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POSSIBILITIES OF LIQUID BIOPSY IN CLINICAL PRACTICE

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Summary

Liquid biopsy represents innovation in the field of oncology diagnosis. It refers to non-invasive blood collection method with isolation of circulating tumor cells, cell-free DNA (with circulating tumor cell free DNA fraction), circulating exosomes or micro RNA. Although requires specific methods for isolation and analysis of targets of interest, liquid biopsy could be of benefit in preoperative prediction od overall survival of patients with carcinoma, for monitoring of disease and progression, along with surveillance of therapy response and early recurrence of cancer. The review describes methods for liquid biopsy analysis, together with concerns and achievements in implementing liquid biopsy in clinical practice.

KEY WORDS: *liquid biopsy, circulating tumor DNA - ctDNA, circulating tumor cells – CTCs, circulating microRNA – cmiRNA, exosomes, clinical practice*

MOGUĆNOSTI TEKUĆE BIOPSIJE U KLINIČKOJ PRAKSI

Sažetak

Tekuća biopsija predstavlja inovaciju na području onkološke dijagnostike. Neinvazivna je metoda kojom se iz uzorka krvi izoliraju cirkulirajuće tumorske stanice, slobodna cirkulirajuća DNA (uz frakciju tumorske DNA), cirkulirajući exosomi ili mikroRNA. Iako zahtjeva specifične metode izolacije i analize pojedinih elemenata od interesa, tekuća biopsija se može primijeniti u predoperativnoj predikciji preživljenja pacijenata s karcinomom, za praćenje tijeka i progresije bolesti, kao i za praćenje odgovora na terapiju ili ranog povratka bolesti. Članak se bavi opisom do danas poznatih metoda analize komponenata tekuće biopsije, kao i problematikom i postignućima u uvođenju tekuće biopsije u kliničku praksu.

KLJUČNE RIJEČI: *tekuća biopsija, cirkulirajuća tumorska DNA - ctDNA, cirkulirajuće tumorske stanice – CTCs, cirkulirajuća mikroRNA - cmiRNA, egzosomi, klinička praksa*

Cancer incidence worldwide counted 14,1 million new cases in 2012, and the number is predicted to overgrow 23 million cases each year by the 2030 (1). Early-stage tumor diagnosis represents leading problem in diagnosis of cancer within asymptomatic population, considered „healthy". Great number of tumor biomarkers are being examined nowadays, but not many have reached clinical practice. Sensitivity and specificity of these biomarkers remain suboptimal for early-stage, or residual tumor diagnosis. Consequently, interest in tumor molecular diagnostics is rapidly increasing. Up to these days tissue biopsy has been the gold standard for cancer diagnosis, but its limitations and potential risks of complications lead to comprehensive search for less invasive techniques. The discovery of cell free DNA (cfDNA) by Mandel and Metais in 1948 (2) and circulating tumor cells (CTCs) in bloodstream revealed the new era of molecular diagnostics of tumor diseases. Liquid

biopsy refers to less invasive technique which provides CTCs, cfDNA, cell free tumor DNA (ctDNA), circulating microRNA (cmiRNA) and exosomes from patient's plasma. Development of molecular technologies such as PCR (polymerase chain reaction) or NGS (next generation sequencing) made analysis of ctDNA possible, in despite of low concentration in plasma.

