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Research Article

Liquid Biopsy and Next-Generation Sequencing Technology to Improve Cancer Diagnosis, Prognosis, and Treatment

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Abstract: Cancer is a disease that has positioned itself as the second cause of death globally. However, there is a deficiency in diagnostic tools and treatment. Traditionally, biopsy involves tissues extraction to be examined. But, it is an invasive procedure and can lead to complications in patients. Liquid biopsy has been developed as a non-invasive technique, with lower cost and allowing a better diagnosis. As a complement, new-generation sequencing (NGS) allows information sequencing and analysis from DNA. The combined use of these tools can result in identification of genetic aberrations in cancer, improving of patients´ prognosis, and use of personalized therapies when studying an individual´s genomics, epigenomics, and proteomics profile. Despite these advances, there are elements to improve procedures sensitivity, a better understanding of biochemistry and immunological response in tumorigenesis processes, and integration of this data through computational strategies for the development of tumor profiles.

Keywords: cancer, genome, liquid biopsy, next generation sequencing, PCR.

INTRODUCTION

The genome is all genetic material of an organism, which allows it to grow and develop. By studying the genome, it could help to understand its formation and a treatment response in diseases such as cancer. This pathology occurs when body's cells begin to divide without control and spread to various tissues. In addition, they can form masses or tumors (National Cancer Institute, 2015, 2019a).

Traditional biopsy consists of an extraction of cells or tissues to be examined. Among the types is the incisional, where a portion of the apparent tumor is removed, and the excisional, in which the entire tumor and part of the tissue are removed. These can be painful and put the patient at risk (Winship Cancer Institute, 2019).

Next-generation sequencing (NGS) describes a technology that has revolutionized genomic research, because the entire human genome can be sequence in just one day (Behjati *et al.*, 2013). There is a wide variety of NGS platforms that use different sequencing technologies through which different processes such as replication, transcription, translation, and DNA methylation can be studied (Buermans *et al.*, 2014).

NGS has the ability to sequence giga base pairs of DNA in an efficient way with high performance, which is not possible to achieve through original Sanger sequencing. Their techniques are based on bound nanoclusters and fluorescently labeled nucleotides incorporation through DNA polymerases. They have the advantage of determining sequences through amplifications of simple DNA fragments. However, it has limitations such as its high cost, its short reading frames (35 to 500 base pairs) compared to original sequencing (1,000 to 1,200 base pairs), and large amounts of data generation, requiring specialized software (Ansorge, 2009) (Frese *et al.*, 2013).

This massively parallel sequencing has a wide variety of applications, including: sequencing of entire genome of a species, non-invasive prenatal testing, identification of genes related to a disease, precise analysis of RNA sequences, identification and analysis of DNA regions that interact with regulatory proteins in gene expression, among others (Buermans *et al.*, 2014) (Ansorge, 2009).

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Regarding cancer, with this technology the entire genome can be study, improving its diagnosis, prognosis, and treatment. In addition, individualized sequencing of a certain tumor can provide a therapy of the same type, improving treatment efficacy and patient safety (Behjati *et al.,* 2013). Therefore, the objective of this research is to provide information about liquid biopsy and NGS technology for improving cancer diagnosis, prognosis, and treatment.

HUMAN GENOME HISTORY

In 1953, Francis Crick and James Watson discovered the three-dimensional structure of DNA, thanks to the X-ray diffractometry performed by Rosalind Franklin. Later, in 1965 Robert Holley and some colleagues managed to produce the first complete nucleic acid sequence of *Saccharomyces cerevisiae*. On the same dates, Frederick Sanger developed a technique based on radiolabelled fragments detection after a two-dimensional fractionation. This technique allowed to produce the first complete sequence of protein coding genes by Walter Fiers in 1972 (Heather *et al.,* 2016).

