

# Concurrent Detection of Circulating Tumor Cells and Circulating Tumor DNA in Triple-negative Breast Cancer

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**Objective:** Circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) provide tumor information in breast cancer. Our objective was to characterize CTCs, and contrasted them with ctDNA PIK3CA mutation in 24 triple-negative breast cancer (TNBC).

**Methods:** CTCs genes were characterized by AdnaTest protocol and ctDNA by digital PCR.

**Results:** We found CTCs genes in 37.5% and ctDNA PIK3CA mutations in 29.16%. Three cases with CTCs genes had concurrent ctDNA PIK3CA mutations. MUC1 or GA733-2 were found in 4 cases, and 3 of them had concurrent ctDNA PIK3CA. CTCs ALDH1/TWIST1 were found in 2 cases, AKT2 in one and PI3K $\alpha$  in another, and none had concurrent ctDNA PIK3CA mutations. There was no correlation between CTCs and ctDNA detection. All 3 cases with CTC & cDNA concurrent finding underwent death during follow-up.

**Conclusion:** Infrequent concurrent detection of CTC and ctDNA presence suggests that both represent independent processes in TNBC patients, and could identify worst prognosis cases.

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## Introduction

The prognostic value of liquid biopsy for metastatic breast cancer (BC) was first introduced more than a decade ago [1]. Two of the most studied liquid biopsies are circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) [2]. CTCs are viable cells actively migrating into the circulation as potential seeds of metastasis and are associated with reduced survival and lower response rates to systemic therapies [1,3,4]. Molecular features of CTCs can also be evaluated through gene expression and predict patient outcomes [5,6]. On the other side, ctDNA appears to be derived from necrosis, apoptosis, and secretions of tumor cells, and its presence has also been associated with shorter survival and lower response to therapy. Different molecular tumor biomarkers like PI3KCA-mutations can be evaluated through ctDNA [7,8].

Triple-negative breast cancer (TNBC) is a biologically undifferentiated and aggressive tumor [9,10], and it appears that most CTCs have TNBC phenotype [11]. However, few publications have analyzed both ctDNA and CTCs from the same patients and they are not constricted to TNBC phenotype [2,12,13].

The purpose of the present study was to evaluate the relationship between gene expression profile of CTCs and presence of PIK3CA mutations in ctDNA in a TNBC series.

## Materials and Methods

### Study population

The study was conducted at the Instituto Nacional de Enfermedades Neoplásicas in collaboration with MD Anderson Cancer Center. Blood samples from 24 TNBC patients were collected between July 2016 and March 2017. Clinicopathological information was obtained from patient files.

### Enrichment of CTCs

Blood samples were analyzed for CTCs using the AdnaTest EMT-2/StemCellSelect™ (QIAGEN Hannover GmbH, Langenhagen, Germany) that enriches CTCs via antibody-coated magnetic beads, targeting EpCAM, EGFR, and ERBB2 following the manufacturer's instructions and were subsequently lysed. The cell lysate was stored for a maximum of two weeks at -80°C until further processing.

### Detection of CTCs, extraction and analysis of mRNA expression

mRNA was isolated from the cell lysate of pre-enriched tumor cells using the AdnaTest EMT-2/StemCellDetect™ (QIAGEN Hannover GmbH, Langenhagen, Germany). Reverse transcription was performed using the Sensiscript Reverse Transcription Kit™ (QIAGEN, Hilden, Germany). The analysis of the cDNA was performed by multiplex PCR for the following transcripts: stem cell marker ALDH1 (PrimerMix StemCell), and EMT markers PI3Kα, Akt-2 and Twist1 (PrimerMix EMT-2). Thermal profiles were used as per the recommendations of the supplier (AdnaGen) in the Mastercycler Nexus Gradient (Eppendorf). We also used the PrimerMix BreastDetect to amplify three Breast cancer-related genes (MUC-1, HER- Visualization of PCR fragments was carried out with the Agilent 2100 Bioanalyzer, with limit of detection (LOD) of  $\geq 0.15$  ng  $\mu$ l<sup>-1</sup>.

