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Mini Review

Liquid Biopsy: The Future Work for Clinical Pathologist

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Abstract

Surgical and/or biopsy specimens are generally used to detect the tumor-associated alterations in traditional clinical pathology. However, because of the high heterogeneity of tumors, Tumor cells always shows intra-tumor heterogeneity and clones within the tumor may arise showing different behavior. Traditional biopsy or surgical procedure of overt metastases is difficult to accord with the clinical requirement. Recently, a new term, "liquid biopsy", would provide a comprehensive real-time picture of the whole tumor burden in an individual patient. Liquid biopsy was originally introduced to include Circulating Tumor Cells (CTCs), circulating tumor DNA (ctDNA) as well as for, and so on. This review will focus on CTCs, ctDNA and EVs with the aim to discuss current challenges and future perspectives of the liquid biopsy in clinical pathology.

Keywords: Liquid biopsy; Circulating Tumor Cells (CTCs); Circulating tumor DNA (ctDNA); Extracellular vesicles (EVs)

Introduction

Clinical pathology is a medical specialty that is concerned with the diagnosis of disease based on the laboratory analysis of bodily fluids, such as blood, urine, and tissue homogenates or extracts using the tools of chemistry, microbiology, hematology and molecular pathology. In the past, Microscopic analysis on tissue biopsy and surgical specimens are the mainly activity of the pathologist. It is uneatable that the standard biopsy is the best method on tumor diagnosis.

However, in the last decades, while we are moving to the "personalized medicine" based on the molecular characterization of the tumor [1]. The molecular characterization of cancer through Next Generation Sequencing (NGS) still holds many surprises [2]. But because of the high heterogeneity of tumors, Tumor cells never reach a steady state. They are continuously changing and they acquire new mutations to survive in an adverse environment. It always shows intra-humoral heterogeneity and clones within the tumor may arise showing different behavior. It is known that different metastatic sites harbor different genomic aberrations and biopsy of one or two accessible metastases may not be representative. So, Biopsy of any cancer is difficult to accord with the clinical requirement. At the same time, traditional biopsy or surgical procedure of overt metastases is an invasive procedure limited to many reasons (e.g. co morbidity of the patients, the lack of a safe access to the lesion) and not easily acceptable in the clinic.

In recently, a new diagnostic technique----"liquid biopsy" [3], it offer the chance to analysis of therapeutic targets and drug resistance conferring gene mutations on circulating tumor cells and cell-free circulating tumor DNA released into the peripheral blood from metastatic deposits through a on-invasive and simple blood tests. Liquid biopsy can easily isolate biomarker from many body fluids (blood, saliva, urine, as cites, pleural effusion, etc.) and, as well as a tissue biopsy, a representative of the tissue from which it is spread. The reality is that liquid biopsy has potential to replace standard biopsy [4].

Liquid Biopsy

Currently, Except for The gold standard measurement of protein tumor markers in serum, Liquid biopsies [5]was originally introduced to include Circulating Tumor Cells (CTCs), circulating tumor DNA (cDNA) as well as for Extracellular Vesicles (EVs) [6], and so on. They may be obtained from almost all body fluids (blood, serum, plasma, urine, pleural effusion, as cites, etc.…). Here, we will focus on CTCs, cDNA and EVs with the aim to discuss current challenges and future perspectives of the liquid biopsy in clinical pathology.

Circulating tumor cells (CTCs)

CTC were first described in the scientific literature by Ashworth in 1869. Paget described his theory of "seed and soil" based on his observations of metastatic breast cancer in 1889. It is clear that CTCs released from the primary tumor mass or the migration secondary sites into the lymphatic and bloodstream, and to adhere to endothelial cells and give birth to distant metastases through the Epithelial-Mesenchymal Transition (EMT) [7].

The separating and enriching CTCs is the first step in CTCs analysis. A wide range of Methods for isolation and enriching are available or under development, include CTC microchips, filtrations systems, and immunomagnetic bead based capture. in which, the Cell Search technology licensed by the US Food and Drug Administration (FDA) for using in breast cancer to monitor treatment. This system uses immunomagnetic purification with antibodies against epithelial cell adhesion molecule (EpCAM) from peripheral blood. The specificity of Cell Search® isolation is only based on EpCAM expression on CTC surface [8-11]. Recent studies suggest that CTCs show considerable molecular heterogeneity, and capture of cells that have an altered phenotype is challenging. Consequently, CTCs with low or absent expression of EpCAM cannot be detected with

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Cell Search® technology. At the same time, automated lab-on-chip technologies and micro fluidic cell isolation and biomarker (mostly molecular, multiplexed) analysis provides affordability for pathology laboratories and is key to the uptake of these methods in liquid biopsy.