In 1977, Sanger developed a DNA sequencing technique, which caused interest in discovering and knowing human genome. This was based on sequencing a DNA strand complementary to a single template strand with DNA polymerase, deoxynucleotides, and dideoxynucleotides. The dideoxynucleotides are modified deoxynucleotides that lack an OH group at their 3'-end, preventing adjacent addition of another nucleotide. When one is incorporated by the polymerase, synthesis of the new strand is interrupted. Among its advantages were simple use and accuracy. The invention of technologies such as recombinant DNA and polymerase chain reaction (PCR) contributed to its development. However, Sanger sequencing allowed fragments of less than 1 kilobase to be sequenced. This was improved with techniques such as shotgun sequencing, in which overlapping DNA fragments are cloned and sequenced separately (Heather *et al.,* 2016).

Due to the development of the Sanger method, the United States Government initiated the Human Genome Project (HGP) in 1988. In February 2001, results of 90% of its base pairs were published, being found to contain around 3 billion of base pairs and 30 thousand genes within the chromosomes (National Human Genome Research Institute, 2016a).

Human Genome Importance for Cancer Understanding

Cancer has come to position itself as the second leading cause of death globally, surpassed only by ischemic heart disease and strokes. There are more than 100 types, depending on the organ or cell type it forms. One of the problems is that its diagnosis is usually made in advanced stages. In addition, there is a lack of diagnostic and treatment tools (World Health Organization, 2018).

Globally, in 2018 18,078,957 new cases were reported, most common being lung and breast cancer. Also, 9,555,027 people died from this disease (majority presented lung and colorectal cancer). For Central American area, there was a similar incidence in terms of sex. However, it was higher for women compared to men, as well as mortality (Global Cancer Obervatory, 2018).

With regard to this pathology, cell growth is a normal process in organs and tissues development, and in remodeling after suffering wounds. However, it can suffer alterations in growth and differentiation regulation, causing appearance of neoplasms that are usually confined in a specific tissue, without systemic manifestations or health effects. Cancer refers to advanced neoplasia forms that include tissue invasion, destruction, and a progressive biological process capable of culminating in systemic diseases and death (Moasser *et al.,* 2019).

Tumorogenesis can generate a diversity in molecular and phenotypic attributes of tumor cells (Moasser *et al.,* 2019). In spite of it, essential alterations have been proposed that help to understand the neoplastic affectations. These are called hallmarks. So far the following have been proposed: genome instability and mutation, tumor-promoting inflammation, angiogenesis induction, cell death resistance, cellular energetics deregulation, sustaining proliferative signaling, growth suppressors evasion, inmune destruction avoidance, replicative immortality, and invasion and metastasis activation (Hanahan, 2011).

BIOPSY AS TRADITIONAL METHOD FOR CANCER DIAGNOSIS

Biopsy is the analysis of a portion of tissue or organ removed from a living being to establish a pathologist diagnosis (Classe, 2016). It is widely employed to detect if a tumor is carcinogenic (National Cancer Institute, 2019b).

There are several procedures to perform traditional biopsy. Incisional is one where a tissue sample is removed. It is recommended when tumor sample needs to be large, to remove it completely, or because tumor location is complicated. As opposed, excisional removes entire mass or suspicious area and is the most invasive. These techniques can be performed by puncture (deep skin sample), scraping (in the dermis), or a needle (in internal organs) (Winship Cancer Institute, 2019).

Traditional biopsy is an accurate method, which offers complete information about tumors. If this is done by excisional method, it may be the only

surgery required for its removal (Susan G. Komen, 2016). However, it is invasive, expensive, and can present significant clinical complications in patients, breaking natural barriers and leaving wounds that can cause infections or unwanted scars. In addition, it requires a longer processing time, causing DNA to crosslink and making the sample not suitable for the information intended (Diaz Jr. *et al.,* 2014). It also does not allow response evaluation of the treatment to be administered to the patient (Winship Cancer Institute, 2019).

Liquid Biopsy

In recent years, non-invasive techniques for diseases detection have acquired great relevance, due to the advantages they have over traditional biopsy. They carry a minimal risk for the patient and have a lower cost (Brock *et al.,* 2015).

Within these, liquid biopsy is especially important in Oncology, since differences in genetic profiles significantly affect treatment efficacy in a certain tumor. They are based on detection of mainly two components: circulating tumor cells (CTC) and circulating tumor DNA (ctDNA) (Brock *et al.,* 2015).

