Notes for developing a molecular test for the full characterization of circulating tumor cells

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Abstract: The proved association between the circulating tumor cell (CTC) levels and the patients’ survival parameters has been growing interest to investigate the molecular profile of these neoplastic cells among which hide out precursors capable of initiating a new distant metastatic lesion. The full characterization of the tumor cells in peripheral blood of cancer patients is expected to be of help for understanding and (prospectively) for counteracting the metastatic process. The major hitch that is hampering the successful gaining of this result is the lack of a consensus onto standard operating procedures (SOPs) for performing what we generally define as the “liquid biopsy”. Here we review the more recent acquisitions in the analysis of CTCs and tumor related nucleic acids, looking to the main open questions that are hampering their definitive employ in the routine clinical practice.

Keywords: Circulating tumor cells (CTCs); single-cell analysis; liquid biopsy

Submitted Mar 02, 2015. Accepted for publication Aug 17, 2015.
doi: 10.3978/j.issn.1000-9604.2015.09.02

View this article at: http://dx.doi.org/10.3978/j.issn.1000-9604.2015.09.02

Introduction

In the past decades, the ever-greater access to screening, the greatest sensitivity and specificity of imaging and the growing number of new molecules have been changing the fate of cancer patients. We can hope now that by taking advantages of treatments tailored to the tumor of individual patient, we will definitively fight cancer. However, just because of the growing number of successful treatments, the number of long-term surviving patients has been increasing, and consequently it has been raising the need of new tools for their follow-up. To address this issue, we should identify a tumor-specific marker that (I) is expressed constantly throughout the disease course; (II) is associated with disease outcome; (III) can reflect “just in time” tumor evolution during its natural history or under any treatments’ pressure; and (IV) is minimally invasive.

The circulating tumor cells (CTCs) meet all these criteria. Indeed, CTCs have been revealed in almost all disease stages (1-3) and their levels have been reported both prognostic (2) and predictive of treatment efficacy (4). Consistently with clinical validity recently confirmed in metastatic breast cancer (5), the quantitative evaluation of CTCs promises to be an appealing tool for revaluing disease conditions throughout the continuum of the care.

Furthermore, just because of the proved association between the CTC levels and the patients’ survival parameters, there has been growing interest to investigate the molecular profile of these neoplastic cells among which hide out precursors capable of initiating a new distant metastatic lesion (6,7). The full characterization of the tumor cells in peripheral blood of cancer patients is expected to be of help for understanding and (prospectively) for counteracting the metastatic process.

To date, the major hitch that is hampering the successful gaining of this result is the lack of a consensus onto standard operating procedures (SOPs) for performing what we generally define as the “liquid biopsy”. This is a wide definition, initially used for indicating the tumor cells in peripheral blood of cancer patients (the also named “leukemic phase” of solid tumors) (8) that is becoming of common use for defining the nucleic acid detectable in plasma samples of cancer patients. Indeed, robust, reproducible and shared procedures are firstly mandatory
for then clinically validating the better methodologies to isolate and/or characterize the tumor burden in peripheral blood and for definitively proving their use (individual or complementary) as companion diagnostic.

Here we review the more recent acquisitions in the analysis of CTCs and tumor related nucleic acids, looking to the main open questions that are hampering their definitive employ in the routine clinical practice.

**Procedures starting from single cell analysis**

In order to study the molecular heterogeneity of the circulating compartment of solid tumors, many different methods have been used. In some cases, the authors preferred CellSearch platform for enriching and counting CTCs and then used all the cartridge content for molecular study (9). Conversely, after CTC enrichment other authors chose to isolate single CTCs using different strategies, including laser micro-dissection, Isolation by Size of Epithelial Tumor cell (ISET), DEPArray or Flow Cytometry sorting. As expected, in these studies a whole-genome amplification (WGA) procedure is required to provide the DNA quantity required for enabling the genomic analysis of a single CTC. To our knowledge, no study combines, with a unique approach, a mapping of copy number variation (CNV) and next-generation sequencing (NGS) techniques, for detecting a nucleotide mutation.

Furthermore, a main challenge is how to control if we are able to isolate successfully single CTCs diluted among millions of normal cells. To address this issue, several authors often use primary tumor tissues or biopsies (10,11) collected at diagnosis for comparing data obtained in CTCs that in turn underestimates the changes of CTC genotype induced by the cancer evolution or under therapy pressure. Here we have been analyzed the pros and cons of these methods, briefly discussing the achieved results by different procedures.

**Sequential use of CellSearch and DEPArray**

The CellSearch platform is the only one method that has completed the clinical validation, thus obtaining the FDA approval to be used in clinical for monitoring metastatic cancer of breast, prostate and colon. The automated system enriches EpCAM-positive cells that are then stained with DAPI (to identify the nucleus) and anti-pan cytokeratin (CK) 8-18 and 19 (to identify epithelial cells), while anti-CD45 serves as specificity control.