Tissue biopsy and liquid biopsy- compared

Tissue biopsy provides enough material for pathohistological and molecular analysis giving information on pathohistological and molecular changes within tumor cells. Although it allows malicious and benign tumor differentiation and classification, it also has shortcomings. Fine or core needle biopsy has risks of inflammation, tumor metastasis induced by biopsy (3), (4), bleeding, or other complications, also it is painful for patient. Further biopsies during treatment are often impossible to be done due to the risks listed. Another disadvantage of tissue biopsy is affordability as it is performed by experienced professional and is usually guided by ultrasound, computed tomography scan (CT scan) or mammography. CT guided fine needle aspiration biopsy cost for lung cancer patients was presented in a study. The cost (including procedure, adverse events and pathology tests) was $4130,09 on average, while blood-based genome test (including blood draw and pathology tests) summed up to $836,45. Another tissue biopsy disadvantage of concern is turnaround time (TAT); while DNA analysis results can be obtained within 71 hours from blood collection, biopsy results are usually given within 1 or 2 weeks from sampling. Molecular analysis obtained from formalin-fixed paraffin-embedded (FFPE) tissue sample has weaknesses; non-standardized preparation methods and toxic fixatives induce DNA degradation, thus DNA integrity verification is necessary prior to molecular analysis (5). As it grows, tumor mutates and tumor tissue becomes molecularly heterogeneous – one needle aspiration can hardly collect all types of tumor cells, and sometimes re-aspiration is needed -this is where liquid biopsy is welcomed. False negative pathohistological or cytological results of patients with early-stage cancer can be consequence of imprecise needle aspiration. On the contrary, ctDNA can be found in some patients with early-stage cancer disease and can detect residual lesions following therapy. High sensitivity and specificity of ctDNA analytical methods can help diagnose and monitoring of invasive cancer in early stage, together with recurrence of the cancer.

Characteristics of liquid biopsy compounds

CTCs-circulating tumor cells

Origin of CTCs can be primary tumor or secondary metastatic sites. Detached cancer cells have a role in metastasis through blood circulation. CTCs found in blood have epithelial /mesenchymal tumor origin and express epithelial markers on their surface (6) for example EpCAM (Epithelial Cell Adhesion Molecules) or CDH2 (Cadherin-2, N-cadherin) – a molecule included in cell-to-cell adhesion and transendothelial metastasis of cancer cells (7). Epithelial tumor cells go through epithelial to mesenchymal transition (EMT) - changing surface molecules and weakening intercellular adhesion. This process affects the success of the isolation of CTCs using antibodies targeting epithelial surface molecules. Transitional cells gain the ability to survive inside bloodstream and invade distance tissue. Adhesion of CTCs and progression of metastatic cancer depend on optimal flow of blood - "mechanic hypothesis" and local microenvironment of the adhesion site – the "seed and soil" hypothesis (8). Great number of researches had shown correlation evidence between blood flow pattern and metastasis of primary tumor cells. CTCs (mean diameter of 12 to 20 μm) are usually trapped inside capillaries of lungs and liver (diameter 3-8 μm), or other capillary perfused organs (7). CTCs need to escape anoikis (programmed cell death that occurs when cells detach from the surrounding extracellular matrix) and immune system defence prior to metastasis (9). Some of these mechanisms include clustering with other cells, such as platelets -"cloaking effect" while other mechanisms are yet to be described. Platelets and monocytes (found in CTC clusters) and macrophages mediate adhesion and thrive the metastatic progress (10,11). Value of CTCs number clinically correlates with invasive progression of cancer, tumor mass and decreased overall survival in patients with different cancer varieties. Using different platforms circulating tumor cells can be sufficiently

separated from other blood cells, despite the low count in which they are found (1 CTC per 10^6 – 10^8 white blood cells or 0-10 CTC/mL of blood (12)). Single tumor cells can be isolated using EpCAM expressed on their surface, or can be separated by size using microporous filter membranes, since CTCs are bigger in size in relation to blood cells. Isolated cells can be characterized by immunostaining of cytokeratin or other specific molecules expressed in tumor cells. On the other hand, clusters of CTCs, which have higher metastatic potential, can be separated physically using microfluidic bifurcating traps (13). Morphological characteristics define best isolation methods to be used in particular research purposes.

