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The curious phenomenon of dual-positive circulating cells: Longtime overlooked tumor cells

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Abstract

The presence in the blood of patients with solid tumors of circulating cells expressing both epithelial and leukocyte markers (dual-positive cells, DPcells), has often been reported, though it has never been investigated in detail. A recent study suggested that DPcells are hybrid cells derived from the fusion of tumor cells with macrophages. Such fusion hybrids acquire macrophage-associated features endowing them with accelerated growth, increased motility, enhanced invasion activity and thus, a higher efficiency in metastasis formation. However, no direct evidence proving the tumor origin of circulating DPcells was provided in patients.

Here we contribute a review of literature data on DPcells and on the hybrid theory with the aim of putting the current evidence both in a biological and clinical perspective and to generate new hypotheses on the mechanisms underlying tumor progression. To add further biological and clinical context to our literature review, we also report some preliminary data from our laboratory on the identification of DPcells in several solid tumors and confirmation of their malignant genotype, thus classifying them as DP-CTCs.

ABBREVIATIONS
CHC, circulating hybrid cells
DPcells, dual-positive cells
CTC, circulating tumor cells
CK, cytokeratins
CAML, cancer associated macrophage-like cells
EMT, epithelial-mesenchymal transition
WBC, white blood cells
CNA, copy number alteration

DP-CTC, dual-positive circulating tumor cells

Key words: circulating tumor cells (CTC); the hybrid theory; dual positive circulating cells; fusion-cells, metastasis

INTRODUCTION:

A recent paper (*1*) addressed the biological role of heterotypic cell fusion for enhancing cellular diversity. In tumors, cell heterogeneity represents a well-known culprit for the onset of progression and treatment-resistance. However, the description of tumor heterogeneity as a genetic-only driven phenomenon leading to emergence of distinct clones that foster tumor evolution is somewhat limited, hereby forgetting the fundamental contribution of heterotypic cell fusion in enhancing cellular diversity during development (*2-5*).

In their comprehensive paper, spanning from established preclinical models such as cell cultures and tumor-bearing mice moving to patient samples, Gast and colleagues provided compelling evidence for the spontaneous fusion of tumor cells with macrophages, thereby producing hybrid cells that are detectable both at the tumor site and in the blood stream (circulating hybrid cells, CHCs). Their preclinical data suggest that hybrid cells acquire functional behaviors from both cell types thus increasing tumor phenotypic diversity that is connected to a higher ability in overcoming selection pressures and driving tumor progression. In keeping with their strong pre-clinical evidences on the role of CHC, the authors report that, in a small cohort of patients with pancreatic cancer, the presence of CHCs (identified as nucleated cells positively staining for both cytokeratins and CD45) in the blood was inversely correlated with overall survival.

The presence of cells simultaneously expressing epithelial and leukocytes markers, circulating in blood of tumor patients, was already reported by several groups since 2011 (*6-11*). Nonetheless, the significance of these cells (the so-called dual-positive cells), together with their cell of origin has never been investigated. The study by Gast et al. was the first one proposing and experimentally supporting a mechanism for the origin of dual-positive cells (DPcells), although with some caution as the authors comment that "unlike in murine models, the etiology of human CHCs, while consistent with cell fusion, cannot be conclusively determined". Indeed, the heterotypic fusion mechanism for CHC is in contrast with the well-known detection of DPcells in healthy donors' blood reported in literature (*7,8,11,12*).

Therefore, to solve this conundrum, there is still the need for evidences directly proving the tumor origin of CHC/DPcells.

Here, as a contribution to the understanding and to the reconsideration of the role of heterotypic cell fusion in promoting tumor progression we are:

- reviewing literature data on circulating DPcells
- reviewing literature on the hybrid theory
- reporting a short insight including original data obtained in our laboratory that demonstrate the presence of cells with malignant genotype and hybrid phenotype in the blood of patients with solid tumors.