At the end of CellSearch procedure, some authors use then DEPArray system to obtain single-cell samples. DEPArray is an automated system for creating a dielectrophoretic (DEP) cage around the cells. After imaging, the operator gently transfer cells of interest, one by one, to specific locations on the cartridge (as a parking area) and finally recovers them in a PCR tube, for further molecular analyses.

The combined procedure, CellSearch plus DEPArray, is time consuming (from 4 to 6 hours depending on the CTC number/sample) and requires highly trained operators. Indeed, different transfer efficiency has been reported depending on the study. The range varies from 85% (median, 77%; standard deviation, ±49%) obtained by Klein et al. (12) to lower level, as reported by Peeters and colleagues (13), which observed a recovery rate close to 62% (CV 19%) of cells counted by the CellSearch system that were then available for cell sorting after loading into the DEPArray cartridge.

By using this procedure, some authors documented a mutational status of CTCs for TP53 in breast cancer. In particular, in two patient affected by TNBC with high number of CTCs Cristofanilli et al. (10) showed the presence of different cancer sub-clones in the peripheral blood. Furthermore, Fabbri et al. (11) found a mutational discordance between KRAS primary tumor and CTCs in CRC patients, revealing KRAS wild type (WT) CTCs in patients harboring mutated primary tumor, but also the contrary.

Conversely, in SCLC CTC pool enriched by CellSearch Hodgkinson et al. (14) compared genomic profiles of CTCs isolated from the parallel enumeration of blood samples, revealing that the CTCs from a patient with extensive-stage SCLC are largely homogeneous.

**Combined use of CellSearch plus cytometry**

In the presence of high numbers of CTCs, the fluorescence-activated cell sorting (FACS) technology allows an automated collection of single CTCs (15,16). However, different studies reported a loss rate of 40% to 50% in comparison with the cell number as identified by the CellSearch system (17).

Indeed, due to the fact that CTCs are rare events in the great majority of the patients the feasibility of flow cytometry for enumerating CTCs is matter of debate (18).

By using flow cytometry technology, many researchers examined the expression of EGFR and its phosphorylated
counterpart, aldehyde dehydrogenase 1 (ALDH1), CD44, CD47, MET, and heparanase (HPSE) (15,19-21). Additional advantages offered by flow cytometry methods include: (I) the possibility to examine the level of expression using quantitative flow cytometry; and (II) the feasibility of multi-marker analysis on a single sample.

The main disadvantages include: (I) limitations concerning assay sensitivity even when flow cytometry is combined with pre-enrichment steps (22,23); and (II) the inability to confirm visually that results that results are from CTCs and not due to leukocyte contamination into the same well. Starting from CTCs enriched by the CellSearch system and sorted by FACS, Swennenhuis et al. (24) recover and amplify DNA with an overall efficiency of 20%. In particular, the authors reported they could use this DNA for calling of variant, but not for quantitative measurements such as copy number detection.

By using immune-magnetic enrichment, FACS sorting and aCGH analysis of CTCs in metastatic prostate cancer (mPCa) patients, Magbanua et al. (25) showed copy number gains in the AR region of chromosome X in CTCs, including high-level gains in 78% of the samples which were successfully profiled. AR amplification is not a common event in primary prostate cancer, but it has been implicated in hormone resistance observed in CRPC (26,27). In the two patients with matching archival tumor and subsequent CTC specimens, the authors observed high-level gain in the AR region in the CRPC CTCs but not in the archival tumors. The gain in AR copy number between tumor tissue obtained at initial diagnosis and CTCs subsequently obtained during the CRPC phase may reflect selective pressures towards amplification of the AR in response to androgen deprivation therapy. It may reveal evidence for AR amplification, which has been associated with disease progression in CRPC.

Molecular studies using specific FISH probes on CTCs from advanced prostate cancer patients have reported gains in AR and MYC, losses in PTEN, and evidence for ERG gene rearrangement (28,29).

Isolating single-cell CTCs by ISET

The ISET can identify directly CTCs or circulating tumor micro-embolis (CTMs). Without using tumor-associated markers, the method exploits selective filtration of CTCs/CTMs because of their larger size compared to leukocytes; the filtration module is equipped with a polycarbonate track-etch-type membrane with cylindrical calibrated pores of 8-μm-diameter (30).

The main strength of ISET is its ability to use laser micro-dissection onto the membrane that allows the recovery of nucleic acids from single CTCs for downstream molecular analysis and characterization.

The method is compatible with immune-labelling, RNA/DNA analysis and fluorescence in situ hybridization (FISH) to characterize the malignant profile and the invasive potential of CTCs/CTMs.

Pinzani et al. also extracted DNA from CK broad-spectrum immune-stained cells recovered by laser micro-dissection from breast cancer patients; they then measured HER-2 amplification in these cells by real-time PCR (31).

