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REVIEW ARTICLE

Pharmacogenetic and liquid biopsy: The new tools of precision medicine in cancer

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Abstract

The main difficulty in the treatment of cancer lies in the already known mechanism of resistance to conventional chemotherapy. It is mainly due to the expression of the multidrug transport systems known as ABC transporters, both in neoplastic cells and in excretory organs that reduce the chemotherapeutic concentration. The cancer cell proliferation by activation of growth factor receptors induces their intrinsic tyrosine kinase activity, and their intracellular signaling pathways involved in such activation. Tumor proliferation can respond to the direct action of growth factors on their respective receptors, or due to somatic mutations in different steps of their signaling pathway, in an independent manner of growth factor stimulus. Pharmacogenetics studies have been performed to identify these drivers' mutations and their detection enables targeted inhibitory therapies against their abnormal proteins. The design of new molecules capable of inhibiting these signals has opened a new line of treatment for each type of tumor, thereby enabling tumor growth control and giving rise to the precision medicine approach. It is possible that mutations of sensitive and resistant to these targeted therapies coexist in the same tumor, from the start of therapy or as a consequence of the first-line treatment. The mutations in circulating DNA in body fluids, which are detected and quantified by droplet digital polymerase chain reaction-assisted liquid biopsy, are the ideal biomarkers for the evaluation of pharmacological response, which is crucial for facilitating the change of therapeutic strategy involving second- or third-generation drugs. The quantification of these mutations can be used to assess minimal residual disease in the therapeutic follow-up of each case.

Keywords: Liquid biopsy; Droplet digital polymerase chain reaction; Pharmacogenetics; Somatic mutations; Sensitive mutations; Resistant mutations; Minimal residual disease

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1. Multidrug resistance (MDR) in cancer

Drug resistance in cancer is a common occurrence that refers to therapeutic failure characterized by tumor relapse with absolute refractory pharmacological behavior to classic chemotherapeutics, after the effectiveness of the chemotherapeutics reduces over time. One of the most characterized mechanisms of this type of response is driven by a family of genes that encode the MDR proteins or ATP-binding cassette (ABC)-transporters (ABC-t).

These membrane proteins are capable of expelling a wide spectrum of drugs out of cells, even though the drugs have highly diverse molecular structures and are directed

against different therapeutic targets. There are specific resistance mechanisms for each type of drug, which presupposes the possibility that such resistance can be avoided simply by changing the drug to be administered. However, this first drug can often induce the expression of this powerful ABC-t system, and consequently, the cancer cell will later reject any other therapeutic agent, and even combinations of several drugs administered simultaneously. Interestingly, these patients will be "non-responders" not only to the recommended doses, but even to high doses, constituting the classic cases that we call "drug-resistant." Unfortunately, these high doses will be ineffective for tumor treatment, and engender toxic effects on other tissues, forcing the suspension of the medication. The discovery of this powerful multidrug drug resistance system is due to the pioneering work of Ropert Juliano and Victor Ling in the late 1970s[1] (Figure 1).

2. First pharmacogenetics-based detection of driver's mutations of tumor growth

In recent decades, the development of new molecular methodologies has allowed the detection and characterization of the genetic profile of most tumors with high precision and specificity, as well as in a personalized way for each affected individual. In such a way, this information makes it possible to identify the presence of somatic mutations that drive the proliferation of tumor cells, and many of the mutations are pharmacologically actionable. This new theranostic reality is a gigantic breakthrough to overcome the multidrug-resistance mechanism described in the above. In this way, the