ctDNA – cell free tumor DNA

ctDNA can be isolated from plasma or serum of cancer patients, together with other cell-free DNA. Short half-life of ctDNA in blood (~ 2 hours) reflects real-time tumor stage(14), and can be clinically used to monitor tumor progression or treatment efficacy, while some protein biomarkers circulate in bloodstream for several weeks. Concentration range of cfDNA in healthy individuals is 0-35 ng/mL while numbers can exceed 1000 ng/mL in cancer patients group. Depending on cancer stage and metastasis ctDNA varies from 0,01% to >90% of cfDNA concentration (15). ctDNA can be either single- or double-stranded DNA, with apoptotic or necrotic tumor cell origin, or can be spontaneously released into the bloodstream by living tumor cells in the form of nucleosomes. Different studies find ctDNA shorter than non-cancer cell free DNA, while some find it opposite – different conclusions can be linked to the variations of detection methods. The approximal length of ctDNA fragment (around 166 bp) indicates its apoptotic origin, during apoptosis DNA is cleaved in between nucleosomes at ~ 180 bp intervals (the endonuclease cleavage leaves "ladder-like" pattern on gel electrophoresis). Other evidences support hypothesis of CTCs as one of the ctDNA sources in blood, since mutations found in ctDNA are identical to the ones found in CTC from the same patient sample(16). Tumor tissue biopsy results can prove ctDNA origin; point mutations, copy number variations or chromosome aberrations as well as epigenetic molecular changes (DNA methylation, sulfonation) or cancer derived viral sequences found in ctDNA will be identical to tumor cell-DNA biopsy, if the samples were taken properly. Garcia-Olmoand coworkers established the hypothesis of genometastasis in 1999 with remarkable discovery of cancer transformation of cultured cells induced by addition of plasma taken from tumor bearing rats (17). The following studies demonstrated oncogenic transformation of susceptible cultured cells caused by addition of plasma containing ctDNA obtained from colorectal carcinoma (CRC) patients (18). Trejo-Becerril et al. brought evidence to the hypothesis of genometastasis with research showing the ability of ctDNA to prompt horizontal tumor progression in an immunocompetent colon-carcinogenesis rat model (19). Oncogenic transformation of cells did not occur when serum and supernatants were depleted of DNA, thus confirming tumorigenic characteristics of ctDNA. The mechanism of ctDNA uptake by the host cells remains unclear. Few studies point out circulating apoptotic bodies as primary source of ctDNA, while others suggest DNA/RNA complexes ("virtosomes") as one of the forms in which ctDNA is taken by host cell (15).

Exosomes

Exosomes are microvesicles (3-200 nm in size) containing RNA, proteins and lipids inside lipid membrane. Physiological function of exosomes circulating in blood is communication and transfer of molecules between distant cells. Tumor cells use exosomes for stimulation of cell proliferation, angiogenesis and metastasis. On the molecular point of view, exosomes released by tumor cells carry highly specific molecular patterns and contribute to the detailed study of cancer metabolism. A study presented specific molecule – glypican-1 (GPC1(+)) isolated from circulating exosomes found in serum of patients with pancreatic cancer. This molecule had absolute specificity and sensitivity for distinguishing healthy subjects and patients with a benign pancreatic disease from patients with early- and late-stage pancreatic cancer (20).

cmiRNA – circulating micro RNA

cmiRNA can be found inside exosomes or in a free-form released into bloodstream by tumor or other cells (21). RNA is fragile molecule, and requires RNase inhibitors and RNase free equip-

ment in order to avoid destruction during isolation and sample manipulation. However, miRNAs are relatively stable in blood containing RNases (necessary for viral RNA degradation), but the mechanism of RNase circumvention remains indistinct (21). miRNAs are single-stranded, non-coding RNA molecules that are 20-23 nucleotides long and have a role in silencing mRNA and post-transcriptional regulation of gene expression, while specific cmiRNA patterns can be found in cancer patients (22) such as circulating miR-21 which can be used as potential tumor biomarker in CRC (23).

TEP-tumor educated platelets

One of the cells that also participates in tumor growth and dissemination and affects their gene expression are platelets. RNA profile of blood platelets is influenced by tumor type, progression and metastasis as tumor alerts premRNA splicing. TEPs provide specific information on the location and characteristics of primary tumor. In 283 patients, Best et al. (24) have done TEPs mRNA sequencing and differed patients with primary tumor and metastasis from healthy ones with 96 % accuracy. Location of primary tumor was correctly identified in 71% of patients and different mutations were described. TEPs isolation should be done within 48 hours after blood collection, but the risk of change in mRNA profile increases during that period. Diseases other than cancer can also alert platelet mRNA profile, however, larger studies are required on this subject.

