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SURGERY

VOLUME 19

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Recent Advances in SURGERY

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Puneet

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CHAPTER 7

Liquid Biopsy: The Future of Cancer Detection

Pranab Dey

INTRODUCTION

Since decades, the surgeons are habituated to do excision and incisional biopsy for histopathological evaluation of the tissue. The biopsy is considered the gold standard for the diagnosis and classification of the tumor. The biopsy is often difficult to collect as it is an invasive technique. The repeated surgical biopsy is also not convenient for continuous monitoring of the patient. Moreover, many times the early tumor is difficult to detect as they may not be visible by radiology or other techniques. Recently, a newer technology known as "Liquid biopsy"[1,2] is becoming popular.[1,2] It is defined as "A type of laboratory test which is done on the blood, urine, or other body fluids to study the circulating cancer cells or circulating DNA, RNA or other molecules that are released by the tumor cells."[3] Unlike conventional biopsy, the liquid biopsy can supply vast amount of essential information regarding early tumor diagnosis, detection of minimal residual disease (MRD), identification of drug resistance, personalized therapy, monitoring of therapy, and early recurrence. It is now the cornerstone of the oncology service, particularly in precision medicine. The current study is focused on the detection and isolation of the various components of liquid biopsy along with its precise clinical applications.

CONVENTIONAL BIOPSY VERSUS LIQUID BIOPSY: WHY TO DO LIQUID BIOPSY?

In case of conventional biopsy, the surgeon takes incisional or excisional biopsy from the tumor tissue **(Table 1)**. The surgical technique is invasive and needs specialization for the procurement of deeper tissue from the internal organ. Unlike the conventional biopsy, the liquid biopsy is totally noninvasive and can be repeated at different intervals. So, it may detect the emergence of treatment resistant clone. The liquid biopsy can be done without the presence of visibly detected mass. The conventional biopsy represents the part of the tumor tissue that has been taken for examination. The tumor is usually heterogeneous that means the distinct genetic and other molecular techniques in the different areas of the tumor and biopsy may not represent the true nature of the tumor. In contrast, the liquid

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biopsy avoids heterogeneity of the tumor. In the deeply situated tumor, the liquid biopsy is very effective as there is no need to access the tumor mass because simple collection of blood and/or body fluid contain the circulating tumor cells (CTCs) or circulating DNA, RNA, or other molecules is enough. So, the liquid biopsy has emerged as a game-changer in cancer diagnosis and management.

The difference between the conventional and liquid biopsy is mentioned in **Table 1** and **Figure 1**.

TABLE 1: Conventional biopsy versus liquid biopsy.

Features	*Liquid biopsy*	*Conventional biopsy*
Technique	Noninvasive	Invasive
Collection of sample	Indirect. Collection of blood, urine, or body fluid	Direct. The tissue is directly collected from the tumor
Presence of tumor	No need of visible tumor	Visible tumor is needed
Multiple sampling	Multiple sampling at different time intervals is possible	Multiple sampling is not always possible
Tumor burden	Tumor burden is directly proportional to size of the primary tumor.	Quantity of CTC/ctDNA is directly proportional to tumor burden
Tumor heterogeneity	Not affected by tumor heterogeneity	Affected by tumor heterogeneity
Mode of processing	Difficult to process and needs special skill	Easy to process
Cost	Very costly	Low cost

(CTC: circulating tumor cell; ctDNA: circulating tumor DNA)

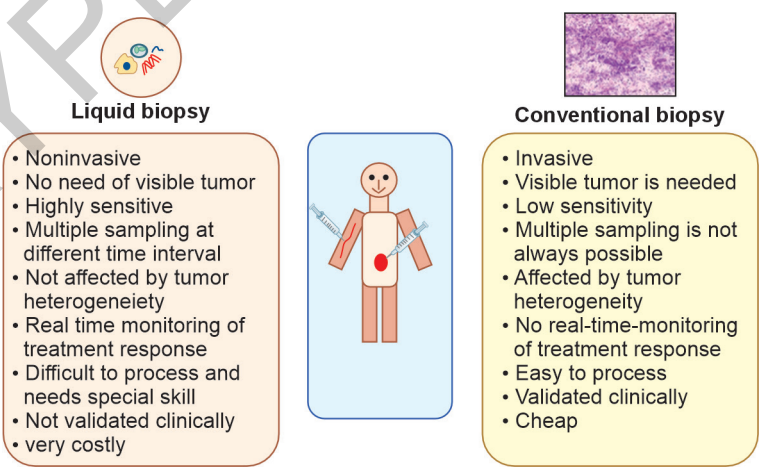

Fig. 1: Comparison of liquid biopsy and conventional biopsy.

