Review

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Liquid biopsy: a new diagnostic modality

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Abstract: The liquid biopsy is the most promising technology in the modern days. It plays a significant role in the diagnosis, management and finding out the minimal residual disease in carcinoma. There are three components of the liquid biopsy: circulating tumour cells (CTC), cell-free DNA (cf-DNA), and exosomes. These components carry vital information, and with the help of sophisticated technology, one can extract a large amount of data from them. The detection of the tumour-specific gene mutation in the CTC or cf-DNA may guide the clinicians about the selection of the appropriate chemotherapeutic agents. The liquid biopsy is still not applied for routine clinical use, and more research is needed in this field. In this review, the techniques and applications of liquid biopsy have been discussed.

Keywords: cell-free DNA; exosomes circulating tumour cells; liquid biopsy;

The conventional incision or excisional biopsy is the most popular technique for histopathological examination. Recently a newer technology is introduced, which is known as “liquid biopsy”. The exact meaning of “liquid biopsy” is the examination of the blood sample for the study of DNA from the circulating cancer cells (CTC) or cell-free DNA (cf-DNA) of the cancer cell [1]. Unlike conventional biopsy, liquid biopsy is a noninvasive technique. It can provide a plethora of valuable information on the detection of the tumour, therapeutic response, and recurrence of the tumour. In this review, the techniques and applications of liquid biopsy have been discussed.

Conventional biopsy versus liquid biopsy

There are several advantages of liquid biopsy over the conventional biopsy. The liquid biopsy is noninvasive and simple blood or peritoneal fluid may provide a lot of information. Unlike conventional biopsy, liquid biopsy can be done repeatedly and even after the resection of the main tumour. The conventional biopsy is often difficult to do from the small and inaccessible deep-seated tumour, but the liquid biopsy overcomes this problem as it can be done from the blood or body fluid.

Moreover, it is unrelated to tumour heterogeneity. The major drawback of the liquid biopsy is its cost. Liquid biopsy is also a very advanced technology and needs a lot of skill to do. Table 1 shows the merits and demerits of liquid biopsy compared to conventional biopsy.

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The constituents of liquid biopsy

The predominant components of liquid biopsy include (Figure 1): 1) circulating tumour cells (CTC), 2) cell-free DNA (cf-DNA), and 3) extracellular vesicles.

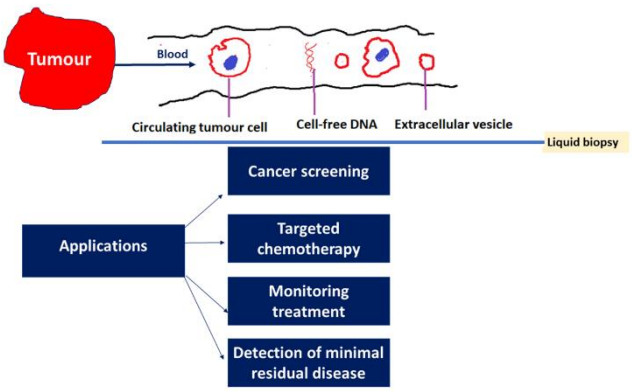

Figure 1: The components of the liquid biopsy and their clinical applications.

1) Circulating tumour cells:

The sources of CTC are either from the primary tumour or rarely from the metastatic tumour. The life span of CTC is short, and the half-life of it is just one to two hours. These cells are quickly removed from the circulation by reticuloendothelial cells of the liver, and spleen. The circulating tumour cells are opsonized by IgG antibody. The Fc region of IgG binds with Fc receptor of the histiocytes, and the tumour cells are phagocytosed.

As the number of cells is less than 1 per 10^6 polymorphs, so it may be challenging to identify and separate these cells from the circulation. The presence of specific antigens on the cell surface of CTCs is helpful to identify and isolate the cells from the blood circulation. The epithelial cells express EpCAM (epithelial cell adhesion molecule), and a cocktail of cytokeratin. Besides, the tumour specific antigen is also used for the identification of the CTC in blood circulation.