Methodologies in liquid biopsy analysis

The selection of method should be made regarding the purpose of the analysis; it is hard to find optimal method to be used in clinical routine due to complex nature of liquid biopsy analytes. Different methods may be required for early-stage recognition and monitoring therapy efficacy. For example: CTCs can be isolated intact and cultivated for further therapy analysis but the method could be time consuming, while ctDNA concentration analysis, which can help monitoring tumor progression, could be done within hours. In the field of liquid biopsy, numerous researches are struggling with method optimization and pre-analytical phase requests, which have to be met for successful analytical phase. Nowadays, next generation sequencing (NGS), pyrosequencing and other similar methods bring improvement and advances in cancer research and post-analytical phase, making it easier for clinicians to interpret.

Pre-analytical phase

The Biomarkers Consortium Workshop held in 2010 brought together the experts in the field of CTC research to share experience and compare the performances of most promising assay technologies with aim to take initial step in clinical validation of these methods (25). In order to enter clinical practice, pre-analytical phase variables should be standardized for each assay – sample acquisition, processing, storage and transport conditions should be defined.

CTCs pre-analytical requirements:

Liquid biopsy requires 5 to 10 mL of blood taken on anticoagulant EDTA tubes used for blood collection need to be processed within 24 hours of blood collection to minimize cell degradation. Procedures can differ regarding stability, size and concentration in plasma in dependence of examined CTCs and method used.

cfDNA/ ctDNA pre-analytical requirements

Since ctDNA builds up a fraction in circulating cell free DNA, cfDNA should be isolated in order to analyse ctDNA. Isolation from plasma or serum sample is performed using isolation kits; shipping, storage or quality of sample, however, can affect isolation's efficiency. Blood cell degradation in pre-analytical phase can contaminate the sample with genomic DNA. Manufacturers give instructions on sample acquisition, processing and storage - tested and optimized in studies that preceded putting the isolation kit on market. Some manufacturers even examined the influence of shaking and storage conditions in a way of contamination with genome DNA in plasma collected in preservative tubes which they made for this purpose especially, and compared it to other commercially available K_2EDTA tubes.

cmiRNA pre-analytical requirements

Studies on miRNA confirmed advantages of plasma over serum samples (26). During clot process required for serum, non-specific miRNA is

released from platelets, lysed red blood cells, leukocytes, and circulating tumor cells, contaminating the cmiRNA. Plasma is collected in tubes containing standard blood anticoagulants: EDTA or sodium citrate followed by centrifugation. Heparin anticoagulant might interfere with analytic methods (inhibits polymerase and reverse transcriptase enzymes used in PCR), therefore, heparin tubes are not recommended (27). Standard protocol for sample handling and storage of miRNA samples have yet to be made, however, cryopreservation on -80°C reduces degradation during long-term storage. All limitations listed can affect the interpretation of miRNA results; therefore, standardized protocols are indispensable.

Analytical phase

Clinical practice has set the bar high when discussing analytical phase requirements; high sensitivity (with low limit detection) and specificity are of main importance, especially for tumor biomarkers since diagnostic criteria and therapeutic protocols depend on biomarker results. Reportable ranges have to be set prior to clinical use of tumor marker, unfortunately, cancer diagnosis is sometimes hard to set due to complexity of cancer, and tumor biomarkers cannot be easily put in between range boundaries. Assay reproducibility and robustness required for routine work represent one of the main difficulties in implementing liquid biopsy in everyday clinical practice.

