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REVIEW ARTICLE

LIQUID BIOPSY: A NON-INVASIVE TOOL TO DETECT CANCER AT AN EARLY STAGE

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Abstract

Cancer is one of the greatest threat facing our society, being the second leading cause of death globally. Currents strategies for cancer diagnosis consist of the collection of a solid tissue from the affected area. But solid biopsy is associated with various risky complications such aspainful tissue collection, time-consuming and expensive processes, in some cases, the extracted tissue is not sufficient for the molecular study of cancer. New alternatives that overcome these drawbacks of solid biopsy are rising up nowadays, such as liquid biopsy. Liquid biopsy provides an opportunity to detect, analyze, and monitor cancer in various body effluents such as blood, saliva, stool, and urine, which are composed of different biological matrices such as circulating tumor cells (CTCs), circulating tumor-specific nucleic acids (circulating tumor DNA (ctDNA), circulating tumor RNA (ctRNA), microRNAs (miRNAs), long non-coding RNAs (lnRNAs)), exosomes, and autoantibodies. These circulating biomarkers play a key role in understanding tumorigenesis andmetastasis, which provides a better insight into the evolution of the tumor dynamics during disease progression and treatment. Herein, we provide a comprehensive overview of the biogenesis, their current detection methods, and importance of each biomolecule in liquid biopsy technique.

Introduction:-

Cancer is defined as extensive genetic changes associated with abnormalities in gene function and activity [Nakagawa et.,al2018].Cancer is one of the major diseases in the world with a higher mortality rate. The new cancer cases are estimated to increase to 19.3 million per year by 2025, due to modified lifestyle along with social and economic changes.Early, sensitive, and accurate diagnosis can be considered as an essential requirement in cancer management, as it can lead to effective therapeutic interventions, reducing the treatment cost and substantially improving patient outcome and overall survival (OS)[Gorgannezhadet., al 2018].Tissue biopsies are the current method to access the molecular information of the tumor and required foridentification of cancer such as type, gene and mutational expression, and screening[Shtivelmanet., al2018].However, it is associated with issues in acquiringsurgical extraction of tumor, which could cause discomfort, pain, and risk for the patient[Marrugo-Ramírezet., al 2018.]

One promising alternative to tissue biopsy is obtained by understanding the microenvironment and its communication with cancer cells or any other cancer related biomolecules (cells, nucleic acids, proteins, microvesicles, etc).The capture of cancer or disease-related biomarkers in a fluid sample is defined as liquid biopsy.Early detection of circulating biomarkers (CBS) inaccessible body fluids such as blood or urine has the potential to improve survival for individuals burdened with cancer [Marrugo-Ramírezet., al 2018].

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The four most common tumorCBs with excellent diagnostic, prognostic and therapeutic potential are circulating tumor cells CTCs[Umeret.,al2018], circulating tumor-specific nucleic acids (ctDNA, ctRNA, miRNAs, lncRNA)[Gorgannezhadet.,al 2018;Masudet.,al2019]extracellular vesicles (exosomes, apoptotic bodies, etc)[Boriachek K et., al 2018], and autoantibodies[Yadavet.,al2019](Fig:1). During the last few years, much research has been carried out to find out new cancer biomarkers which allow non-invasive assessment, screening, disease classification, and monitoring, to reduce cancer mortality[Marrugo-Ramírezet.,al2018].In this regard study on liquid biopsygaining popularity in tumor biology due to its non –invasiveness, low consumption of reagents, and ease of use[Tadimetyet.,al 2018].Circulating marker analysis in cancer patients will also facilitate to develop personalized medicine for tumor-specific treatment[Bardelli&Pantelet.,al2017].

Liquid Biopsy In Cancer:

A few years ago, the term "liquid biopsy" was coined [Lianidouet.,al2010; Pantel& Alix-Panabièreset.,al 2010]to describe the utility of circulating tumor cells (CTCs) as candidate tumor biomarkers in breast cancer.The existence of CTCs is not new, as their presence in the bloodstream was already documented in the middle of the 20th century [Hcet.,al1955].Liquid biopsy is now extended for the analysis of many components released by the tumor in body effluents (mainly blood) including cell-free circulating nucleic acids (DNA, mRNA, non-coding RNA such as micro-RNA or long non-coding RNA), "tumor educated platelets" (TEPs) or vesicles such as exosomes (Fig:1)[Pouletet.,al2019].Owing to the recent advances in the development of highly specific gene-amplification and sequencing technologies liquid biopsies can access more biomarkers relevant to cancer. Thus, the molecular profile of liquid biopsies is a promising field for cancer biomarker discovery [Sodaet.,al2019].It provides an insight into tumor biology and potentialityto differentiate metastatic and indolent lymphoma. Thus, an opportunity to identify reliable CBs(Cancer biomarkers) mirroring tumorbehavior, via fully or minimally non-invasive liquid biopsy, represents a great paradigm shift in personalized clinical care.