DUAL-POSITIVE CELLS IN LITERATURE

Circulating tumor cells (CTCs) present in the blood stream of patients with early- or late-stage tumors, are directly implicated in distant dissemination thus providing an unprecedented opportunity for understanding the metastatic process and an easy accessible source for biomarker

assessments. Already fifteen years ago, Allard and Terstappen provided the first report on accuracy and reproducibility of the CellSearch™ system for CTC enumeration (*13*), setting this way the premises for the following demonstration of the clinical validity of CTC counts in numerous solid tumors (*14*). One, among the many key factors responsible for the success of the CellSearch™, is the strict definition of CTCs as nucleated cells enriched from blood using EpCAM coated ferrofluids and expressing cytokeratins (CKs), but lacking CD45 expression by immunofluorescence.

However, despite the unquestionable value of such a rigorous definition, growing evidences are being collected on a possible role of other types of cells not identified or clearly reported by the use of CellSearch™ CTC-enrichment strategy and/or identification criteria. Yu and colleagues (*15*), for instance, reported an association between CTCs with a mesenchymal phenotype (lacking therefore the expression of epithelial markers) and treatment resistance, while in a study on metastatic breast cancer patients, cancer associated macrophage-like cells (CAMLs, cells expressing cytokeratins but also CD45, characterized by large-atypical/multiple nuclei and by an oblong or amorphous shape), did provide additional prognostic information over CTC counts alone both for progression and for survival (*16*).

There is however, one more subpopulation of circulating cells which is excluded by CellSearch™ identification criteria: the so-called dual-positive (DP) cells characterized by a double staining for epithelial markers and CD45 and by a leukocyte-like shape, clearly different from CAMLs.

Over the past years, different studies reported the presence of DPcells in the blood of patients with breast, prostate and lung cancer (*6-11*) with variable detection frequencies ranging from less than 5% (*10*), up to 100% (*11*) in the blood samples analyzed. DPcells were observed in studies using different approaches for CTC-enrichment spanning from the classic CellSearch-immunomagnetic system (*7*), to the innovative herringbone chip (*8*) and to immunomagnetic negative depletion (*11*), a finding that enforces their actual presence in the blood (Table 1).

Although frequently described, these cells have never been directly investigated. One of the main reasons for this is that DPcells were observed also in healthy donors' blood (*12*), albeit in lower numbers (*11*), suggesting that these cells could be artifacts due, for example, to a false-positive staining for epithelial markers (*7*). However, in analyses specifically run to exclude dead cells and cell doublets, DPcells were still present at comparable frequencies, thus excluding technical artifacts (*11*). As a possible explanation for the double-positive staining, Nel and colleagues proposed that CD45 expression could be acquired by tumor cells during dormant stay in the bone marrow or through trogocytosis (*9*) and underlined the importance of studying this subpopulation of circulating cells.

Also Haber and colleagues suggested the need to improve the understanding of DPcells (*8*) using their CTC-chip method. Nonetheless, their study was limited to description of DPcells histological characteristic to report the cell morphology after hematoxylin and eosin staining. Since the majority of those cells presented a leukocyte-like morphology, they concluded that DPcells were "appropriately excluded from CTC enumeration" even though some of them showed an ambiguous staining pattern, more consistent with CTCs.

Recently, a new hypothesis regarding circulating DP-cells was proposed by Gast and colleagues (*1*). Their study was focused on understanding the role of cell fusion in enhancing tumor heterogeneity and increasing migratory and invasive properties of tumor cells. In particular, they analyzed hybrid cells derived by the fusion of a tumor cell with a macrophage (fusion hybrids). By performing *in vitro* and *in vivo* experiments the authors showed that fusion hybrids could acquire macrophage-associated features resulting in an accelerated growth, an increased motility and invasion activity and a higher efficiency in metastasis formation. Then, by *FISH* analysis, they demonstrated the presence of fusion hybrids in human tumors exploiting tumor biopsies derived from female cancer patients who previously received a sex-mismatched bone marrow transplant and subsequently

developed a secondary tumor. In fact, they could detect cytokeratin-positive cells containing Y chromosome in tissue biopsies from patients with pancreatic ductal adenocarcinoma, renal cell carcinoma, head and neck squamous cell carcinoma and lung adenocarcinoma. Finally, in a cohort of 18 patients with pancreatic ductal adenocarcinoma, they reported the presence of circulating hybrid cells (CHCs) defined as cells expressing both CD45 and CK (resembling, therefore, the characteristics of DPcells). CHCs were more frequent than canonical CTCs and, notwithstanding the limited number of considered patients, they were associated with a statistically significant increased risk of death (no correlation between canonical CTCs and overall survival was instead observed).