Table 1: The merits and demerits of liquid biopsy compared to conventional biopsy

Factors	Liquid biopsy	Conventional biopsy
Procedure	Noninvasive	Invasive
Approach	Easy and always approachable	Difficult in deep-seated small tumour
Presence of tumour	Not dependent on the presence of tumour	It cannot be done if the tumour is already resected
Tumour heterogeneity	Mostly independent of tumour heterogeneity	A small biopsy may not represent the tumour proper
Multiple biopsies	The repeated biopsy can be taken as an only blood sample is needed	The repeated biopsy is not feasible
Cost	Very high cost	Cheap
Technological skill	Only specially trained technologist is able to perform this complicated technique	Any technologist can process the histopathology section

2) Cell-free DNA (cf-DNA):

Cell-free DNA (cf-DNA) is another critical component of liquid biopsy. The predominant sources of cf-DNA in the blood are mainly from the primary or metastatic tumour, CTC, and even from normal healthy cells of the body. The cf-DNA comes into circulation by 1) active release from these cells, 2) apoptotic death, and 3) necrosis (Figure 2).

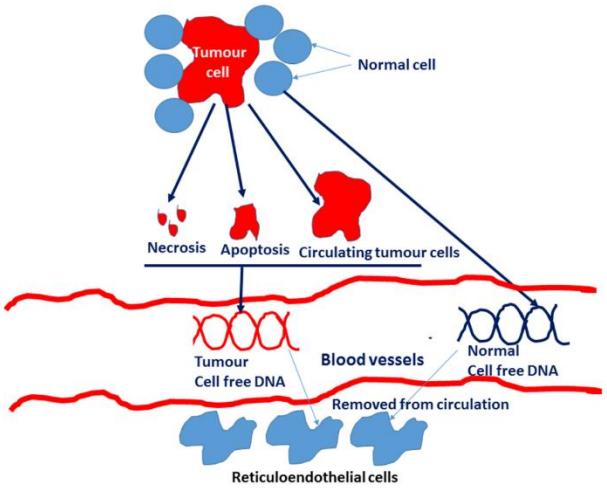

Figure 2: Schematic diagram showing the details of cell-free DNA.

The half-life of cf-DNA is very short (5 to 30 minutes) and these are rapidly cleared from the circulation by the liver, spleen and kidney [2]. The exact mechanism of removal of cf-DNA is still a mystery. The plasma DNase has a limited role in the removal of cf-DNA from the plasma because of the protection of the cf-DNA by the entangled nucleosomal protein complex. The methylation status of DNA also bears little effect in the clearance of the circulating DNA. The cf-DNA is mostly fragmented DNA, and so the molecular weight of such DNA is lower than the normal cellular DNA. It is important to note that not only tumour cells, but normal healthy cells also release cf-DNA. In a healthy individual, the cf-DNA value ranges from 2 ng/ml to 27 ng/ml [3]. The range may be widely variable, and the mean value of cf-DNA may vary with a very high standard deviation (450ng/ml). The extremely variable cf-DNA level may be due to the lack of proper standardization of the measurement and absence of valuable information regarding the structure and function of the cf-DNA. In malignancy, the level of cf-DNA depends on the 1) tumour size, 2) tumour grade, and 3) tumour aggressiveness and 4) metastasis. As there is no reference value of cf-DNA, so one has to careful in the application of its use in clinical practice.

Cell-free RNA (cf-RNA): The importance of cf-RNA was ignored for many years. It is challenging to study cf-RNA in circulation as the half-life of it is only 15 seconds. The RNA is relatively unstable in the circulation because of its rapid degradation by minor changes of pH and also by hydrolyzing enzymes. Unlike cf-DNA, the cf-RNA comes to the circulation by active release instead from the death of the cells. The cf-RNA derived from the tumour cells are noted in the blood, sputum, urine etc. Recently it has been shown that the tumour-derived m-RNA plays a vital role in cancer progression. The cf-RNA as a cancer biomarker is still not properly explored [4].

