Review Article

"Liquid biopsy"—ctDNA detection with great potential and challenges

Mingwei Ma[1*], Hongcheng Zhu[2*], Chi Zhang[2], Xinchen Sun[2], Xianshu Gao[1], Gang Chen[3]

[1]Department of Radiation Oncology, Peking University First Hospital, No.7, Xishiku Str., Xicheng District, Beijing 100034, China; [2]Department of Radiation Oncology, The First Affiliated Hospital, Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, China; [3]Department of General Surgery, Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing 210008, China

*These authors contributed equally to this work.

Contributions: (I) Conception and design: X Gao, G Chen; (II) Administrative support: None; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: None; (V) Data analysis and interpretation: None; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Xianshu Gao, MD, PhD. Department of Radiation Oncology, Peking University First Hospital, No.7, Xishiku Str., Xicheng District, Beijing 100034, China. Email: gao7777@139.com; Gang Chen, MD, PhD. Department of General Surgery, Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing 210008, China. Email: gulou_hospital@163.com.

Abstract: Circulating tumor DNA (ctDNA) is now being extensively studied as it is a noninvasive "real-time" biomarker that can provide diagnostic and prognostic information before, during treatment and at progression. These include DNA mutations, epigenetic alterations and other forms of tumor-specific abnormalities such as microsatellite instability (MSI) and loss of heterozygosity (LOH). ctDNA is of great value in the process of cancer treatment. However, up to date, there is no strict standard considering the exact biomarker because the development and progression of cancer is extremely complicated. Also, results of the studies evaluating ctDNA are not consistent due to the different detection methods and processing. The major challenge is still assay sensitivity and specificity for analysis of ctDNA. This review mainly focuses on the tumor specific DNA mutations, epigenetic alterations as well as detecting methods of ctDNA. The advantages and disadvantages will also be discussed.

Keywords: Circulating tumor DNA (ctDNA); biomarker; DNA mutation; DNA methylation; cancer

Submitted Aug 18, 2015. Accepted for publication Aug 25, 2015.
doi: 10.3978/j.issn.2305-5839.2015.09.29
View this article at: http://dx.doi.org/10.3978/j.issn.2305-5839.2015.09.29

Introduction

Cancer is a leading cause of death globally which needs appropriate diagnosis methods. "Solid biopsies" cannot always be performed since it has invasive characteristic and cannot reflect current tumor dynamics or sensitivity to the treatment. Therefore, it is of great value to develop noninvasive detecting methods that could monitor the real-time dynamics of cancer. "Liquid biopsy" of circulating nucleotide acids [circulating tumor DNA (ctDNA), circulating RNA or microRNAs, etc.] may be an ideal one for patients with cancer (1). This review aims at describing the current contribution of ctDNA detection in cancer patients and to analyze the advantages and disadvantages.

ctDNA mutations

Cancer cells often rely on the activation of dominant oncogenes for proliferation and survival. The presence of specific gene alteration can have diagnostic value, reflect patient's responsiveness to the treatments and predict survival (2). Tumor DNA can be detected by tracking tumor-specific mutations or aberrant rearrangements. Since multi-site biopsies repeated sequentially is unpractical, a non-invasive DNA detection approach from peripheral blood may help reflecting dynamic changes in cancer cells. At present, advanced technologies have turned precise ctDNA mutation detection to reality (3).

Detecting methods

Genetic mutation in cancer and genome-wide association analysis

Genetic mutation profiles

Variety types of cancer are sensitive to inhibition of a certain kind of molecular pathway signaling. Somatic mutations and epigenetic alterations in known components of the signaling network might influence treatment efficacy. Moving beyond the target gene, tumor cells can develop therapy resistance through acquisition of mutations (4). Therefore, additional pathway signatures might be involved in prediction of individual response.

Multigene test moved the clinical pharmacogenomics tests away from conventional sequencing of single genes and marked a huge step towards more comprehensive analysis of cancer genome ever since. It allows the oncologists to identify the targetable genetic aberrations and direct the patients to the targeted therapy if a potential drug is available (5,6). However, cancer is a multigene disease which arises as a result of the mutational activation of oncogenes coupled with the mutational inactivation of tumor suppressor genes. These genetic alterations can synergize or antagonize each other and often occur according to a preferred sequence.