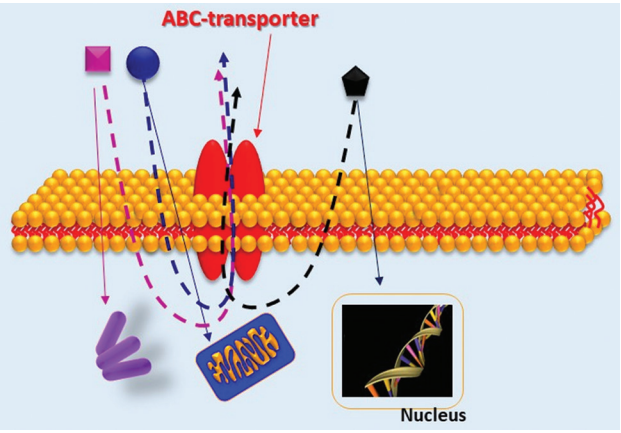


Figure 1. ATP-binding cassette transporters related to multidrug-resistance phenotype, such as P-glycoprotein (P-gp), breast cancer resistance protein, and multidrug resistance-associated proteins (MRPs), form an active ATP- and Ca^{2+}-dependent drug pumping system, which is capable of expelling a broad spectrum of substances (including drugs with different structures and directed to different targets) from the interior to the exterior of the cells, preventing the access of the drugs to their therapeutic targets.

administration of drugs directed exclusively against the mutated proteins, responsible for tumor proliferation, has meant an extraordinary advance in the therapy of neoplasia.

The pioneer in this new field of pharmacology was the advent of imatinib, an inhibitor of the tyrosine kinase (ITK) activity of the BCR-ABL fusion protein in chronic myeloid leukemia (CML), which contributes to an overall survival of 93%[2]. However, as a consequence of the pharmacological pressure exerted, new neoplastic clones with genetic variants resistant to said initial therapy may emerge. This concept is applicable to all the molecules introduced into the therapeutic arsenal of targeted drugs. Consequently, the molecular identification of these resistant-mutations, could allow alternative therapeutic strategies directed towards these new genetic variants.

In this regard, despite the very high percentages of remission achieved with this therapy in CML, it did not take long for a minority number of non-responders to arise, due to different mechanisms. Among them are the presence of ABC-t and the increase in the expression of BCR-ABL by leukemic cells, which necessitate administration of staggering doses culminating with only suboptimal therapeutic responses[3], and the appearance of cell clones with mutations in the BCR-ABL protein, which prevent the imatinib from taking effect and keep the activated tyrosine kinase intact[4].

Perhaps, the best example of how important this new modality of mutation detection and specific drug therapy is clearly described in CML. The appearance of different imatinib-resistant mutations in the *BCR-ABL* gene (L248V, F317L, G250E, H396R, M244V, T277A, F311I, M318T, Q252H, F359A, F359I, or Y326H) can be inhibited with second- and third-generation of ITKs drugs, such as dasatinib, nilotinib, and bosutinib[5]. Furthermore, the T315I mutation that confers resistance to imatinib and the second- and third-generation ITKs is sensitive to another ITK called ponatinib, allowing for the drug resistance in this leukemia to be overcome, and prolonging survival of the patients[6]. In addition, polymorphisms in the *MDR-1*/*ABCB1* gene, such as C3435T, may favor the overexpression of the transporter[7], which is associated with the poor prognosis with imatinib in CML[8].

All these mechanisms are not mutually exclusive and can simultaneously contribute to therapeutic failure with ITKs. More recently, the possible concomitance of mutations in the *JAK2* gene has been suggested, capable of activating the BCR-ABL clone, even under the pharmacological pressure of the corresponding ITK, and which requires the co-administration of a second ITK specific for JAK2[9,10].

3. Pharmacogenetics in cancer

The use of the molecular genetic methods that allows early detection of clones harboring driver mutations has achieved immediate and widespread in all aspects of clinical oncology, allowing the identification of a large number of mutations with pharmacological interest in most solid tumors. This also promotes the development of a wide spectrum of new therapeutic molecules directed at each of these genetic variants. Thus, this methodology allows early detection of tumor and enables informed decision-making in the selection of specific therapies that can control tumor growth, recurrence and metastatic progression. These stratagems of selective therapies only act on somatically mutated targets expressed in neoplastic cells, with no effect on normal cells. These developments ushered in the new era of the so-called personalized therapies, and more recently "precision medicine" in cancer. The new anti-cancer medications known as "targeted therapies" have emerged and aroused great interest in recent years. In this group of agents, there is a wide range of inhibitors of different tyrosine kinases or serine/threonine kinases, which are purportedly responsible for motorizing tumor growth, either by their stimulated (dependent) activation by extracellular growth factors, or by the presence of somatic mutations that activate said kinases in an independent manner on their corresponding growth factor. Several kinase inhibitors (KI) emerged as molecules with a high capacity to penetrate the active site of the kinase, preventing ATP access to that site, and thus inhibiting its tyrosine kinase activity with concentration values at the picomolar level[11-13].