3) Extracellular vesicles (EV):

The EVs are another critical part of liquid biopsy. These are small vesicles encased by a membrane. The EVs are derived from the tumour cells and are present not only in blood but also in saliva, urine and effusion fluid. EVs contain exosomes (EXO), apoptotic bodies and micro-vesicles.

The EXOs are vesicle of 50 to 100 nano micron diameter and are generated by the budding of the plasma membrane. EXO contains mRNA, DNA, and micro RNA (mi-RNA). As previously believed, they are not the simple waste products of the cells; it is proved that EXOs carry vital molecules from one cell to the other distant cells [5, 6]. EXOs take an essential role in the tumour metastasis.

These EXOs are protected from various enzymes (DNase and RNase) due to the lipid bilayer. So, they give valuable information about tumours such as tumour diagnosis, progression, response to therapy and recurrence.

Detection and enrichment of the constituents

As mentioned before, the CTCs, cf-DNA and EXO are present in a meagre amount in the blood or body fluids. Therefore, it is a great challenge to identify and enrich those components for different molecular tests. Table 2 highlights the different techniques to isolate and enrich the CTC. The

predominant ways to isolation and enrichment of the CTCs are:

1) Immunological method: The magnetically tagged antibody is used to identify the cells [7]. The epithelial tumours are usually separated with the help of EpCAM (epithelial cell adhesion molecule). It is preferable to use the combination of cytokeratin (CK), and EpCAM antibody as these two antibodies detect most of the epithelial cells. The isolated epithelial cells are further confirmed by the various cytokeratins such as CK 8, CK 18, and CK 19. In many tumours, there is a transition of epithelial to mesenchymal cells (EMT). Due to the EMT, the tumour cells may not express the epithelial markers (CK and EpCAM). Moreover, the non-tumour epithelial cells may also come into the circulation, creating the confusion. Due to these difficulties, the immunological methods have been replaced by the other techniques.

Table 2: Techniques to detect and enrich the circulating tumour cells

Enrichment technology	Methods	Comments
Immunological method	The magnetic tagged specific antibodies are used for the tumour cells.	CTCs are removed by the immunogenic method. EpCAM and pan-cytokeratin for carcinomas. Besides, the epithelial cells are negative for CD45.
Leukocyte removal	The leukocytes are removed from the sample.	Immunologic separation of leukocytes by using CD45 tagged magnetic bid. Alternatively, batch cell lysis can be done.
Microfluidic chips	Immunomagnetic based microfluidic chips can separate the CTC.	The target cells from the minimal volume of fluid can be isolated
Physical properties	The several physical properties such as size, density, electrical charges etc. are used to separate the cells.	Physical filtration technique, density gradient or microfluidic can be used to separate CTCs.

2) Leucocyte depletion method: Leukocytes are removed from the sample by cell lysis or by magnetically tagged antibody [3]. CD 45 antibody is useful to detect the leucocytes in the blood.

3) Microfluidics: Immunomagnetic based microfluidic chips isolate the CTCs and

4) physical methods.

Besides, nanotechnology is also used by applying nanomaterials that can enhance the interaction of CTC and antibodies which subsequently changes the electrical property of the cells [8]. Cf-DNA isolation: The cf-DNA are mainly isolated by silica-gel membrane technique or fluorometric technique.

Exosomes isolation: The EXOs are commonly isolated with the help of Differential centrifugation, size exclusion chromatography, Ultrafiltration, Polymer-based precipitation, and Immunological separation [9].

The molecular techniques applied in liquid biopsy

The various molecular tests can be applied in the DNA or RNA of the liquid biopsy samples that include:

1) Polymerase chain reaction (PCR) based techniques: The PCR technologies include Droplet Digital PCR (*ddPCR*), beads, emulsion, amplification, and magnetics PCR (BEAMing PCR), and enhanced ice-cold PCR (E-ice-COLD-PCR) [10–14].