CTCs

CTCs assay technologies used in process are determined by target cells and assay outcome-characterization, enumeration or enrichment. Immunoassays are widely used in detection and enrichment of rare CTCs. Specific antigen-antibody reaction allows isolation of EpCAM+, CD45-, HER2/neu+ or other tumor cells, following purification can be performed with immunomagnetic beads or columns. Downstream processes usually include fluorescent antibodies staining and labelling of intracellular components and confocal microscopy. Other technologies are based upon CTC size, for example, CTC Membrane Microfilter is designed to exploit cell size differences between normal blood cell and tumor cells and following characterization of the tumor cell is done by quantum dot-based immunofluorescence detection (28). If viable, intact cells are required for cultivation or other analysis, size based vacuum filtration would be a method of choice, e.g. ISET (Isolation by Size of Epithelial Tumor cells) and Nucleopore Assay methods. Flow cytometry is also one of technologies with wide application in CTC analysis. Some technologies took a step forward and exploit differences in density and dielectric properties for differentiation of cells (Dielectroforetic Field Flow Fractionation – DFFF). Extraordinary assays use nanodetectors binding EpCAM+ cells *in vivo*, or even noninvasively count CTCs by injection of tumor-specific fluorescent ligand followed by multiphoton fluorescence imaging of superficial blood vessels to quantitate the flowing CTCs. CTC undergoing EMT can be separated using negative enrichment – red cells are lysed and CD45+ cells (leukocytes) removed by immunomagnetic beads, but still have to be distinguished from remaining epithelial cells. Manifold technologies are used in but still have not reached clinical practice.

cfDNA

cfDNA is isolated from plasma using specialized DNA isolation kits prior to ctDNA analysis– numerous articles compared different commercially available kits (29) (e.g. QIAamp® circulating nucleic acid kit (QIA - Qiagen), PME free-circulating DNA Extraction Kit (PME - Analytik Jenna), MagTMcfDNA Isolation Kit (NpM - BiooScientific)). Steps following isolation include measurement with quantitative PCR – qPCR (based on fluorescence of probes attached to amplified DNA segments), digital droplet PCR (ddPCR), quantitative kits for measuring cfDNA concentration (based on fluorescence and standard curve) or sequencing of cfDNA fragments (using NGS, pyrosequencing or other methods). Sequencing usually requires pre-amplification of cfDNA and library construction using multiple-primer reaction. Ion Torrent™ technology by ThermoFisher Scientific is platform that allows generating gigabases of sequence per day, and enables new experiments on complementary DNA (cDNA) sequencing. This technology directly translates chemically encoded information (A, C, G, T) into digital information (0, 1) on a semiconductor chip, resulting in simpler, faster, more cost-effective and scalable next-generation sequencing. Technology evolution and

widening of gene databases contributes to screening and early recognition of cancer diseases; cfDNA/ctDNA and NGS have potential to dramatically change diagnostic, monitoring and treatment of cancer once used in clinical practice, as concluded on Circulating Tumor Cells (CTC) and Cell-Free DNA (cfDNA) Workshop 2016: Scientific Opportunities and Logistics for Cancer Clinical Trial Incorporation conference (30).

cmiRNA

Previous isolation of RNA included multiple extraction steps with analyte loss and long duration of the process. Phenol-chloroform extraction (Trizol) required a large sample volume and included working with toxic reagents, but recently several isolation kits become commercially available for research purposes. MiRCURY™ RNA Isolation Kit (Exiqon, Denmark), mirVana™ PARIS™ (Life Technologies, Grand Island, NY, USA) and miRNeasy® (Qiagen, Venlo, Limburg, Belgium) made isolation of miRNA affordable, easy and practical. The amount and quality of isolated material could not be compared between these kits since the reports on this subjects haven't been done yet. Quantification of cmiRNA extracted can be done using spectrophotometer, but due to low amounts of cmiRNA, more sensitive technologies are required. Agilent Technologies 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) uses capillary electrophoresis to assess miRNA concentration (31). The integrity of isolated RNA represents another problem in miRNA analysis – degraded RNA is not a suitable analyte for NGS or microarray analysis. Reverse transcriptase PCR (RT-qPCR) is a method of choice for miRNA analysis measuring changes of gene expression to a suitable internal control. This is the "weak point" of RT-qPCR method, since few endogenous microRNAs can be used as an internal control to evaluate cmiRNA multiplication. This is due to variations of microRNA expression in several diseases and among individuals (32). Exogenous control refers to artificial microRNAs added to the samples prior to RNA extraction, but can contaminate primary samples, and it is difficult to control the amount of external RNA added to each sample. These disadvantages can be circumvented using standard curves for each microRNA. Unfortunately, this is an expensive, time-consuming method with questionable stability of stock sample used in generating curves. Microarray assay methodologies allow wide gene profiling, but require software systems for imaging and data analysis (31) and cannot be used for miRNA quantification due to low specificity and sensitivity. 3D-Gene® microarray produced by Toray (Tokyo, Japan) is improved, highly sensitive and reproducible assay expanding the horizon for miRNA analysis. Massive parallel sequencing was a complex method of miRNA analysis including RNA extraction and size fractionation of the small RNA, conversion to cDNA and PCR multiplication together with library construction. It is replaced by the HTG EdgeSeq system (HTG molecular, Tuscon, AZ, USA) which relies on the specificity of the pre-designed probes. Currently it is ongoing validation to determine specificity and sensitivity in detecting cmiRNA. The advantages of HTG EdgeSeq: highly automated protocol (with reduced user variations), adjusted sample preparation and input requirements, large number of targeted miRNAs (>2000), needless RNA extraction and absence of molecular library made it revolutionary in miRNA analysis.