The non-small cell lung cancer (NSCLC) affects almost 17% of Western patients that have an activating epidermal growth factor receptor (*EGFR*) gene mutation, with Del19 and L858R being the most common ones. These mutations are sensitive to ITK erlotinib (Tarceva; 60% response rate) or gefitinib (Iressa). However, a new clone carrying the drug-resistant *EGFR* T790M mutation emerges as a consequence of the pharmacological pressure exerted by first-line ITK treatment. Although there are drugs that specifically inhibit the second mutation, clear evidence has already emerged that various mechanisms of resistance activated by other or downstream signaling pathways, including RAS, RAF, and MAPK pathway[14].

Because the mutation of EGFR and KRAS are mutually exclusive, detection of *KRAS* mutations in patients with non-mutated *EGFR* could provide insights into other therapeutic options. In this regard, recently, the U.S. Food and Drug Administration (FDA) has approved sotorasib (AMG 510, Amgen), the covalent inhibitor on the more common *KRAS* mutant (G12C), for the treatment of NSCLC, and it is the first KRAS inhibitor to reach the market and enter clinical use[15].

The example provided by the first experience with imatinib and BCR-ABL allowed the development and rapid clinical use of new inhibitors of these mutated kinases to block different intracellular signals of proliferation, immortality, and metastasis present in different types of tumors, such as breast cancer, NSCLC, colon carcinoma, and melanoma. Many of these tumors proliferate at the expense of the stimulation produced by growth factors. However, the same tumors can also proliferate without the need for growth factor stimulation due to the presence of activating mutations of the proteins of the signaling cascades responsible for tumor growth (Figure 2).

The presence of sensitive mutations, which favor the insertion of ITKs in the active ATP binding site in the ATPase pocket of the enzyme, is a therapeutic opportunity that has significantly changed the prognosis and evolution of different types of tumors. Furthermore, analysis of circulating tumor DNA profiling has also enabled tracking of clonal variations in patients with colorectal carcinoma, assisting the monitoring of tumor progression and therapeutic resistance against EGFR blockade in real time[18].

On the other hand, the presence of mutations in the same gene but that modify its protein conformation, preventing the action of the corresponding ITKs mentioned above, makes it necessary to look for new second-line ITKs specific for this type of mutation, such is the case of sensitive and resistant mutations present in the *EGFR* in NSCLC. In these patients with NSCLC, a large number of different sensitive mutations but a smaller number of resistant mutations have been described[19,20].

To date, several EGFR tyrosine kinase inhibitors (TKIs) such as afatinib, erlotinib, gefitinib, and osimertinib have already been approved for first-line treatment of patients with advanced NSCLC harboring tumor growth driver mutations in *EGFR* gene. However, the correct indication of these drugs requires the identification of the corresponding sensitive and/or resistant mutations in this gene[21]. The most common sensitive mutations are deletions in exons 18, 19, and 21 (5%, 45%, and 45% of cases, respectively). Meanwhile, among the resistant mutations (5% of cases), the most frequent corresponds to the T790M mutation in exon 20, which can be treated with a specific ITK as second-line therapy. However, whether a significant number of mutations detected in *EGFR* gene are pharmacologically actionable remains to be explored[22].

The presence of these mutations necessitates personalized therapy with the corresponding ITK, but the