2) Next-generation sequencing (NGS) based techniques: Several types of NGS-based technologies are used in liquid biopsy. These are

a) Tagged amplicon deep sequencing (Tam-Seq): Here, the parallel amplification was done in the multiple regions of the genes [15].

b) Cancer personalized profiling by deep sequencing (CAPP-Seq): It is a "capture-based NGS" technology to detect cell-free DNA [16].

c) Safe sequencing system: It is a unique technology that reduces the error rate of NGS to less than 1%. A unique identifier is added to the template molecule, and the molecules of the same unique identifier family are recognized [17].

3) Epigenetic study: Methylation-specific polymerase chain reaction protocol is applied to identify the DNA hypermethylation. For the global assessment of DNA methylation, the "Microarray-based DNA methylation assay technology" is used.

Clinical implications of liquid biopsy

Table 3 highlights the details of genetic and epigenetic changes of the common carcinomas that may help in the diagnosis and management in related to liquid biopsy.

Cancer screening: The total quantity of cf-DNA is much higher in cancer patients, and many studies suggest that quantitation of cf-DNA may be useful in cancer screening [3, 18]. However, the metanalysis study shows that the cut-off value of cf-DNA is not effective due to the different factors play a role in the cf-DNA level [3]. So simple quantitation of cf-DNA may not be useful in the screening for carcinomas. The circulating tumour DNA may show specific changes that include mutation of an oncogene, microsatellite changes, loss of heterozygosity and hypermethylation of promoter genes. The demonstration of

qualitative changes of tumour specific DNA may be helpful in the detection of the tumours. However, the amount of cf-DNA in the circulation may be very less in comparison to the normal circulating cf-DNA, and therefore it may be challenging to detect tumour [19].

detect the MYCN amplification in advanced neuroblastoma cases [33].

Minimal residual disease (MRD): The most potential application of the liquid-based biopsy is the detection of MRD. The identification of the single or multiple tumour

Table 3: Tumour specific genetic changes in different cancers that are helpful for liquid biopsy

Tumour type	Genetic and epigenetic alterations	
	Early diagnosis and screening	**Therapy and management**
Breast cancer	• The detection of mutation in the gene PIK3CA is related with early diagnosis of breast cancer [21] • Higher expression of cancer antigen15 to 3 (CA15-3) is related to cancer screening and early relapse [21] • DNA amplification of oncogene *BRF1*, *MTA1*, and *JAG2* [22]	• Mutations in the *PIK3CA* gene related to resistance to HER2-targeting therapy [23] • Microsatellite DNA changes associated with aggressive course [24] • High level of Lysine-specific demethylase 1 (LSD1) expression in metastasis that can be blocked by chemical LSD1 blockers [18]
Colorectal cancer	• Mutation of APC KRAS, and PIK3CA for detection of carcinoma [25] • Methylated SEPT9 related to early cancer screening [26]	• Mutated *KRAS* genes may escape anti-EGFR therapy [27] • Methylated BCAT1 and IKZF1 gene detection related to the detection of minimal residual disease [26] • somatic structural variants associated with prognosis and monitoring [26]
Lung cancer	• Lung cancer-specific methylation sites may help in the early detection of lung cancer such as *SHOX2* gene hypermethylation is related to squamous cell carcinoma of lung [28]	• Higher expression of Ep-CAM and other genes (*PIK3CA*, *AKT2*, *TWIST* and *ALDH1*) is related with metastasis and aggressiveness [28] • EGFR T790M mutation in non-small cell lung cancer causes resistance of EGFR inhibitor therapy [29]
Prostate cancer	• GSTP1 promoter methylation helps in prostate cancer screening [30]	• Androgen receptor amplification, mutations are related to primary drug resistance [30] • Androgen receptor L702H and AR-T878A mutations related to acquired drug resistance [30]

Management and prognosis: Liquid biopsy play a vital role in the treatment and assessment of prognosis of the tumour. The CTC is the direct 'dynamic diagnostic tool', and the count of CTC in the circulation before and after therapy indicates the response of the therapy [20].

