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Editorial

Liquid Biopsy : An emerging concept for diagnosis and management of cancer

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Tumour diagnosis is conventionally done by radiological findings and invasive surgical biopsy. Of late non-invasive technique where blood sample, urine and body fluids are used to extract circulating tumour cells (CTC) and genetic material for cancer diagnosis and treatment which is called as "Liquid Biopsy".[1,2] In this technique the liquid sample is used to isolate CTC, circulating tumour DNA (ctDNA), RNA, Exosomes and proteins which are shed by tumour cells into blood circulation, body fluids or urine in most of the cancers depending on the site of the cancer. This technique enables non-invasive profiling of solid tumours, the results which can be comparable with that of tissue biopsy.[3,4,5,6,7] As tissue biopsy is single biopsy, it gives only spatially and temporary snap shot of genetic makeup of cancer tissue unlike liquid biopsy, where samples can be taken at repeated intervals and it reveals the dynamic and heterogeneity of the cancer tissue.[8] Originally liquid biopsy was used to analyze CTC. At present it mainly analyzes ctDNA. However CTC and ctDNA are complementary technologies which can be used in parallel. As ctDNA is a potential surrogate for the entire tumour genome, it is many times referred as "Liquid Biopsy".[9,10] The different components of liquid biopsy are CTC, ctDNA, RNA, Exosomes, Proteins and Platelets.

Liquid biopsy has evolved slowly at different phases. Scientists isolated tumour cells in blood for the first time in 1869.[11] In 1948 Mandel and Metais isolated ctDNA and RNA in blood in healthy individuals. The neoplastic characteristics of these genetic material was defined after 30 years by Leon et al and 10 years later by Stroun et al.[8,10] The CellSearch technique to isolate the CTC was approved by US FDA in 2004.[11] Tissue DNA test was available by 2012.[7] Exosome diagnostic laboratory launched CLIA certified test for isolation of exosomes in 2015.[12] US FDA approved Cobas EGFR Mutation test v2 in 2016.[2]

Circulating Tumour Cells (CTC)

CTC are defined as cells having viable nucleus and shows positive expression for cytokeratin, epithelial cell adhesion molecule (EpCAM) and negative expression for CD45. They can be isolated in healthy persons and in patients with non-malignant lesions. However it is more common in cancer patients. In cancer patients it arises either from primary tumour or from the metastatic sites. The tumour cells enter into blood circulation by active and passive mechanism. The CTC may express down regulation of EpCAM and other epithelial markers indicating epithelial mesenchymal transition (EMT), become discohesive and acquire the property of motility by which it enters the blood vessels by active mechanism. Once the cells reaches the organ at distant site, the tumour cells either remain with mesenchymal cell property or undergo mesenchymal epithelial transition (MET) to proliferate in the new microenvironment. If CTC get frozen in mesenchymal state, it cannot proliferate at metastatic site unless and until it transforms into epithelial state. The CTCs having maximum EMT / MET are highly aggressive having property of increased potential of tumour dissemination and tumour growth at metastatic deposits. The tumour cells may also enter the blood vessel by passive mechanism travelling across basement membrane and endothelial cells, reaching the organ at distant site. The tumour cells may form tumour emboli within the lumen of the vessel and block the blood supply.[1,9,13,14]

The CTC once reaches the distant organ may remain dormant for a few years, proliferate in the microenvironment or enter into blood circulation and form disseminated tumour cells (DTC). The DTC may again get deposited in various organs or may reach the primary site and are called as "tumour self-seeding" or "cross seeding". These cells are highly aggressive, start proliferating and present as local recurrence.[13]

CTC provides information at both cellular and genetic level.[1] Studies have reported CTCs in some COPD cases, where the patients presented with lung cancer later within 1-4 years.[15] Metastatic tumour deposits more likely releases the CTCs amenable to

isolation. However their frequency is low, i.e. ~1-10 CTCs per mL of whole blood. CTCs should be isolated immediately from the blood sample as CTCs are fragile and tend to undergo degradation especially when collected in standard evacuated blood collection tubes. Hence it is advisable to drawn blood into the Cellsave preservative tube which is a component of CellSearch test. The CellSearch CTC test, a US Food and Drug Administration (FDA) approved test, where the blood sample is processed within 96 hours of collection to prevent degradation of CTCs.[14] CTCs are isolated and enriched by various techniques as CTCs occur in very low concentration with millions of blood cells in blood samples. However it needs to be validated to consider for clinical application.[13] Biomarkers are detected on cell surface which helps in treatment and assess the prognosis. [6] Detection of EMT or MET in CTCs is a challenge as Vimentin biomarker is positive in blood cells and hence cannot be used as EMT / MET marker.[13]