The future of liquid biopsy in clinical practice

Liquid biopsy has been introduced in clinical practice through prenatal screening tests, and consequently showed great potential to be implanted in other fields, especially for diagnosis and monitoring of cancer disease. Detection of specific mutations, overall survival prediction, recurrence of disease, cultivation of CTCs for further pharmacotherapy and therapeutic targeting, as well as monitoring of therapy response can be provided from ctDNA and CTCs – the most frequently used liquid biopsy compounds.

CTCs are detectable in 60 % of metastatic breast and prostate cancers, while lower absolute number is found in metastatic CRC cancer (~ 30 – 40% of cases) (33). The explanation for this event can be found (could be) in circulatory anatomy of intestine and hepatic drainage, which prevents/disables CTCs to reach peripheral blood. However, in patients with advanced solid tumors, ctDNA was present even when CTCs were not detectable. The detection and

enumeration of CTC cells can be used in prognosis of disease and prediction of disease-free and overall survival. FDA approved CellSearch® (Veridex, New Jersey, USA) Circulating Tumor Cell Kit for enumeration based on EpCAM+ antibodies. Specificity of antibodies, however, can vary and the detection rate can be greatly changed (34). This has to be considered when interpreting the result. Consequently, serial CTC testing using CellSearch® should be used together with other clinical methods for monitoring metastatic breast, colorectal, and prostate cancer. CTC go through dynamic changes in tumor progression, therefore characterization of EMT features can detect tumor cells resistance to given therapy (35). CTCs obtained from patient before surgery can help predict the overall survival, but more importantly CTCs obtained after surgical therapy can help guide therapeutic decision (36), which represents an endless challenge for oncologists worldwide.

ctDNA gives real-time information on molecular changes inside growing tumor including copy number aberrations and other somatic mutations and deletions. Clinical correlation of ctDNA and tumor burden depends on tumor stage, localization and type. Another study detected ctDNA in 48-73% patients with gastrointestinal cancer, while all patients with metastatic GI cancer had higher levels of ctDNA (36). Circulating tumor DNA can be used in screening for CRC; FDA approved EpiProColon blood-based test that identifies methylated Septin 9 DNA. High preoperative ctDNA concentration is associated with poorer prognosis and shorter overall survival. Postoperative ctDNA is more reliable and sensitive for detection of tumor recurrence than other tumor biomarkers, such as CEA (carcinoembryonic antigen) in CRC or CA 15-3 (cancer antigen 15-3) in breast cancer. Patients were followed for 2 years after surgery: the disease reoccurred in 85% patients with detectable postsurgical ctDNA levels while 100% patients with undetectable ctDNA were recurrence-free. ctDNA can identify patients with higher risk of recurrence and help guide adjuvant therapy. Chemotherapy or radiotherapy cause extensive ctDNA release, but it is unclear if ctDNA is released from dying cells or higher levels of ctDNA are consequence of cancer resistance to therapy. Another disadvantage of ctDNA is discordance between expressed phenotype and detected genotype (not all of the mutations found take part in cancer pathogenesis). From analytical point of view, ctDNA is reliable, stable and easily isolated analyte, but requires a priori knowledge and target of interest for clinical practice.

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