Specific mutational changes of genes such as p53, K-ras and APC are highly correlated with the stage of cancer. It is particularly true in cases of breast, ovary and colonic carcinomas. Liquid biopsy can show these mutational changes in CTC and may predict the tumour stages [19]. It was shown that microsatellite DNA changes demonstrated in cf-DNA are related to a more aggressive course in carcinoma of the breast [24]. In the resectable tumour, the liquid biopsy may not have much role to play. However, in the case of advanced cancer, a liquid biopsy may have a significant role. The presence of K-RAS mutation in non-small cell lung carcinoma (NSCLC), predicts poor prognosis. This mutational change s can be demonstrated in the cell-free DNA of the plasma of the patient [31]. The development of EGFR T790M mutation in NSCLC causes resistance of EGFR inhibitor therapy. Therefore, the identification of such mutational changes in lung carcinoma cases in the blood of the patient by liquid biopsy may help to decide to start 'third-generation EGFR inhibitors' therapy [29]. The amplification of 'MYC related oncogene (MYCN)' is related to the aggressiveness of neuroblastoma [32]. This particular information is essential for the decision to start immunotherapy. Liquid biopsy can successfully

specific mutational changes are helpful to detect the recurrence of malignancy. Diehl et al. detected mutant gene fragments in the cf-DNA in the plasma of operated case of colorectal carcinoma by BEAMing PCR method. They identified the recurrence of colorectal carcinoma with 100% sensitivity and specificity [19]. The various other studies also highlighted the importance of the demonstration of tumour specific mutational changes in cf-DNA to identify the recurrence of breast, lung and oral carcinomas [34, 35].

Conclusions and future perspectives

The liquid biopsy is a relatively new area with vast potential. It may help significantly in the early detection of cancer, individualized therapeutic management, treatment response, prognosis and identification of minimal residual disease. However, there are several challenges in this field. As the number of CTCs is very less in circulation, so the technology to identify and enrich these cells should be robust. Till date, FDA has approved only two systems for CTC enrichment: 1) CellSearch assay (Menarini Silicon Biosystems, Italy) and image cytometry [36]. The second challenge is the cost of the liquid biopsy, which is several thousand times higher than conventional biopsy. Moreover, the technology is too advanced to implement in routine laboratories.

The third challenge is the interpretation of the data generated from CTC and cf-DNA. We need to know the important genetic alteration of the tumour in the course of

the tumour progression. Therefore, a thorough knowledge of the dynamic alteration of CTC, and cf-DNA are needed [37].

The fourth challenge in this filed is variable sensitivity and specificity of liquid biopsy. The various method of separation of the components of liquid biopsy and application of different sophisticated PCR and NGS technologies are often responsible for variable results. Therefore, proper standardization and validation are necessary for the implementation of liquid biopsy in laboratory [37].

In brief, the liquid biopsy is the most promising technology in the diagnosis, and monitoring of therapy of carcinoma. Till now, there are many hurdles for the routine application of liquid biopsy as most of the studies are for research purposes. The more number of studies are needed to solve many important questions and also to standardize and clinically implement newer technology.

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References

[1] Liquid biopsy (2020). https://www.cancer.gov/publications/dictionaries/cancer-terms/def/liquid-biopsy (accessed 28 July 2020).

[2] Tamkovich SN, Cherepanova AV, Kolesnikova EV, Rykova EY, Pyshnyi DV, Vlassov VV, et al (2006). Circulating DNA and DNase activity in human blood. *Ann N Y Acad Sci*; 1075:191–196. https://doi.org/10.1196/annals.1368.026

[3] van der Vaart M, Pretorius PJ (2010). Is the role of circulating DNA as a biomarker of cancer being prematurely overrated? *Clin Biochem*; 43:26–36. https://doi.org/10.1016/j.clinbiochem.2009.08.027

[4] Zaporozhchenko IA, Ponomaryova AA, Rykova EY, Laktionov PP (2018). The potential of circulating cell-free RNA as a cancer biomarker: challenges and opportunities. *Expert Rev Mol Diagn*; 18:133–145. https://doi.org/10.1080/14737159.2018.1425143

[5] Pan BT, Johnstone RM (1983). Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell*; 33:967–978. https://doi.org/10.1016/0092-8674(83)90040-5

[6] Harding CV, Heuser JE, Stahl PD (2013). Exosomes: looking back three decades and into the future. *J Cell Biol*; 200:367–371.