CTC in liquid biopsy is real time test, which demonstrates specific sub-population of cells which revolutionize cancer detection, management and targeted therapy. Metastatic cells (DTC) has specific genotype and phenotype characteristics compared to primary tumour (CTC) as metastatic subclone are smaller in size and has additional genomic characteristics over time. Analysis of CTCs and metastatic cells (DTCs) in specific is required to stratify patients for therapy as metastatic cells may escape from targeted therapy.[13] CTCs are also useful to extract DNA, RNA and proteins for further analysis of cancer cells.[9]

Circulating Tumour DNA (ctDNA)

Majority of circulating DNA is often not of cancerous origin. Increased concentrations of circulating DNA have also been detected in physiological and noncancerous pathological conditions. ctDNA is an effective alternative of CTCs as it is easily isolated and analyzed. It is derived by primary tumour or metastatic tumour by active or passive mechanism. The active mechanism is, some tumour cells spontaneously release small pieces of genetic material or DNA into circulation. The passive mechanism is ctDNA is released by tumour cell apoptosis or necrosis. The ctDNA is also derived by rupture of CTCs in circulation. A single human cell contains 6 pg of DNA and there is an average of 17 ng of DNA per ml of plasma in advanced stage cancers. Therefore, if CTCs were the primary source of ctDNA it would require over 2,000 cells per ml of plasma. In reality, there are on an average less than 10 CTCs per 7.5 ml of blood. Hence ctDNA is derived by other mechanisms other than the CTCs. The genetic mutation vary from time to time in a single tumour and also vary from person to person.[1,2,8,10] ctDNA helps to know the level of ctDNA tumour specific mutation and its structural changes.[16] The ctDNA is analyzed for tumour-associated genetic aberrations, including somatic mutations, loss of heterozygosity and chromosomal aberrations.[8]

Normal cells DNA are roughly 100–200 base pairs long, and are still wound around proteins called histones. Length of the DNA may indicate the organ from which it is derived. Tumour DNA (like fetal DNA) is shorter than normal blood DNA fragments. High degree of fragmentation of ctDNA occurs through apoptotic mechanisms. Large concentrations of very short fragments have been linked to metastasis. This genetic information helps clinicians to make an informed guess as to where to look for a tumour. Clearance mechanisms of ctDNA seem to be rapid, is executed by spleen, liver and kidneys. [10,11,14]

ctDNA alterations are noted in very low concentration with the frequency of less than 0.4% of total DNA in the circulation. But the accuracy of liquid biopsy remains high.[12] The optimal DNA concentration for the detection of mutations (using a Sequenom® Mass ARRAY® platform) is approximately 30ng/ml in the plasma.[8] ctDNA is not detectable in all tumours uniformly. It is approximately detectable in 82% of all solid tumour outside brain. It is detectable in less than 50% of cases of meduloblastoma, metastatic cancers of kidneys, prostate, thyroid and less than 10% of cases in gliomas. ctDNA is detectable in 55% cases of localized tumours of any type. ctDNA is detected in 75% cases of advanced cancer of ovary, colon, rectum, bladder, gastroesphageal tract, pancreas, breast, liver, head & neck and melanomas. Detectable cfDNA levels increases with tumour stage with mutated DNA fragment.[5,16]

The cfDNA is highly informative. Pattern of genetic changes in ctDNA is comparable to that of tissue biopsy DNA as 94 – 100%.[12] In a study, ctDNA sensitivity clinically was 86%, 83%, 85%, and 78%, in lung, breast, colorectal and other cancers respectively which correlated with those in tissue biopsy except resistance mutations. The overall accuracy of ctDNA sequencing comparing that of tissue was 87% and it was 98% when the interval between tissue biopsy and blood sample was less than six months.[4] Concordance between primary tumour tissue and plasma DNA is reported as 95% to 100% in one study.[8] Abnormalities or mutations in EGFR, BRAF, ALK, KRAS, RET and ROS1 in liquid biopsy was comparable with that in tumour tissue biopsy.[12] Detec-

tion of KRAS, NRAS and BRAF mutation with high levels of ctDNA indicates decreased survival of colorectal carcinoma (87.2% sensitive, 99.2% specificity) and this was associated with resistance to EGFR antibodies.[16] Emergence of KRAS mutations is a mediator of acquired resistance to EGFR blockade.[13]

