The innovative approach to combat cancer: Liquid biopsy and immunotherapy

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Abstract. Liquid biopsy, as an advanced examination approach, has revealed its powerful function, since it could monitor development of cancer synchronously. Sampling for liquid biopsy usually is blood, so the sampling is feasible, non-invasive and repetitively, which overcomes traumatically sampling of conventional biopsy. Moreover, immunotherapy is considered as a novel treatment of cancer. It aims to enhance the immune system response to cancer. One of immunotherapy called chimeric antigen receptor T cell therapy (CAR-T) has accomplished great success in cancer treatment. The data from recent study has shown the remarkable antitumor activity of CAR-T to hematologic malignancies. Therefore, this review summarized the principles of liquid biopsy and the evaluation of liquid biopsy. Also, the review introduced immunotherapy, general mechanism of CRT-T, structure of CAR and its limitations.

Keywords: Cancer; liquid biopsy; immunotherapy; CAR-T cell therapy.

1. Introduction

Cancer is able to grow at various positions, causing considerable death of patients every year around the world. Since the 1990s, as hygienic conditions have improved, noncommunicable diseases have become a major source of global disease[1]. According to the International Agency for Research on Cancer, cancer, one of the top contributors of death among noncommunicable diseases, caused an estimated 10 million deaths in 2020. The human beings have been seeking the solutions for centuries and appreciatively the modern medical science brought the hope. The developing technology of examination could locate cancers accurately and currently mature surgery and treatments, such as chemotherapy and radiotherapy, have rescued countless lives. Eradicating early stage of cancers become feasible. With existing technology, the innovative examination approach and therapy may overcome the drawbacks of conventional pattern. This review aims to introduce the diagnostic technology, called liquid biopsy, and recently distinguished treatment called immunotherapy. Besides, it will evaluate their improvement from traditional technology respectively.

2. General mechanism of liquid biopsy

The current technology of cancer diagnosis is still dominant by tissue biopsy. As the name implying, tissue biopsy normally requires surgical approach to obtain the tissue, but meanwhile, risk of bleeding, infection and tumor dissemination may occur when obtaining the tissue. Tissue biopsy is also a non-repeatable and time-consuming technology and is possible to misdiagnosis attributed to tumor heterogeneity. [2] [3] Consequently, due to the drawbacks of tissue biopsy, the advanced concept of liquid biopsy emerged. [3] With the development of microfluidics, computational biology and genomics, it has attained tremendous progress recently. [4] Liquid biopsy denoted the inspected samples were obtained from any bio-substance derived from or related to tumor cell. [5] The tumour-related material contains nucleic acids produced by degradation of tumour cells, as well as proteins and antibodies produced in reaction to the tumour. Hence, liquid biopsy focused on investigation of circulating tumor cells (CTC), circulating tumor DNA (ctDNA), methylated DNA, circulating ribonucleic acid, circulating exosomes and tumor-macrophage fusion cells. [6] [7] The common

technologies for supporting liquid biopsy so far were CellSearch® system and Next-generation Sequencing (NGS), based on the analysis of CTC and cfDNA correspondingly.

CTCs could be explained that tumor cells were administered from original tumor cluster to bloodstream. [8]Many studies have verified CTCs were the one of major factors to trigger metastasis in diverse categories of solid cancers. Various equipment for CTC capture and isolation was developed. There is a system for counting and detection of CTC called CellSearch®, which was the only one liquid biopsy based on CTC approved by FDA. [9]this approach captures CTCs by coating iron nanoparticles with a polymer film containing biotin mimics and conjugating them with antibodies against epithelial cell adhesion molecule (EpCAM). Isolated cells are connected to an analyser, which captures images of isolated cells after they've been stained with fluorescent antibody conjugates. Utilizing ferrofluid nanoparticles and a magnet, this technique initially augments tumour cells immunomagnetically. The cells are then stained with fluorescent antibody conjugate against CD45, which is a leukocyte marker). Besides, cytokeratins 8, 18, and 19. (Epithelial markers) are also labelled with similar method. CTC has been defined as a cell with nucleus, positive for cytoplasmic cytokeratin and negative for the CD45, with a diameter more than 5μm. A blood sample is positive if the total amount of tumour cells matches the aforementioned criteria is 5 or more. [10]