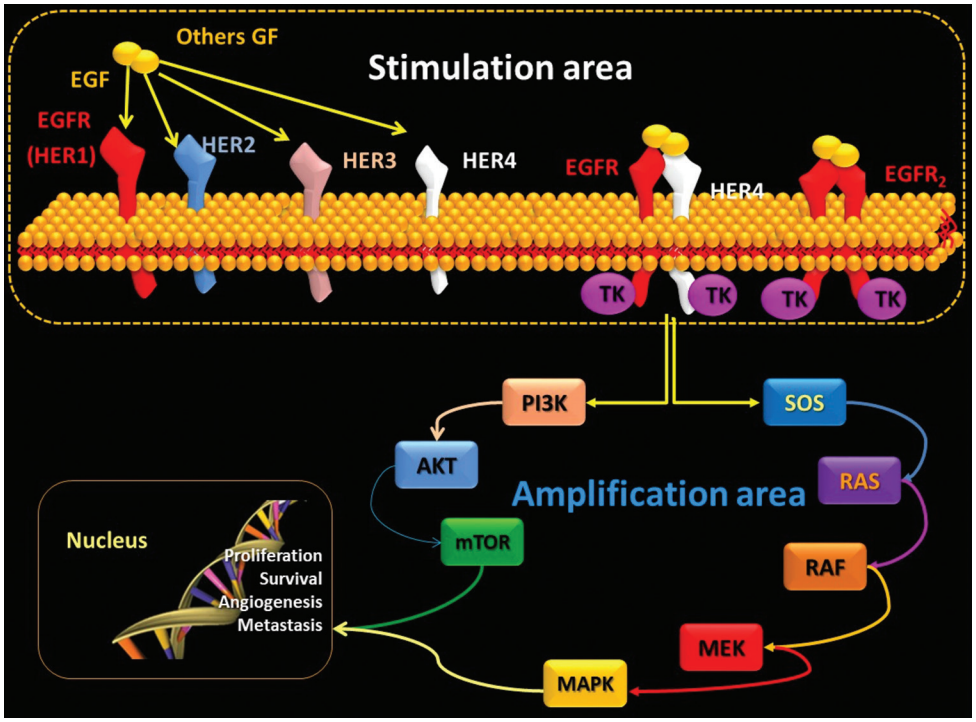


Figure 2. Schematic representation indicating how a growth factor stimulates its corresponding receptor, which contains an intrinsic tyrosine kinase (phosphorylated) in its intracytoplasmic domain and triggers a specific signal cascade, sequentially activating different proteins, until reaching the executing phase of the effect on the nucleus, activating cell proliferation[16,17]. Mutations found in the tyrosine kinases of growth factor receptors as well as on signaling pathway intermediaries are the entry points for therapy since they are sensitive to the action of first-line specific inhibitors. The presence of resistant mutations in the same tyrosine kinases forces the use of second- or third-generation drugs directed against said mutations. The identification and quantification of both types of somatic mutations, which are sensitive and resistant to first-line ITKs, are essential for identifying patients amenable to the treatment and assisting with the monitoring of tumor evolution.

presence of both types of sensitive and resistant mutations is an indication of the heterogeneity of the tumor population that may warrant joint treatment with both types of ITKs simultaneously. If this is the case, we are in the presence of two pharmaco genetically distinct populations, which occupy different percentages of the tumor mass. This duality can exist from the very moment of molecular diagnosis, or arise as a consequence of the pharmacological pressure exerted by treatment with first-line ITKs that will lead to gradual apoptosis in the cell clone carrying the sensitive mutation, giving place or biological space to the growth of the cell clone carrying the resistant mutation.

In the event that both the *EGFR* gene and its entire specific signaling cascade do not contain any somatic mutations that drive tumor growth, the therapeutic opportunity will lie in the use of monoclonal antibodies that inhibit and/or block growth factor binding with its specific receptor. Although it has not been possible to obtain positive results with this therapy in the case of NSCLC, it is usually effective in colon cancer[23,24].

Typically, in colon cancer, the *EGFR* gene does not have mutations of any kind, but mutated and activated tyrosine kinases are found downstream of the signaling pathway arising from EGFR. Thus, the mere presence of any of these mutations in the *KRAS* or *NRAS* genes prevents therapy with the aforementioned anti-EGFR monoclonal antibodies. This prompts the search for pharmacologically actionable mutations on other G proteins that are downstream of the KRAS/NRAS signaling, such as BRAF, MEK, and mTOR[25-27]. The therapy combining an anti-EGFR monoclonal antibody and an ITK directed at the tyrosine kinase mutations of the G-proteins has shown encouraging responses in colon cancer, even in the presence of *KRAS* mutations[28]. However, these strategies have failed to show any benefit in NSCLC with the same mutation background because the mutations in *KRAS*, *NRAS,* or *BRAF* (exclusive of each other) may drive the tumor growth, and they are genetic markers of drug resistance, which requires treatment with conventional chemotherapies[19,20]. It is also possible that we find EGFR gene mutations, whose biological and/or pharmacological action is unknown[22].