[7] Schneck H, Gierke B, Uppenkamp F, Behrens B, Niederacher D, Stoecklein NH, et al (2015). EpCAM-independent enrichment of circulating tumor cells in metastatic breast cancer. *PLoS One*; 10:1–23.

[8] Viswanath B, Kim S, Lee K (2016). Recent insights into nanotechnology development for detection and treatment of colorectal cancer. *Int J Nanomedicine*; 11:2491–2504. https://doi.org/10.2147/IJN.S108715

[9] Momen-Heravi F, Balaj L, Alian S, Mantel PY, Halleck AE, Trachtenberg AJ, et al (2013). Current methods for the isolation of extracellular vesicles. *Biol Chem*; 394:1253–1262.

[10] Tost J (2016). The clinical potential of enhanced-ice-COLD-PCR. *Expert Rev Mol Diagn*; 16:265–268.

[11] Board RE, Ellison G, Orr MCM, Kemsley KR, McWalter G, Blockley LY, et al (2009). Detection of BRAF mutations in the tumour and serum of patients enrolled in the AZD6244 (ARRY-142886) advanced melanoma phase II study. *Br J Cancer*; 101:1724–1730.

[12] Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D (2006). BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nat Methods*; 3:551–559.

[13] Little S (2001). Amplification-refractory mutation system (ARMS) analysis of point mutations. *Curr Protoc Hum Genet*; Chapter 9:Unit 9.8. https://doi.org/10.1002/0471142905.hg0908s07

[14] Oxnard GR, Paweletz CP, Kuang Y, Mach SL, O'Connell A, Messineo MM, et al (2014). Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. *Clin Cancer Res*; 20:1698–1705. https://doi.org/10.1158/1078-0432.CCR-13-2482

[15] Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DWY, Kaper F, et al (2012). Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci Transl Med*; 4(136):136ra68. https://doi.org/10.1126/scitranslmed.3003726

[16] Newman AM, Bratman SV, To J, Wynne JF, Eclov NCW, Modlin LA, et al (2014). An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nat Med*; 20:548–554. https://doi.org/10.1038/nm.3519

[17] Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B (2011). Detection and quantification of rare mutations with massively parallel sequencing. *Proc Natl Acad Sci U S A*; 108:9530–9535. https://doi.org/10.1073/pnas.1105422108

[18] Chang HW, Lee SM, Goodman SN, Singer G, Cho SKR, et al (2002). Assessment of plasma DNA levels, allelic imbalance, and CA 125 as diagnostic tests for cancer. *J Natl Cancer Inst*; 94:1697–1703. https://doi.org/10.1093/jnci/94.22.1697

[19] Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al (2008). Circulating mutant DNA to assess tumor dynamics. *Nat Med*; 14:985–990. https://doi.org/10.1038/nm.1789

[20] Alix-Panabières C, Pantel K (2016). Clinical

applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. *Cancer Discov*; 6:479–491. https://doi.org/10.1158/2159-8290.CD-15-1483

[21] Alimirzaie S, Bagherzadeh M, Akbari MR (2019). Liquid biopsy in breast cancer: a comprehensive review. *Clin Genet*; 95:643–660. https://doi.org/10.1111/cge.13514

[22] Shaw JA, Page K, Blighe K, Hava N, Guttery D, Ward B, et al (2012). Genomic analysis of circulating cell-free DNA infers breast cancer dormancy. *Genome Res*; 22:220–231. https://doi.org/10.1101/gr.123497.111