RNA

RNA is highly fragile. Cell-free RNA in blood get immediately degraded. RNA is released into the blood by tumour cells as microvesicle dependent or independent mechanism. RNA are stable in microvesicle. The RNA can be analyzed as ctDNA quantitatively or qualitatively for various aberrations which help in diagnosis, progression and prognosis of cancer. Mutations in RNA can be detected in cancer and is strongly linked to cancer development and progression.[14]

Exosomes

Exosomes are actively released tiny vesicles (carrying RNA, DNA and protein) that are shed by all living cells, including tumours. Tumor cells can shed tens of thousands of vesicles into circulation. They are synthesized by two mechanisms. In classical exosomes biosynthesis, first the endosome is formed with inward bulging into the cytoplasm resulting in multivesicle bodies having many intraluminal vesicles containing cytoplasm of the cells having nucleic acids and proteins. The vesicles are liberated from surface of cells by fusion of multivesicle bodies with plasma membrane. In second method, the exosomes are synthesized as out pouching of cytoplasmic membrane which get detached from cytoplasmic membrane subsequently leading to release of membrane bound vesicles.[11, 14] The size of exosomes ranges from 30-200 nanometers in diameter. They can be isolated from all biofluids, including serum, plasma, saliva, urine and cerebrospinal fluid. They are stable carriers of genetic material and proteins from their cell of origin. They are highly stable packages of RNA which are used for detection of mutation. DNA is also a component of exosomes.[14]

The exosomes act as inter-cellular Messengers. They stimulate tumor cells growth, suppress the immune response, induces angiogenesis, takes part in the metastasis of tumour cells and detection of tumor specific mutations. Exosomes monitor therapeutic response and resistance.[14] Exosomes carry surface markers. The surface markers are used for enrichment strategies as in CTCs.[14] . In January 2015, a blood test was developed by Exosome Diagnostics of Cambridge, Massachusetts, to detect lung cancer by analyzing tumour-exosome RNA. This test is certified for laboratory use under the Clinical Laboratory Amendments (CLIA) quality programme in the United States.[11]

Proteins

Proteins are released from tumour cells. The measure of serum /plasma specific proteins is used clinically since decades for diagnosis, monitoring progress and prognosis of cancer. The classical examples are serum HCG and PSA in choriocarcinoma and carcinoma of prostate respectively. Many kits are available in market for clinical use. However one should be careful in sampling process as drawing blood, separation of serum /plasma, storage and use of standard method of analysis.

Platelets

Platelets has role in tumour growth, spread, invasion, intravasation, migration, extravasation and distance metastasis. Tumour cells release biomolecules into blood which are absorbed by platelets. Tumour cells cause platelet aggregation. Platelets swallow vesicles loaded with tumour RNA and provide a diagnostic treasure of information when the RNA in platelets are isolated and analyzed from cancer patients and may work well in combination with tests on other biomarkers such as circulating DNA. Hence platelet is one of the blood components in non -invasive liquid biopsy for monitoring of biomarkers in cancer.[11]

Advantages of liquid biopsy

Liquid biopsy is simple, safe, reliable, cost effective, quick, convenient, minimally painful and defines the highly sensitive and specific biomarker for the disease.[3,10,14] Liquid biopsy is minimally invasive procedure and an alternative method in cases where tissue biopsy is not available, insufficient or difficult to obtain or cannot be obtained safely, site difficult to assess, primary tumour spreads to bone / brain / lung / other organs which are difficult locations. Liquid biopsy can be done when genetic analysis of archived tumour samples is not possible.[3,8,10,12,16] The utility of liquid biopsies is likely to be limited in resectable tumours. However it is useful in advanced-stage and unresectable cancers.[8] Mutations exist on both exosome RNA (living process) and ctDNA (dying process). Combining the information of both exosome RNA and ctDNA has the advantage of increasing the detection sensitivity for low frequency mutations tumours.[14]

Tumour related mutations have been observed in healthy individuals and smokers indicating genetic aberrations which might be present at low frequencies even in the absence of cancer.[8] Liquid

biopsy assess the risk of acquiring specific cancer in future and hence can be used for screening of cancer.[3,16]