Although Gast and colleagues proposed an intriguing hypothesis supported by strong preclinical evidences, they did not demonstrate the tumor nature of CHCs detected in patients, nor did they comment on the presence of DPcells in healthy individuals. This leaves an open question about the tumor origin of such cells, which requires further investigations to be adequately addressed.

THE HYBRID THEORY

Cell fusion occurs during normal development and differentiation of specific tissues (*17-19*) and plays a role in regeneration after tissue damage (*20-22*). Already in 1911 it was hypothesized, for the first time, that cell fusion between tumor cells and cells of hematopoietic lineage also plays a role in tumor metastatic progression by creating hybrid cells capable of dissemination and new tumor growth (*2*). Later, in 1974, Goldenberg and colleagues provided karyological evidence of *in vivo* fusion of transplanted human cancer cells with hamster cells, resulting in the formation of extremely lethal neoplasms (*23*).

Since then, the hybrid theory has been put aside in favor of other hypotheses on mechanisms driving cancer progression extensively studied during last decades, including i) the accumulation of genetic and epigenetic changes, ii) the epithelial to mesenchymal transition (EMT) and iii) the existence, within the heterogeneous tumor cell population, of cancer stem cells (*24*). However, more recently different groups have retabled the hybrid theory providing *in vitro* and *in vivo* evidences that cell fusion between tumor and normal cells can give rise to hybrids characterized by a more aggressive behavior than unfused tumor cells (*1, 4, 25-29*). Indeed, in these studies, fusion hybrids proved to have accelerated proliferation, stronger tumorigenicity, increased motility and enhanced metastatic potential compared to parental cancer cells.

For instance, in ovarian and lung cancers, fused cancer cells exhibited dramatic up-regulation of the promigratory marker CXCR4 granted by the myeloid compartment cells and resulting in a higher migratory potential (*25*). Similarly, in a melanoma model, fusion hybrids were characterized by macrophage features including an up-regulated activity of GnT-V (*26*) a glycosyltransferase able to accelerate metastatic dissemination by altering cell surface receptors (*30*). Two groups independently working on non-small cell lung cancer (*28*) and hepatocellular carcinoma (*29*) reported the acquisition of EMT and stem cell markers by cancer cell lines through the fusion with bone marrow-derived cells. In the first study, hybrids were characterized by a fibroblast-like appearance and by increased pneumosphere-forming capacity. Moreover, consistently for 3 different lung cancer cell lines, hybrids injected in mice were more efficient in forming tumors than parental cells: all hybrid cell lines, when injected in the same mouse for the same period of time, formed significantly larger tumors (*28*). Also in the second study (*29*), fusion hybrids exhibited a mesenchymal morphology and, after orthotopic injection in the liver of mice, formed poorly differentiated invasive tumors, compared to well-differentiated and noninvasive tumors of the parental cell line. In fact, tumors generated by fused cells produced a higher number of lung metastasis, compared to un-fused ones, indicating increased metastatic potential. The increased metastatic potential of fusion hybrids was further confirmed by independent studies in mouse models of colon adenocarcinoma (*1*) and melanoma (*27*).

Whereas numerous evidences testify the increased tumorigenic potential of fusion-derived cells in animal models, it is still unknown whether fusion is a common phenomenon in human cancers. Nonetheless, the presence of fusion hybrids between tumor and normal cells have been observed in primary tumor biopsies from patients with different types of cancer, including renal cell (*31*), ovarian (*25*), pancreatic (*1*), colorectal (*32*) and breast (*33*) cancers. Furthermore, in 2 cohorts of 101 patients with colorectal and 127 women with breast cancer, Shabo and colleagues reported an association between the presence of tumor-macrophage hybrids in primary tumors and both early recurrence and poor survival (*32, 33*). The observation of fusion hybrids also at metastatic sites (*34, 35*), further supports a possible role of cell fusion in cancer progression and metastasis formation. However, the direct contribution of hybrids at primary tumor site to the process of metastasis still needs to be clarified. Beside this crucial question, another open question regarding fusion hybrids in humans is how and why they are originated. In fact, though it has been proposed that inflammation (*36, 37*) and apoptosis (*38, 39*) could be involved in promoting fusion, the mechanisms directly involved in this phenomenon have not been demonstrated yet.