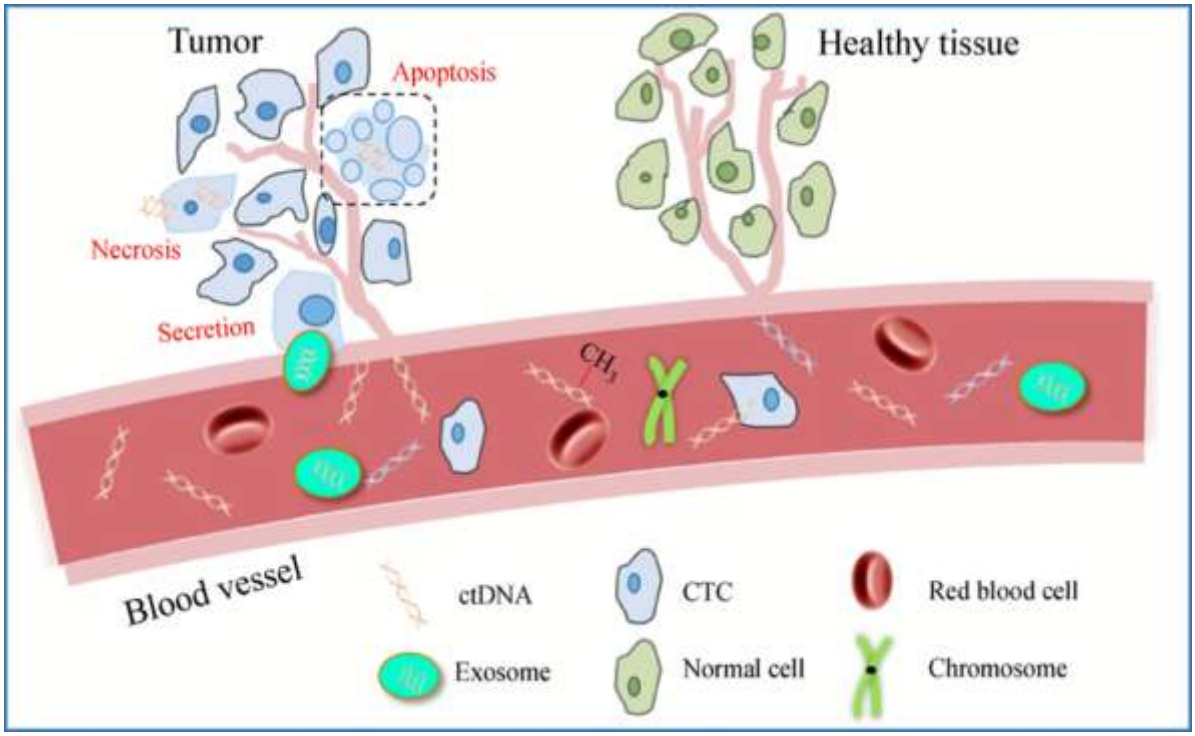

Fig. 1:- The above diagram represents the blood of cancer patients composed of CTC, ctDNA, ctRNA, Exosomes etc[Wang*et., al* 2017]

Because of immense research on liquid biopsy, it has undergone an exponential evolution with a fast implementation in clinical practice. Some authors and clinical associations have proposed the introduction of liquid biopsy in diagnosis [Gregget.,al2016]and treatment protocols[Oxnardet.,al2016] and different commercial systems have received government approval for clinical use. In this review, we mainly focus on each constituent of liquid biopsy ina detailedview such as their biogenesis and the detection methods of CTC, ctNA, and exosomes.

1. CTC (Circulating Tumor Cells)

CTCs are described as cells shed by a primary tumor into vasculature and they keep circulating in the bloodstream of cancer patients[Yapet.,al2014].The earliest detection of CTCs in peripheral blood was reported by Australian physician, Thomas Ashworth by using a microscope in 1860 [Ashworthet.,al1869].

1.1. Biogenesis Of CTC:

Majority of the CTCs are found to be entered accidentally into the circulation by means of external forces such as tumor growth and mechanical stress during surgical operation either passively or actively[Marrugo-Ramírezet.,al2018].Recent reports have demonstrated that CTCs infiltrate from the blood circulation at an early stage and later spread to potential metastatic regions as single cells or clusters upon shedding from the primary site[Pantelet.,al2016;Micalizziet.,al2017].Numerous metastasis studiesin the 20th century indicated that clusters or aggregates of tumor cells contain higher metastatic potential than that of single cells[Comanet., al 1951; Zeidmanet., al 1957;Fidler et., al 1973].The analysis of CTCs is therefore centered on the evaluation of mechanisms of cancer metastasis[Soda et., al 2019].