CTCs are tumor cells released from primary tumor or from metastatic sites into the bloodstream. It is not known whether this process occurs spontaneously or as a result of biological processes. However, in the bloodstream the conditions for these cells are severe, so their life time is short (1 to 2 hours) (Alix-Panabières *et al.,* 2016). Its early detection in patients with solid tumors (lung, breast, prostate, colon) provides better opportunities for early diagnosis of recurrence detection (Zhang *et al.,* 2015).

The difficulty of its detection lies in the low concentration of these cells in the bloodstream. Likewise, their identification and characterization require extremely sensitive and specific analytical methods, based on enrichment and detection procedures (Pantel *et al.,* 2013).

Currently, detection methodologies of CTC need blood samples to be processed quickly after collection, preventing long-term storage. In addition, these cells are fragile, because they tend to degrade when collected in standard blood collection tubes (Brock *et al.,* 2015).

Tumor DNA can also be released from primary tumors, CTC, micrometastases, or apoptotic cells. So, it is used for identification of various subtypes of tumor cells (Heitzer, 2019). Under normal physiological conditions, infiltrating phagocytes are responsible for cleaning necrotic and apoptotic residues, making cfDNA levels in healthy individuals low (approximately 1% of total cfDNA). However, in late stages of the disease, these levels can increase up to 40% (Crowley *et al.,* 2013) (Neumann *et al.,* 2018).

When comparing the use of cfDNA and CTC, the advantages of the first is that it can be analyzed from fluids stored as frozen plasma. As a complement, when comparing detection of mutations between one method and another, a greater abundance in ctDNA is determined (Crowley *et al.,* 2013).

Analytical Methods for Liquid Biopsy

Polymerase Chain Reaction

PCR is an enzymatic assay that allows amplification of a specific DNA fragment from a sample of the same genetic material. It can be done using DNA of a variety of tissues and organisms, including peripheral blood, skin, hair, saliva, and microorganisms. Only minimal amounts of DNA are needed to generate enough copies that will be analyzed by conventional laboratory methods (Garibyan *et al.,* 2014).

This technique process can be summarized in three steps: denaturation, annealing, and extension or elongation (Science Learning Hub, 2017).

In the first step, as with DNA replication, two double helix chains must be separated. Separation occurs by raising the mixture temperature (greater than 90 °C), causing hydrogen bonds between complementary chains to break (Bolivar *et al.,* 2014) (Nature Education, 2014).

Next, primers bind to complementary 3' zones that flank the fragment to be amplified. This annealing can only occur once the solution temperature has been lowered (50 to 75 °C) (Ahrberg *et al.,* 2016) (Weil, 2018).

Finally, in elongation step new strands are synthesized, which are simple chains that by bases complementarity with the template strand generates double helix fragments. DNA polymerase enzyme incorporates phosphate deoxynucleotides in 5' to 3' direction by the order determined by nucleotide sequence in the original DNA chain or template (Ahrberg *et al.,* 2016) (Weil, 2018).

Therefore, the result of a PCR cycle is two double stranded sequences or the desired double DNA helix. Each contains a newly created strand and an original one. The cycle is repeated (usually from 20 to 30) and takes 2 to 3 hours to obtain one billion copies (Garibyan *et al.,* 2014) (National Human Genome Research Institute, 2019b).

Ultraviolet-visible spectroscopy (UV-Vis)

UV-Vis spectroscopy has been used in determining solutions concentrations through Beer-Lambert law (Velasco Aparicio, 2015). This allows quantifying proteins, establishing enzymatic activity, and evaluating cell viability. Therefore, it is very useful in diseases such as cancer (Maldonado Cubas *et al.*, 2018).