It is finally important to mention that the combination of 2 genomes during cell fusion produces unpredictable, unique and heterogeneous hybrids (*37*). By using established cell lines, Rachkovsky et al. tested, *in vivo*, the metastatic potential of 35 distinct hybrids lines generated by the fusion of macrophages with Cloudman S91 melanoma cell line and found a striking heterogeneity among hybrids, with some of them producing no metastasis at all in mice (*27*).

Cell fusion itself is indeed a trigger of genomic instability and such an effect might be even magnified in human tumors which are intrinsically heterogeneous, due to the presence of numerous subclones. Theoretically, hybrids generated by distinct cells of the same tumor can exhibit a wide range of different behaviors from being extremely aggressive and more capable of adapting to selective pressures, up to being non-metastatic or even non-viable cells (*40*).

For this reason, a simple phenotypic evaluation of hybrids by marker expression is not sufficiently informative to define their biological and clinical significance. Therefore, an analysis of hybrids' genomic traits is mandatory to both clearly define the tumor nature of these cells and to help clarifying the biological processes underlying this poorly understood phenomenon.

Overall, lessons coming from the hybrid theory together with detection of DPcells in the blood of cancer patients seem to support the involvement of fusion in cancer progression and metastasis formation. But the hypothesis of a direct link between cell fusion, DPcells and cancer formation and dissemination is blurred by the fact that i) DPcells are present also in healthy donors' blood and ii) there is no direct evidence proving the tumor origin of DPcells in cancer patients (Figure 1). In this context, the demonstration of the tumor nature of cancer patients' DPcells, would be instrumental to definitely embrace a role of fusion in cancer.

DETECTION OF DPcells IN PATIENTS WITH SOLID TUMORS: SOME PRELIMINARY DATA FROM OUR LABORATORY

We analyzed 53 blood samples from 36 patients with different malignancies (kidney cancer, breast cancer, gynecological tumors, cholangiocarcinoma and salivary duct carcinoma). Blood samples were processed for CTC detection by using a previously described protocol (*41*) that includes a marker-independent enrichment approach, followed by identification and recovery of single cells labeled with a cocktail of antibodies against epithelial (CK, EpCAM, EGFR) and leukocyte (CD45, CD14, CD16) markers. By using this approach we were able to detect 3 types of cells: i) white blood cells (WBC) expressing leukocyte but not epithelial markers, ii) CTCs expressing only epithelial markers and iii) DPcells positive for both classes of markers (Figure 2). DPcells were

frequently noticed and did not display any specific morphological difference with respect to WBC and canonical epithelial CTCs.

Noteworthy, of 53 blood samples analyzed, only 12 (23%) contained at least 1 CTC (range: 1-4), whereas DPcells were detected in 40 samples (75%, DPcells range: 1-23). In particular, among the cancer patients, DPcells were observed at different frequencies depending upon the specific type of tumor, with rates ranging from 38% (gynecological tumors) to 86% (kidney tumors) and were unrelated to the presence of CTCs (Figure 3 A, B). Nonetheless, with the exception of gynecological tumors, samples containing DPcells were more frequent compared to those lacking this type of cells. In all analyzed tumor types, DPcells were more prevalent than CTCs.

After visualization and counting, DPcells were collected as single cells. This allowed us to deeper investigate on DPcells and to perform, for the first time to the best of our knowledge, a single-cell low-pass whole genome sequencing of amplified DNA from DPcells for copy number alteration (CNA) analysis. Single-cell CNA profiling allows unambiguous discrimination between a normal cell (with a diploid, flat profile) and a malignant one (characterized by an aneuploid genome with an altered CNA profile), thus offering direct evidence for the origin of each single cell (Figure 4A).