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LIQUID BIOPSY: CONSTITUENTS

Sample: The liquid biopsy samples are taken from (1) Blood, (2) Broncho-alveolar lavage, gastric lavage, urine, effusion fluid, sputum, or any other body fluids.

The components of liquid biopsy are as follows **(Fig. 2)**:

- Circulating tumor cells
- Circulating tumor DNA (ctDNA)
- Cell-free DNA (cfDNA)
- Cell-free RNA (cfRNA)
- Extracellular vesicles (EVs)

Circulating tumor cells: In recent years, it has been noted that the initial level and rising number of CTC is related with the evolvement of cancer and metastasis. CTCs are heterogeneous in nature and carry the specific genetic characteristic of the tumor cell. CTC comes to blood circulation mainly from primary tumor tissue. Occasionally it is derived from the metastatic site. CTCs have a very short life span of 1–2 hours and they are quickly cleared by the liver and spleen. CTCs are remarkably less in blood circulation ranging from 1 to 10 CTCs per mL of blood.[4] It is a challenge for the pathologists to isolate CTCs from the circulation. The isolation of CTC is done by many complex technologies. The presence of the cell surface antigen is an important way to recognize the tumor cells and isolation. The cell membrane of CTCs contains epithelial cell adhesion molecule (EpCAM). The antibody against EpCAM binds to the cell membrane of CTCs and so helps to isolate CTCs.

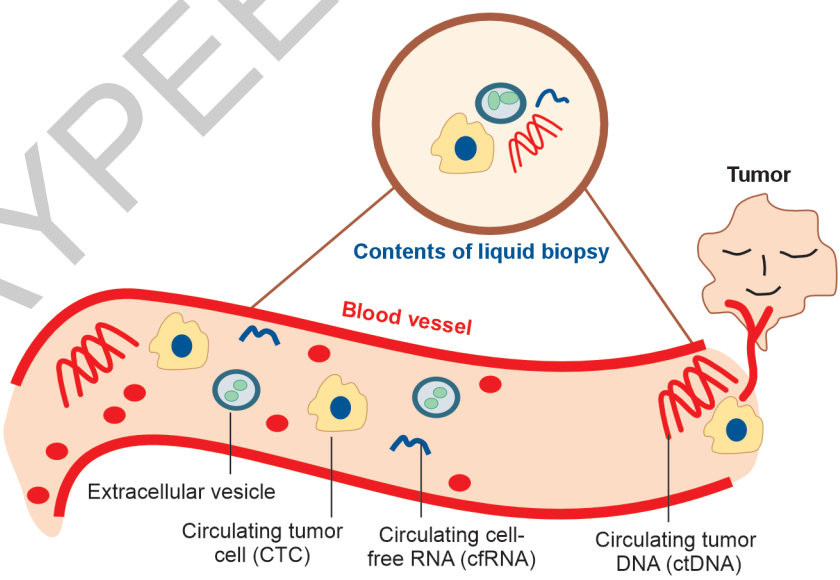

Fig. 2: Schematic diagram showing major constituents of the liquid biopsy.

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Circulating tumor DNA: ctDNA comes to the circulation by cell death (both apoptosis and necrosis) and active secretion by normal and cancer cells. ctDNA is a type of circulating extracellular nucleic acids derived from tumor. The concentration of ctDNA is only <0.01% of total cfDNA and constituted of small DNA fragments (180–200 bp). It has a short half-life of few minutes to couple of hours because it is rapidly cleared from circulation through nucleosomes in the liver (liver macrophages), or by circulating nucleases and in kidney. Circulating tumor DNA (ctDNA) is one of the important components of liquid biopsy. ctDNA may be either double-stranded or single-stranded and carries all the genetic information as that of CTCs. The rising level of ctDNA is an important marker of metastasis. ctDNA shedding in circulation depends on various factors such as tumor site, disease burden, and tissue cell turnover.