Liquid biopsy helps in diagnosing cancer at early stage. It assures highly individualized health care and will revolutionize cancer diagnosis and treatment.[1,2,8,16] With advanced stage of cancer, as in the case of bone metastases, some pancreatic cancers and deep pelvic masses primary are detected.[8]

Liquid biopsy has marked diagnostic and clinical implications. ctDNA is highly informative and 83% of cancer cases shows ctDNA. It captures entire heterogeneity of the tumour as tumour genotype is highly unstable and changes with multiple factors. Liquid biopsy provides an accurate snapshot of the genomic landscape of the tumour. Serial samples in liquid biopsy monitor change in genome in real time, gives more information than tissue biopsy, helps in treatment decision in nearly two thirds of patients, stratify the patients to treat with FDA approved drugs or clinical trial and ensures that the treatment is relevant. Hence it provides right tailored treatment targeting the correct molecular aberration and proteins.[1,2,3,5,7,12,16] It detects new genetic alteration, emergence of new / rare mutations which causes resistance to the targeted treatment, development of heterogeneous subclonal population of tumour cells during the course of progression of cancer, clue regarding the stage and spread of cancer.[7,8,12,16] Liquid biopsy helps in identifying signaling pathways which causes tumour invasiveness and development of metastatic competence.[1] Tumour-associated aberrations can be lost or gained over the monitoring period or in response to drug. Presence or absence of a single genetic alteration in tumour DNA is currently employed for clinical decision making for a number of targeted agents (for example, EGFR mutations for gefitinib in NSCLC, BRAF mutations for vemurafenib in melanoma, KRAS mutations for cetuximab or panitumumab in colorectal cancer, ALK rearrangements for crizotinib in NSCLC).[8]

Genetic changes detected in liquid biopsy closely mirrors those identified by traditional tissue biopsy.[2] Liquid biopsy is more effective and alternative to gold standard tissue biopsy. The mutations detected in liquid biopsy paralleled that in tissue biopsy as in EGFR, ALK and TP3 gene alterations. In some cancer cases liquid biopsy could capture mutations not detected in tissue biopsy especially as disease progressed. They reveal molecular signatures which are targeted for chemotherapy.[6] It correlates with tumour burden also.[8]

The association between the levels of tumour -specific genetic aberrations and stage of cancer requires thorough evaluation of various cancer types which are done in many studies. Significant correlation is reported between disease stage and the presence of tumour associated new genetic aberrations with resectable breast cancer, ovarian cancer, pancreatic cancer, colorectal cancer and oral squamous-cell carcinoma. In breast cancer, following mastectomy and follow up of cases showed that, the vascular invasion, metastasis of more than three lymph-node and high histological grade at diagnosis had persistent tumour-associated microsatellite DNA alterations as detected in plasma extracted DNA by PCR. However there are conflicting studies correlation between stage and levels of tumour -associated genetic aberrations which may be due to limited sample, improperly designed studies and technical differences.[8]

Persistence of tumour-associated genetic aberrations in ctDNA after surgery in cases of incomplete resection of breast cancer, lung cancer and oral squamous cell indicates residual disease.[8] Liquid biopsy detects minimal residual disease which is undetectable by imaging.[13] Stratification of patients with minimal residual disease and to identify patients likely to relapse are reported. In addition identification of cases with dormant disease which cannot be detected by standard methods is reported. As DNA is cleared from circulation within 30 minutes, the presence of DNA might reflect persistent dormant cells cycling between replication and cell death. Tumour specific copy number aberrations persisted up to 12 years post diagnosis. Significant reduction and not eradication in tumour derived DNA following surgery is also reported.[8]

Liquid biopsy can be used periodically to monitor disease progression after potentially curative treatments by periodic sampling. It systematically tracks genomic evolution and prognosis. It can detect treatment response and recurrence after surgery. The genetic changes in ctDNA can be detected much earlier than clinical signs and radiological findings of cancer progression.[8,10,12] Imaging cannot be used for frequent monitoring. Imaging techniques have limited sensitivity of detection of micrometastases. Monitoring tumour -specific aberrations in the plasma of patients with colorectal cancer identify disease recurrence with almost 100% sensitivity and specificity. An association is reported between disease recurrence and the reappearance of certain tumour aberrations, including KRAS, APC and TP53 mutations as well as allelic imbalances.[8]