The underlying principle of cancer genomics is cancer derived from somatically acquired mutations, hence cancer is attributed to disease of the genome. [11] Several studies indicated that ctDNA was discharged from tumor cells into the circulation, and it could be detected from plasma (blood depleted with cells), blended with DNA fragments, collectively called cell-free DNA (cfDNA). Since ctDNA was the potential alternative of whole tumor genome, it has been verified the possibility of ctDNA to rebuild the entire tumor genome. [12]The existing multiple techniques for isolating cfDNA were time-consuming, complex and expensive, Sonnenberg et al. has devised an advanced electrokinetic system to isolate cfDNA straight from blood sample. The system utilized an AC electrokinetic device to separate ctDNA from untreated blood sample. The interest ctDNA was isolated into dielectrophoretic high-field regions and other constituent of blood would be removed through fluidic wash. Fluorescence was used to identify concentrated ccf-DNA, and further quantification and PCR were completed by elution. The time consuming of the blood sample to PCR testing was less than 10 minutes. [13] Firstly, the DNA sequencing libraries were amplified in vitro by clonal amplification. Next, the DNA sequence would be established through nucleotides supplement with the complementary strand. Ultimately, without requirement for additional physical separation process, the amplified spatially separated DNA templates were then synchronously sequenced in an enormously parallel pattern. [1]

These improvements have commercially shared regarding high-throughput sequencing platform, but each platform employs a somewhat different technique. For instance, the first commercialized next-generation sequencing platform was developed by Jonathan Rothberg and colleagues in 2005, called 454 Genome Sequencer. Creating a DNA library was the initial phase of this sequencing platform, which included single-stranded DNA or PCR amplicon. This DNA library implied adaptor sequences that beads could be immobilized and captured by the DNA library fragments. Afterwards, the adaptor-modified DNA fragments, PCR reagents, and capture beads were firstly emulsified in a water-in-oil combination and then automatically being physically separated into individual aqueous micro-reactors, as shown in Figure 1(A). [1] To assure that each bead averagely has one clonally amplified DNA molecule, the reaction mixture was added the correct stoichiometric quantity of the DNA library. After amplification, the solvent was supplemented to break emulsions, and magnetic beads coated with streptavidin were incubated with the beads to preferentially filter biotin-labeled amplified product. The DNA attached to the beads was annealed to a sequencing primer, and the beads were located onto a fiber-optic "picotiter" plate comprising massive individual wells. Every well had rough diameter of one bead, to confirm that one sequence could be read by one well on the plate. [1]

Pyrosequencing took use of the pyrophosphate molecule produced during the extension phase by adding a dNTP. Through the activity of sulfurylase, pyrophosphate was transformed to ATP, and

luciferase then used ATP to promote luciferin transformed to oxyluciferin. The light generated from this interaction might be monitored and quantified by the instrument's extraordinarily sensitive camera. [1]

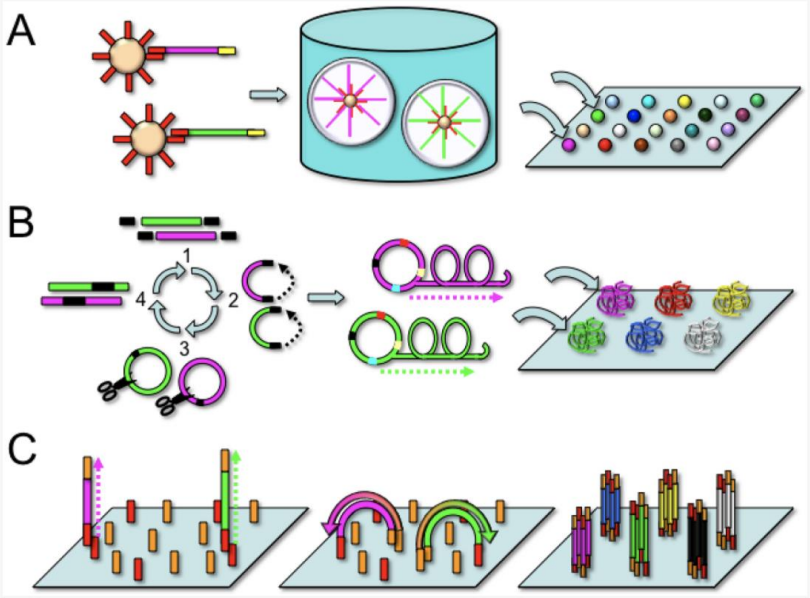


Figure 1. PCR emulsion, Nanoballs with DNA and Amplification of isothermal bridge.