Cell-free DNA: cfDNA is primarily derived from normal leukocytes and stromal cells, released in the blood circulation. Unlike ctDNA, the cfDNA are derived both from normal and tumor tissue. They also may be nuclear or mitochondrial origin.

Circulating cfRNA: cfRNA consists of messenger RNA (mRNA), microRNA (miRNA), long non-coding RNA (lncRNA), small nuclear and nucleolar RNAs (snRNA, snoRNA) and circular RNA (circRNA). This cfRNA is actively released from the tumor tissue and also from apoptotic and necrotic cells.

Extracellular vesicles: Extracellular vesicles are membrane covered particles of size around 30–120 nm in diameter. The vesicles have lipid bilayer membrane and carry proteins, DNA, RNA, small non-coding RNAs [microRNA (miRNA)], and lipids. There are three types of EV: (1) exosomes, (2) microvesicles, and (3) apoptotic bodies. In recent years, the exosomes and its products (nucleic acid and metabolites) are the focus of research attraction.

Tumor-educated platelets (TEPs): The platelets in cancer patients have a different RNA and protein profile that are called TEPs. TEPs may be related with metastasis and progression of solid tumors. It may be used as one of the components of liquid biopsy. At present, the exact mechanisms of the involvement of platelet RNA are incomplete and further research is needed.

microRNA and Long Non-coding RNA

The miRNA is a small single-stranded RNA molecule that gives important information in liquid biopsy. lncRNAs are not associated with transcripts. lncRNAs are related in the development of many cancers. In future, lncRNAs may be used in liquid biopsies.

Circular RNA

Circular RNAs are circular in shape with closed ends. These are also nonprotein-coding transcripts and resist degradation by nuclease enzyme.

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CircRNAs work as proto-oncogenes, and are involved in the growth, proliferation, invasion, and metastasis of cancer.

Circulating Tumor Cell Enrichment: Searching "a Needle in Haystack"

In the advanced cancer, the concentration of CTC in the blood is about 1 CTC per 10^7 of WBC. So, it is a challenge to find out and collect CTC from this extremely low concentration.[5] CTC enrichment can be done by: (1) on the basis of immunological method, (2) physical properties, and (3) microfluidic immunocapture. **Table 2** highlights the different types of cell enrichment technique.

- *Immunological method:*
  - *Positive selection:* CTCs are usually epithelial cells that are coated with EpCAM. So, antibody against EpCAM is used to identify and subsequently separation of CTC. The magnetic nanoparticles coated with anti-EpCAM antibodies are used **(Fig. 3)**. Subsequently, the

TABLE 2: Different types of cell enrichment technique.

Category	*Commercially available techniques*	*Mode of enrichment*	*Capture efficiency (%)*	*Comments*
Immunological	Cell search, Adna test, magnetic-activated cell sorting (MACS test)	Target antibody against epithelial cell adhesion molecule (EpCAM) is used to separate the cells	40–90	• Only EpCAM-positive circulating tumor cells (CTCs) are detected • Cell search system is Food and Drug Administration (FDA) approved
Physical property based Density based	• OncoQuick • AccuCyte	• Density separation • Buffy coat preparation to separate CTCs	• 25–80 • 90	• Low recovery rate • Multiple samples can be processed
Microfluidic immuno-capture	• CTC-Chip • HB-Chip geometrically enhanced differential immunocapture (GEDI chip)	The sample flows in an antibody coated channel to capture the CTC	60–90	Only EpCAM-positive cells are recovered