Liquid biopsies helps in early detection of treatment resistance and thus spare the patient from

unnecessary treatment and toxicity of the drug.[1,5,8] Detection of the emergence of resistant clones, by the presence of tumour associated genetic aberrations in the blood, identifies treatment resistance up to 10 months before radiological methods.[8] 50% of NSCLC patients became resistant to tyrosine kinase inhibitor therapy through an epidermal growth factor.[14]

TP53 mutations and loss of heterozygosity correlated with overall survival or disease-free survival. KRAS mutations in NSCLC predict poor prognosis in cases receiving first-line chemotherapy. In pancreatic cancer, emergence of KRAS mutations had a significantly reduced probability of survival. In Neuroblastoma, amplification of gene MYC-related oncogene is a hallmark of aggressive disease. Liquid biopsies can accurately detect MYC-N amplification in the serum of stage III to IV patients with neuroblastoma with a sensitivity and specificity of 75–85% and 100%, respectively.[8]

Thus liquid biopsy is useful in various stages of development of cancer as; risk of cancer, screening of cancer, early detection of cancer, cancer diagnosis, therapy guidance, therapy management, predict response to treatment, monitoring therapy success, early detection of resistance to therapy, recurrent monitoring of disease progression and death, risk stratification and treatment is more personalized at microlevel. Hence biomarkers in liquid biopsy act as surrogate markers.[3,8] Longitudinal monitoring with broader molecular understanding is very much required for cancer treatment to be successful. Clinical applications of liquid biopsies have significantly improved in recent times.[14]

Disadvantages of Liquid Biopsy

Preanalytical factors as blood sampling, processing, storage, DNA extraction and quantification can strongly affect DNA yield. The amount of DNA isolated in the blood sample may be very low especially in some tumours as glioblastoma which can be due to blood brain barrier where ctDNA finds difficult to cross blood brain barrier and reach the blood circulation.[12] There is lack of harmonization of quantification methods as these different methods produce different results because these measurements target either total or only amplifiable DNA. Hence the method in liquid biopsy has to be standardized, reproducible, approved, validated and cost-effective before it enters the market.[2,8,10] Isolation of ctDNA are costly, time consuming and complex.[10] The liquid biopsy has concern of accuracy to be used in clinical practice. Not all results consistently support the application of ctDNA to the patient as increased concentrations of ctDNA have also been detected in physiological and noncancerous pathological conditions. Confounding events might also contribute to the release of ctDNA, e.g., nonmalignant diseases, heavy smoking, pregnancy, exercise, and heart dysfunction which has to be accounted. During the time periods between sampling and clinical application, there can be alteration in genetic composition.[2,8,10] However ctDNA appear to be a better prognostic marker than CTC count.[20] Some authors consider analyzing ctDNA is like finding needle in a haystack.[14]

Future of Liquid Biopsy

There is a big hurdle between theoretical robustness, laboratory data, translational experience and the real possibility of a clinical application which requires 'formal validation' in liquid biopsy. There should be standardized and approved sampling technique (blood collection, processing and storage), extraction of DNA, quantification, analysis and reporting. Validation should be in multicenter clinical studies with respect to disease free or overall survival. ctDNA testing have been so far developed for research or investigational purposes only and should proceed to CLIA (Clinical Laboratory Improvement Amendments) certification before implementation in clinical trials.[1,5,8,13]

To localize tissue of origin especially the small and occult lesions, organ-specific metastatic signatures in CTC cells should be evolved which guide diagnostic / therapeutic strategies and decrease cancer mortality.[13] The sensitivity of the technique has to be increased by which one can detect the genetic changes even in low frequency cfDNA.[12]

Liquid biopsy will be elegant, promising, reliable, robust non-invasive platforms for the diagnosis, patient stratification and to monitor treatment response.[6,14] Hopefully in future liquid biopsy helps in decision making in cancer treatment replacing extensive imaging and invasive biopsy procedure. In addition liquid biopsy may screen cancer before it is visible on imaging.[16] It will be novel life saver technique and boon for human community.[3] However cost for test needs to go down and sensitivity needs to rise. At the moment, liquid biopsies are mostly confined to basic and clinical research.[11] In future liquid biopsy may give invaluable information for research and clinical management in oncology. It will transform clinical practice, becomes integral part of precision medicine, revolutionize cancer care and will be hallmark of cancer care.[1]

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