As shown in Figure 1 (A) PCR emulsion. Through the first PCR step of ligation, adaptor sequences, labeled by red and yellow, were integrated into DNA fragments of template, marked by pink and green. Next, the adaptor sequences were hybridized by capturing oligonucleotides and covalently attached to beads. Carefully mixing the template molecules to assist beads to obtain one molecule of template for each bead averagely. Afterwards, the beads were emulsified in a mixture of water and oil to separate PCR microreactors for each combination of bead-template. The surface of each bead was covered with clonally amplified DNA template during emulsion PCR. Then, the beads were allocated onto a microplate to ensure every clonally amplified template might be sequenced individually and spatially separated. As shown in Figure 1 (B) Nanoballs with DNA. Step 1, green and pink meaning target DNA fragments were connected to adaptor oligonucleotides (black) on each side. Step 2, the fragments were circularized by linking the adaptors oligonucleotides together on both sides. Step 3, restriction endonucleases break the circles. Step 4 adaptor sequences have been embedded into the template DNA sequence. By repeating the above method with integrating new adaptor oligonucleotides, showing in red, blue, and yellow, it could obtain a circular template containing 4 adaptor sequences to drive the reaction of sequencing. Then, DNA polymerase was utilized to produce several duplicates of template DNA connected together to form DNA nanoballs, which were allocated onto the surface in an array manner. It meant separated spatially for sequencing. As shown in Figure 1 (C) Amplification of isothermal bridge. Green and pink template DNA fragments were connected to orange and red adaptor oligonucleotide and they were denatured to generate single stranded DNA. Then, they could be hybridized with capturing oligonucleotides complementarily through covalently attached to the flow cell surface. The templates were duplicated by utilizing the capture oligonucleotides as primers, and then denatured. The new integrated DNA molecules could curve downward to hybridize with a capture oligonucleotide primer, which was considered as the subsequent primer of DNA synthesis. On the flow cell's surface, repeated above procedure until bunches of numerous clonal template copies were produced. Derived from M.W. Anderson, et al., *Genes*, 2010.

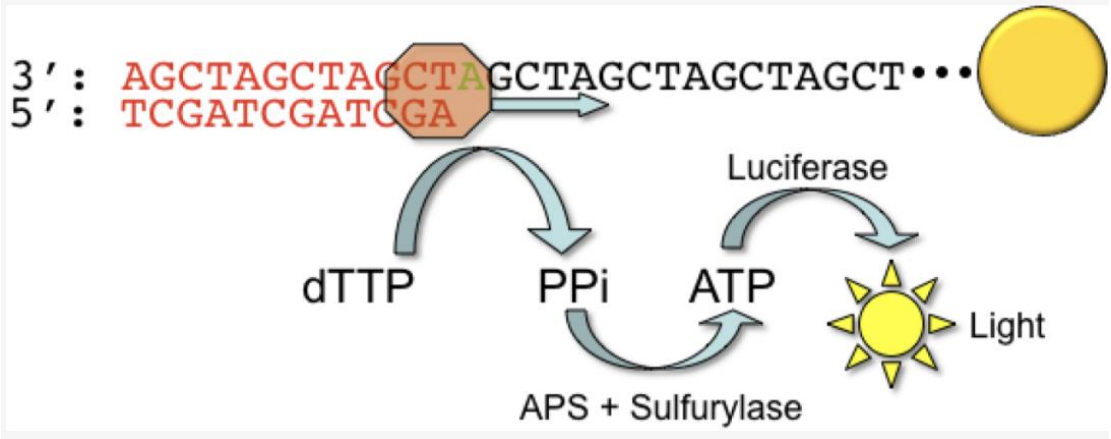


Figure 2. Chemistry related to pyrosequencing.