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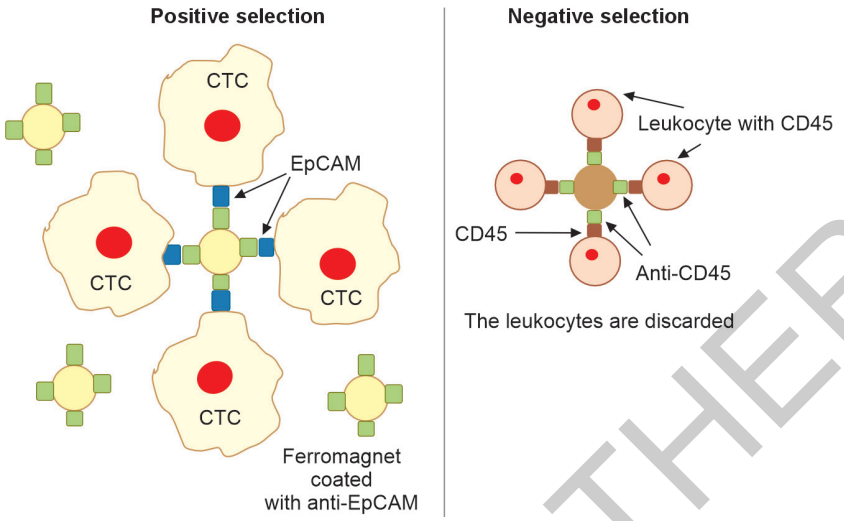

Fig. 3: Immunological method of cell enrichment. In positive selection, magnetic nanoparticles coated with anti-epithelial cell adhesion molecule (anti-EpCAM) antibodies are used to capture the tumor cells. In negative selection, the CD45 positive cells are removed from the sample.

magnetic field is applied to isolate those CTC. Cell-search system is based on this principle.

- *Negative selection:* The leukocytes have CD45 antibodies on the surface and so by using anti-CD45 antibody, they can be isolated **(Fig. 3)**.

- *Physical properties:* The CTCs can also be separated on the basis of various physical properties such as large size, deformation of CTCs, density, and surface electrical charges **(Fig. 4)**.
- *Microfluidic-based enrichment:* The sample flows in an antibody-coated (anti-EpCAM) channel to capture the CTC **(Fig. 4)**.

ANALYSIS OF LIQUID BIOPSY: MOLECULAR TECHNIQUES

The different types of molecular techniques are shown in **Flowchart 1**.

Genetic Study

Mutational detection: The demonstration of the mutation in ctDNA provides valuable information. The following techniques are used to detect the mutations.

Polymerase Chain Reaction-based Technologies

- *Droplet digital polymerase chain reaction (ddPCR):* It is an extremely sensitive technique which can quantify the target nucleic acid with high precision.[6]

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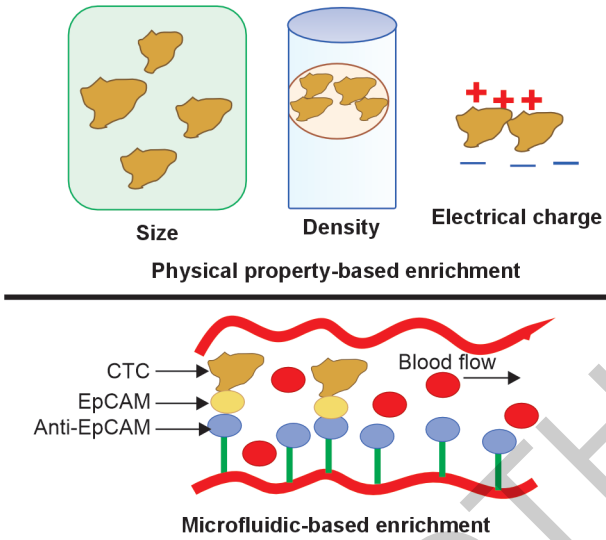
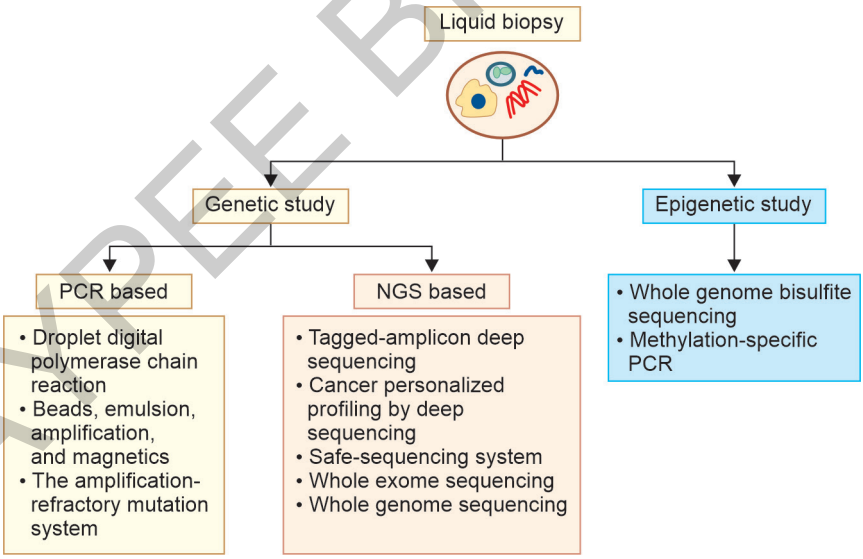