A particular situation arises in the case of the V600E mutation of the BRAF gene, where it was found that this

same mutation is good news for patients with melanoma, since it can be actionable with specific ITKs with very good results, but it becomes a biomarker of poor prognosis in colon cancer, where said ITKs have no effect[29]. Moreover, V600E is present in all hairy cell leukemia cases, serving as a marker of minimal residual disease of said hematological neoplasia[30]. The pharmacogenetic characterization of a tumor, from the very moment a biopsy sample is obtained and prior to the start of any treatment, makes it possible to obtain the so-called genetic fingerprint and identify its possible therapeutic targets. Given the large number of molecular variants described, high-throughput methodologies such as next-generation sequencing are the most appropriate tools for obtaining said fingerprint of each tumor, even from within each cell subclone of the same tumor[31,32]. In the aforementioned genetic footprint, we can, in turn, establish a biomarker map that provides us with different levels of information, such as tumor identity, aggressiveness, prognosis, drug sensitivity and resistance, and monitor its therapeutic evolution due to decay (sensitivity) of the initial clone, or its relapse due to the appearance of a clone with new escape mutations (resistance) to the pharmacological pressure exerted.

In turn, we must remember what was initially mentioned about the ABC-t. ITKs are substrates of ABC-t. A series of preclinical and clinical studies have shown that ABC-t can influence the bioavailability of several TKIs, modifying their pharmacokinetics and also playing a role in the development of resistance to this type of therapy, but that in turn, the ITKs can also inhibit ABC-t[33,34].

ABC-t gene polymorphisms can induce significant differences of therapeutic responses in the same pathology with the same TK mutations treated with the same ITK.

4. Liquid biopsy (LB), the ideal biomarker

Since the early report by Vietsch *et al.*, circulating tumor DNA and micro-RNA (later named as "LB") have been used as cancer diagnostic tools[35]. Today, there are more than 12,000 reports on LB on PubMed database.

The process searching for the ideal biomarker provides us with extensive information regarding the pathology of cancer. For this, the ideal biomarker must provide diagnostic, prognostic, and therapeutic information. The genesis and stability of the biomarker must reflect the kinetics of cancer evolution, and it should be quantitatively representative of tumor size or mass. In addition, its sampling must be accessible and repeatable without involving invasive, risky procedures on the patients. Importantly, the ideal biomarker must be highly sensitive and highly specific, surpassing the qualities seen in the classical clinical biochemical methods (Figure 3).

A great difficulty in the therapeutic follow-up of solid tumors is the limitation in repeatedly acquiring tissue biopsies from the same patient during the course of tumor evolution, especially if the site of tumor reappearance is clinically critical and there is no adequate access to it, or surgery acted on the tumor would leave serious sequelae or even put the patient in life-threatening situation.

A new biomarker concept known as "LB" can partially solve this dilemma. LB is a reflection of the genetic information possessed by all types of cells (normal and pathological) or generated by all tissues, and that is poured into the bloodstream and fluid in our body. Like all biological materials, it will have its elimination kinetics since it will be continuously degraded and replaced in the circulation as a result of the balance between production and elimination. The quantity, quality, and identity of this biological material provide an idea of its origin and clonality, and are proportional to the mass of the tissue that produces it. It can come from normal senescent cells, or from necrotic cells destroyed by the immune-macrophage system, and be detected as free circulating genetic material (microRNA, DNA, RNA)[36]. Genetic material may be presented in an encapsulated form within microvesicles, known as exosomes, actively secreted by both normal and tumor cells, which travel laden with adhesion molecules, enzymes, structural proteins, and specific genetic material [37] (Figure 4).

