schedule). Written consent was obtained from all patients prior to enrolment in the study and the Ethical Committee of the NCI approved the protocol in accordance with once the ethical guidelines of the 1975 Declaration of Helsinki.

Enumeration and characterization of CTCs. This procedure has been previously described (26). Briefly, 15 ml of anti-coagulated blood were centrifuged at 400 ×*g* for 35 min and buffy coats were collected into 50-ml conical tubes. Enrichment of disseminated carcinoma cells from peripheral blood was performed by positive selection of cytokeratin-7/8 expressing cells. For direct immunomagnetic labeling of intracellular cytokeratin-7/8, cells were permeabilized with MACS CellPerm Solution, fixed with MACS CellFix Solution and incubated with MACS Cytokeratin MicroBeads in MACS CellStain Solution (Carcinoma Cell Enrichment and Detection Kit, Miltenyi Biotec Inc., Bergish Gladbach, Germany). The magnetically labeled cells were enriched on a positive selection column in the magnetic field of a MACS separator. For immunocytochemical detection of carcinoma cells in the MACS-enriched cell fraction, the cells were first incubated with Fluorescein Isothiocyanate (FITC)-conjugated antibody to cytokeratin and then with anti-FITC antibody conjugated to alkaline phosphatase. These staining steps were performed in suspension before magnetic enrichment. After MACS enrichment, cells of the magnetic fraction were spun on slides and incubated with alkaline phosphatase substrate.

TGF-β and CXCL1 enzyme-linked immunoassay (ELISA). Plasma samples from patients and healthy donors were assayed for levels of TGF-β and CXCL1 by a sandwich ELISA assay (Quantikine Human TGF-β and Quantikine Human CXCL1 Immunoassay; R&D Systems, Inc., Minneapolis, USA) according to the manufacturer's recommendations. The absorbance of the solution produced was measured at 490 nm. The absorbance is directly proportional to the

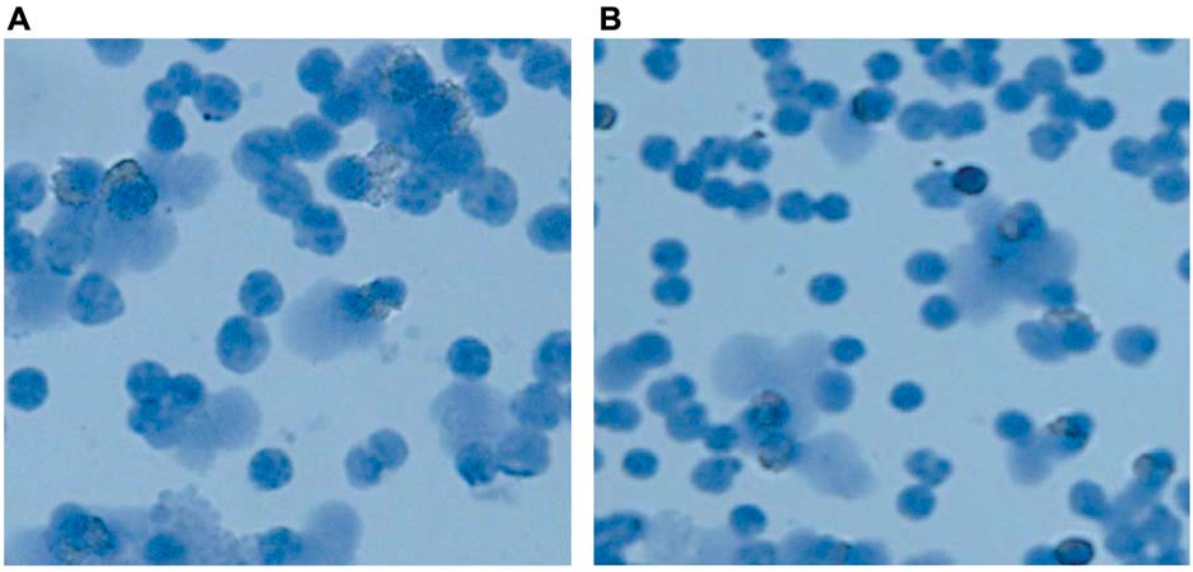

Figure 1. *Representative circulating tumor cells found in the peripheral blood of patients with metastatic breast cancer. Cells from the enriched fraction were immunocytochemically-stained using a FITC-conjugated anti-cytokeratin antibody in combination with anti-FITC antibody-conjugated alkaline phosphatase and substrate (×40).*

amount of TGF-β and CXCL1 present in the sample. A standard curve was constructed by plotting the mean absorbance value measured for each standard *versus* its corresponding concentration. The minimal detection limit was 4.61 pg/ml for TGF-β, and 10 pg/ml for CXCL1.