Fig. 4: Physical and microfluidic-based technique of cell separation.
(CTC: circulating tumor cell; EpCAM: epithelial cell adhesion molecule)

Flowchart 1: Different technologies to detect genetic and epigenetic abnormalities in liquid biopsy specimen.



(NSG: next-generation sequencing; PCR: polymerase chain reaction)

- *Beads, emulsion, amplification, and magnetics (BEAMing):* In this technology, the PCR is combined with flow cytometry. BEAMing is also very sensitive and can identify 0.01% mutation.[6]

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- *The amplification-refractory mutation system (ARMS)*: ARMS can identify the single base change mutation or deletion of DNA.[7]

Next-generation Sequencing-based Techniques

- *Tagged-amplicon deep sequencing (TAm-Seq):* TAm-Seq uses unique tag (barcode) to label DNA molecule. It is a very specific and sensitive technique and can low allele frequencies (0.1–2%).[8]
- *Cancer personalized profiling by deep sequencing (CAPP-Seq):* CAPP-Seq is a hybrid-capture-based targeted deep sequencing. It is very powerful NGS technique and can detect mutations in ctDNA with high sensitivity and specificity.[9]
- *Safe-sequencing system (Safe-SeqS):* Safe-SeqS has ultra-high sensitivity and specificity and it is able to detect mutations at <0.1% allele frequency.
- *Whole exome sequencing (WES):* WES captures all the exons (coding) regions of the DNA. It is relatively less sensitive. However, it provides the comprehensive mutation profiling.[10]
- *Whole genome sequencing (WGS):* WGS sequences both coding and noncoding regions of ctDNA. It has low sensitivity in detecting point mutations.

Epigenetic Study

Whole-genome bisulfite sequencing (WGBS-Seq): It is considered as gold standard method for assessing DNA methylation. It gives epigenetic profiling of ctDNA.[11]

Methylation-specific PCR (MSP): MSP can do rapid detection of DNA methylation at specific CpG sites of ctDNA. It is highly specific and of low cost.

Flowchart 1 shows different technologies to detect genetic and epigenetic abnormalities in liquid biopsy specimen.

- *Study of RNA (miRNA and small RNA):* These studies are done in exosomes by quantitative reverse transcriptase PCR.

POTENTIAL CLINICAL APPLICATIONS OF LIQUID BIOPSY

The potential applications of liquid biopsy are following:

- *Early diagnosis and screening of cancer:* Early diagnosis of cancer before any symptoms, visible tumor, imaging in radiology, and tumor biomarkers is of intense clinical interest. Is it possible that the liquid biopsy can fulfil this expectation? The main challenges in early detection by liquid biopsy are as follows:
  - Low shedding of tumor cells in the circulation and extremely low amount of ctDNA in the early cancer

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- Limited cancer-specific mutations
- Demonstration of driver gene mutations in aged individual has added complication.[12]

Early detection of cancer and cancer screening can be done by the following ways:

- *Quantitation of cfDNA:* There was attempt to assess the presence of cancer by quantitating cfDNA/ctDNA in the blood. Numerous studies have shown the raised cfDNA/ctDNA level in the cancer patients compared to normal healthy persons.[13-15] In a meta-analytic study on prostate carcinoma, it was noted that the sensitivity and specificity of quantitative cfDNA is higher than serum prostate-specific antigen (PSA). So, cfDNA can be for prostate cancer screening.[16]

  The sensitivity of cfDNA to detect various cancer varies widely from 50 to 90%. The wide range of variation of sensitivity may be due to the mode of detection techniques and variable cut off value for the diagnosis in different studies. Moreover, cfDNA concentration may also depend on the tumor type, tumor volume, and metastasis.[17] So to determine threshold of the cancer detection needs more studies. The large case–control studies are needed before the clinical application of cancer detection by cfDNA quantitation.
- *Tumor-specific mutation:* The cancer specific DNA mutation has been highlighted by various studies. The detection of those specific mutational changes may be helpful in early diagnosis of cancer or cancer screening **(Table 3)**.[18-23] In addition, epigenetic changes such distinct DNA methylation patterns, DNA hydroxymethylation, DNA fragment length are also related in identification of cancer.[24]
- *Tumor-specific miRNA and s-RNA:* Tumor-specific miRNA and s-RNA are helpful in the detection of carcinomas.[23] Mensah et al.[25] studied miRNA in plasma by microfluidic cards and quantitative real-time PCR for screening of lung carcinoma cases. They suggested miRNA-based liquid biopsy in lung cancer screening. Association of several small RNAs is found with colorectal adenomas and can be used as useful biomarker to detect colorectal adenoma.[24]

Recently, the Food and Drug Administration (FDA) has approved some liquid biopsy tests for the cancer screening, such as the Epi proColon® for colorectal cancer (CRC). Epi proColon® detects methylated *SEPT9* DNA in CRC. The Epi proColon® is a qualitative test and uses real-time PCR. It is recommended in the patients with high risk of CRC and those patients who have not undergone in CRC screening.[26]

The CancerSEEK helps in screening of eight cancers (breast, colon, esophagus, lung, liver, ovary, pancreas, and stomach). It detects the mutation of 16 genes and combines both genetic and protein biomarkers analysis.[27]

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TABLE 3: Cancer-specific mutation.

Tumor	*Common mutations*	*Early diagnosis and screening*	*Early diagnosis and management*	*References*
Lung	EGFR, KRAS, ALK, BRAF, and TP53	*SHOX2* gene hypermethylation is related to screening of squamous cell carcinoma	• *EGFR T790M mutation in NSCLC:* Resistance to EGFR inhibitor therapy • *Greater expression of Ep-CAM, PIK3CA, AKT2, TWIST* and *ALDH1*: Metastasis and aggressiveness	19
Breast cancer	PIK3CA, TP53, ESR1, and HER2 (ERBB2)	*PIK3CA* gene mutation: Associated with early diagnosis of breast cancer	*PIK3CA* gene mutation: Resistance to HER2-targeting therapy	20
Colorectal cancer	APC, KRAS, BRAF, TP53, and PIK3CA	Methylated SEPT9 related with early cancer screening[22]	Mutated *KRAS* genes may escape anti-EGFR therapy[23]	21
Prostate cancer	AR, TP53, PTEN, and BRCA1/2	*GSTP1 promoter methylation:* For prostate cancer screening	Androgen receptor L702H and AR-T878A mutations related with acquired drug resistance	22

- *Targeted therapy by assessing specific molecular alterations:* In case of solid tumor, the demonstration of certain mutations indicates targeted chemotherapy. CTC and ctDNA/cfDNA contain all the molecular footprints of the tumor. So instead of going for traditional conventional biopsy, these mutations can be demonstrated by liquid biopsy. FDA has given approval for several tests on ctDNA to detect the actionable mutations. In cases of carcinoma of unknown primary (CUP), the actionable genetic mutation in ctDNA of liquid biopsy has been demonstrated in more than two-thirds of the patients, and so, they can be treated by specific therapy.

  The Food and Drug Administration (FDA) has approved several diagnostic tests on ctDNA such as Cobas EGFR mutation test V2 in non-small cell lung cancer, the therascreen PIK3CA RGQ PCR kit to detect PIK3CA mutation in carcinoma of the breast, and Guardant 360 for the detection of molecular profiling. It is a challenge to decide on chemotherapy protocols in the CUP and many times empirical chemotherapy regimens are given.