CTCs are extremely present at low concentrations in body fluids,and provide information regarding diagnosis, prognosis, and follow-up of therapeutic responses to treatments[Krebset., al 2014]. CTC phenotype is preclinical model to provide valuble information regarding molecular therapies in the last decade [Krebs et., al 2014;Polzer et., al 2014;Tellez-Gabrielet., al 2018].However, with technical limitations,there is a delay in the CTC analysis and clinical practice. Only in the last decade, the technical solutions would allow us to investigate these rare cells in circulation. The characterization of CTCs could help in dissecting the complex clonal architecture of cancers and provide insight into tumor heterogeneity. Highly accessible CTCs are potential resources for molecular information of cancerin clinical assessment and provide an opportunity to facilitate the development of more effective personalized therapies [Zhanget.,al2016].Therefore, technologies that can effectively isolate a single CTC from the background of several blood components are essential. A considerable number of novel technologies have been developed to efficiently isolate, quantify and characterize rare CTCs[Sodaet., al 2019].

1.2.Isolation and enrichment methods of CTCs:

Isolation of CTC's involves enrichment of CTC's population apart from surrounding blood cells by using immunomagnetic properties and morphological properties of CTC's [Thieleet., al 2017; Harigopalet., al 2020].

1.2.1.1.Immunomagnetic Isolation:

Specific cancer biomarkers are indispensable in most biological detection methods [Gribkoet., al 2019]. Cancer biomarkers refer to the molecular changes that are measurable and helpful in differentiating normal and cancerous tissues of patients[Gribkoet., al 2019] and Identifying these biomarkers in capturing and isolating CTCs from the blood circulation. Immunomagnetic method is most important in isolation of CTC's. The method consists of an attachment of antibodyto a magnetic bead in order to target an antigen. The formed antigen-antibody complex can be isolated after exposing the sample to magnetic field. The targeted moleculeswere captured by using two different approaches in immunoselection.They are positive selection by using antibodies against tumor-associated antigens and negative selection against the common leukocyte antigen[Marrugo-Ramírezet.,al 2018] (Fig:2A,2B).

1.2.1.1. Positive Selection

EpCAM(Epithelial cell adhesion molecule) based assay is the only method approved by FDA in CellSearch where positive immunoselection approach in CTCs detection will involve in identifying the of epithelial markers such as CD45, EpCAM+, CK 8+, and 19+(Fig:2A)[Sodaet.,al2019].

1.2.1.2. Negative Selection:

The 'negative markers' which are used to identify and eliminate questionable cells such as leukocyte markers CD45, CD66, and CD15, platelet marker CD61, and apoptosis marker M30 (Fig:2B) [Gribkoet., al2019; Castro-Giner&Acetoet., al 2020].

1.2.2. Based On Physical Properties:

The physical properties such as size, deformability, density, adhesion, and dielectric properties were considered in isolating CTCs from other blood cells [Thieleet., al 2017; Zou et., al 2018]. Several methods are developedbased on these physical differences to increase recovery rates to enrich CTCs [Zouet., al 2018].Some of them are discussed below.

1.2.2.1.Size-Based Enrichment and Isolation Technologies:

Size of the cells is a basic foundation for physical separation of CTCs enrichment in peripheral blood [Gabrielet., al 2016]. Size-based filtration is the most common enrichment strategy for CTCs separation [Haoet.,al2018]. By understanding the physical differences such as geometrical and mechanical properties between CTCs and blood cells,various isolation techniques were discovered.They are 1.AnaccuCyte-CyteFinder assay[van der Toomet.,al2018]2.high-throughput Vortex HT chip[Cheet.,al2016], 3.single-useScreenCellCyto device[Desitteret., al 2011] have been developed by understanding the CTCs from the remaining blood cells based on size. These techniques would separatee the CTCs from the remaining blood cells based on size.

1.2.2.2. Separation Based On Density:

Density gradient centrifugation can be used to separate CTCs from other blood cells based on the differences in densities of different cell typesof CTCs can be separated by using Ficoll-Hypaque® (Cytiva) and OncoQuick® (Greiner Bio-One)[Huanget., al 2018;Yapet., al 2019]density gradient centrifugation methods are the most popular in preclinical and clinical research(Fig:2D) [Gabrielet., al 2016;Costa et., al 2020].However, the limitations in these methods overcome by using RosetteSep™ CTCs Enrichment Cocktail (StemCell Technologies Inc.) in combination with Ficoll-Hypaque® (Cytiva) will help in better separation with enrichment and purity of CTCs [Druckeret., al 2020].