One assay to assess cell viability is metabolic activity. In it, tetrazolium salts are added to cells in culture. Only viable ones will be able to reduce the salt to formazan, which is more stable by tautomerization. This reduction is catalyzed by mitochondrial dehydrogenases, as well as endoplasmic reticulum enzymes, plasma membrane, and lysosomes. Formazan has electronic transitions that generate absorption in the visible region. Signal intensity will be proportional to viable cells number in the culture, salts concentration, incubation time of the culture, and cellular metabolic activity (Maldonado Cubas *et al.*, 2018).

This technique is quick and simple, since complete test can be performed on a single plate. However, results depend on metabolic rate and amount of viable mitochondria in cells. In addition, there are several intermediate steps before absorbance measurement, capable of affecting the results (Maldonado Cubas *et al.*, 2018) (Vargas *et al.*, 2006).

NEXT-GENERATION SEQUENCING

NGS has proven to be an efficient technique for DNA sequencing and for obtaining information from it. It is based on analysis of several million short sequences of DNA in parallel, followed by a sequence alignment with a reference genome or a *de novo* sequence assembly (Elazezy *et al.*, 2018). The latter refers to the fact that genome reconstruction is based only on information obtained from sequencing, without prior knowledge of its organization (Aguilar-Bultet *et al.*, 2015).
There are different NGS techniques. Each of them is explained below.

Pyrosequencing

It is considered the first NGS technique. It was born as a result of the proliferation of DNA sequencing projects and led to the search for alternative methods to reduce time and cost associated with these projects (Margulies *et al.*, 2005). It is based on an enzymatic reactions cascade that leads to light production by luciferase enzyme. Light amount is proportional to nucleotides number incorporated in the synthesis. It is cataloged as a non-electrophoretic bioluminescence method (Abbadi *et al.*, 2017) (Metzker, 2010).

The method can be summarized in four general steps: library preparation and genetic material (DNA) fragmentation, oligonucleotide or adapters ligation to each end of the fragments, clonal amplification by emulsion PCR (emPCR), and pyrosequencing (Applied Biological Materials Inc, 2019) (Bozan *et al.*, 2017).

At the beginning, a library is prepared by isolating DNA sample and its respective fragmentation. Fragments of between 300 and 800 base pairs are generated. Subsequently, each fragment is marked with two adapters (A and B), one at each end of the fragment. With the first (A), the fragment adhere to an agarose microsphere. The second (B) acts as a primer in the amplification and sequencing phase (Applied Biological Materials Inc, 2019).

The next step is clonal amplification by emPCR. To do this, DNA fragments (including both adapters) are mixed with agarose microspheres, which contains an oligonucleotide complementary to one of the adapters. Interaction between DNA fragments of the library and microspheres causes a fusion (Heather *et al.*, 2016). In parallel, an emulsion is prepared. In the aqueous phase, all reagents necessary to perform a PCR are added. Next, microspheres and emulsion are mixed. The result is an encapsulation of microsphere-DNA complexes in simple aqueous drops, isolated by the oil phase, which separates them from other microspheres in the medium (Metzker, 2010).

Each drop contains the reagents necessary to carry out a PCR process inside, exponentially increasing the number of DNA fragments identical to the template. Up to two million copies can be reached, which will adhere to the sphere surface until it is covered by all clones generated in the PCR (Kanagal-Shamanna, 2016). The emulsion is then undone and coated microspheres of DNA copies are distributed on a solid phase sequencing substrate. It has 1,600,000 wells, and each one can contain a microsphere and additional reagents, such as polymerase, luciferase, ATP sulfurylase, and apyrase (Metzker, 2010) (Huse *et al.*, 2011).

Then, pyrosequencing is carried out. Two enzymes are found in each well: ATP sulfurylase and luciferase. Nucleotides are added to make bases complementarity with the copies generated in the PCR. Bases added to the medium are attached to the strand mold with the help of the polymerase. When base binding occurs, inorganic pyrophosphate is generated and is metabolized by an ATP sulfurylase, converting pyrophosphate into ATP. This ATP is used by luciferase to transform luciferin into oxyluciferin. This generates a light photon, causing luminescence (Metzker, 2010) (Bozan *et al.*, 2017) (Huse *et al.*, 2011).