In addition, the circulating tumor cells (CTC) are part of the concept of liquid biopsy. CTC undoubtedly contain 100% of the genetic information of each tumor, but their presence is relatively rare in the circulation and it is extremely difficult to detect them. Together, all the circulating genetic material can be isolated, amplified, and properly deciphered, providing much of the information corresponding to the cells present in the tumor of origin.

LB provides very valuable information regarding the presence of somatic mutations that are pharmacologically actionable and mutations that are resistant to current available therapeutics. These genetic information can be used as a biomarker of minimal residual disease, and as a theranostics tool to evaluate therapeutic behavior, such as optimal, suboptimal, or null response. In addition, LB allows for early detection of tumor or its relapse of tumor harboring genetic variants resistant to the first treatment, even before the onset of clinical symptoms and/or its detection by imaging methods. This early detection reduces the risk of tumor evolution, improving the patient' life quality, and increase the event-free survival time. However, the main limitation of LB is that it cannot identify the site where the tumor is growing, or whether it is a primary or metastatic tumor.

Blood or other bodily fluids can be used in the early detection of information about a tumor that perhaps cannot yet be detected by imaging procedures. The high sensitivity of molecular detection methodologies allows LB to become the ideal tool for monitoring therapeutic responses. Since the circulating genetic material can be found as free form in plasma, cerebrospinal fluid, urine, pleural fluid, and ascites, or as RNA adhered to (protected by) the platelet membranes. Circulating genetic material may also be quantifiable. Thus, the "molecular charge" or number of copies of given genes detected by multiplex droplet digital polymerase chain reaction (ddPCR) procedure is proportional to the tumor mass that is producing it[38]. In this way, it is possible to verify the drop in the number of copies of sensitive mutations, but at the same time, detect resistant mutations after a period of treatment with first-line ITKs. Clearly, this simultaneous information gives us an idea of the degree of efficacy of the first treatment, and documents the presence of a tumor relapse at the expense of a change in the pharmacogenetic identity of the emerging clone of said tumor. These "mutational changes" produced in the original clone, largely to be expected after the pharmacological pressure is exerted, provide new therapeutic targets that can be acted upon with other second- or third-generation drugs in some cases. Thus, targeted therapy could be started long before the cancer is clinically evident or detected by imaging. In general, when the images appear, it is because there is an important tumor mass and often consistent with stages of dissemination.

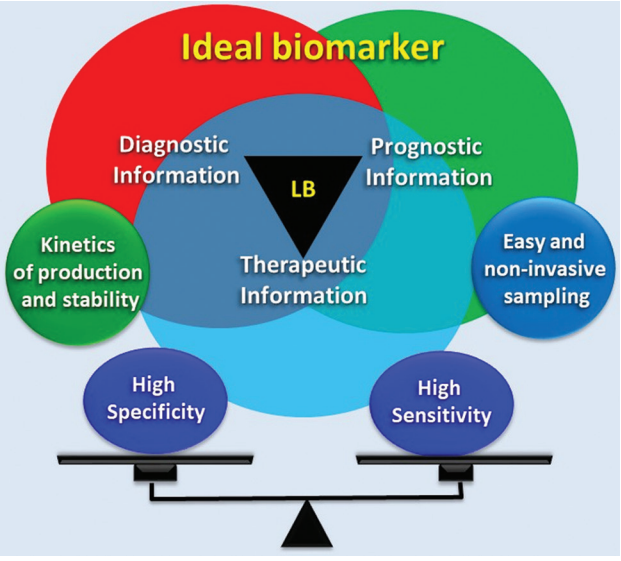


Figure 3. Liquid biopsy, implemented with droplet digital polymerase chain reaction, manifests the characteristics of an ideal biomarker. With high specificity and sensitivity, it can detect mutations of clonal lineage as well as sensitivity and resistance to treatment. The biomarker should be quantifiable, giving an idea of the magnitude of the tumor size, as well as serving as an indicator of minimal residual disease.