1.2.2.3. Based On Di-electricity Of CTCS:

In addition to size, density, and deformability, the dielectricity of CTCs can also be harnessed to distinguish CTCs from other blood cells (Fig:2F)[Waheedet., al 2018].In this separation method, different cell populations can be isolated based on different levels of polarization generated by using a non-uniform alternative electric field [Murlidharet., al 2016].ApoStream™ (ApoCell) is the commercially available dielectrophoresis separation method where 45-85 kHz AC signals were used to filter the cancer cells (~30-40 kHz) and peripheral blood cells (90-140 kHz) [Guptaet., al 2012;Nasiri et., al 2020].

1.2.2.4. Filtration:

Ultrafiltration and micro-filtration were also used to separate CTCs in the whole blood. Microfiltration-based methods like track-etched polymer filters[Vonaet., al 2000; Marrinucciet.,al2012]and flexible micro spring array (FMSA) devices[Harouakaet., al 2014], are the known filtration methods for CTCs separation from whole blood (Fig:2E).

1.2.2.5. Inertial Sorting:

By using hydrodynamic forces CTCs and blood cellswere separated at distinct streamlines in fluid.In inertial sorting technique use of inertia and fluid dynamics were usedto separate different particles within a channel (Fig:2G) [Sollieret., al 2014].The recent advances in engineering and technology, several other next-generation microfluidics platforms have been developed by advanced engineering and technology to enrich CTC's from other blood cells[Marrugo-Ramírez et., al 2018].

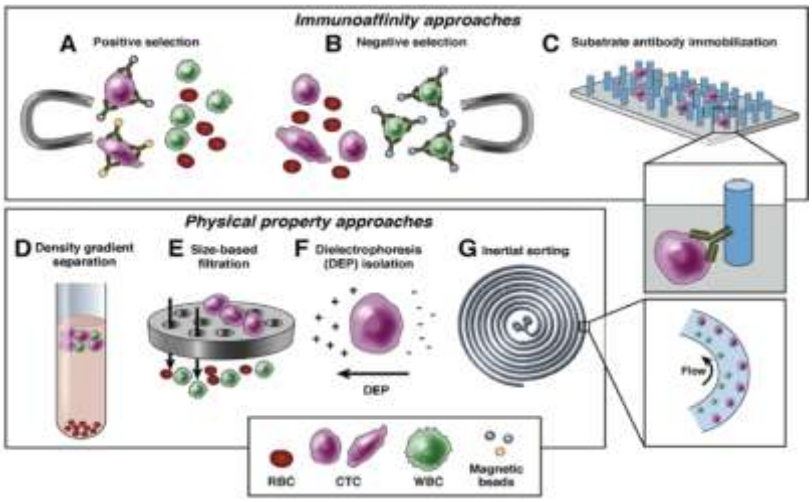

Fig. 2:- Schematic representation ofisolation of CTs by using different methods (A) positiveselection (B) negative selection (C)Immobilization of CTCs(D)Separation based on density(E)Through filteration(F)Dielectrophoresis (G) Inertial sorting. Retrieved with permission from ref[Nagrath*et., al* 2016]Copyright 2016, Elsevier.

1.3.Significance Of CTCs:

CTCs are the widely used astumor biomarkers due to their minimal invasiveness and easy detection in liquid biopsy[Nakayaet., al 2013;de witet., al 2014]. Detection of CTCs in liquid biopsy can provide information about the molecular mechanism of cancer and tumor heterogeneity. It provides the basic information to understand mutations and genotypic changes of malignant cells for the best suitable targeted therapy. CTCs are multifunctional biomarkers and enable to assess the patient serially along the treatment journey[Potdar et., al 2015]. They are potential alternative to invasive biopsies for detection, characterization, and monitoring of non-hematological cancers[Krebset., al 2014].At the end, CTCs give biological insights into the disease condition, progression, and treatment prediction. Reports indicate that patients with lesser numbers of CTCs survive longer than the patients with more number of CTCs[Krebset., al 2014].They can even help in the selection of secondary treatment options while the patient has failed to respond to first-line treatments[Balicet., al 2005].Examples of clinical trials testing CTC-based enrichment methods are indicated as table -1 [Dianat-Moghadam et., al]2020.Since CTCs hold such critical information about a tumor and its characteristics; they can definitely, be an important biomarker for cancer diagnosis.

Table 1:- Examples of clinical trials testing CTC-based enrichment methodsas explained by [Dianat-Moghadamet., al 2020.]