  Kato et al.[28] studied ctDNA alteration of the CUP by NGS. They noted that 66% of patients had pathogenic alterations and almost all the patients had actionable alterations.[28] The demonstrations of actionable genetic mutations have also been highlighted in liquid biopsies of esophageal carcinoma, gastric adenocarcinomas gynecological cancers, and non-small cell lung carcinomas. Therefore, liquid biopsy plays a significant role in individualized or precision medicine therapy.[29-32]
- *Monitoring the therapy:* Liquid biopsy plays an important role in monitoring the chemotherapeutic response and also the assessment of drug resistance. The fall of CTC count is directly related with the adequate response of chemotherapy.[33] Variant allele frequency (VAF) indicates ratio of DNA fragments with the specific mutation to the total number of DNA fragments at that locus. The higher pretreatment VAF is related with reduced survival and lower on-treatment VAF means the good chemotherapy response and better survival.[34] Genome instability number (GIN) refers to the degree of genomic instability in a tumor. Continuously reduced level of GIN measured in ctDNA indicates the good response to chemotherapy.[35]
- *Detection of drug resistance:* In colorectal cancer, the clonal evolution of the mutated *RAS* gene is responsible for resistance of anti-EGFR antibody therapy.[36] In breast carcinomas, PIK3CA gene mutation is associated with resistance to HER2-targeting therapy.[20] The loss of function PTEN mutations and ESPR1-activating mutations cause resistance to PI3K-α inhibitor therapy.[37] In non-small cell lung carcinoma cases, EGFR T790M mutation causes resistance to anti-EGFR therapy.[19] The expression of AR-V7 splice variant on the CTCs of metastatic prostate cancer patients strongly predicts the resistance to abiraterone and enzalutamide.

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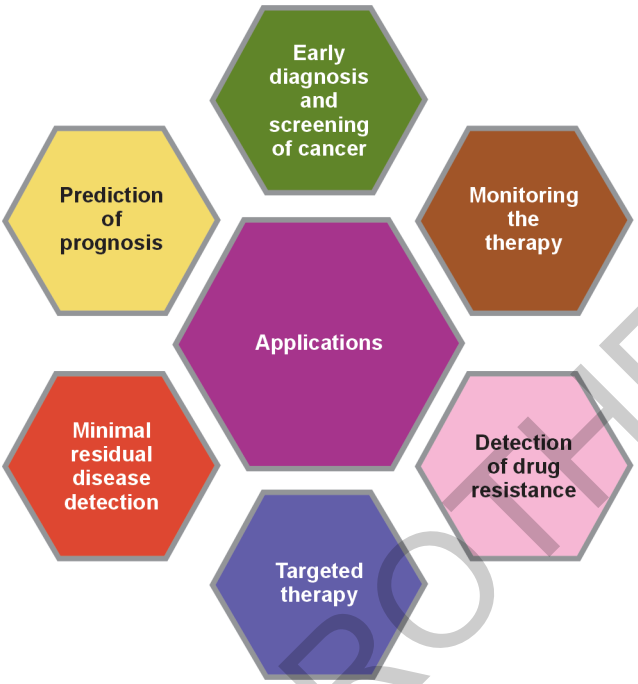

Fig. 5: Major applications of liquid biopsy.

- *Identifying MRD:* Liquid biopsy is useful in the detection of MRDs in various carcinomas.[38,39] Real-time monitoring of CTC and cfDNA may be helpful to detect MRD. Interestingly, the molecular relapse is demonstrated many months before the detection by standard radiological imaging or other biomarkers.[40]
- *Prediction of prognosis:* The preoperative and postoperative level of CTC and ctDNA/cfDNA may help in prognostic assessment. The increased number of CTC in the postoperative period indicates poor disease-free survival.[41] In a study with a variety of cancer, the patients with more than 5% *ctDNA* gene alteration had poor prognosis.[42] Certain mutations such as KRAS hotspot mutations or hypermethylation of CDKN2A in colorectal carcinomas have bad prognosis.[43] **Figure 5** highlights the major applications of liquid biopsy.

