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Detection of EMT, anoikis and stem cell markers in metastatic breast cancer patients under different lines of treatment

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Background

Metastasis are thought to be induced by occult spreading of tumor cells already during the early phases of the disease. Circulating Tumor Cells (CTCs) are regarded as precursors of distant metastasis, while detaching from the primary tumor and originating micrometastases in distant organs. Recent evidences pointed to CTC heterogeneity, showing that CTCs can present different phenotypes. Goal of this study was to identify in metastatic breast cancer (mBC) patients EpCAM negative CTCs and to further characterize them for epithelial (EM), mesenchymal (MM), EMT (EMT-TF), TrkB1 anoikis resistance (AM) and cancer stem cell (CSM) marker expression. A correlation between marker overexpression and clinical parameters was also evaluated.

Methods

Blood samples were obtained from mBC patients under different lines of therapy. Patients' characteristics are summarized in Table 1. All blood samples, withdrawn at different time points during treatment, were beads depleted of EpCAM$^+$ cells and CD45$^+$ white blood cells (Figure 1) (Mego *et al*., Int J Cancer 2012;130(4):808-816) and marker expression was analyzed by qRT-PCR in the EpCAM/CD45 depleted fraction. Beta-actin was used as housekeeping (HK) gene and cDNAs were quantified with the delta-Ct method using the formula: ½^Ct (target-HK). The highest expression level for each marker found in healthy donors (n=11) was used as threshold value to measure gene overexpression in mBC patients. All blood samples were analyzed in parallel for the presence of EpCAM$^+$ CTCs with the CellSearch™ system (Veridex, Raritan NJ) (Table 2).

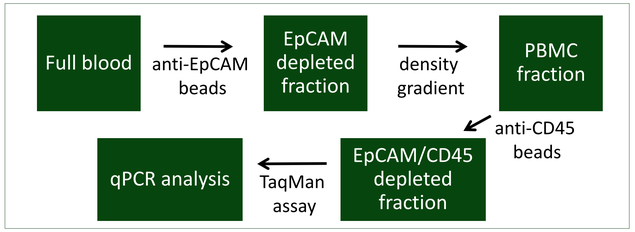

Figure 1: Sample processing workflow

Median Age	62	
Range (years)	35-79	
pT (%)	T1(b-c)	8 (28.6%)
	pT2-4	19 (67.8%)
	n/a	1 (3.6%)
pN (%)	pN0	9 (32.1%)
	pN1-3	13 (46.5%)
	n/a	6 (21.4%)
Histology (%)	G1	0 (0.0%)
	G2-3	23 (82.1%)
	n/a	5 (17.9%)
Metastasis localization	Viscera	8 (28.6%)
	Bone	8 (28.6%)
	Viscera+Bone	11 (39.2%)
	n/a	1 (3.6%)
HER2 status	Negative	20 (71.4%)
	Positive	7 (25.0%)
	n/a	1 (3.6%)
Hormone-receptor status	Negative	2 (7%)
	Positive	25 (93%)
	n/a	1 (3.6%)
Therapy line	Endocrine	22 (78.5%)
	Chemotherapy	5 (17.9%)
	HER2	7 (25.0%)
	Chemo. + HER2	1 (3.6%)
	Anti-angiogenic	4 (14.3%)

Table 1: Patient characteristics (n = 28)

CellSearch™ Analysis (n=28)	
CTC positive	9 (32%)
CTC negative	19 (68%)

Table 2: EpCAM$^+$ CTC detection

Results

In the EpCAM/CD45 depleted fractions derived from the 9 CellSearch™ positive patients, 6 (66%) of the samples were positive for at least one EM, one MM or one EMT-TF (Table 3). Simultaneous overexpression of EM and EMT-TFs in CTCs was observed in 2 (22%) patients. There was no upregulation of TrkB1 or SCM in this group. In the EpCAM/CD45 depleted fractions derived from the 19 CellSearch™ negative patients, 5 (26%) samples were found positive for at least one EM, 2 (10%) for at least one MM and 4 (21%) for at least one EMT-TF, notably with Zeb1 overexpressed in 3 (16%) samples. No overexpression was found for TrkB1, suggesting that anoikis repression might be regulated by a different mechanism. Interestingly, 9 (47%) samples showed an overexpression of at least one SCM. Co-expression of EM and EMT-TF, EM and SCM or SCM and EMT-TF was observed in 3 (16%), 4 (21%) and 4 (21%) patients respectively, while co-expression of EM, EMT-TF and SCM was found in 3 (16%) patients. A significant association (chi-squared test, p=0.039) was found between SCM and endocrine therapy of the primary tumor: patients presenting SCM overexpression were found less likely to have received an endocrine therapy for their primary tumor (3 out of 9; 33%) as compared to patients with no SCM overexpression (14 out of 18; 78%). No significant association was found in the same group between SCM overexpression and hormone receptor status (p=0.103). Finally, CTCs overexpressing MM were found significantly (p=0.016) more often in patients with a pT4 tumor (3 out of 5; 60%) as compared to patients with pT1 (0 out of 8; 0%), pT2 (1 out of 10; 10%) or pT3 tumors (0 out of 4; 0%). No other correlations between marker expression and clinical parameters were found.

Conclusions

These preliminary results suggest that mBC patients undergoing different lines of therapy present heterogeneous CTCs populations. 12 (63%) patients with undetectable EpCAM$^+$ CTCs were found positive for different markers in different combinations in the EpCAM/CD45 depleted blood fraction. These findings underline a high grade of heterogeneity in the CTC population which could explain why mBC patients, at first responding to treatment, might finally relapse, probably because of resistant CTCs subpopulations selection.

EpCAM / CD45 depleted fractions			
Markers	CellSearch™ positive (n=9)	CellSearch™ negative (n=19)	Total (n = 28)
EM	2 (22%)	5 (26%)	7 (25%)
MM	2 (22%)	2 (10%)	4 (14%)
EMT-TF	2 (22%)	4 (21%)	6 (21%)
AM	0 (0%)	0 (0%)	0 (0%)
SCM	0 (0%)	9 (47%)	9 (32%)
EM + EMT-TF	2 (22%)	3 (16%)	5 (18%)
EM + SCM	0 (0%)	4 (21%)	4 (14%)
SCM + EMT-TF	0 (0%)	4 (21%)	4 (14%)
EM + EMT-TF + SCM	0 (0%)	3 (16%)	3 (11%)

Table 3: Expression levels of the different markers in EpCAM/CD45 depleted cellular fractions derived from CellSearch™ positive and negative samples (EM: EpCAM, E-Cadherin, Cytokeratin 18 and 19; MM: N-Cadherin, Vimentin; EMT-TF: Twist1, Snail1, SLUG, Zeb1, FoxC2; AM: TrkB1; SCM: CD24, CD44, CD133)

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