CNA profiling was performed on 59 single DPcells recovered from 18 blood samples from 16 patients (4 with kidney cancer, 10 with cholangiocarcinoma and 2 with gynecological tumors). Thirty DPcells displayed a flat CNA profile, while 29/59 cells had aberrant profiles indicating their tumor origin and were defined DP-CTCs. Examples of representative CNA profiles from a normal DPcell and 2 aberrant DP-CTCs are reported in Figure 4.

DP-CTCs were detected in all tumor types, in particular in 4/4 kidney cancer patients, in 7/10 cholangiocarcinoma patients and in 1/2 patient with gynecological tumor. Among the 18 blood samples, CNA in DPcells were observed in 13 cases (thus defined as DP-CTC^{+ve}), whereas in the remaining 5 blood samples DPcells did not present CNA. The median numbers of DPcells identified in DP-CTC^{pos} vs. DP-CTC^{neg} samples were 4 and 1, respectively. Indeed a statistically significant direct correlation was observed between the numbers of DPcells and of DP-CTCs (r_s= 0.9, P<0.001, Spearman's rank correlation) suggesting that samples with higher numbers of DPcells are more likely to contain DP-CTCs. No statistically significant correlations were instead observed between the number of CTCs and DP-CTCs (r_s=-0.28, p=0.91) or DPcells (r_s=0.13, p=0.61).

Conclusion:

Overall, by combining our results with the literature data on the hybrid theory that we have reviewed here, some inspiring suggestions on future research avenues can be drawn.

First of all, it is becoming clearer and clearer that although the classic definition of CTC is a milestone in cancer research, it is no longer possible to neglect the increasing number of reports on non-conventional CTCs, mainly including cells that have undergone a complete or partial EMT, but also embracing cells lacking epithelial and leukocyte markers (43). Now, DP-CTCs too, should be included among the promising information derived from liquid biopsies, as a new subpopulation of CTCs whose study may raise high expectations due their peculiar origin.

This novel subpopulation of CTCs offers, for the first time, an evidence connecting the hybrid theory and the process of metastatization, a connection that was supported by many *in vitro* and *in vivo* studies (*1,4,25-29*) but has never been directly documented in cancer patients. Indeed whereas most preclinical studies have shown the high tumorigenic potential of hybrid cells, the presence of a mixture of DPcells with normal or aberrant genotypes in cancer patients' blood samples urges getting a confirmation of the malignant nature of these cells in order to clearly identify only DP-CTCs and to define their clinical relevance. In fact, heterotypic fusion can also occur between 2

normal cells, as demonstrated by the presence of DPcells in healthy individuals (*7,8,11,12*). Moreover, besides ascertain the malignant nature and the metastatic potential of DP-CTCs, their molecular characterization will also offer the possibility to gain new hints on the mechanisms involved in fusion-mediated metastatic progression, a phenomenon that still needs to be understood.

Once assessed that DPcells with CNA can be defined as DP-CTC, it remains to clarify the origin of this cells. In consideration of their detection using different approaches (1,6-11) (thus rebutting the hypothesis of staining artifacts), we favor an explanation relying on the hybrid theory which is further supported by the results of Gast et al. showing that circulating hybrid cells can be generated by the fusion of a tumor cell with a macrophage (*1*). But the mechanisms leading to hybrids formation still needs to be investigated.

Moreover, if fusion between a normal cell and a tumor cell is among the possible triggers of tumorigenesis and cancer dissemination, the mechanisms underlying this process could provide possible new treatment targets. Their analysis will therefore broaden our armamentarium of therapeutic tools by adding a completely new target to mutations, cell signaling molecules, and by opening our mind to reconsider the microenvironment under a different perspective.

Finally, the fact that i) DPcells cells are more prevalent than CTCs in all analyzed tumor types (a finding reported also by other groups: *1,7,8,11*) and that ii) they were detected in 75% of samples (compared to 23% for CTCs) makes them particularly interesting for liquid biopsies studies in those cancers where the detection of conventional CTCs is not efficient.

When trying to introduce the liquid biopsy approach as an achievable patient-friendly alternative to tissue biopsy, the opportunity to relay on several CTC subpopulations considerably increases both the number of patients with detectable CTCs and the number of single CTCs detected in each blood sample that may be used for molecular analysis, thus increasing the possibility of obtaining clinically relevant information in a broader range of cancer patients.