This technique allows luminescence to be directly related to the amount of nucleotides sequenced (Applied Biological Materials Inc, 2019). Complementary to this process, aggregated nucleotides

that did not bind to the template strand are degraded by apyrase. It is an enzyme that hydrolyzes pyrophosphate bonds in nucleoside di- and triphosphates (NDPs and NTPs) in presence or absence of bivalent metal ions, to form nucleoside monophosphates (NMPs) (Pavankumar *et al.,* 2018). Thus, it can continue with its incorporation into the environment and continue chain elongation (Applied Biological Materials Inc, 2019).

The reaction is recorded with a detector called charge-coupled device (CCD) (Huse *et al.,* 2011). It is an integrated circuit that contains a certain number of linked or coupled capacitors. Luminescence is channeled by a beam of optical fibers that affects the microarray and is converted into an equivalent digital value. It is an arrangement cooled by a Peltier element (plate generating cold from electricity), to reduce thermal noise (Bustamante Mejia *et al.,* 2014) (García-Álvarez *et al.,* 2012).

The recording of luminescence intensity when adding a nucleotide is analogous to a chromatogram. Normally, curves are shown in the order of T, A, C, and G residues or as they have been added for binding with DNA sequencing template (Huse *et al.,* 2011).

Sequencing by synthesis

It consists of DNA polymerase or ligase methods to extend numerous parallel strands of DNA. They can be categorized into two types: based on a single molecule (only one molecule is sequenced) or based on assembly (multiple copies of a DNA molecule are sequenced and amplified together on isolated surfaces or in spheres) (Fuller *et al.,* 2009).

It is necessary to amplify fragments to obtain sufficient copies, so that a signal strong enough to detect incorporated nucleotides is produced. This can be done on the surface of the sequencing fluid device or indirectly on the surface of a spherical support. In either case, solid support is covered with synthetic DNA adapters, which are complementary to those used in the process of building the fragment library (Mardis, 2017).

This sequencing requires identifying incorporated nucleotides without interrupting the process. To do this, optical or physical separations are used to differentiate those in solution free of label with respect to those bound and incorporated in the synthesis (Fuller *et al.,* 2009).

Sequencing by ligation

It is used in instruments with Sequencing by Oligonucleotide Ligation and Detection (SOLiD). A single stranded DNA primer is placed and starts the sequencing. Next, short oligonucleotides are synthesized, which possess any of the nitrogen bases (A, C, T or G) in a specific position or a combination of two of them. Then, the oligonucleotide is labeled with a fluorescent dye. Finally, a hybrid is made with single stranded DNA template and DNA ligase binds it to the primer. Fluorescent signal is tracked to identify the incorporated nucleotide. Then, the fluorophore is cut and the process is repeated until it reaches 7 cycles (Ravin, 2010).

Ion semiconductor sequencing

This technique analyzes changes in the concentration of hydrogen ions (Bleidorn, 2016) (Quail *et al.,* 2012). Each time polymerase incorporates a nucleotide into DNA chain, a hydrogen (or proton) is released. Its release is measured in real time by ion-sensitive field-effective transistors (Bleidorn, 2016).

The instrument used has no optical components. It is mainly composed of an electronic reader board that interacts with the chip, a microprocessor for signal processing, and a fluidics system to control reagents flow on the chip (Rothberg *et al.,* 2011).

These microchips contain sensors that have been manufactured as individual electronic detectors, allowing to read one sequence per sensor. However, technology has escalated and has gone from having one million sensors in the first generation of chips (Ion 314) to seven million in the second generation (Ion 316), maintaining same runtime (one to two hours) (Myers *et al.,* 2011).

DNA treatment is similar to that described in pyrosequencing technique. First, DNA is fragmented, obtaining a library of DNA fragments. Subsequently, these fragments are linked to primers, which will be specific to fragments sequences generated in the libraries. Finally, this complex (DNA-primer) is clonally amplified (Rothberg *et al.,* 2011). Amplification is performed by emPCR on the surface of acrylamide spheres of 2 to 3 µm in diameter, known as magnetic beads (Quail *et al.,* 2012) (Rothberg *et al.,* 2011).