Molecular cytogenetic expectation

The methods of molecular cytogenetic, such as spectral karyotyping, fluorescence in situ hybridization (FISH) and chromosome-based comparative genomic hybridization (CGH) improved resolution and genome coverage compared with conventional karyotyping based on the visualization of metaphase chromosomes. However, the most substantial developments in obtaining increasingly more detailed and comprehensive characterizations of tumor genomes have been described during the last decade. The emergence of a range of new technologies including "omics" profiling, microarray-based CGH and single nucleotide polymorphism (SNP) analysis, and next-generation sequencing (NGS) enabled to interrogate the tumor genome and proteome in a more unbiased way and led to remarkable insight into tumor biology (7).

Chip-based microarray

Genome-wide scan using microarray platform are applied to identify genetic variants, including Array-based CGH, which is one of the approaches to improve the detection of structural variation affecting many base pairs. This technique is based on the principle of complementary hybridization between the array of oligonucleotide probes immobilized on a slide and two differentially fluorescently labeled test and reference DNA samples (8). The principles of SNP array are similar and based on the hybridization of fragmented single-stranded DNA to arrays containing unique nucleotide probe sequences immobilized on solid surface (9). The specialized equipment can measure the signal intensity associated with each probe and its target after hybridization. SNP array platforms contain oligonucleotide probes that interrogate both copy number and SNP sites. Genome-wide association studies (GWAS) became possible by the availability of chip-based microarray technologies for analysis of more than one million SNP (10,11).

Mutation detection using cutting-edge technologies

Targeted plasma re-sequencing (TAm-Seq)

Forshew *et al.* reported that *de novo* mutation can be detected through TAm-Seq noninvasively in 2012, which they termed as TAm-Seq (12). It allowed the re-sequencing of approximately 6,000 nucleotides whilst maintaining high depth analysis. The authors conducted a proof-of-concept experiment by tracking ctDNA from an ovarian patient, which had been re-sequenced tumor tissue from a right oophorectomy specimen and identified a TP53 mutation. TAm-Seq analysis revealed the emergence of an EGFR mutation in plasma samples, as the cancer progressed, which was not found in the original specimen. Further investigation identified low frequencies of EGFR mutation from initial samples. Forshew *et al.* hypothesized that as chemotherapy regimens restrained the growth of other clones, the resistant EGFR clone, which was initially present only at low frequency, gained in dominance. They demonstrate that plasma analysis can identify heterogeneous clones from different sites of the body.

Massively paralleled sequencing (MPS)

Personalized analysis of rearranged ends (PARE) was developed by Leary *et al.* to detect unselected genetic events that span across the whole genome (13). Similarly, another MPS named "Shotgun" was used by Chan *et al.* in 2013 (14). They identified copy number variations and single nucleotide variants (SNVs) of the whole genome from the plasma of 4 patients with hepatocellular carcinoma (HCC). Furthermore, they demonstrated the ability of MPS to track ctDNA level changes pre- and post-surgery. Interestingly, shotgun MPS of the plasma was also able to distinguish between tumor types in a patient with synchronous breast and ovarian tumors. The above studies

illustrate that ctDNA analysis, through de novo mutation detection, can continue to track disease burden as tumors evolve, without the need for re-biopsy.

Whole-genome sequencing (WGS)

WGS enables detecting ctDNA in patients prohibitively expensive, regarding the limit analysis of whole genome MPS to a small number of samples due to expense (15). Although low depth, and therefore reduced cost, WGS approaches have been successful at detecting copy number variations, a higher depth of coverage is often required to detect rearrangements at high resolution or SNVs directly from plasma DNA. Furthermore, where low mutant: wild type allele frequencies exist, e.g., in early stage disease, an even higher depth of coverage would be necessary to detect ctDNA fragments. In addition, WGS approaches detect a higher ratio of intronic or passenger mutations than targeted re-sequencing (16). The clinical significance of passenger mutations is currently unknown and often not targetable.

Whole exome sequencing (WES)

To make routine analysis of de novo mutations in serial plasma samples possible, WES was performed to track tumor evolution in response to therapy. Murtaza *et al.* used this approach in a proof-of-concept study involving 6 patients with metastatic tumors. Plasma samples were collected at the beginning of treatment and at the time of relapse. Subsequent re-sequencing and variant analysis revealed that by comparing the relative representation of mutations in pre- and post-relapse samples, one could identify enrichment of mutations that may drive resistance WGS can screen a larger spectrum of the genome but is currently too expensive for routine use to detect SNVs, whereas WES approaches allow more in-depth interrogation of multiple regions but is less sensitive to identifying copy number changes (17). This work demonstrated a much more cost effective way for mutation sequencing.

