Liquid Biopsies

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49.1 A Bit of History

For years now, the diagnosis and the study of a tumor mass in patients is based exclusively on the use of computed tomography that allows to analyze every part of the body through X-ray beams. At the discovery of a suspected mass, depending on specific cases, it is possible to decide to proceed with different exams types in order to evaluate the entity of the same. The gold standard is tissue biopsy; however, it represents a snapshot limited in space and time, poorly reflecting the clonal heterogeneity and evolution of the tumor and not always feasible.

In 1948, a first manuscript was published about circulating cell-free DNA (ccfDNA) and RNA in the bloodstream [1] and, in 1977, Leon SA et al. correlated level of DNA in the serum with cancer [2]. In 1997, Dennis Lo, a professor of Hong Kong University, discovered the free fetal circulating DNA in the maternal bloodstream of a pregnant woman [3]. This led to the concept of "liquid biopsy," i.e., the sampling and analysis of biomarker in non-solid biological tissue (blood, urine, saliva, cerebrospinal fluid, etc.). From that moment on, based on analysis of cfDNA, the non-invasive prenatal testing (NIPT) was born, i.e., a new prenatal method of determining the risk that the fetus will be born with certain genetic abnormalities.

The possibility of measuring and analyzing cfDNA from peripheral blood presents great opportunities not only in prenatal screening but also in clinical oncology. The discovery of cfDNA has revolutionized the way to treat cancer patients thanks to the comprehension of the genetic bases underlying tumor development and progression [4]. Liquid biopsy falls fully into the new era of a patient-centered care management, namely *Precision Medicine*, that makes use of patient information to drive treatment options.

Several components with distinct characteristics are contained in liquid biopsy: circulating cell-free DNA or RNA (cfDNA or cfRNA), circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), circulating endothelial cells (CECs), and exosomes. These components may be used alone or in combination and can all be measured quantitatively in the bloodstream.

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49.2 Cell-Free DNA

Tumor cells release circulating tumor DNA into the blood, but the majority of circulating DNA in body fluid is often of no-cancerous origin. During the normal apoptosis processes (programmed cell death) or necrosis (cell trauma—premature death), the cell undergoes a series of morphological changes. The cytoskeletal proteins begin to be degraded and cell takes a roundish shape, the chromatin begins to be condensed and degraded into small fragments, approximately 200 bp in length. At the end of the process, the cell is phagocytized or divided in more vesicles called apoptotic bodies, which will later be phagocytosed by necrophagous phagocytes such as macrophages. All cells are therefore linked by the same destiny: cell death. When this happens, in the bloodstream, the DNA fragments known as cfDNA (cell-free DNA) can be found; it is the free circulating DNA not encapsulated inside the nucleus cell that will be degraded by the scavengers of our organism. The amount of dividing cells in an organism affected by neoplasia is enhanced during cell cycle, therefore the number of apoptotic events and the quantity of cfDNA found in the bloodstream will be greater than in healthy organism. Given the greater amount of free circulating DNA in neoplastic individuals compared to healthy individuals, not the complete totality of this fragmented DNA will be digested by macrophages and it is possible to observe a greater cfDNA quantity in advanced neoplastic phase organism. Another way with whom DNA is released inside the bloodstream seems to be the spontaneous release by living cancer cells perhaps to influence the oncogenic transformation of susceptible cells on other sites. Thanks to the presence of cancer cell-free circulating DNA, it is possible to perform liquid biopsy (Fig. 49.1).

During the last years, cfDNA has been analyzed using different methods such as real-time PCR, digital PCR, and next-generation sequencing. It can be measured quantitatively or qualitatively. The increasing in amount of cfDNA as well as the finding of key tumor-specific mutations allows for early disease detection and assessment of therapy response/resistance in order to promptly take action on treatment options.