Farace et al. demonstrated that they could reliably detect ALK rearrangement in CTCs of all patients with ALK-positive NSCLC. In this group of patient ALK-rearranged CTCs were positive for mesenchymal markers, vimentin and N-cadherin, with a moderate expression level that was significant but generally lower than that of hematopoietic cells. CK markers were not detected in ALK-rearranged CTCs. Expressions of CKs or of both CKs and vimentin were detected in CTCs bearing a native ALK status in the one ALK-negative and six ALK-positive patients (32,33).

Procedures starting from free DNA

The molecular characterization of “liquid biopsy” promises to facilitate the access of cancer patients to targeted therapies. To this purpose, as an alternative to investigate CTC’s DNA many authors have proposed to study Circulating Tumor DNA (ctDNA).

Indeed, small DNA fragments have been previously reported in the blood stream of healthy donors at low concentration. The range is from 1.8 up to 44 ng/mL in plasma, although this level can greatly increase following exhaustive exercise, in pregnant women, in elderly patients suffering from acute or chronic disease and in individuals with premalignant lesions, inflammation or trauma (34-36). Despite this well-known limits of specificity, in cancer patients’ ctDNA often show the same genetic alteration present in tumor biopsy, hence this assay remains a promising minimally invasive test for the follow-up of malignancies.

To date, several different methods have been reported for detecting ctDNA, but none has been reached until now the FDA approval. The main trouble is the pre-analytical phase of the procedure to whom some authors attribute the lack of comparable results, while a lack
of standardisation and appropriate controls is stressed by others researchers (37-40). The SOP for sampling of ctDNA is being developed by the CEN/ISO and is expected to get approved for Europe in the next few months.

However, the concentrations of ctDNA in plasma show good correlation with the disease status in gastric cancer. Furthermore, some authors observed a decrease in ctDNA levels after surgical resection. Tie et al. (41) analysed 136 metastatic tumors originating from 14 different tissue types; by using patient-specific rearrangements the authors demonstrated that recurrence of stage II colorectal cancer after surgical resection might be predicted by ctDNA.

Moreover, ctDNA is often used as a DNA source to detect cancer cell-derived mutations (42), promoter methylation (43,44) and loss of heterozygosity (45).

To date, multiple methods have been developed to enable the assessment of ctDNA, including digital droplet PCR methodology, “BEAMing” (beads, emulsion, amplification, and magnets) and other approaches based on PCR and NGS (42,46,47). Early reports using PCR-based methods to identify specific tumor-associated mutations in ctDNA demonstrated that these mutations could be detected.

In a selected patient population with unusually high ctDNA levels, wide coverage exome NGS detected ctDNA mutations appearing at the time of treatment resistance (48). However, a significant proportion of mutations detectable in tumor biopsies were undetectable in plasma. This data contrasts with a metastatic ovarian study that reported recovery of most tumor mutations from plasma using more limited, but still multiplexed NGS (49).

**Interpreting data coming from high throughput technologies**

The feasibility of high throughput technologies for comprehensive analysis of the cancer genomes promises to arouse an information flood to which we need to attribute a biological significance, if we want to translate the effort into translational objectives. The development of tools and methods that can be used on such large datasets need to proceed in parallel with the improvement of deep sequencing.

To date, it seems that we are more able to produce an unimaginable amount of data rather than organizing the information in an integrated view of physiological significance. To address this issue, there are kicking off several collaborative consortia that bring together biologist, statisticians, bioinformatics and computational engineers; some of them have been producing datasets and analytical tools that are free accessible, whereas in other cases, for protection of patients’ privacy, more strict access policy is required.

Several of large scale projects are accurately summarized in the review of Chin et al. (50) [including the Cancer Genome Project (CGP) at Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/genetics/CGP), the Cancer Genome Atlas (TCGA) Research Network (http://www.cancergenome.nih.gov/), and ICGC (http://dcc.icgc.org)] but almost every month is undertaken a new initiative in this field. Indeed, the feasibility of ever-greater large datasets promises to enhance our ability to unravel molecular alteration in cancer, especially in case of rare mutated genes, when high numbers of samples are required to perform analyses of adequate statistical power.

For example, by the end of 2015, the TCGA plans to have achieved the ambitious goal of analyzing the genomic, epigenomic and gene expression profiles of more than 10,000 specimens from more than 25 different tumor types; the data, along with tools for exploring them, are publicly available. Now TCGA Research Network has launched the Pan-cancer analysis project (51) that aims to compare the first 12 tumor types profiled by TCGA. The hope is to connect different tumor types on molecular signature basis for discriminating tissue-independent components of the disease. This will offer the opportunity to interrogate cancer about common pathways, involved in the disease pathogenesis and to extend the indications of targeted drugs already used in some malignancies to others that share common mechanistic alterations.