Tumor-specific gene mutations

Pancreatic cancer

Pancreatic cancer has the distinction of being the first solid tumor associated with a specific mutation in ctDNA. This is partly because the *KRAS* gene is frequently mutated and easy to detect. Sorenson *et al.* used allele-specific amplification to assay for mutations in codon 12 in the plasma or serum of pancreatic adenocarcinoma patients (18).

The sensitivity of detecting primary pancreatic cancer on the basis of ctDNA is mostly 30% to 50% while the specificity is generally higher (approximately 90%) (19). A variety of detection methods including restriction digestion and single-stranded conformational polymorphism have been used. In one study, sensitivity was improved when CA199 was measured in combination with DNA measurements (20). However, although at a lower frequency (5% to 15%) than adenocarcinoma, pancreatitis cases also showed *KRAS* gene mutilations (21). Most studies have focused on KRAS mutations in pancreatic cancer because of their prevalence, other approaches have been tried. The advents of higher-throughput methods, such as NGS and digital PCR, have had a profound effect on this field. For instance, one recent study using this method showed that pancreatic duct cancer had a high rate of ctDNA than other malignancies, more so in metastatic disease than non-metastatic disease. In summary, for clinical and biological reasons, pancreatic cancer is an ideal candidate for the diagnostic and prognostic use of detection of ctDNA (22).

Colorectal cancer

Plasma or serum mutation status of KRAS, APC, and TP53 which have a high mutation frequency with colorectal cancer is correlated with diagnosis, prognosis, and also treatment response (23,24).

The overall detection rates of KRAS mutations in serum or plasma of patients with colorectal cancer were 25% to 50% (24). Also, KRAS mutations in ctDNA have been reported to have the highest level in patients with more advanced stage (25). Besides, KRAS mutations in ctDNA are also associated with a higher risk of recurrence after surgery (26,27). Analysis of circulating mutant DNA could also monitor response to monoclonal antibody therapy for colorectal cancer, which makes repeatedly monitoring patients during treatment possible (28).

The exploration for APC mutations in ctDNA has focused on exon 15, which is a hotspot for APC mutations in colorectal cancer. The rate of APC mutation detection in primary ctDNA is approximately 45%. As for TP53, the mutation rate has been identified in ctDNA in about 40% of cases. Most studies focused on portions of TP53 between exons 4 and 8, the most commonly locations of TP53 mutations in colorectal cancer (24).

Unless targeting ctDNA alterations in hotspots of certain genes, a panel targeting mutations of the *KRAS*, *TP53*, and *APC* genes enabled the detection of at least one gene mutation from approximately 75% of colorectal cancer tissue. However, those mutations could only be detected in

the serum of 45% of these patients (29).

ctDNA methylation—epigenetic changes

Unless DNA mutation, there is also gene methylation which affects their expression that can be found in ctDNA. Tumorigenesis is regulated not only by genetic but also by epigenetic alterations (2). In fact, as for detection, there are a variety of genes mutated in tumors, even when a gene is consistently mutated in a particular cancer, the gene mutations may be spread over large region that makes evaluation difficult. DNA methylation tends to occur in CpG dinucleotides in the promoter region of tumor suppressor genes that leads to expression silencing (30). Therefore, methylated ctDNA in recent years is becoming an emerging target and shows promising results.

Methylation detection methods

Methods for methylation detection emerge in an endless stream. Generally it is divided into 3 categories: (I) methylation content: high-performance liquid chromatography (HPLC) or high-performance capillary electrophoresis (HPCE); (II) candidate gene: methylation-sensitive restriction endonuclease-PCR/Southern (MSRE-PCR/Southern), bisulphite sequencing, methylation-specific PCR (MS-PCR), MethyLight, etc.; (III) methylation pattern and methylation profiling: restriction landmark genomic scanning (RLGS), amplification of inter-methylated sites (AIMS), Methylated CpG-island amplification (MCA) and so on. The most common method at present studies is usually MS-PCR.