Individual beads are loaded into chip wells. Then, they are flooded in cycles with DNA polymerase and with one type of nucleotide at the same time, doing washes with each interaction (Bleidorn, 2016). When added nucleotide in the flow is complementary to the template base, it is incorporated directly downward with respect to the sequencing primer. The chain increases sequencing primer length on a base or more, causing hydrolysis of incoming nucleotide triphosphate and net release of a single proton for each nucleotide added to the sequencing chain (Bleidorn, 2016) (Rothberg *et al.,* 2011).

Proton release produces a change in the pH of the surrounding solution. This is proportional to the nucleotides number incorporated in the flow (0.02 pH units per base incorporated). This change is detected by the sensor at the bottom of each chip well, converted to

voltage, and digitized in response to nucleotide binding (Rothberg *et al.,* 2011).

It is important to note that there are different analytical and technological methods, such as PCR or UV-Vis spectroscopy that are used simultaneously to obtain results with the NGS, depending on the study being done (Kamps *et al.,* 2017). NGS techniques improve efficiency in cloning of genetic material, but do not exclude the use of older methods for biological samples analysis.

USE OF LIQUID BIOPSY IN ONCOLOGY

Cancer diagnosis

Recently, liquid biopsy, in which CTC and /or ctDNA are extracted and analyzed by NGS, has been the technique used in many cases to determine genetic causes of different cancer types. It has also been used for early detection, disease progression, and metastasis process (Neumann *et al.,* 2018) (Kamps *et al.,* 2017).

Patients with chronic obstructive pulmonary disease (COPD) have high risk of developing lung cancer. Through a CTC analysis, it was detected in 3% of patients with this condition. These individuals were monitored annually. One to four years after CTC detection, they presented nodules. This allowed an early stage diagnosis of lung cancer (Alix-Panabières *et al.,* 2016).

The ctDNA can also be used to examine clonal evolution of cancer. In one study, more than 75% of patients with advanced breast cancer had ctDNA. These levels showed a greater correlation than CTC with changes in tumor burden. Therefore, this method could be considered to detect disease progression (Alix-Panabières *et al.,* 2016).

On the other side, diagnosis resulting from CTC analysis revealed data about additional mutations that were present in primary lesions only at a subclinical level. This was indicative of the origin of tumor metastasis (Heitzer *et al.,* 2013).

An example was an investigation in which a mutation in epidermal growth factor receptor (EGFR) gene was found in CTCs. It was not detected in initial physical biopsy in ovarian and breast cancer (Forshew *et al.,* 2012).

As a complement, some studies have proven the effectiveness of CTC use. A study carried out in 27 patients obtained that 28 of 29 mutations identified in a metastatic biopsy were detected in a liquid one. These biopsies were analyzed by NGS. In addition, another mutation was detected in comparison with the other procedure and in seven patients mutations detection failed, because metastatic biopsy did not work, due to obtaining inappropriate material (Lebofsky *et al.,* 2015).

In hematologic cancer, NGS also has various utilities. Some of them are establish guide diagnosis, subclassification, and prognosis. In multiple myeloma, it has allowed a better understanding of disease progression and determination of gene mutations with greater clinical relevance, including: KRAS (gene involved in EGFR pathway) (National Cancer Institute, 2019c), NRAS (gene involved in EGFR pathway) (Genetics Institutes of Health, 2019), TP53 (tumor suppressor gene) (National Cancer Institute, 2009d), BRAF (gene that controls cell reproduction) (National Cancer Institute, 2009e), and FAM46C (gene involved in interferon type 1 response) (Mrozeck *et al.,* 2017).

As for lymphomas, there is not yet an NGS panel available to be used in the clinical practice. However, it is intended to identify specific sequence of circulating tumor in peripheral blood of patients with Hodgkin´s lymphoma (Serratì *et al.,* 2016).