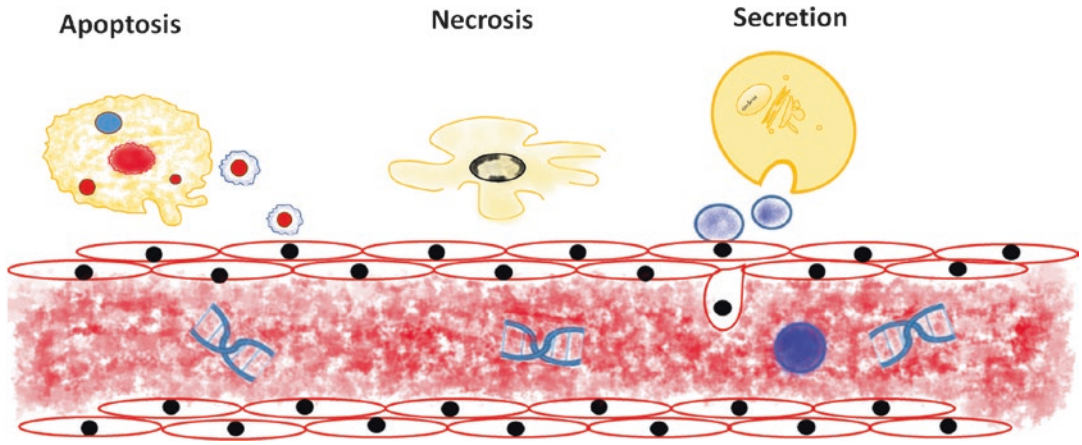

Fig. 49.1 Biomolecular components of liquid biopsy. cfDNA is released into the bloodstream by apoptotic cells as well as by necrotic cells. Into the bloodstream it is possible to find endothelial cells who lose contact with adjacent cells becoming circulating endothelial cells and cell secretion products, such as exosomes, also spill in the bloodstream

49.3 Circulating Tumor Cells, Circulating Endothelial Cells, and Exosomes

CTCs are tumor cells derived from solid tumors and detectable in cancer patients' peripheral blood. When a tumor mass is removed surgically, it may happen that some tumor cells remain latent and after years they can be found around the bloodstream going to affect other organs and give rise to metastases, and it is hypothesized that these circulating cells may underlie tumor recovery. Tumor cells can be recognized thanks to their shape or their physical (size, density, electric charges, and deformability) and biological characteristics (cell surface protein expression and viability) [5]. Some studies highlight a discrepancy between the number of CTCs and the quantity of cfDNA in the blood. A single human cell contains 6 pg of DNA and there is an average of 17 ng of DNA per ml of plasma in advanced-stage cancers; therefore, if CTCs were the primary source of ctDNA it would require over 2000 cells per ml of plasma. In reality, there are, on average, less than 10 CTCs per 7.5 ml blood [6] but definitive evidence for this mechanism has not been reported.

The endothelial cells constitute the blood vessels wall, being in close contact with the bloodstream on one side and with all the other tissues on the other. It seems that after vascular damage the circulating endothelial cells (CECs) can be found in the bloodstream, but it is still not known precisely whether CECs are actually derived from the endothelial cells. However, since their discovery, CECs have been described in many diseases that share the same vascular damage bases [7].

Another liquid biopsy starting platform seems to be represented by the exosomes. The exosomes are material transport vesicles from inside cell to outside through active or passive mechanisms to guarantee normal cell homeostasis. These vesicles carry a lot of information on the outside of cells, such as nucleic acids, proteins, RNA, miRNA, signaling pathways products, drugs, and viruses. The exosomes are secreted both by normal and neoplastic cells and in the latter case we can exploit information derived from them to study the tumor cell characteristics (Fig. 49.1).

All these components (CTCs, CECs, and exosomes) are now used to study tumor growth and evolution in a non invasive way.

49.4 Clinical Use of Liquid Biopsy

The characterization of the genetic status in a precise tumor type (e.g., KRAS or EGFR in non-small cell lung cancer—NSCLC) allows the patient selection to treatment with targeted agent [8]. Whereas, for all those patients in which the molecular analysis is not feasible or does not provide any information, the strategy is to apply the standard treatment for their disease (e.g., cisplatin per NSCLC). In these patients, liquid biopsy may represent a valid non-invasive approach to characterize tumor clones in order to detect therapeutic targets allowing clinicians to adopt appropriate and alternative treatment strategies.

The non-invasive sampling of plasma ctDNA isolation allows not only the driver mutation detection with impact on treatment decisions but also the opportunity to dynamically monitoring tumor burden and the occurrence of tumor relapse or resistance mechanisms as well as the genetic changes that tumor cells undergo during treatment to repeatedly evaluate tumor genetic characteristics and response to therapy.