However, when attempting to introduce DPcell assessment into the pathway to translate biologically relevant findings to the clinics, a note of caution needs to be raised on the type of enrichment that is chosen, since DPcells are reported to be more common among EpCAM -/CK+ samples than EpCAM+/CK + samples (*11*). If confirmed, this would limit the use of CellSearch™ even in the case a phenotypic marker for DP-CTCs were identified.

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Figure legends

Fig. 1. Overview of literature data on the hybrid theory and DPcells detection.
Cartoon summarizing why the malignant genotype of each isolated DPcell needs to be confirmed in order to definitely understand the link between fusion hybrids, presence of DPcells in the blood of cancer patients and metastatic dissemination.

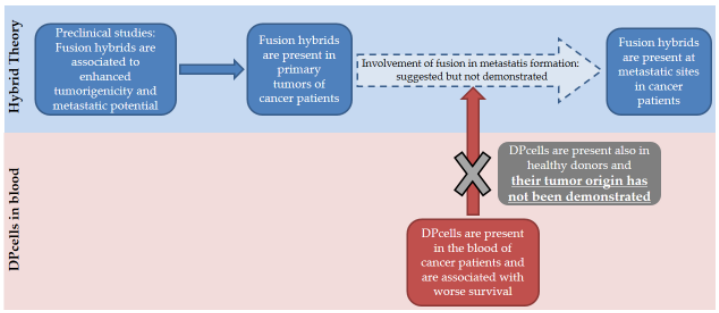


Fig. 2. Image galleries of cells isolated from patients' blood using the DEPArray platform. Immunofluorescence staining for epithelial markers (pan-CK, EpCAM, EGFR), leukocyte markers (CD45, CD14, CD16) and DAPI. (A) One CTC matching the conventional identification criteria (epithelial markers+, leukocyte markers- and DAPI+). (B) Three white blood cells expressing leukocyte markers and negative for epithelial markers. (C) Representative images of double-positive cells (epithelial markers+ and leukocyte markers+). Magnification 10X; each side of the grid in the 'brightfield column' represents 20 μm.

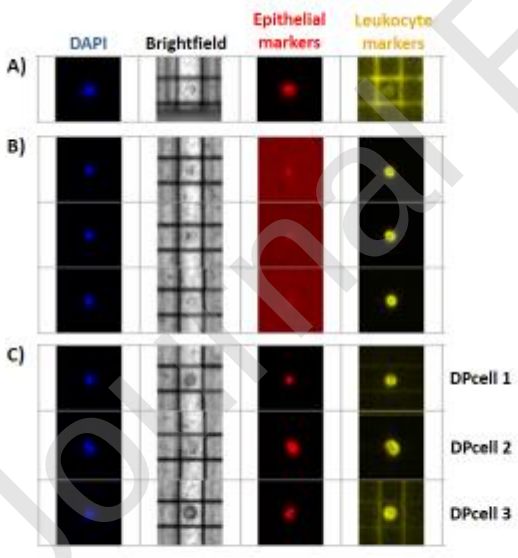
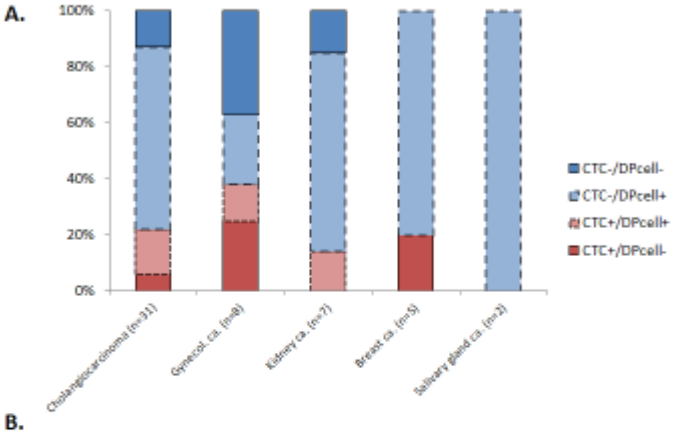


Fig. 3. Detection of CTC and DPcells in clinical samples. (A) Histogram showing detection frequencies of CTCs and DPcells in 53 samples collected from patients with different solid tumors. (B) Table reporting the absolute number of samples positive/negative for CTC and DPcells for each tumor type.