Cancer Prognosis

To evaluate an individual´s prognosis, a biomolecular and histopathological characterization of the sample must be done. It is also necessary to define its stage and to take into account the patient´s clinical observations. However, disease stage is one of the most important factors for disease prognosis (Crowley *et al.,* 2013) (Esfahani *et al.,* 2015) (Babayan *et al.,* 2018) (Cohen *et al.,* 2018) (Wheler *et al.,* 2015).

A correlation between cancer stage and genetic aberrations presence associated with blood tumors has been observed in patients with breast, ovarian, pancreatic, and colorectal cancer. Some of them are TP53, KRAS (Crowley *et al.,* 2013) (Cohen *et al.,* 2018) (Wheler *et al.,* 2015), APC (tumor suppressor gene found on chromosome 5q21) (CIDEGEN, 2019), and Ki67 (Bustreo *et al.,* 2016).

Liquid biopsy has a limited utility in prognosis of resectable tumors (those that can be removed by surgery) (National Cancer Institute, 2019b), due to their little clinical application. However, it could be helpful in unresectable tumors. In a study of lung cancer, their prediction about prognosis was low.

Yet, it did show a good prediction in a study with pancreatic cancer. This indicates that the method must still be validated in different patients and in distinct types of cancer (Crowley *et al.,* 2013).

Treatment against Certain Types of Cancer

-Lung cancer

Most prominent application of liquid biopsy is personalized medicine (treatment strategy based on an individual´s genomic, epigenomic, and proteomic profile) (Mathur *et al.,* 2017) is frequent sampling for detection of resistance development to targeted therapy. In a study with patients presenting with lung cancer,

evaluation of mutations in EGFR by CTC analysis with NGS was valuable for pharmacological treatment optimization. The majority of patients treated with EGFR tyrosine kinase inhibitors (gefitinib, erlotinib or afatinib) showed better results in overall response rates, progression-free survival, and quality of life compared to chemotherapy. Unfortunately, most of them develop acquired secondary resistance within a year, with T790M mutation in EGFR gene being the resistance mechanism in 60% of cases. For patients who presented this mutation, treatment was changed to osimertinib (Sorber *et al.,* 2017).

Analysis of ctDNA with NGS also allowed to identify mutations, amplifications, and translocations in EGFR, MET (sends signals of growth and cell survival) (National Cancer Institute, 2019f), ALK (anaplastic lymphoma kinase, which participates in cell formation) (National Cancer Institute, 2019g), and ROS proto-oncogene 1 (receptor tyrosine kinase that participates in cell growth) (National Cancer Institute, 2019h). This has clinical relevance for personalized treatment, because patients presenting MET amplifications were likely to develop resistance to targeted therapy with EGFR tyrosine kinase inhibitors, specifically erlotinib and afatinib (Kamps *et al.,* 2017) (Sorber *et al.,* 2017).

-Prostate Cancer

Prostate cancer is characterized by high genetic variability, significantly affecting clinical response of patients to treatment (Barbieri *et al.,* 2013). CDK12 is a cyclin-dependent kinase. When associated with cyclin K, form heterodimers involved in cellular processes. In one study, it was determined that prostate cancer has recurrent mutations in this kinase, related to high cancer variability. Among main alterations was a change from an oxidative metabolism to a glycolytic one, which is a marker of various cancer types (Wu *et al.,* 2018).

In addition, prostate cancer has numerous mutations in the process of repairing DNA damage. These mutations are in BRCA2, BRCA1, CDK12, ATM, FANCD2, and RAD51C genes, with those in BRCA2 gene being the most common. These changes cause more aggressive cancer evolutions and lower response to treatments (Karami *et al.,* 2013) (Nombela *et al.,* 2019).

-Breast cancer

An investigation was conducted on detection of mutations in estrogen receptor 1 (ESR1) in patients with metastatic breast cancer. It was found that mutations in this receptor generated resistance to treatment with tamoxifen, a selective modulator of the estrogen receptor with specific tissue activity (DrugBank, 2019a). Therefore, it was recommended to change to fulvestrant or other drugs (Guttery *et al.,* 2015).

