BACKGROUND

Liquid biopsy is an ingenious innovative technique which has the potential to provide information about cancers without invasive biopsy, using circulating biomarkers. A progressing cancer features heterogeneity and constant evolution, posing a significant challenge to its eradication. Whereas tissue biopsies samples only have a small fragment of the tumour thus capturing a fraction of this heterogeneity, liquid biopsy represents a minimally invasive and more sustainable alternative to interrogate cancer cells longitudinally. All stages of cancer therapy have the ability to benefit from this, starting with screening for cancer before it is clinically apparent. During treatment of metastatic disease, it is advantageous to predict response and monitor disease progression. It involves sampling of non-solid biological tissue, generally blood for isolation of tumour derived components. Circulating tumour cells (CTC), circulating tumour DNA (ctDNA) and eventually other tumour-derived material (e.g. exosomes) alongside existing and new protein markers. In this review, we summarise main techniques and applications of liquid biopsy in oncology.

KEYWORDS

Liquid biopsy, Circulating Tumour Cells (CTC), Circulating Tumour DNA (ctDNA), Tumour-Derived Material, Exosomes

Cytokeratins (OK), Specific tumour markers such as TTF-1 (Thyroid transcription factor 1) for lung and thyroid cancer, PSA (Prostate specific antigen) for prostate cancer, HER-2 (Human epidermal growth factor receptor-2) for breast cancer. Various methods of isolation for CTCs are tabulated in Table-1.12

**Isolation of CTCs**

The combination of differential methods is essential to isolate functionally heterogeneous CTCs. After their isolation genomic and proteomic profiles of CTCs are analysed for detection of molecular targets for developing targeted therapy. CTCs can be used to study mutations, alternative splicing, chromosomal rearrangements, gene overexpression and down regulation, protein expression and drug sensitivity. The most common method used to isolate CTCs is the Cell Search system; an automated detection system for CTCs that uses anti-EpCAM antibodies, anti-CK antibodies and anti-CD45 antibodies.13

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**Clinical Applications of CTCs**

CTCs detection has diagnostic, prognostic as well as therapeutic significance. It can virtually substitute tissue biopsy for diagnosis of cancer. Their early detection helps in stratification of patients according to risk for recurrence which has got prognostic significance. The absolute number of CTCs in a 7.5 ml blood sample is significantly associated with prognosis. (>5 CTC /7.5 ml of blood has poor prognosis). Prediction of response or resistance to patient can also be assessed.12 Limitations of CTCs include their scarcity making them difficult in detection, absence of a reliable and efficient marker to distinguish CTCs from other blood born cells. CTCs are often untraceable in patients with dysplastic or early malignant lesions, thus limiting their utility for early diagnosis or surveillance.13 Most isolation methods include multiple operative processes, such as erythrocyte lysis, cell centrifugation and washing, which leads to insufficient capture or cell damage. All the isolation techniques are time consuming and expensive procedures.

**Circulating Tumour DNA (ctDNA)**

Cell free DNA, cfDNA is derived from apoptotic normal cells and contains a minor fraction of DNA from cancer cells named as cell free circulating tumour DNA (ctDNA). ctDNA is released by the process of apoptosis, necrosis or by active secretion from tumour cells. In normal subjects, the concentration of cell free DNA (cfDNA) ranges from less than 10 ng/ml to more than 100 ng/ml (half-life 16 min-2.5 hour).14 Circulating tumour DNA (ctDNA) represents a small percentage of total cfDNA, varying from less than 0.1% to over 10% according to tumour burden, cancer stage, cellular turnover and response to therapy.15 CtDNA has virtuous clinical significance and can be used to study specific gene mutations (e.g. KRAS, EGFR), structural chromosome rearrangement, de novo ctDNA mutations, somatic copy number variations (CNVs) and analysis of entire genome and exome. Tumour genesis is regulated not only by genetic but also by epigenetic alterations. The cytosine (C) base in DNA and lysine residue in histones tails can be methylated and this methylated ctDNA could be a biomarker for early detection of cancer.