49.5 What About Liquid Biopsy Advantages and Disadvantages?

To date in order to know the nature of a suspected neoplasia, it is possible to perform invasive procedures. In fact, before proceeding to tumor mass removal, a needle aspiration is performed to obtain cytological specimens [9]. This tissue biopsy is therefore an invasive procedure, not always applicable, and not able to give us a complete picture on the real tumor heterogeneity. Given the multiclonal tumor origin, the removed mass is often not representative of all the tumor cells and this may lead to underesti-

mate the complexity of tumor genomic landscape. The aspirated needle use does not guarantee to take a sample that is cellularly representative of the entire tumor mass (Fig. 49.2). This could led to analyze a single tumoral cell type and in the same way underestimate the presence of differently mutated cells. Nowadays, even if the tumor heterogeneity is well known, the aspirated needle represents great limitations; Even magnetic resonance imaging, performed to evaluate the progress of the tumor mass, is unable to provide any information on its heterogeneity and how it evolves over time. Moreover, it is difficult to obtain a tissue biopsy when tumor is located in places not easily accessible by surgery. Considering the invasiveness of a tissue biopsy, the impossibility of performing it in certain tumors, the inadequacy of the technique to evaluate tumor heterogeneity and the tumor extreme dynamism, it is necessary to move towards techniques that allow not only to monitor neoplasia evolution, but also to evaluate in real way the entire tumor mass heterogeneity, guiding towards a targeted and personalized therapy. This is possible thanks to liquid biopsy.

The liquid biopsy consists of a simple peripheral blood sampling and for this reason it is a non-invasive or minimally invasive procedure (Fig. 49.2). Liquid biopsy allows to monitor the lesion over time and to evaluate which mutations are positively selected at the expense of others that instead regress (Fig. 49.3). It is also a very

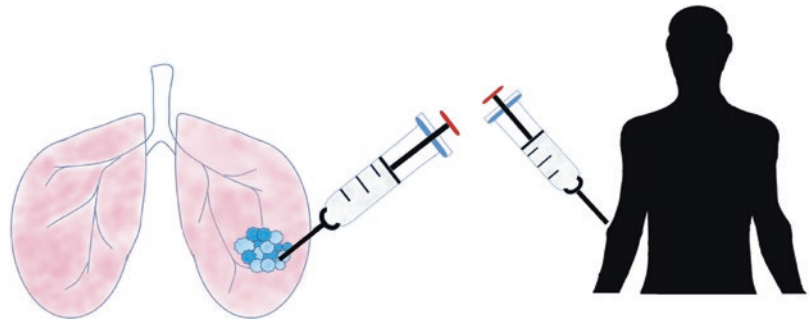

Fig. 49.2 Cancer biopsies. Representation of invasive needle aspiration procedure into a lung heterogeneous tumor mass vs a non-invasive blood sample