Device	Enrichment/Detection method	Condition	Status	Primary Endpoints	Trail
GILUPI CellCollector®	Immunoaffinity (anti-EpCAM Ab)	Locally advanced breast cancer	Completed	PFS, OS	NCT03732339
ISET® technology	Immunocytochemistry (PD-L1 expression analysis)	Lung cancer	Recruiting	Not provided	NCT02827344
	FISH analysis of ALK	Lung Neoplasms	Active	Validation of ALK analysis	NCT02372448
CellSearch®	Immunoaffinity (anti-EpCAM Ab)	Prostate cancer	Recruiting	EMT markers, PFS and OS	NCT04021394
		Metastatic breast cancer	Completed	CTC-Endocrine Therapy Index	NCT01701050
CTC-Chip	Size or Immunoaffinity	Prostate cancer (Prostatectomy)	Recruiting	Examine chromosome translocation	NCT01961713
Culture system	Affinity-based WBC deletion	Early detection of cancer	Recruiting	Early diagnosis and screening	NCT03843450
Parsortix™	Cellular size and deformability	Ovarian neoplasms	Completed	Estimate risk of cancer	NCT02785731
EMT-marker based ferrofluid device	N-cadherin or O-cadherin based analysis	Metastatic breast and prostate cancers	Completed	Clinical stage, Screening	NCT02025413
IsoPic™ microfluidic system	Flow rate, surface interactions, plasticity, and elasticity	Unknown primary cancer	Recruiting	Prediction of molecularly targeted therapies	NCT04025970
Flexible Micro Spring Array (FMSA)	Filtration (or size-exclusion of viable CTCs)	Stage IV colorectal cancer	Completed	PFS, OS, response to therapy	NCT01722903
Ficoll enrichment	Density/ PCR	Pancreatic ductal adenocarcinoma	Completed	PFS, OS, response to therapy	NCT02150746

2.Circulating Tumor Nucleic Acids:

2.1. Circulating Tumor DNA (ctDNA):

Circulating tumor DNA was first reported by Mandal and Metails in 1948[Mandelet., al 1948]. CtDNA is a fraction of cell-free DNA which is released by tumor cells into the circulation. Cell-free DNA comprises short nucleic acid fragments (B166 bp) found in almost all body fluids, including plasma, and is likely derived from apoptotic cells[Alix-Panabièreset., al2016;Canzonieroet., al 2016].

2.1.1.Biogenesis:

In cancer patients, the large number of tumors leads to the release of circulating tumor DNA[Sodaet., al 2019].ctDNA is composed of DNA fragments released from primary tumors, CTC, micro metastases into the bloodstream of cancer patients[Ficiet., al 2019].Furthermore, phagocytosis of necrotic or neoplastic cells by macrophages also play a role in the release of tumor DNA fragments [Ficiet., al 2019]. Previous studies have confirmed a high level of concordance between genomic alterations detected in plasma ctDNA and those found in tumor tissues[Higginset., al2012;Wyatt et., al 2017].Therefore,considering ctDNA as a key biomarker in the diagnosis of cancer will guide in understanding the mutational and epigenetic alterations occur in the primary tumor tissue.

2.1.2. Detection Methods Of ctDNA:

Due to limitations in terms of sensitivity of the standard sequencing approaches (e.g., Sanger sequencing, pyrosequencing), the ctDNA analysis is limited only in patients with heavy tumor burden and high levels of ctDNA[Leunget., al 2016;Siravegnaet., al 2017].However, advancements in technology over the past ten years have led to the development of molecular assays, making it possible to analyzetumor gene sequences in ctDNA more sensitively.Based on the small size and low concentration of ctDNAs in blood, highly sensitive and target-specific techniques have been developed in order to detect them.

Various PCR-based techniques have been developed to improve the sensitivity including digital PCR (dPCR), the microfluidic system BEAMing digital PCR of single molecule on microparticles in water and oil emulsion, and the microfluidic droplet digital PCR (ddPCR),Allele-Specificamplification (AS-PCR)[Thierryet., al 2014],Peptide Nucleic Acid-Locked NucleicAcid (PNA-LNA) PCR[Kimet.,al 2013] and co-amplification at lower denaturation temperature (COLD-PCR)[Freidinet.,al2015]. These methodological advancements increase the sensitivity of the ctDNA assays, respectively, up to 0.01%, and between 0.05% and 0.001%, but for limited detection from 1 to 2 by dPCR up to 5–10 target sequences by ddPCR[Ficiet., al 2019].Non-target procedure, as the conventional next-generation sequencing (NGS), shows a less sensitivity, around 2–5%, and this allows massive parallel sequencing to detecthigh number of molecular aberrations such as SNPs, insertion, and deletion, simultaneously through massive parallel sequencing[Moatiet., al 2021].DNA methylation analysis of ctDNA is the untargeted approach which involves site-specific detection and genome-wide methylation detection[Wartonet., al 2016].The methylation study involves bisulfite conversion and Methylation-specific PCR(MSP) [Hermanet., al 1996].By using the discussed methods and other conventional methods the mutational changes, genetic aberrations, and epigenetic changes were studied in ctDNA of cancerous individuals.In an unpublished data in our lab we observed the release of methylated genes in the blood of tumorigenic mice at an early stage of cancer.