Statistical analysis. The association between the detection rate of CTCs and clinical parameters was investigated with the Chi-square test, whereas the association between CTCs and plasma levels of TGF-β and CXCL1 was analyzed with unpaired *t*-test and ANOVA test. Spearman correlation was used for the correlation analysis and the survival analysis was evaluated with log-rank test. Progression-free survival (PFS) and overall survival (OS) were defined as the time between the date of the blood sample drawing and the date of clinical progression or death or the last follow-up examination, respectively. A *p*-value ≤0.05 indicates statistical significance. All statistical analyses were performed by the Number Cruncher Statistical System-Power Analysis and Sample Size Software 2007 (NCSS-PASS, 329 North 1000 East Kaysville, Utah. USA).

Results

CTCs isolated from peripheral blood and circulating levels of TGF-β and CXCL1 were assayed in 61 patients with metastatic breast cancer. As shown in Figure 1, only one type of CTCs, those circulating as single cells, were identified by microscopy. As part of the single CTCs and according to the number of cells, we classified three groups of patients: i) patients at low risk, with number of CTCs <10 ($CK1_{+/-}$) n=21; ii) patients at medium risk, with CTC number between 10 and 50 ($CK2_{+}$) n=17; and iii) patients at high risk, with CTC number ≥50 ($CK3_{+}$), n=23. No cytokeratin-7/8-positive CTCs were identified in any of the 20 healthy donor samples.

Correlation between CTCs and clinical parameters of patients with metastatic breast cancer. The association between clinical features and the number of CTCs in patients with metastatic breast cancer is shown in Table I. A high number of CTCs was significantly associated with negative HER-2/NEU status ($p<0.0001$, Chi-square test), Ki-67 index greater than its cut-off value (≥15) ($p=0.003$, Chi-square test) and Carcinoma Antigen 15-3 (CA-15.3) ≥40 U/ml ($p=0.004$, Chi-square test). Elevated levels of CTCs were also associated with the presence of lung metastases. In fact, in patients with lung metastases, we observed a greater number of CTCs compared to patients with only bone or visceral metastases ($p=0.01$, Chi-square test).

Association between CTC positivity and plasma TGF-β and CXCL1 levels. The circulating plasma levels (mean±SD) of TGF-β and CXCL1 are reported in Table II. The mean values of these cytokines were much higher in patients than in control individuals ($p=0.0001$, *t*-test). We found that the circulating levels of TGF-β and CXCL1 significantly correlated with an increased the number of CTCs ($p<0.0001$ and $p=0.02$, respectively, ANOVA). In addition, when the TGF-β and CXCL1 plasma levels were compared as continuous variables, a significant direct correlation between the two cytokines was found (Spearman correlation r=0.3, $p=0.01$) (Figure 2). Finally, a positive correlation was observed only for TGF-β when the number of CTCs was compared with the plasma concentrations of each cytokine (Spearman correlation r=0.01, $p=0.67$) Figure 3A and B).

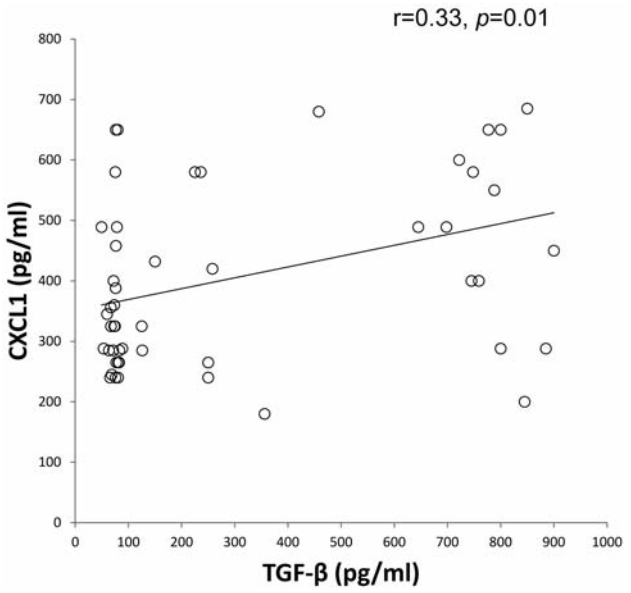

Figure 2. *Scatter plot showing correlation between serum levels of transforming growth factor-beta (TGF-β) and Chemokine (C-X-C Motif) Ligand-1 (CXCL1).*