[23] Ibrahim EM, Kazkaz GA, Al-Mansour MM, Al-Foheidi ME (2015). The predictive and prognostic role of phosphatase phosphoinositol-3 (PI3) kinase (PIK3CA) mutation in HER2-positive breast cancer receiving HER2-targeted therapy: a meta-analysis. *Breast Cancer Res Treat*; 152:463–476. https://doi.org/10.1007/s10549-015-3480-6

[24] Silva JM, Garcia JM, Dominguez G, Silva J, Miralles C, Cantos B, et al (2002). Persistence of tumor DNA in plasma of breast cancer patients after mastectomy. *Ann Surg Oncol*; 9:71–76. https://doi.org/10.1245/aso.2002.9.1.71

[25] Peyressatre M, Prével C, Pellerano M, Morris MC (2015). Targeting cyclin-dependent kinases in human cancers: from small molecules to peptide inhibitors. *Cancers (Basel)*; 7:179–237.

[26] Ding Y, Li W, Wang K, Xu C, Hao M, Ding L (2020). Perspectives of the application of liquid biopsy in colorectal cancer. *Biomed Res Int*; 2020:6843180. https://doi.org/10.1155/2020/6843180.

[27] Mostert B, Jiang Y, Sieuwerts AM, Wang H, Bolt-De Vries J, Biermann K, et al (2013). KRAS and BRAF mutation status in circulating colorectal tumor cells and their correlation with primary and metastatic tumor tissue. *Int J Cancer*; 133:130–141. https://doi.org/10.1002/ijc.27987

[28] Revelo AE, Martin A, Velasquez R, Kulandaisamy PC, Bustamante J, Keshishyan S, et al (2019). Liquid biopsy for lung cancers: an update on recent developments. *Ann Transl Med*; 7:349. https://doi.org/10.21037/atm.2019.03.28

[29] Castellanos-Rizaldos E, Grimm DG, Tadigotla V, Hurley J, Healy J, Neal PL, et al (2018). Exosome-based detection of EGFR T790M in plasma from non–small cell lung cancer patients. *Clin Cancer Res*; 24:2944–2950. https://doi.org/10.1158/1078-0432.CCR-17-3369

[30] Riaz I Bin, Wang L, Kohli M (2018). Liquid biopsy approach in the management of prostate cancer. *Transl Res*; 201:60–70. https://doi.org/10.1016/j.trsl.2018.05.004

[31] Nygaard AD, Garm Spindler KL, Pallisgaard N, Andersen RF, Jakobsen A (2013). The prognostic value of KRAS mutated plasma DNA in advanced non-small cell lung cancer. *Lung Cancer*; 79:312–317. https://doi.org/10.1016/j.lungcan.2012.11.016

[32] Brodeur GM (2003). Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer*; 3:203–216. https://doi.org/10.1038/nrc1014

[33] Combaret V, Bergeron C, Noguera R, Iacono I, Puisieux A (2005). Circulating MYCN DNA predicts MYCN-amplification in neuroblastoma. *J Clin Oncol*; 23:8919–8920. https://doi.org/10.1200/JCO.2005.04.0170

[34] Hamana K, Uzawa K, Ogawara K, Shiiba M, Bukawa H, Yokoe H, et al (2005). Monitoring of circulating tumour-associated DNA as a prognostic tool for oral squamous cell carcinoma. *Br J Cancer*; 92:2181–2184. https://doi.org/10.1038/sj.bjc.6602635

[35] Sozzi G, Conte D, Mariani L, Lo Vullo S, Roz L, Lombardo C, et al (2001). Analysis of circulating tumor DNA in plasma at diagnosis and during follow-up of lung cancer patients. *Cancer Res*; 61:4675–4678.

[36] de Wit S, van Dalum G, Terstappen LWMM (2014). Detection of circulating tumor cells. *Scientifica (Cairo)*; 2014:1–11.

[37] Castro-Giner F, Gkountela S, Donato C, Alborelli I, Quagliata L, Ng C, et al (2018). Cancer diagnosis using a liquid biopsy: challenges and expectations. *Diagnostics;* 8:31.