2.1.3. Significance Of ctDNA In Cancer Detection:

Early diagnosis of cancer is vital as it provides guidance towards effective therapeutic interventions and significant improvement in patient survival[Siravegnaet., al 2017].Over the past few years, detection of ctDNA by non-invasive means has made an exceptional contribution towards the pursuit of potent and credible biomarkers. Predictive biomarkers play a pivotal role in guiding treatment decisions [Sodaet., al 2019].By using restriction fragment-length polymorphism and polymerase chain reaction (RELP-PCR) assays we were able to detect KRAS mutations in circulating DNA of healthy subjects up to 2 years before cancer diagnosis[Gormallyet., al 2006].Mutations in common cancer-associated genes are attractive candidates,as they can be measured in plasma cfDNA from patients with early-stage cancer through the use of established methodologies[Bettegowdaet., al 2014; Newmanet., al 2016 Phallenet., al 2017]. Another important epigenetic aberration which is a potential diagnostic biomarker detected in ctDNAwith a proven track record in the setting of primary cancer screening is DNA methylation.These studies revealed that ctDNA may be a more effective method for timely medication recommendations for patients with metastatic disease, and many of these patients subsequently had ctDNA analysis to monitor disease progression and therapeutic response.

2.2.Circulating RNA:

The other group within the ctNAs is the ctRNA, which comprises two types of noncoding RNAs (ncRNAs): Long noncoding RNAs (lncRNAs)—longer than 200 bp, and microRNAs (miRNAs)—shorter than 200 bp [Peterset., al 2011;Phallenet., al 2017]. These two types of circulating RNAs play an important role in cancer progression.

2.2.1.Long Non-Coding RNAs:

LncRNAs are a class of long transcribed but not translated RNAs that are longer than 200 nucleotides[Mercer et., al2009;Perkelet., al 2013].The main function of lncRNA is to serve as a molecular signal to regulate transcription in response to various stimuli. Many lncRNAs have been functionally associated with human diseases, in particular, cancers [Gutschneret., al 2012].Dysregulation of lncRNAs has been implicated in glioblastoma [Wanget., al 2012;Elliset., al 2014; Yao et., al 2015], breast cancer [Guptaet.,al2010] colorectal cancer [Kogoet., al 2011;Elliset.,al 2012],liver cancer[Yang et., al 2011;Quagliata et., al 2013]and leukaemia[Yildirimet., al 2013].Commonly, dysregulation of lncRNAs exerts impact on cellular functions such as cell proliferation, resistance to apoptosis, induction of angiogenesis, promotion of metastasis, and evasion of tumor suppressors[Brunneret., al 2012].

Unfortunately, very little information is known about lncRNAs and only a fraction of them have been experimentally analyzed. Tumor-related lncRNAs could be used as a diagnostic biomarker and are considered one of the "newly" investigated biomarkers to provide several therapeutic approaches [Shiet., al 2013].

2.2.1.1.Detection Methods:

The lncRNA can be identified by detecting the transcription of unannotated genomic regions. This can be done by a number of techniques, including tiling array, serial analysis of gene expression (SAGE),cap analysis gene expression (CAGE) [Shirakiet., al 2003],and the most powerful RNA-seq techniqueue, which prompts the development of multiple RNA-seq based pipelines for identifying lncRNAs[Sunet., al 2012; Chen et., al 2016].Furthermore, chromatin immunoprecipitation (ChIP) technology, either ChIP-chip or Chip-seq can also identify novel lncRNAs indirectly by studying genomic regions with protein or histone modifications[Guttmanet., al 2009; Cottrell et., al 2016].

2.2.1.2. Significance Of lncRNA:

lncRNA deregulation in primary tumor tissues is clearly mirrored in various bodily fluids, including whole blood, plasma, urine, saliva, and gastric juice[Reiset., al 2012; Tanget., al 2013;Sartoriet., al 2014; Shaoet., al 2014].Therefore the detection of circulating cancer-associated lncRNAs in body fluids could be served as an excellent indicator in the assessment of cancer progression and in differentiating healthy individuals from cancerous ones. Along with evaluating the risk of tumor metastasis and recurrence after surgery,it may also be possible to predict the prognosis of tumor and operation success rate[Shiet., al 2016].

2.2.2. Micro RNA:

MicroRNAs (miRNAs) are a family of small non-coding RNAs that regulate a wide array of biological processes including carcinogenesis[Penget., al 2016].Dysregulated miRNA can affect various cellular pathways leading to tumor development and progression.The underlying mechanisms include chromosomal abnormalities, transcriptional control changes, epigenetic changes, and defects in the miRNA biogenesis machinery.Hence, miRNA can be used in the diagnosis and management of cancer[Liet., al 2015; Zhouet., al 2016; Matinet., al 2016].