This property even makes it possible to verify the presence of metastasis in the central nervous system in cases of patients duly treated with first-line ITKs, where the original tumor is inactive, and is no longer releasing genetic material into the plasma, but as a consequence of the pharmacological pressure, sensitive mutations are still present in circulating DNA in the cerebrospinal fluid. Under this circumstance, the originally "sensitive" tumor managed to generate systemic metastases in different organs that can be abrogated by specific therapy, except those in the central nervous system since the access of the drugs is limited by the blood-brain barrier due to the high expression of the ABC-t of MDR. All positive results in the detection of one or several of these somatic mutations can identify and characterize a tumor constitute quantifiable markers, which can be used to detect minimal residual disease. Other important information is the presence of hypermethylated DNA fragments in CpG islands of promoter regions of tumor suppressor genes, such as *SEPT9* (colon cancer) or *SHOX2* (lung cancer). This type of epigenetic silencing, which inhibits tumor apoptosis, serves as an excellent marker of tumor lineage (Figure 5).

5. ddPCR applied to LB

At present, the latest ddPCR amplification techniques increases the sensitivity of detection, obtaining positive results even when the copy load of each of the mutations sought is extremely low. Under this circumstance, the tumor mass will be $<10^4$ cells, and the imaging studies will

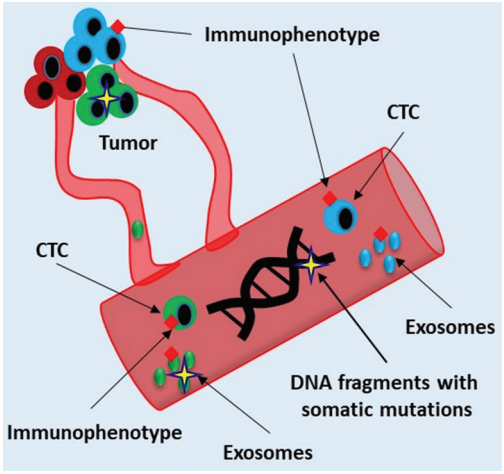


Figure 4. Every tumor usually presents a heterogeneous cell mass, and its genetic information will be present in the circulation. Circulating tumor cells, exosomes and different DNA fragments can be detected, quantified and identified according to the specific information they carry (i.e., immuophenotype and somatic mutations). The same information can be detected in virtually all bodily fluids. Furthermore, larger DNA fragments without specific genetic information are derived from cell turnover in normal tissues.

remain negative since they require around 10^9 cells to be clinically detectable, and the patient probably does not show clinical signs or symptoms of this incipient relapse. In this way, a positive LB result achieves a significant diagnostic anticipation of the presence of a primary tumor, and allows both monitoring of therapeutic efficacy and early detection of the emergence of resistant variants[39-41] (Figure 6). Today, LB is being applied to the detection of most tumors, and the use of gene panels provides extensive information on the genetic fingerprint of each tumor, with high levels of sensitivity and specificity.

In our experience, we have been able to show how a LB sample from a patient with NSCLC, which was positive for the L858R susceptibility mutation, became negative in the 1:125 dilution of the sample measured by real time PCR, but remained positive when the sample was processed by ddPCR in upto the 1:1250 dilution (Figure 7).

This difference in sensitivity allows very early detection of the presence of sensitive and/or resistant mutations, which have great value in anticipating the diagnosis of relapses and allows for informed decision-making regarding the early installation of therapies according to the detected mutations.

The combination of the use of LB and ddPCR results in a strategy of great diagnostic and prognostic value in the therapeutic monitoring of cancer with target drugs, and allows for the identification and quantification of biomarkers of minimal residual disease.