As shown in Figure 2, for each round of sequencing, DNA templates attached to a capture bead, the yellow circle, were exposed to a single nucleotide. Particularly, a dTTP nucleotide is integrated via the chemical action of brown DNA polymerase and it generated the production of inorganic pyrophosphate (PPi), which chemically reacted with adenosine 5'-phosphosulfate (APS) and sulfurylase to form ATP. Therefore, ATP was subsequently used as a substrate to be catalyzed by luciferase in order to produce light, which could be measured. Derived from M.W. Anderson, et al., *Genes*, 2010.

3. Evaluation of liquid biopsy

Early screening and detection are likely the most promising application of liquid biopsy, while they may the most complicated. Early-stage lung cancers were smaller in bulk and released less DNA than mature tumors. The detection of ctDNA with low concentrations was beyond the capabilities of existing ctDNA techniques. Furthermore, the amount of DNA sequencing and the size of the analysis populations required to demonstrate the clinical validity and effectiveness of early detection assays were considerably greater than what was necessary for tumor genotype in advanced disease. [14] In spite of this, two noteworthy trials had recently been conducted to investigate liquid biopsy–based multicancer early detection (MCED) assays on a vastly large scale of population as a supplement to current cancer screening assays. [15] [16] When supplemented with proteomics and/or imaging, MCED has showed the capability to detect over 50 forms of cancer. [4] A research of tumor DNA methylation patterns revealed 99.3 percent specificity and 54.9 percent sensitivity for detecting all forms of cancer. 93% of samples through this technique have accurately localized the tissue of source, without imaging supplement. [16]

Despite liquid biopsy cannot determine the stage, it might have a significant place in diagnosis. Biopsy has been frequently utilized in treatment of advanced non-small-cell lung cancer (NSCLC), where the necessity for molecular diagnostics was driven by imaging results indicating advanced illness at diagnosis or progressing disease after treatment. [4] Regarding a subgroup of NSCLC patients, taking tissue sample was impractical at the time of diagnosis or progression; in these cases, liquid biopsy could offer substantial therapeutic usefulness. [17]even in instances where tissue sampling was feasible, the average time of plasma analysis for oncogenic factors has been confirmed to be much reduced, resulted in the beginning of appropriate targeted therapy occurring more quickly. [18] Furthermore, liquid biopsy, and more especially cell-free DNA (cfDNA), might be advantageous for evaluating tumor heterogeneity. cfDNA generated from tumor cells was dispersed in the whole body. Consequently, cfDNA analysis could possibly detect numerous contemporaneous diverse resistance mechanisms in specific patients while single-lesion tumor tissue biopsy might not be competent to achieve it. [2] Although the current situation was numerous studies have demonstrated the therapeutic value of liquid biopsy for a variety of cancer types, its clinical effect was only beginning to be implemented in clinical applications. [9]

4. Novel treatment for cancers: Immunotherapy

Conventional cancer therapeutic options, such as radiation, chemotherapy, and surgery, face significant challenges in terms of invasiveness, tumor recurrence, and patient compliance. Because of their potential to avoid recurrence and lack of discernible adverse effects, immunotherapies are therefore of considerable scientific interest. [19] [20] Janes Allison and Tasuku Honjo were awarded the 2018 Nobel Prize in Physiology or Medicine for their remarkable achievements for immunotherapy, which established immunotherapy as an innovative strategy in cancer treatment. [21]

In a healthy human body, nascent tumor cells could be recognised and removed by an immune surveillance mechanism called host immunity. It is comprised of tumor antigen release, antigen uptake by antigen-presenting cells (APCs), APC maturation, activation of T cell, infiltration of T cell, and eventually tumor cell demise. [22] However, the many morphologies of tumors empower them with the potential to evade immune surveillance, which is mostly attributable to the extensive tumor signaling pathways which disorder the immunity-oncology cycle. [23] In order to reinvigorate immune surveillance, immunotherapy must be enhanced. As an illustration, certain cancer regulatory mechanisms (immune checkpoints), including those programmed cell death protein 1 (PD-1) and its ligand PD-L1, have been intensively explored. With the rise of checkpoint blockade immunotherapy (CBI), this prompted the successful development of tumor immunology. [24] Nevertheless, CBI was only efficient in immunogenic microenvironment of tumor, which were normally mentioned as "hot tumors". Even though there were no formal criteria for designating "hot tumors," it was considered that they are characterised by a dense infiltration of T cells and a high-level expression of PD-L1. Likewise, "cool tumors" referred to tumors with low immunogenicity that reside in an immunogenic microenvironment (TME).