Table I. *Association between clinical characteristics and presence of circulating tumor cells (CTCs) in patients with breast cancer.*

Patients' characteristics	N (%)	$CK1_{+/-}$ (n=21)	$CK2_{+}$ (n=17)	$CK3_{+}$ (n=23)	*p*-Value[a]
Steroid hormonal receptor status					0.9
ER_{+}/PgR_{+}	31 (51)	9	9	13	
ER_/PgR_	22 (36)	9	6	7	
ER_{+}/PgR_{-}	8 (13)	3	2	3	
Nodal status					0.6
Negative	14 (23)	6	4	4	
Positive	47 (77)	15	13	19	
HER-2/NEU					<0.0001
Negative	36 (59)	6	10	20	
Positive	25 (41)	15	7	3	
Ki-67					0.003
<15%	16 (26.2)	11	3	2	
≥15%	45 (73.7)	10	14	21	
CA-15.3					0.004
<40 U/mL	27 (44.2)	15	7	5	
≥40 U/mL	34 (55.7)	6	10	18	
Metastatic site					0.01
Bone	15 (24.5)	8	3	4	
Visceral	20 (32.7)	12	5	3	
Lung	26 (42.6)	1	9	16	

ER: Estrogen receptor, PgR: progesterone receptor. HER-2/neu: human epidermal growth factor receptor, Ki-67: marker of proliferative index, CA-15.3: carbohydrate antigene. [a]All *p*-values were calculated with Chi-square test.

Table II. *Association between plasma levels of trasforming growth factor-β (TGF-β) and chemokine (C-X-C Motif) ligand-1 (CXCL1) in relation to circulating tumor cells (CTC) positivity and to clinical characteristics in patients with metastatic breast cancer.*

	N (%)	TGF-β (pg/ml)	*p*-Value	CXCL1 (pg/ml)	*p*-Value
Control group	20	39.5±9.8	0.0001[a]	58.4±12	0.0001[a]
Patient	61	309±311		407±147	
CTC positivity					
$CK1_{+/-}$	21 (34.4)	70±9	<0.0001[b]	352±120	0.02[b]
$CK2_{+}$	17 (27.8)	216±203		394±148	
$CK3_{+}$	23 (37.7)	714±202		482±156	
Hormonal receptor status			0.2[b]		0.6[b]
ER_{+}/PgR_{+}	31 (51)	319±321		423±144	
ER_/PgR_	22 (36)	242±258		386±154	
ER_{+}/PgR_{-}	8 (13)	456±383		400±142	
Nodal status			0.9[a]		0.3[a]
Negative	36 (59)	458±321		396±152	
Positive	25 (41)	460±350		432±175	
HER-2/NEU			0.08[a]		0.07[a]
Negative	36 (59)	391±367		349±132	
Positive	25 (41)	243±241		417±158	
Ki-67			0.5[a]		0.6[a]
<15%	16 (26.2)	273±307		390±113	
≥15%	45 (73.7)	322±315		412±157	
Metastatic site					
Bone	15 (24.5)	90±52	<0.0001[b]	125±100	<0.0001[b]
Visceral	20 (32.7)	210±123		326±135	
Lung	26 (42.6)	653±208		456±189	

ER: Estrogen receptor, PgR: progesterone receptor. HER-2/neu: human epidermal growth factor receptor, Ki-67: marker of proliferative index. [a]*p*-values Calculated with unpaired *t*-test; [b]calculated with ANOVA test.