Now, the enumeration of CTCs is considered relevant as a surrogate marker for tumor growth as well as for defining tumor aggressiveness in several major cancer types, including breast, prostate, colorectal and lung cancer. In this regard, the increase in the number of cells predicts tumor progression. Furthermore, the total count of CTCs before chemotherapy initiation is associated with staging (higher detectable number of cells in stage IV patients) and the Progression-Free Survival (PFS) and Overall Survival (OS). In the future, therapy decision should also be made taking into account CTC analysis, especially for the selection of those patients who might benefit from adjuvant therapies.

Furthermore, CTCs contain intact DNA, RNA and proteins, and arguably represent clones within the tumor with metastatic potential. So the isolated and enriched CTCs may be stained for cytopathological analysis or subjected to RNA or DNA analyses may have considerable diagnostic potentials [12,13].

It is clear that liquid biopsy has potential to replace standard biopsy in future, but is hampered by lack of biomarker [14]. So it must be embedded within patient pathways and cross-validation with existing tests is a prerequisite for implementation.

Cell-free Nucleic Acids (cfNAs)

The presence of Circulating Nucleic Acids (CNAs) in blood from cancer patients as biomarkers of cancer has been known since the 1970s. Thus far, CNAs include cell-free DNA (cfDNA), circulating miRNAs and mRNA [15]. Due to their stability and easy isolation, the majority of research has focused on cfDNA. cfDNA is released from both healthy and cancer cells. The tumor-released cfDNA has been identified as circulating tumor DNA (ctDNA), which is increased in cancer patients. ctDNA permits the detection of gene mutations and methylation, which can be used to guide therapy.

ctDNA fragments mainly originate from apoptotic or necrotic tumor cells which discharge their DNA into the blood circulation. ctDNA released from necrotic malignant cells varies in size, whereas DNA released from apoptotic cells is uniformly truncated into 185-200-bp fragments. Because the main source of cfDNA in healthy individuals is from apoptotic cells, the longer DNA fragments could be a marker for malignant tumor detection [16].

To successfully detect the presence f ctDNA in plasma or serum, quantitative analysis and analysis based on DNA-specific mutation detection are two main methodologies. Real time PCR allows DNA quantification of cfDNA including tumor and non-tumor derived DNA [17]. Never the less PCR-based mutation detection systems and other several methods, such as "Beaming" (beads, emulsion, amplification, and magnetic), digital PCR, NGS or single-molecule sequencing, which have been demonstrated to provide relevant information about the mutations in their tumors. Recently, Dawson and other authors used whole-genome sequencing to quantify ctDNA in serially collected plasma samples. The results show a good correlation between ctDNA and CTC levels in patients with higher CTC counts [18,19].

So, ctDNA analysis is the opportunity to study "tumor dormancy" phenomenon. It has been demonstrated that T790M mutations are detectable in the blood before radiological and symptomatic relapse. ctDNA might offer the opportunity to monitor patients with no clinically detectable disease after surgery and standard therapy. Thus in the near future tumor monitoring might also be integrated with ctDNA testing and CTCs molecular characterization [20].

In contrast to DNA, RNA is often thought of as very labile and difficult to work with. But the signature profile of circulating miRNA has good sensitivity and specificity for cancer diagnosis. Recently the other non-coding RNA (ncRNA), the long-non-coding RNA (lncRNA) is being investigated as a novel biomarker in cancer patients.

Additionally, mRNA from plasma CTCs can be used for RNA sequencing, permitting the identification of fusion genes from RNA as well as DNA. While mRNA from blood leukocytes may become of considerable future importance, particularly for patients on immunotherapy.

Extracellular vesicles (EVs)

This part describes a new liquid biopsy technique to sensitively detect disease specific circulating Extracellular Vesicles (EVs) [21-23]. These EVs are more than simple exophytic budding of cell membrane. They are small membranous vesicles that differ in their cellular origin, abundance and biogenesis, and are naturally secreted by almost all cell types to transport bioactive molecules intercellular. Molecules contained within these small vesicles can therefore move through the bloodstream and reach distant sites, enabling the exchange of "information and materials" between different sites [24].