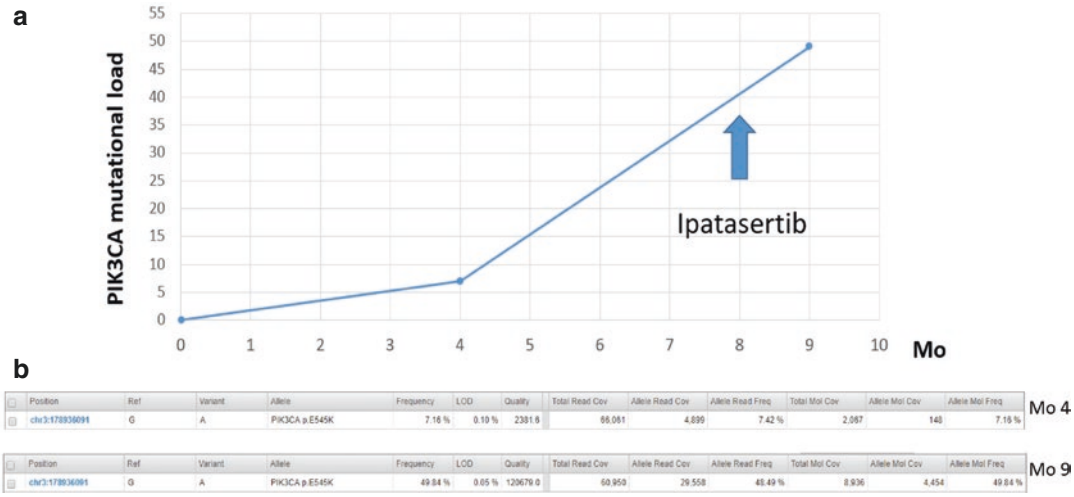

Fig. 49.3 Mutated clone monitored by liquid biopsy. Graphical representation of evolution of PIK3CA mutated clone in a patient affected by metastatic breast cancer (**a**). At time month 4 the mutational load is about 7%, and it grows up to 49% at month 9 corresponding to the clinical worsening of the patient. In such case, personalized treatment with PIK3CA inhibitor could be envisaged. The mutation is observed by liquid biopsy assay (Oncomine pan-cancer cell-free assay—Ion Reporter Software) (**b**). Mo = months

sensitive technique able to detect the presence of a limited number of mutated circulating molecules and to identify possible therapeutic options vs specific mutation and therefore specific to the patient.

With regard to the disadvantages to date, they seem to be far less than liquid biopsy advantages but one of them is the uncertainty. With this term, in fact, we want to summarize all the challenges that scientists have yet to continue to face in order to improve and guarantee the reliability of the technique. Therefore, to date there is not a consensus in technical approaches of choice and the procedure is waiting the CE-IVD (In Vitro Diagnostics) certification.

49.6 Method

Presently, the most commonly used protocols to obtain cfDNA require approximately 3–4 ml of plasma (8 ml of blood). Blood sampling must be collected in specific tubes, containing anticoagulant which prevents the white blood cells lysis and genomic DNA release that would make very difficult cfDNA detection present in a lower quantity. Cells and cellular debris are removed by a double centrifugation and the supernatant, the plasma, is recovered. Circulating DNA is then extracted from the plasma using commercially available kits. After cfDNA extraction, its quantity and quality are evaluated (Fig. 49.4) and it is sufficient to obtain 20 ng of cfDNA in 10 µl of eluate to detect mutations in a very low percentage. The obtained cfDNA is used to set up the so-called "libraries." Preparing a library of cfDNA means to increase, with PCR cycles, the number of cfDNA fragments and to bind same barcodes to each unique molecule for the same sample, which allows to distinguish, at the end of the procedure, which molecule belongs to which sample. The enriched libraries are loaded into the chips and sequenced with the next-generation sequencing techniques (Norton).

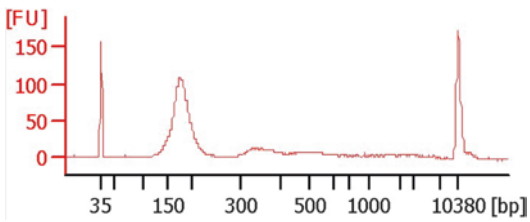

Fig. 49.4 cfDNA quality analysis. Quality evaluation results of cfDNA with Bioanalyzer Assay. Note extract cfDNA obtained is well represented by length fragments about 150 bp, readable on the abscissas axis. The fluorescence (FU) gives us an indication of cfDNA quantity at 200 fragment length (bp). The higher the peak, the greater is its quantity

49.7 Liquid Biopsy for Melanoma

Melanoma is one of three skin cancers with greater prevalence in Western society, together with basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) [10]. The latter two, unlike melanoma, are not lethal and can be treated surgically. Melanoma is the skin cancer that causes more deaths in the world; in fact, the survival rate in patients with fourth-stage melanoma is below 15%. Melanocytes, epidermis cells responsible to pigment production involved in color skin, are subject to change, especially by UV rays presence [7] and their genome mutation determines the formation of a malignant tumor cell.

Melanoma is the cutaneous tumor responsible for the highest number of deaths and with an ever increasing incidence [11–16]. Circulating tumor DNA (ctDNA) can be used to monitor advanced melanoma stages and identify the mutations that can be labeled pharmacologically but in the same way also circulating tumor cells (CTCs) represents a good strategy for tumor cell detection thanks to membrane markers that are only found on melanoma surface cells and not on normal lymphocyte cells surface. Transcriptomic expression and genomic mutations in melanomas are very heterogeneous and therefore liquid biopsy represents to date the best monitoring as well as clinical treatment [10], before and after surgery when it is feasible.

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