### Detection of PIK3CA mutations in ctDNA and tumor

Circulating cell-free DNA (ccfDNA) or ctDNA were extracted using affinity-based binding to magnetic beads according to the manufacturer's instructions (Maxwell® RSC ccfDNA Plasma Kit, Promega). Template DNA was mixed with TaqMan® Assay primer/probe mix and QuantStudio™ 3D dPCR Master Mix (Life Technologies) according to the manufacturer's instructions. PCR solutions were then loaded into QuantStudio™ 3D dPCR 20 K chips and the PCR reaction was performed. The data were analyzed with the QuantStudio™ 3D AnalysisSuite™ v1.1.3 (Life Technologies, Carlsbad, CA) for mutation search and quantification of the DNA copies in the plasma with LOD of  $\geq 1$  copy  $\mu$ l. Custom PIK3CA mutations (PI3KCA 760: c.1624 G (VIC) >A (FAM), PI3KCA 763: c.1633 G (VIC) >A (FAM), PI3KCA 775: c.3140 A (VIC) >G (FAM), Thermo Fisher scientific). Tumor mutations were evaluated through TaqMan- based RT-PCR analysis in formalin paraffin samples archived at the pathology department. The process was conducted using a Light Cycler® 96 RT-PCR System (Roche Applied Science, Mannheim, Germany) to detect the three "hot spot" PIK3CA mutations (H1047R, E545K and E542K).

## Results

The cohort consists of 24 women with TNBC phenotype, and with NST histology except one metaplastic lesion. PIK3CA mutations in tumor were analyzed in 10 cases and 1 case was positive (H1047R mutation). Median follow-up time was 39 months (1.4-44.4 months). Eight cases died during this period. All patient characteristics are listed in Table 1.

Features	n= 24	%
Age		
Median	52.5	
≤53	13	54.2
>53	11	45.8
Histology		
Ductal	21	87.5
Metaplastic	1	4.2
Aprocrine	1	4.2
Medular	1	4.2
Histologic grade		
1	2	8.3
2	1	4.2
3	21	87.5
Ki67 Median		
≤60	13	54.2
>60	11	45.8
Tumoral PIK3CA (n=10)		
Negative	9	90
Positive	1	10
Stage		
I	3	12.5
II	12	50
III	4	16.7
IV	5	20.8
Plasma drawn time		
Early disease undergoing surgery	9	37.50%
Locally advanced before NAC	6	25%
Locally advanced after NAC	3	12.50%
Metastatic disease	6	25%
CTC AdnaTest-positive		
Yes	9	37.50%
No	15	62.50%
ctDNA-positive		
Yes	7	29.20%
No	17	70.80%
Death	8	33.30%

**Table 1. Clinicopathological Features for Triple-negative Breast Cancer Patients.**

We detected CTCs in 9 cases (37.5%) with presence of at least one Adnatest marker. Three of them had concurrent presence of ctDNA PIK3CA mutations and they were the only CTC-positive cases who died during follow-up. TWIST1, PI3K $\alpha$ , AKT2 or ALDH1 expression in CTCs were found in 4 cases (co-expression in one). Cases with expression of AKT2 or PI3K $\alpha$  in CTCs were negative for PIK3CA mutation in ctDNA. Four cases expressed GA733-2 (epithelial cell adhesion molecular or EpCAM) or MUC-1 at CTCs (3 with concurrent ctDNA PIK3CA mutation). No one of our cases expressed HER2 in CTCs (Table 2).

PIK3CA mutation in ctDNA was found in 7 cases (29.1%), including 1 case with proved mutation in the primary tumor (H1047R). Four ctDNA-positive cases died during the follow-up.

## Discussion

CTC presence was detected by at least one of the Adnatest markers in 37.5%. Which is close to the previously described finding of more than 30% detection in larger series of metastatic breast cancer [11,14]. Three of these cases had a concurrent positive ctDNA detection and all were the only cases in our series who died during the follow-up (Table 2).