2.2.2.1. Detection Methods:

In the case of miRNA analysis, RT-qPCR offers several merits such as sensitivity, a wide dynamic range, less sample input, and better accuracy [Bustinet., al 2002].Since the discovery of miRNA, numerous RT-qPCR-based miRNA expression analysis strategies have been developed.The TaqMan technology[Robertset., al 2014]is commonly used method for miRNA analysis which utilizes a stem-loop reverse transcription primer system to reverse transcribe the RNA and amplify cDNA. NGS is capable of identifying and quantifying both known and unknown, sequences simultaneously and also allows multiplexed expression analysis of miRNA from various sample sources in a single experiment.

2.2.2.2.Significance:

Circulating miRNA biomarkers provide several advantages in liquid biopsy such as high stability, early detection, and minimal invasive means for monitoring cancer. Compared to other circulating RNAs such as mRNA and lncRNA, miRNAs are more stable and exhibit sturdy expression patterns in clinical samples [Tavallaieet., al 2015]. Recently, a lot of attention has been focused in the exploration of diagnostic significance of miRNA in cancer[Kupryjańczyket., al 2003;

Hayeset., al 2014; Gupta et., al2010. Different miRNA profiles could provide valuable information to advance drug regime selection in cancer therapy MacFarlaneet., al 2010.

3. Exosomes:

Exosomes are a subgroup of membrane-bound microvesicles released by normal and diseased cells into interstitial spaces and body fluids. In the late 1980s, exosomes were first identified in extracellular spaceJohnstoneet.,al 1987. Recently it was found that extracellular vesicles are functional vesicles that carry a complex cargo of proteinsSimpsonet.,al 2009, lipidsVidalet.,al 1989, and nucleic acids Waldenströmet., al 2012; Valadiet.,al 2007;Simpsonet.,al 2009, which can deliver these cargoes to the encountered target cells, further release of these cargos ultimately reprogram the recipient cell. Therefore exosomes offer a novel approach for intercellular communication, which may play an important role in many cellular processes, such as immune responseGreeninget.,al 2015, signal transduction Gangodaet.,al 2015, antigen presentation Mittelbrunnet.,al 2011. From other multicellular vesicles, exosomes are separated based on their source, method of isolation, sizes, and surface markers. The diameter of exosomes ranges from 30-100nm and they are in spherical to cup-shaped nanoparticles with specific surface molecular characteristics (eg: expression of the tetraspanins CD9, CD81, and CD63). The total no of exosomes per mL of blood is 109 but it usually depends on the tumor burden and stage. Tumor-derived exosomes(TD exosomes) are a subtype of exosomes released by tumor cells into interstitial spaces or lymphatic or pseudo capillaries formed by tumors. Compared to other microvesicles tumor-derived exosomes are more in number and TD exosomes possess different tumor-related molecular characteristics than microvesicles from other sources. Because of these tumor-related molecular characteristics, exosomes may be useful clinically and in translational research by improving the analysis of biomarkers to detect cancer by using liquid biopsyKanwar et., al 2014; Jia et., al2017.Previous reports also provide evidence of secretion of EVs from the embryonic bodies obtained by re-differentiated cancer stem-like cellsJinkaet.,al2016.

3.1.Biogenesis:

The limited multivesicular body(MVBs) membrane undergoes inward budding to form late endosomes, by which, exosomes were formed during the processes of invagination of late endosomal membrane, certain proteins are incorporated into the invaginating membrane to form intraluminal vesicles within large MVBsMinciacchiet.,al2015. The membrane of ILVs upon fusing with the plasma membrane is released into the extracellular space, which is called "exosomes".There are two pathways in the formation of exosomes they are ESCRT (endosomal sorting complex required for transport function) dependent pathway and ESCRT independent pathway. In ESCRT dependent pathway there is intricate protein machinery composed of four separate protein ESCRTs (0 through III) the MVB formation,co-operatively facilitatesd to MVB formation, vesicle budding, and protein cargo sorting Henneet.,al 2011;Hurleyet.,al 2015. The ESCRT mechanism is activated by the binding of ubiquitinated proteins to specific domains of the endosomal membrane via ubiquitin-binding subunits of ESCRT-0leading to the formation of ESCRT-1 and 2complex, which binds to the ESCRT-3 to generate an activated protein complex which is involved in promoting the budding processes. When buds are cleaved to form ILVs, then the ESCRT-3 complex separates from the MVB membrane with energy supplied by the sorting protein VpS4 Henneet.,al 2011.

The alternative pathway to this is the ESCRT –independent pathway, in which raft–based microdomains are involved in the lateral segregation of cargo within the endosomal membrane. In these microdomains, sphingomyelinase is present which can cleave the hydrolytic bond of phosphocholine moiety to form ceramides Airolaet.,al 2013 which induce later phase separation and coalescence of microdomains. Therefore, the cone-shape structure of ceramide may cause spontaneous negative curvature of the endosomal membrane, to facilitate domain-induced budding. Consequently, this ceramide-dependent mechanism emphasizes a key role of exosomal lipids in exosome biogenesis Castroet.,al 2014. Several specialized mechanisms exist to ensure the specific sorting of bioactive molecules into exosomes, either the ESCRT-dependent or -independent mechanism (involved tetraspanins and lipids), which may act variously depending on the origin of the cell type.