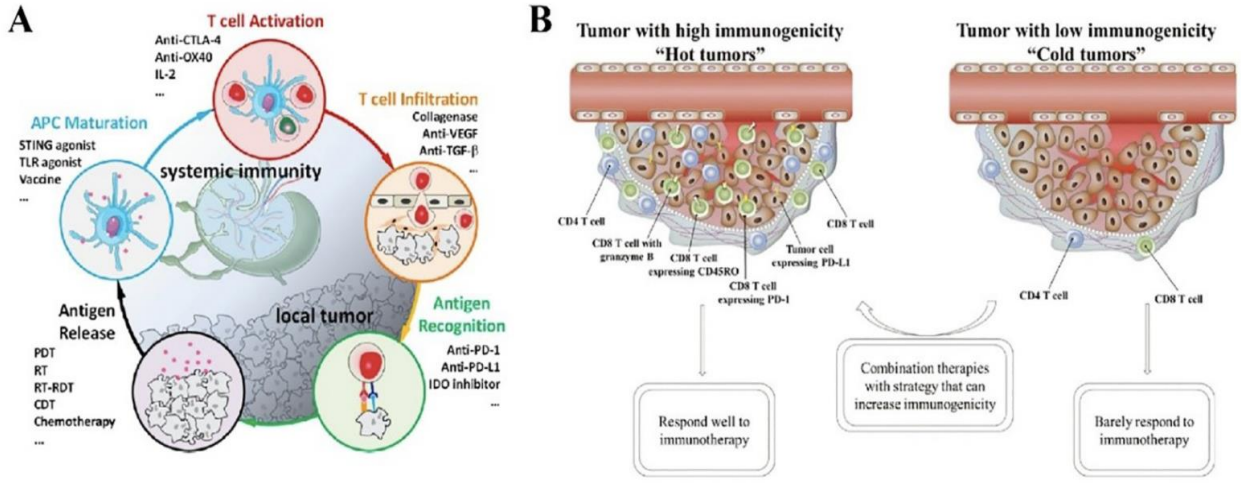


Figure 3. The immuno-oncology cycle and "hot tumors" with high immunogenicity.

As shown in Figure 3 (A), step-by-step depiction of the immuno-oncology cycle and immunotherapeutic use. This cycle consists of tumor antigens release by tumor cells, antigen presentation by mature APCs, activation of T cell in lymph nodes, and T cell infiltration into the tumour parenchyma. As shown in Figure 3 (B), "hot tumors" with high immunogenicity were characterised by many infiltrations of T cell and strong expression of PD-L1, whereas "cool tumors" had minimal immunogenicity. Derived from Q. Li et al., *Journal of Controlled Release*, 2022.

Besides therapeutic chemicals and proteins, immunotherapy included cell-based treatment strategies. Adoptive cellular therapy (ACT) is an encouraging treatment for infection, autoimmune illness, and cancer that involves the transitory transfer of disease-targeting immune cells. Chimeric antigen receptor (CAR) T cell therapy, which denoted the transfer of allogeneic or autologous T cells that have been engineered to express a CAR, was the most used ACT technique. The CAR was first introduced by Eshhar et al. in 1993. [8] It allowed modified T cells to produce an immune response with antigen specificity against cells containing the CAR target antigen independent of the major histocompatibility complex (MHC). [26] To manufacture CAR-T cells, T-cells were extracted from

the patient's or a healthy donor's blood and genetically modified to express artificial receptors by viral or non-viral transfection techniques. Proliferated CAR-expressing T-cells are subsequently administered into the patients. Prior to receiving CAR-T cells, patients need to undergo chemotherapy, to exhaust immunosuppressive cells and promotes CAR-T cell growth.[27]