EVs have also been found in the blood of cancer patients and therefore provide a novel type of biomarker for various diseases [25,26]. Perhaps EVs include Exosomes, secretome, oncosomes and other micro vesicles from cells, they are one same structure [27,28].

EVs show highly different distributions of size, shape and electron density when analyzed by transmission electron microscopy, each having typical characteristics. EVs can be identification and characterization via electron microscopy, flow cytometry and Western blot or immunoblotting or enzyme-linked immunosorbent assay (ELISA) assays, On-Chip Immunoelectrophoresis [29]. These methods are impractical in most clinical settings. Currently, the characterization and concentration of nanoparticles in liquid can be obtained through the NanoSight™ platform. Additionally the exome screen is a highly sensitive and rapid analytical technique for detecting CD147 and CD9 double-positive EVs from patient blood that can be used to identify biomarkers of colorectal cancer. ExoScreen can be a tool for detection of EVs from as little as 5 ml serum of cancer patients to detect circulating cancer-derived EVs without the need for purification [30]. And it is superior for the detection of EVs to conventional methods, immunoblotting and ELISA.

Exosomes: Exosomes were initially isolated from the peripheral circulation of patients with cancer in 1979. Exosomes is one type of EVs, which are small (40 to 100 nm) membrane derived vesicles that are released extracellular following the fusion of multi vesicular bodies or mature endosomes with the cellular lipid bilayer membrane to protect biological molecules [31]. Exosomes are rich in a variety of

molecules such as signal proteins and/or peptides, double-stranded DNA fragments (exoDNA), microRNAs (miRNA), mRNAs, and lipids proteins. Because Exosomes provide signals to distant cells, they can act as a nanoparticles-based communication system and play a central role in cell-to-cell communication by a direct activation of surface-expressed ligands or by transferring molecules between cells. Interestingly, Exosomes can either manipulate the local and systemic environment allowing cancer growth and dissemination or modulate the immune system to elicit or suppress an anti-tumor response in cancer. Murine mRNA-containing Exosomes can be taken up by human cells resulted in the synthesis of mouse proteins. Tumor Derived Exosomes (TDE) plays a crucial role in regulating the changes of tumor niche and in driving tumor progression.

Conventional Exosomes diagnostic methods comprise a two-step procedure, including Exosomes extraction from body fluids, and followed by the specific detection of harbored biomarkers. Currently, the most commonly used approach for Exosomes isolation is based on ultracentrifugation. Recently the increased application of proteomic technologies has significantly contributed to a deeper understanding of the protein profiles of Exosomes from a wide variety of cultured cells and body fluids (such as plasma, urine and malignant effusions) [32]. A global proteomic analysis of highly purified Exosomes provides new insight into the multiple functions of Exosomes in cancer progression and will aid in the development of novel diagnostic tools for NSCLC.

Secretome: The term "secretome" was introduced by Tjalsma [33]. It is referred to as the rich, complex set of molecules secreted from living cells. More loosely the term also includes molecules shed from the surface of living cells. Exosomes can be considered as "operating/active secretions" of cancer cells. Secretome proteins play a key role in cell signaling, communication and migration. The cancer secretome was first mentioned in 2006. the fact show that secretome participate in various physiological processes such as immune defense, blood coagulation and cell signaling and also play crucial roles in pathological processes including cancer angiogenesis, differentiation, invasion and metastasis [34].

In a word, EVs analysis in cancer is a new field and there are still many questions about their function. Thus, the analysis of molecules contained in EVs can provide additional information about the tumor biology. Although the potential of EVs as cancer biomarkers has been promising, the identification and quantification of EVs in clinical samples remains challenging. There is still a gap between in vitro EVs testing and clinical applications [35,36].

Conclusion

Liquid biopsy is a rapidly advancing field and the subject of intense interest from both academia and industry. Liquid biopsy can provide a comprehensive real-time picture of the whole tumor burden in an individual patient [37]. So it has potential to replace standard biopsy in tumor patients for early diagnosis, estimation of the risk for metastatic relapse, stratification and real-time monitoring of therapies, identification of therapeutic targets and resistance mechanisms.

Nevertheless there are still several limitations for the introduction of liquid biopsy in a clinical setting. Except for CTC enumeration with the Veridex system (FDA-approved) [38], there are no validated techniques both for ctDNA and EVs analysis. But hopes are high to see them included in clinical practice.

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