Mean survival in correlation to CTC positivity, circulating levels of TGF-β and CXCL1. The median follow-up was 12 months (range=0-24 months) from blood sampling. The median PFS and OS in relation to CTC positivity for cytokeratin 7/8 and in relation to plasma concentrations of TGF-β and CXCL1 above their median value (of 110 pg/ml and 380 pg/ml, respectively) are reported in Table III. For all patients, median PFS and OS were 10 and 17 months, respectively. When considering CTC positivity, PFS was 11 months for $CK1_{+/-}$ patients *vs.* 9 and 7.4 months for $CK2_{+}$ and $CK3_{+}$ patients, respectively (p=0.001, log-rank test). The median OS was much higher in $CK1_{+/-}$ patients (19.5 months) compared to that of $CK2_{+}$ (16.5 months) and $CK3_{+}$ patients (12.6 months), (p=0.001, log-rank test). In 22 patients (36.2%) with elevated plasma levels of TGF-β (median value: ≥110 pg/ml) and CXCL1 (median value: ≥380 ng/ml), the median PFS was 7.3 months *vs.* 10.7 months observed in 19 patients (31.1%) with non-elevated levels of TGF-β and CXCL1 (p=0.001, log-rank test). The OS for these patients was 15.2 *vs.* 18.6 months (p=0.001).

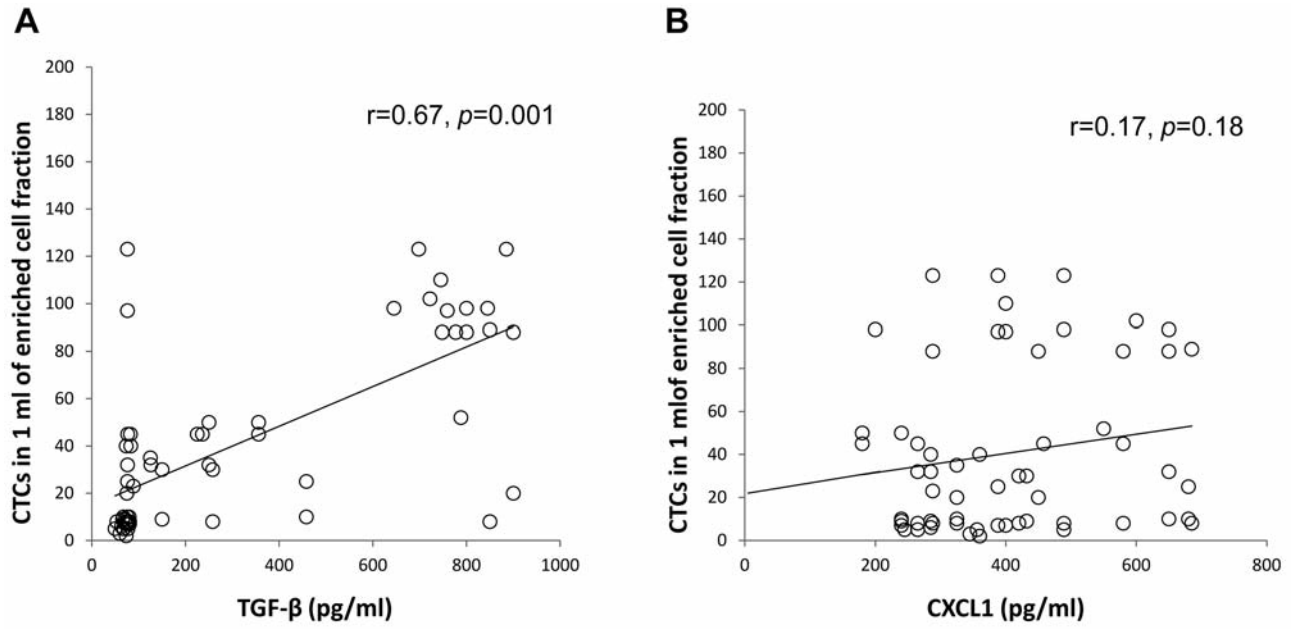

Figure 3. *Scatter plots showing correlation between tumor cell counts and serum levels of transforming growth factor-beta (TGF-β) (A) and Chemokine (C-X-C Motif) Ligand-1 (CXCL1) (B).*