Case	CTC	Age	HG	Ki67%	Subtype	Tumor mutation	ctDNA	Sample taking	Disease status	Follow-up
1	EMT-2 AKT-2	53	3	70	Metaplastic	(-)	(-)	ED	Absent	Alive
2	EMT-2 PI3K $\alpha$	40	3	70	Ductal	NE	(-)	ED	Absent	Alive
3	Stem Cell ALDH1, EMT-2 TWIST1	54	3	80	Ductal	NE	(-)	A-NAC	Absent	Alive
4	Stem Cell ALDH1	32	3	60	Ductal	(-)	(-)	A-NAC	Absent	Alive
5	Breast GA733-2	45	3	40	Ductal	(-)	E545K	ED	Active	Death
6	Breast GA733-2, Breast MUC1	67	2	25	Ductal	NE	E545K	MD	Active	Death
7	Breast MUC1	64	3	60	Ductal	NE	(-)	ED	Absent	Alive
8	Breast MUC1	78	3	70	Ductal	(-)	E542K, E545K	ED	Active	Death
9	Breast MUC1	54	2	50	Ductal	NE	(-)	ED	Absent	Alive

**Table 2. Clinicopathological Features of CTC-positive Cases.**

MD, Metastatic disease; A-NAC, Locally advanced after neoadjuvant chemotherapy; ED, Early disease without previous chemotherapy; HG, Histologic grade; NE, Not evaluated.

This poor prognosis of the co-detection of both biomarkers CTCs and ctDNA had been previously studied by Ye et al. They detected CTCs through enumeration CellSearch System and ccfDNA through RT-PCR in blood samples from 117 metastatic breast cancer, and found that higher levels of both markers had an extremely high death risk ( $p < 0.001$ ) [2].

Triple negative breast cancer is an undifferentiated tumor that over-express molecules related to stem cells and EMT processes [10,15]. Metaplastic histology is associated with higher expression of EMT markers and we found that our only case with metaplastic histology had detectable CTCs (with the EMT marker AKT2) (Table 2). Despite different series indicate that EMT and stem cell phenotype is related to advanced disease and poor outcome [6,16,17], we found that our 4 cases with these markers (ALDH1, PI3K $\alpha$ , Akt-2 and Twist1) were not metastatic and disease-free during follow-up. Similar to our results, the large series of Kasimir-Bauer et al. did not find any prognosis activity in early breast cancer [11]. Regarding activation of PI3K pathway in CTCs (overexpression of PI3K $\alpha$  or AKT2), they were found in 2 cases. However, they had no presence of PIK3CA mutation in ctDNA and no tumor mutation in the cases with available tissue. This suggest that activation of the pathway in CTC is not related to the presence of an activating tumor mutation but represent physiopathological processes happening when the malignant cells are circulating through the blood. A few studies have evaluated the activity of PI3K pathway in CTCs and have found a phosphorylated status in PI3K $\alpha$ /Akt2 molecules as well as higher expression levels in advanced

stages [16, 18].

On the other side, the finding of 7 cases (29%) with PIK3CA mutation in ctDNA is in the range found in breast cancer with advanced stages and high proliferative rates [9,10,19]. SOLAR1 trial found that presence of ctDNA PIK3CA mutations can predict response to the drug targeting the PI3K pathway, alpelisib [8].

Regarding the evaluated CTC breast cancer-related genes (MUC-1, HER- 2 and GA733-2), the absence of HER2 expression in CTCs indicates that this pathway doesn't participate in the metastatic process of TNBC cells. MUC1 (Carcinoma Antigen 15-3) was found in 4 cases and 2 of them coincide with presence of ctDNA. However, MUC1 is overexpressed not only in CTCs but in activated circulating leukocytes [11].

In conclusion, infrequent concurrent detection of CTC and ctDNA presence suggest that both represent independent processes in TNBC patients, however, their concurrence appears to carry poor prognosis.

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### Conflicts of interest

The authors declare no conflicts of interest.

### Ethical statement

The study was approved by the ethics committee of the Institutional of Instituto Nacional de Enfermedades Neoplásicas, approval no. 16-30/INEN. Written informed consent was obtained from each patient.

### Authors' Contribution

HLG, and CAC. contributed to the study conception and design. JMC, JD, MV, JA, HG, LAB and NS. performed material preparation and data collection. CAC, HLG, and MC contributed to statistical analysis and interpretation. Resources were by CAC. CAC and HLG wrote the first draft of the manuscript. All authors have provided critical input to the design of the study and the manuscript preparation. All authors read and approved the final manuscript.

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