3.2. Techniques Used For Detection Of Exosomes:

There is no "gold standard" method for the detection and purification of extracellular vesicles, so we are not able to confirm that there is an optimal method that could be used uniformly. The International Society for Extracellular Vesicles (ISEV) provides protocols for proper planning and maximization of the significance of EV detection Witweret.,al 2013; Lötvallet.,al 2014. The mainly used technique for the separation of exosomes from other EVs is ultracentrifugation. When compared to other techniques ultracentrifugation possess various types of advantages, low cost, and convenience, its spinning capacity of a wide range of volumes, up to 100ml Ramirezet.,al 2018.In addition to advantages, there are some disadvantages associated with ultracentrifugation as they are co-purified along with of

protein aggregates and lipoproteins [Davieset.,al 2012], which are not related to EVs.So the ultracentrifugation is always used with sucrose density gradient for better separation [Wahlundet.,al 2017].

ExomiR is an isolation kit created by Bioo scientific, in which micro-filter and outward pressure are used to remove all cells, platelets, cell debris to enrich exosomes [Vlassovet.,al 2012].ExoQuick is a reagent released by system biosciences, when this reagent is added to the serum or urine, it can precipitate exosomes of size ranging from 60-150nm. But it lacks specificity towards exosomes. Therefore, it can precipitate non-exosomal content of similar size [Taylor et., al2011].

Immunoisolation is another approach for the enrichment of exosomes, in this technique, EVs are subjected to functionalized antibody-coated latex[Cabyet.,al 2005]or magnetic beads [Kogaet.,al 2005] to promote a biomarker-based separation. It can avoid unspecific binding to cell debris or other biological structures because it has specificity to a selected antibody, therefore it will target only EVs with specific surface markers. There was a shred of evidence that the immunoisolation technique can improve the yields of colon-cancer-associated EVs[Tauroet.,al2012] . It has limitations, as like of CTCs and ctNAs they are the targeted surface markers that could also be expressed in several subpopulations of EVs.In order to obtain complete information about the EVs biogenesis and organ of origin,immunoisolation should be used in combination with other enrichment and detection techniques.

Immunoassay-based approaches, such as ELISA can be used to measure the purity of EVs, in which either antigen or protein concentration of them was quantified [Webberet.,al 2013]. Other sophisticated techniques such as dynamic light scattering (DLS), and surface plasmon resonance (SPR) are also used to quantify exosomes [Smithet.,al 2015].

3.3. Significance Of Exosomes:

Exosomes shed from tumor cells can transport oncogenic molecules to recipient cells and regulate their gene expression, thus playing an important role in the progression, metastasis and drug resistance[Raposoet.,al 1996;Logozziet.,al 2009;Lobbet.,al 2017;Lobbet.,al 2017].In recent years, many scientists examined the potential of exosomes in the diagnosis and therapeutic interventions of numerous diseases including cancer, neurodegenerative diseases, infectious diseases, and cardiovascular diseases. A number of studies have recently indicated the significant diagnostic potential of exosomal proteins for breast cancer[Ruppet.,al 2011; Truonget.,al 2017;Sounget.,al 2017]. The establishment of exosomal biomarkers is still in its primacy, but recent studies have reported interesting evidence for the future development of exosomal-based cancer diagnosis. Therefore, cancer-derived exosomes are thought to be an attractive tool for potential diagnostic, prognostic, and therapeutic value, although the clinical utility of exosomes is still in its primacy.

Future Perspectives And Conclusion:-

Blood-basedassays for screening and identifying tumor-relatedmarkers are of great importance and can significantly reducehealthcarecosts.Currently, tumor detection is clinicallyconfirmed using the traditional biopsy,which has some limitations like inaccessible tumor sites in some cases and pain during extraction of tissue.Liquid biopsy could overcome these limitations by analyzing the tumor biomarkers like CTC, ctNA, and Exosomes in the blood of cancer patients. These biomarker tests show optimal sensitivity and specificity in concordance studies. Recent technological advances would lead to many integrated systems for biomarker capture, detection, and analysis using the same assay and also facilitates in studying the molecular level mutations and epigenetic alterations which are actually present in the primary tumor tissue. Real-time monitoring of the evolution of the tumor dynamics is necessary, to broaden the understanding of cancer metastasis and its molecular landscape,liquid biopsy-based approaches provide a valuable tool for minimally invasive diagnosis and monitoring [TadimetyAet.,al 2018].

Declaration Of Competing Interest:

All authors have no conflict of interest.

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