**Isolation of ctDNA**

cDNA is isolated from plasma as opposed to serum, because the latter is contaminated by genomic DNA derived from non-malignant sources such as the lysis of leukocytes and other hematopoietic cells.16 Two major methods are used for isolation of ctDNA: first, an approach which targets specific genetic mutations or structural chromosomal rearrangements in specific genomic regions which are generally found in most malignancies; whereas second is an untargeted approach which relies on WGS (whole genome sequencing) or WES (whole exome sequencing) in which whole genome is analysed for the detection of de novo mutations in ctDNA as well as somatic copy number variations (CNVs). In this second approach, there is no need of any prior knowledge of genetic alteration making it more informative and reliable.17 Targeted approaches include the PCR based techniques (Polymerase Chain Reaction) and NGS (Next Generation Sequencing). The limit of detection using PCR based techniques is 1%-0.001%. The techniques- used are ddPCR (droplet digital PCR) and BEAMing (Beads, Emulsion, Amplification, and Magnetic). NGS (Next Generation Sequencing) is the latest, state of art technology with >2%-0.001% limit of detection. NGS is broadly divided in type sub-types i.e. targeted approach and untargeted approach. Different NGS sub-types based on targeted approach are TAm-Seq (Tagged Amplicon deep Sequencing), CAPP-Seq (Cancer Personalized Profiling by deep Sequencing), Safe-Seq (Safe Sequencing system) and PARE (Personalized Analysis of Rearranged Ends). Untargeted approaches do not require any prior knowledge of molecular alteration; analysis of entire genome and exome is done. It includes the NGS based technologies which allow whole genome sequencing (WGS) and whole exome sequencing (WES).18

**Clinical Applications of Circulating Tumour DNA (ctDNA)**

CtDNA can be used for screening, diagnosis, assessment of minimal residual disease after surgery done with curative intent, response monitoring and follow-up. ctDNA aids in
patient stratification on the basis of variable risk of recurrence after surgery, i.e. for selecting the patients who can get maximum benefit from an adjuvant treatment post-operatively to maximize their cure rate and also for avoiding systemic toxicities in patients who are not going to benefit from adjuvant therapies. Detection of alteration in ctDNA molecular profile is a clear indicator of clinical progression which is detected several months prior to clinical evidence of progression of malignancy, thus playing key role in deciding early intervention and also shedding light into the possible mechanisms of resistance to ongoing therapy. Limitations of ctDNA assessment include the lack of standardization of isolation techniques, cost ineffective, non-feasibility for proteomic & miRNA assessment and no information about tumour heterogeneity.

**Exosomes (EXOs)**

Exosomes are nano-sized vesicles (40–100 nm) released by cells. EXOs are end products of the recycling endosomal pathway and originate from inward budding of the plasma membrane. These are detectable in most body fluids such as plasma, urine, saliva, ascitic fluid etc. EXOs consist of a lipid bilayer which contains both transmembrane and non-membrane proteins, as well as noncoding RNAs, mRNAs, and either single-stranded or double-stranded DNA. They are characterized by a conserved set of proteins independent of their cellular origin; such as- CD63, CD81, and CD9 tetraspanins (can be used for isolation).

**Exosomes Isolation Methods**

There are several methods for isolating exosomes from body fluids. These techniques majorly rely on the principle of vesicle separation in accordance with their biophysical properties which includes size, morphology and density. Other methods of isolation are based on the immunooaffinity capture or altering exosomes solubility to improve their precipitation. To recognize specific exosomal surface antigens, immunocapture approaches, such as microplate-based enzyme-linked immuno-sorbent assay or antibody-coated magnetic particles are used for selective isolation of exosomes. In recent times several user-friendly kits are commercially available which are based on the principle of exosomal precipitation in the presence of water-excluding polymers, but there is a disadvantage with these kits of lacking proper selective isolation mechanism which compromise the purity of yields.

**Clinical Applications of Exosomes**

Tumour-derived exosomes are investigated for their protein expression or genetic profile for revealing diagnostic or prognostic markers after their isolation and investigation by electron microscopy or flow cytometry assays. CAV1+ EXO have been proposed as potential diagnostic markers of melanoma and pancreatic ductal adenocarcinoma patients who are more likely to develop liver metastasis can be identified by detection of serum exosomes enriched with high levels of migration inhibitory factor (MIF), acting as an adverse prognostic factor. Exosomes from prostate cancer cells, collectively called prosastomes comprising PSMA, prostate-specific transglutaminase and prostate stem cell antigen, are detected by prostate-specific membrane proteins, whose serum and urine levels directly correlates with tumour burden or advance stages. Exosomes are also enriched with single- or double-stranded DNA fragments from all chromosomes which are subjugated to detailed examination can reveal specific genetic mutations, for example KRAS and EGFR mutations that are already proven in pancreatic cancer and colorectal cancers.

**CONCLUSIONS**

Liquid biopsy has recently emerged as a highly promising method to diagnose and monitor cancer. Liquid biopsy provides the real time picture of genetic makeup of cancer cells and is capable of generating valuable information about the genetic features of individual tumours, thus playing a role in early detection, prognosis, and monitoring the treatment responses, residual tumour and development of resistance. A more detailed understanding of the biology behind ctDNA, CTCs and exosomes is required to understand if the molecular profiles generated from these sources truly reflect the physiological disease state of the patient and if they can help reliably to detect and monitor the cancer.

**REFERENCES**