Circulating tumor DNA detection predicts the response of drugs such as palbociclib and fulvestrant in metastatic breast cancer.[44] ctDNA detection can also predict the response of neoadjuvant chemotherapy and prognosis in patients with early breast cancer. In patients with advanced pancreatic cancer on therapy, decreases of KRAS mutant ctDNA are considered as early tumor response.

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CHALLENGES IN THE IMPLEMENTATION OF LIQUID BIOPSY: ARE WE READY?

Liquid biopsy is a noninvasive technique that has intense impact of cancer diagnosis, treatment, monitoring, and prognostic prediction. In spite of its vast potential, liquid biopsy faces several technological, biological, and clinical challenges **(Table 4)**.

- *Extremely low concentration:* As mentioned repeatedly, the concentration of ctDNA and CTC is extremely low. The concentration of ctDNA may be <0.01% of cfDNA. Therefore, there may be problem in distinguishing real mutation versus background noise.
- *Lack of standardization:* There are considerable variations in the collection of sample, processing, and interpretation. This may be responsible for significant variation of the result, and so it is difficult to compare results across the laboratories.
- *Fallacy in CTC detection:* CTC is usually detected and isolated on the basis of surface EpCAM of the tumor cells. However, many tumor cells may not show surface EpCAM expression.
- *Sensitivity and specificity:* The sensitivity of detecting ctDNA or CTCs on liquid biopsy is low particularly in low tumor burden and early-stage cancers. Thus, the chances of false-negative reporting are high. The presence of ctDNA from benign tumors and other nonmalignant conditions are responsible for false-positive reporting.
- *Tumor heterogeneity:* The liquid biopsy may not fully capture the tumors genetic heterogeneity and thus, it may lead to incomplete profiling and inappropriate treatment planning.

TABLE 4: Challenges and solutions of liquid biopsy.

Challenges	*Impact*	*Solution*
Extremely low concentration of ctDNA and CTC	Reduction of sensitivity	Deep sequencing
Lack of standardization	Reproducibility is low	Written strict guidelines
Fallacy in CTC detection	Low sensitivity	Multiple epithelial markers
Clonal hematopoiesis of indeterminate potential (CHIP)	Misinterpretation as tumor mutations	Parallel sequencing of WBC to filter out CHIP mutations
Clinical validation	Slow acceptance	More large prospective real-world study
Cost	Reduced accessibility	Cheaper and cost-effective techniques

(CTC: circulating tumor cell; ctDNA: circulating tumor DNA)

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- *Clonal hematopoiesis of indeterminate potential (CHIP):* The presence of driver gene mutations in elderly individuals may be misinterpreted as tumor mutations.[13]
- *Cost and accessibility:* Liquid biopsy is very costly and is inaccessible to many laboratories.
- *Clinical validation:* Many techniques need large-scale prospective validation before its clinical applications.
- *Ethical and psychological concerns:* The application of liquid biopsy in cancer screening is a debatable issue. As no visible tumor is detected, so, the management may be difficult to decide. Informed consent explaining the implications of the liquid biopsy and its limitations should be explained to the patient and also crucial for ethical practice.

CONCLUSION

In conclusion, the liquid biopsy is a cutting-edge technology that has vast potential. This is a noninvasive technique, and the samples are easy to collect independent of the visible tumor. The potential clinical applications of liquid biopsy are the cancer screening, monitoring therapy, precision medicine for individual patients, the detection of MRDs, prognosis prediction. The liquid biopsy provides the sequential information of tumor life and provides new insight of the disease. Currently, there is a great need of strict guidelines in the liquid biopsy. The guidelines are helpful mainly for the maintenance of the standard and interpretation of its results, such as European Society for Medical Oncology (ESMO) recommendations[45] and National Comprehensive Cancer Network (NCCN) guidelines.[46] In spite of huge potential, it is not implemented in the laboratories because of technical complexity, lack of standardization, prospective validation, and high cost. We expect that the rapid progression of technology will make the liquid biopsy cheap and accessible to the laboratories.

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