Discussion

Metastasis results from a complex cascade of events by which cancer cells leave the site of the primary tumor and disseminate to distant organs where they proliferate and form secondary tumor foci (27, 28). During their journey through the blood circulation to colonize distant sites, CTCs undergo a series of events that eventually define their phenotypic fate (29). These events are regulated by a plethora of molecular factors, including cytokines and chemokines released into the microenvironment by both host and tumor cells (30, 31). In this study, we have shown that circulating levels of TGF-β and CXCL1 are useful indicators to predict both the presence of CTCs and their capacity to metastasize to certain preferential sites (*i.e.* the lung) in patients with metastatic breast cancer. Unlike others, we stratified CTC-positive patients expressing cytokeratin 7/8 into three groups according to the number of cancer cells isolated from their peripheral blood: $CK1_{+/-}$ (N<10), $CK2_{+}$ (10≤N<50) and $CK3_{+}$ (N≥50). The latter group, with highest number of CTCs, was associated with negative HER-2/NEU status, Ki-67 and CA 15.3 greater than their cut-off values of 15% and 40 U/ml respectively, and with lung metastasis. As a consequence the analysis of circulating cytokines, TGF-β and CXCL1 had a significant impact on CTCs, as demonstrated by the fact that elevated plasma levels of TGF-β and CXCL1 are predictive of detection of CTCs in patients with breast

Table III. *Mean survival in correlation to circulating tumor cell (CTC) positivity and plasma transforming growth factor-β (TGF-β) and Chemokine (C-X-C Motif) Ligand 1 (CXCL1) levels.*

	N (%)	PFS months	*p*-value	OS months	*p*-value
All patients	61	10		17	
CTC positivity					
$CK1_{+/-}$	21 (34.4)	11.1		19.5	
$CK2_{+}$	17 (27.8)	9	0.001	16.5	0.001[a]
$CK3_{+}$	23 (37.7)	7.4	0.001	12.6	0.001[b]
TGF-β (pg/ml)					
≥110	31 (50.8)	8.4	0.001	15.2	0.001
<110	30 (49.2)	10.3		18.1	
CXCL1 (ng/ml)					
≥380	33 (54.1)	9	0.03	15.7	0.05
<380	28 (45.9)	10		17.6	
TGF-β ≥110 CXCL1 ≥380	22 (36.2)	7.3	0.001	15	0.001
TGF-β <110 CXCL1 <380	19 (31.1)	10.7		18.6	
TGF-β ≥110 CXCL1 <380	9 (14.7)	8.7	0.08	15.7	0.07
TGF-β <110 CXCL1 ≥380	11 (18)	10.0		17.2	

[a]$CK2_{+}$ compared with $CK1_{+/-}$; [b]$CK3_{+}$ compared with $CK2_{+}$. All *p*-values were calculated with log-rank test.

cancer. In fact, we have shown that plasma levels of TGF-β and CXCL1 are positively correlated with the presence of cytokeratin 7/8-positive CTCs in these patients. In patients with late stages of tumorigenesis, TGF-β may favor a more aggressive phenotype by promoting tumor growth and resistance to apoptosis or by enhancing tumor cell motility and eventually metastasis. These features have a negative impact on the prognosis of patients that we have examined. The positive correlation found between CXCL1 and TGF-β would lead us to postulate that CXCL1 modulates the expression of TGF-β within the tumor microenvironment and its releases into the blood circulation by CTCs. The chemokine CXCL1 is constitutively overexpressed in tumorigenic cells and transcribed in normal cells only during growth stimulation (32). Overexpression of the CXCL1 gene is associated with lung relapse in patients with breast tumors and increases the aggressiveness of CTCs (33). Chemokines and growth factors such as CXCL1 and TGF-β are intricately associated with cellular transformation, tumor growth and increase of invasive potential (34, 35). Our study shows that elevated circulating levels of TGF-β and CXCL1 in patients with metastatic breast cancer are associated with a higher propensity of CTCs to seed lung metastases. We can now conclude that expression of chemokine CXCL1 may facilitate cell seeding and outgrowth of metastases at distant sites and that CXCL1 also plays an important role in the recruitment of CTCs to distant target organs in patients with metastatic breast cancer. However, further studies are needed to better-clarify the mechanisms by which CXCL1 and TGF-β regulate the transit of CTCs towards certain metastatic target sites in patients with breast cancer. Targeting these mediators may unveil new therapeutic opportunities to treat or prevent metastatic disease in these patients.

Competing Interests

The Authors declared that no competing interests exist.

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