Generally, the structure of CAR was comprised of four parts. Firstly, extracellular and antigen recognition domains. The extracellular domain recognises and binds to the tumor-associated antigens which are located on tumour cells surface, hence determining CAR specificity. A single-chain variable fragment (scFv) obtained is the most common type of this component from an antibody, despite the fact that it has diverse types. [28]the scFv segment of CAR identifies antigens independent of MHC; therefore, it could identify a broad spectrum of tumor-associated antigens, such as glycolipids, proteins and polysaccharides. [29] Secondly, the extracellular hinge region. This region offers the synapse and plasticity, situated the middle of extracellular and transmembrane domains. [28]Moreover, hinge length is essential for cell proliferation, cytokine production, and CAR-T cell memory formation. [30]thirdly, the transmembrane domain. This domain is crucial for binding the CAR to the plasma membrane and connecting the antigen recognition domains to the intracellular signaling area. Additionally, transmembrane domain facilitates the steady expression of CAR.[31]lastly, the intracellular signaling domain. The intracellular signaling region connected by transmembrane and extracellular regions, resulted in induce of T-cell activation. [29]

In fact, a number of active clinical experiments concentrated on the use of CAR T therapy to treat autoimmune diseases, HIV, and solid malignancies. Surprisingly, CAR T cell treatments have had the greatest success in haematological cancers. [26] For instance, the result of report from Kochenderfer, J. N. et al. in 2015 has indicated the practicality and efficacy of treating B-cell malignancies resistant to chemotherapy with CAR T cells. [32] The achievement of CAR-T therapy was undeniable, CAR-T cell therapy has revolutionized the treatment of paediatric and adult hematologic malignancies. Acute lymphoblastic leukemia is the most prevalent pediatric cancer, and with risk-stratified therapy for B-cell ALL (B-ALL), survival exceeds 90 percent. In contrast, survival rates for adult patients with B-ALL range from 50 to 60 percent. [33] Until now, five CAR T cell therapies for CD19 antigen-expressing haematological malignancies and the treatment of B cell maturation antigen, have been approved by the FDA. [34]

However, patients with relapsed or refractory B-ALL had a negative prognosis. [33]Generally, the failure of CAR T cell therapy was related to antigen escape, in which selection pressure under CAR T surveillance resulted in the formation of antigen-negative malignancies. [35]Furthermore, recurrence could also occur in antigen-positive illness condition, indicating cell-intrinsic variables of CAR-T might result in inadequate antitumor response. The analysis of clinical statistics gathered from chronic lymphocytic leukaemia patients with CD19 CAR-T cells revealed that overall CAR-T cell fitness was predictive therapeutic success. [36] For the further therapy of solid tumours, the capability of CAR T cells to enter solid tumours and successfully destroy target cells are normally restricted by immunosuppressive microenvironment. The tumour microenvironment contains stromal cells and extracellular matrix that constrains the entry of CAR T cells to tumour cells. In addition, immunosuppressive lymphocytes develop in the microenvironment of the tumour to inhibit the effector function of CAR T cells. Through the release of depletion of IL-2 and inhibitory cytokines, tumor-infiltrating immune cells, for example regulatory T cells, create the antagonistic environment to CAR T cells. [34]

5. Conclusion

Liquid biopsy is an extremely informative, effective, and minimally traumatic method for the monitoring cancer patients because it offers information on the molecular properties of the tumour synchronically, thereby capturing the whole complexity of the tumour. Since blood sample may be repeated, liquid biopsy is especially crucial for understanding how the tumour evolves as it develops. Especially when conventional biopsy is infeasible, examination by liquid biopsy could obtain the

essential data as well as normal conventional biopsy did. But liquid biopsy was still in the initial phase of application, it had space to improve in the future. [9] For therapy strategy, immunotherapy has constructed the revolutionary method for cancer treatment, particularly CAR-T therapy. However, the limitation of CAR-T has emerged and unresolved. Although the early clinical experiment of CAR-T cell therapy has achieved great success with haematological cancers, the field is still in constructive and developmental phase. Next-generation sequencing can detect early relapses, but it cannot forecast or prevent them. [33]

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