-Leukemia

Patients with defects in TP53 gene have the worst prognosis in leukemia. Therefore, they need to be directed towards alternative therapies, due to a minimal response to chemotherapy. In addition, chemotherapies combined with rituximab, a chimeric monoclonal antibody that acts on CD20 antigen in B lymphocytes (DrugBank, 2019b), also have a low response. Although, alemtuzumab, a humanized monoclonal antibody specific for mature lymphocyte antigens (DrugBank, 2019c), acts at another site that does not correspond to this gene, treatment effectiveness was very low (Malcikova *et al.,* 2015).

As a complement, another important mutation is fms-like tyrosine kinase 3 (FLT3) gene. It is an important cytokine receptor in normal hematopoiesis that affects more than 39% of patients with acute myeloid leukemia (internal tandem duplication being the most common mutation type). This duplication causes unfavorable prognosis, reducing the duration of complete remission and reduced survival (Schranz *et al.,* 2018).

-Neoantigen-targeted T cell-based treatments

Mutations have significant correlation with carcinogenesis development and tumor growth. These can lead to neoantigens presence (antigens found in tumor cells that have not been presented by thymus epithelial cells). They have been found in many cancer types, including breast, lung, pancreas, ovary, and gastrointestinal. Through NGS, they can be detected from an extracted tumor, and by binding analysis to major histocompatibility complex (MHC), bioinformatics, or autologous dendritic cell analysis, neoantigens epitopes are identified (Yin *et al.,* 2019).

Thanks to NGS, progress has been made in neoantigens identification in various types. This therapy becomes more useful in those who have a high tumor mutational load such as melanoma, non-small cell lung carcinoma, and colorectal cancer. For example, a case of metastatic melanoma was studied and, through this sequencing, a neoantigen epitope called YA14-ARMT1 was identified. This is an advance for vaccines development against them (Nonomura *et al.,* 2019).

Also, specific T lymphocytes can be obtained against a given neoantigen and through NGS, find its specific T cell receptor. Subsequently, through Genetic Engineering it is feasible to produce immune system cells with this receptor (Yin *et al.,* 2019).

COMMERCIAL PRODUCTS

In the market there are different kits to make liquid biopsy studies more accessible to health centers. Among the current options are:

- Cobas® EGFR Mutation Test v2: real-time PCR test that identifies 42 mutations in exons 18, 19, 20, and 21 of EGFR gene, including T790M resistant mutation. It is designed for analysis of tissue and plasma samples with a single kit, allowing laboratories to analyze tissue and plasma simultaneously on the same plate (F. Hoffmann-La Roche Ltd, 2019).
  Formalin-Fixed Paraffin-Embedded Tissue (FFPET) samples are processed with Cobas® DNA sample preparation kit and plasma samples are processed with complementary DNA (cDNA) sample preparation kit of Cobas®. After preparation, amplification, and detection of both types of samples, they can be run together, giving laboratories flexibility to obtain accurate results with both samples (Jenkins *et al.*, 2017).
- Epi proColon®: first blood-based test approved by the Food and Drug Administration (FDA) for detection of colorectal cancer. It is performed by a clinical laboratory. A specific type of DNA called Septin 9 is detected. It is altered more frequently in tumor cells of colorectal cancer than in normal cells (Epigenomics AG, 2019).

CONCLUSIONS

Because cancer is a genetic disease driven by somatic or heritable mutations, liquid biopsies and NGS have an important impact on its detection, prognosis, and treatment. Proof of this are international efforts such as the Cancer Genome Atlas program, which aims to catalog the genomics landscape of different types of cancer (Meldrum *et al.*, 2011). Identification of changes in the complete set of DNA of each type, its genome, and the interaction of these changes for disease progression can be the basis of an individual era for cancer approach (National Cancer Institute, 2019i).

Despite recent advances in liquid biopsy, there are several future challenges. Among them are the understanding of analytes composition in peripheral blood in both healthy people and in those with particular physiological conditions, and knowledge about microenviroment effects and immune response in tumorigenesis processes. In addition, diagnostic tools should be increased for detection of small amounts of tumor-derived components in circulation, integrating computational strategies to generate profiles of the tumor composition.

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