A.



B.

	Cholangio-carcinoma	Gynecological tumors	Kidney cancer	Breast cancer	Salivary gland cancer	Total
Number of samples	31	8	7	5	2	53
$DPcells^{pos}$ CTC^{neg}	4	3	1	0	0	8
$DPcells^{pos}$ CTC^{neg}	20	2	5	4	2	33
$DPcells^{neg}$ CTC^{pos}	5	1	1	0	0	7
$DPcells^{neg}$ CTC^{pos}	2	2	0	1	0	5

Fig. 4. Analysis of copy number alterations (CNA) in dual-positive cells (DPcells). (A) CNA profiles obtained from 3 DPcells individually isolated from one patient blood sample. The first profile (i) is diploid and has no CNA, while the other 2 profiles (ii, iii) show numerous CNA indicating the tumor origin of these cells. The images showing the morphology and the staining of the analyzed cells are reported in fig.1C (DPcell 1, 2 and 3 corresponding to CNA profile A, B and C, respectively). (B) Clustering analysis of CNA profile of 59 single-DPcells showing one cluster of 30 cells with a normal diploid profile (upper part) and a second cluster of 29 cells characterized by altered profiles, indicating their tumor nature (lower part).

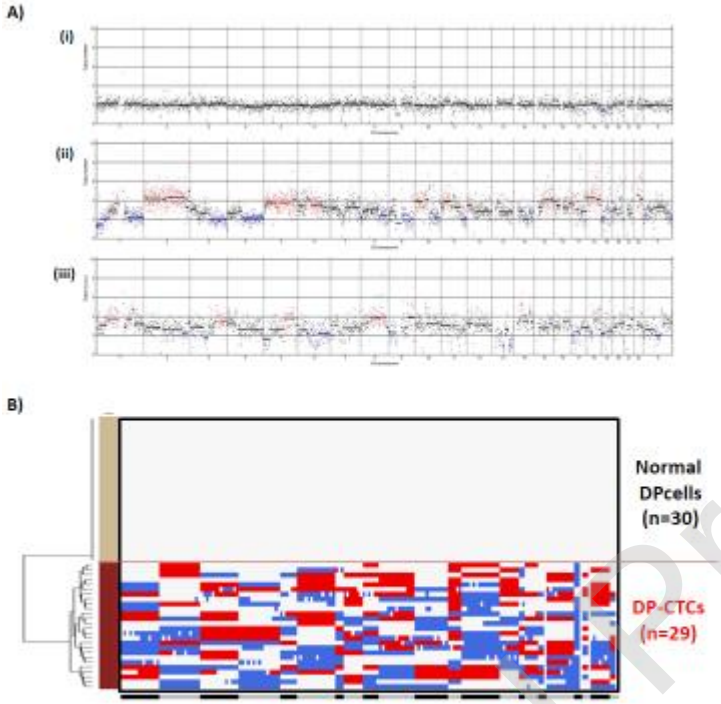


Table 1: Overview of literature reporting circulating DPcells in cancer patients

Pathology	**Enrichment**	**Identification criteria**	**DPcell detection**	**Reference**
Prostate cancer (15 samples)	Herringbone-chip	PSA+/CD45+	DPcells more prevalent than CTCs	Stott *et al.*
Metastatic breast and lung cancers (56 samples)	Spiral microfluidic chip	CK+/CD45+	DPcells detected in <5% samples	Khoo *et al.*
Non-small cell lung cancer	Density gradient centrifugation	EPCAM+/ CK+/CD45+	N.A.	Nel *et al.*

Metastatic breast cancer (32 samples)	Negative depletion	CK+/CD45+	DPcells detected in all samples	Lustberg *et al.*
Metastatic breast cancer (29 samples)	CellSearch	CK+/CD45+	DPcells detected in 28/29 samples	Schneck *et al.*
Pancreatic cancer (20 samples)	Density gradient centrifugation	CK+/CD45+	DPcells detected in all samples	Gast *et al*.