On the “computational” front, the National Cancer Institute (NCI) has recently supported three independent research teams to develop separate compute infrastructures for the analysis of cloud-hosted genomics data generated by large, public projects (https://cbiit.nci.nih.gov/).

However, beyond considerations about what analysis’ algorithms we will use to what purposes, there are evaluations of biological competence that affect a priori the interpretation of cancer genomics data, and that we might better address at level of “liquid biopsy”.

By interpreting raw genomic data, the first key point is surely the quality of the analyzed samples: the standardization of samples preparation, especially regarding the proportion of stromal contamination, is expected to affect our ability to reveal a somatic mutation in the neoplastic counterpart. It is also conceivable that discrimination between tumor and normal cells should be more feasible at single cell level.
We think that the peripheral blood represents a good source of single tumor cells that had lost any relation with the stromal counterpart during the natural history of the disease, provided that we have a consensus definition of CTCs and we are isolating these cells by a robust method with an adequate degree of clinical validation. In others words, despite their rarity CTCs represent the ideal source of highly pure tumor cells for downstream molecular analyses.

A second key point derives from the complexity of cancer genome alterations that come from a puzzle of “drivers” and “passengers” mutations, for discriminating among which a stringent functional validation is required. Genetic engineering approaches can be used to manipulate mammalian gene function (transiently or stably) both in vitro and in vivo, by using appropriately modified cancer cell lines or mouse models. One of the more recent successes of this strategy is the discovery of the transforming ELM4-ALK fusion gene in NSCLC (52): by forcing its expression in 3T3 mouse fibroblast cell line the authors demonstrated both in culture and in nude mice its oncogenic potential that was then validated in a panel of human NSCLC specimens. The treatment with small-molecule ALK inhibitors is now feasible for NSCLC patients harbouring EML4-ALK fusion gene. These encouraging results further strengthen the extensive use of human cancer cell lines, primary human cancer cell cultures and genetic modified mouse models as functional validation assays of any genomic alteration.

However, we cannot forget the limits of the models mainly based onto the cell lines, including a reduced representation of tumor heterogeneity and the loss of interaction with tumor microenvironment, so that we cannot exclude that some genetic functions emerge from an highly forced artificial system and it do not really reflect what it happens in the “spontaneous” malignancies. Exactly for these reasons and especially for mechanistic studies of metastasis, the “liquid biopsy” offers the great advantage to directly interrogate a subset of tumor cells with higher aggressive potential just in the time it’s happening, provided that an association with patients’ outcome has been demonstrated. If associated with the comprehensive study of primary tumors and metastasis lesions, this strategy promises to reduce the required time for functional validation of any genetic alteration discovered in cancer samples.

For carrying out a fruitful survey of cancer genomic in peripheral blood, we shall standardize and validate in clinic consensus analyses of CTCs and (ctDNA)/miRNA.

Among the several consortia focused onto this theme, the CANCER-ID project is recently kicking-off with the final objective of implementing the validated assays of “liquid biopsy” in ongoing or starting prospective clinical studies enrolling patients with NSCLC and breast cancer.

Among the others, the sample size to consider is a key point able of strongly influencing the quality of the obtained results.

Indeed, otherwise classical molecular biology, cancer genome studies collect from thousand to millions assessments of DNA alterations. Hence, statistical power of the study design is mandatory in order to draw informative conclusions. Consistently with previous report, to identify somatic mutation observed in at least 3% of tumors of a given subtype, it was determined that 500 samples would be needed per tumor type, although smaller sample size may be justified for rare tumor (53). Similarly, we should consider of collecting at least 500 CTCs, if we intend to discover a somatic mutation of a gene of interest in at least 3% of the circulating compartment. This raises some doubt that we can base definitive conclusions onto genomic results obtained from circulating compartment, because of the low tumor burden detectable in the great majority of the patients. Recently, new procedures are feasible in vivo that could address this criticism. Indeed, EpCAM conditioned filaments (54) or leukapheresis (55) can collect more large pool of CTCs in vivo and new tools are ongoing to draw CTCs from up to one half of total blood volume via specific markers (http://www.utwente.nl/tnw/ctctrap/).

Conclusions

At the time of the diagnosis, the tumor is a mixed cell population with different somatic alterations. In this heterogeneous landscape, cancer genome sequencing allows identifying the specific and unique change a patient has undergone to develop his/her cancer. Based on these changes, a personalized therapeutic strategy can be hopefully undertaken. Consistently with previous reported data, the presence of CTCs is a tool for stratifying malignancies with different outcome. We think the scientific evidences and the technological tools are now ready to include the CTCs in the genomic studies, because of their peculiar role into the metastatic process.

Acknowledgements

The CellSearch platform is a gift of the association
ASCOM, Padova, Italy.


Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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