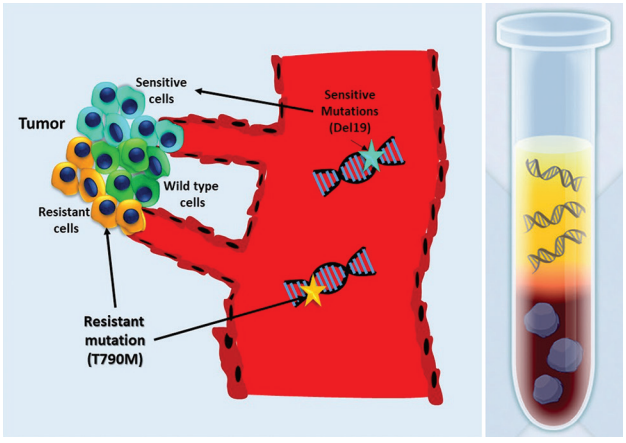


Figure 5. Non-small cell lung cancer. The tumor mass can be heterogeneous, containing a proportion of non-mutated cells (wild type), and another presenting a sensitive mutation, such as the deletion of exon 19 of the *EGFR* gene. Pharmacological pressure following first-line inhibitor of the tyrosine kinase treatment favors the appearance of clones harboring resistant mutations to this treatment, such as the T790M mutation. While the sensitive clone is disappearing, the resistant clone grows until a therapy with second- or third-generation TKIs specific for this tumor variant is started.

6. Strengths and limitations of LB

Due to the reproducibility, high sensitivity and high specificity of the method, LB constitutes a tool of great diagnostic and therapeutic indication value in cancer. Its main advantage is that it can be applied as many times as necessary, and that it can be quantifiable, serving as a parameter for monitoring the therapeutic evolution of a tumor, and for detecting its mutational changes that require modifications in the treatment strategy. In this way, it plays a role in the monitoring of minimal residual disease.

Furthermore, the advantage of using a highly sensitive molecular amplification methodology such as ddPCR significantly increases its sensitivity to detect the presence of tumors in advance compared to conventional techniques. Since it is a molecular biology procedure, the high specificity of the method lies in the design of the

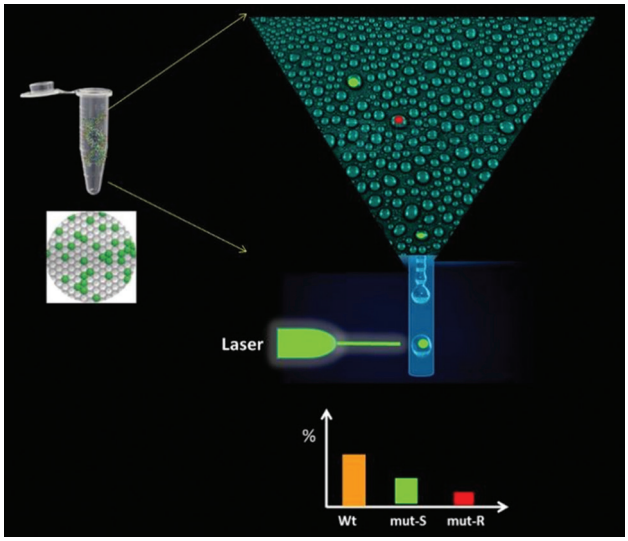


Figure 6. The working principle of droplet digital polymerase chain reaction (PCR) is based on the segmentation of samples using water-in-oil emulsion of the PCR mix to generates 20,000 microdroplets that contain all the components, e.g., genetic materials. After the corresponding cycles, microdroplets are read individually by a laser flow cytometry system, which can quantify mutation burden and sensitivity.

Sample dilution	Real time PCR	ddPCR
Not diluted	Positive	Positive
1:15	Positive	Positive
1:25	Positive	Positive
1:125	Negative	Positive
1:625	Negative	Positive
1:1250	Negative	Positive
1:3125	Negative	Negative

Figure 7. Comparative results of classical quantitative polymerase chain reaction (PCR) and droplet digital PCR.

primers to be used. Collectively, both diagnostic qualities strengthen the utility of liquid biopsy.

These advantages of LB and ddPCR indicate that their combination would provide the best tools in the precision medicine of cancer. However, the main limitation is that the LB cannot define the site of nesting and tumor growth.

7. Concluding remarks

Early detection of pharmacologically actionable somatic mutations by LB-ddPCR makes it possible to establish the corresponding therapy, regardless of the location of the tumor. This strategy allows for the detection of clonal changes that warrant therapeutic modifications, which is instrumental for controlling tumor growth without side effects, and improving the life quality and survival of cancer patients. Hence, LB is an ideal theranostic approach for cancer.

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Conflict of interest

The authors have no conflict of interest.

Author contributions

Conceptualization: Verónica Alejandra Alonso
Writing – original draft: Alberto Lazarowski
Writing – review & editing: Alberto Lazarowski

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Not applicable.

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