Tumor-specific gene methylation

Methylation of ctDNA has been reported for many years. Detections of ctDNA methylation mostly focused on colorectal cancer, lung cancer, breast cancer, pancreatic cancer as well as some other types of carcinoma. Compared to mutations, the consistency of DNA methylation alterations makes it a potential promising biomarker for diagnosis, staging, monitoring response and predicting survival for cancer patients.

Colorectal cancer

Colorectal cancer is the third most common cancer both in men and in women worldwide (31). An easy and quick screening test is essential for the early diagnosis for colorectal cancer. It is reported that the promoter hypermethylation status of SEPT9 was high associated with the development of colorectal cancer. In PCR-based retrospective trials for SEPT 9 promoter methylation, sensitivity and specificity were 72-90% and 88-90% respectively (32-35). Another research from USA showed that methylated SEPT9 DNA in plasma may help screening out 72% colorectal cancer with a high specificity of 93% (34). Furthermore, SEPT 9 methylation could be found in precancerous lesions of colorectal cancer. Church *et al.* conducted a large, prospective trial to assess the accuracy of circulating methylated SEPT9 DNA for detecting colorectal cancer in 7,941 patients using a commercially available assay. It showed a disappointing result that sensitivity and specificity were 48.2% and 91.5%, respectively (36). That may because the population they enrolled were not confirmed cancer patients, which differs to other researches.

Other biomarkers such as well-known novel sequences of APC, RASSF1A and E-cadherin (37,38) as well as novel markers in plasma have also been found to correlate with colorectal cancer. A German study showed that Methylation of helicase-like transcription factor (HLTF) and hyperplasic polyposis 1 (HPP1) in serum significantly correlated with tumor size, stage, and metastatic disease, and were also prognostic factors in metastasized colorectal cancer (39).

Besides, methylation status of H3 lysine 9 (H3K9me3) and H4 lysine 20 (H4K20me3), which are hallmarks of pericentric heterochromatin in healthy donors and patients with colorectal cancer was tested and showed weak correlation between cNUCs and histone methyl marks (40).

Breast cancer

Breast cancer is the most common cancer in women both in more and less developed regions. Numerous studies were conducted aiming to analyze the methylation status of biomarker genes in breast cancer and assess possible clinical value, mostly using the candidate gene testing. That means a majority of the makers are the well-established genes, such as *cyclin D2*, *RARβ2* (41), *ESR1* (42) and so on. Dulaimi *et al.* have found that at least one hypermethylation of APC, RASSFIA or DAP-kinase could be found in 94% serum samples of all the breast cancer patients (43). Scholars from All India Institute of Medical Sciences have conducted a serious of prospective studies including 100 invasive ductal breast cancer patients. Methylation status of multidrug

resistance 1 (MDR1), Stratifin, ERα and PR, DNA repair genes-*BRCA1*, *MGMT* and *GSTP1* were tested. Significant correlation was found between methylation status of the promoter of the above genes in tumor tissue and paired serum. However, the sensitivity of these genes was not high (*MDR1* 50%, *Stratifin* 56%, *ERα* 55%, *PRB* 55%, *BCRA1* 22%, *MGMT* 26%, *GSTP1* 22%) (44-46).

Unlike using the candidate genes, methylation detection in 56 genes (MethDet-56) test was conducted to find novel methylated genes and assess the dynamics of methylation so as to monitor treatment (surgery and hormone therapy). Larger study based on these results was encouraged (41).

Lung cancer

Lung cancer is the leading cause of cancer-related death partly because the absence of early detection approach (47). Up to now there is no ideal early diagnostic method. Changes in DNA methylation may occur on its early stage. DNA methylation detection is expected to be an essential method in early diagnosis of lung cancer. There are more than 80 hypermethylated genes related to lung cancer such as *APC* (48,49), *RARb* (50,51), *RASSF1A* (52), *CDH13* (48,51), *SHOX2* (53), *SHP-1* (54). Unless early diagnosis, study focused on correlation of methylation and survival of lung cancer patients showed that CHFR methylation status correlated the results of second-line chemotherapy or EGFR TKIs in 179 of 366 patients (55).

Over-all, methylation test on ctDNA is a very encouraging and promising method to diagnose or monitor tumor. As a unique biomarker having the sufficient specificity and sensitivity is not available, a panel of multiple genes could be used.

Other forms of tumor-specific abnormalities

Microsatellite instability (MSI) and loss of heterozygosity (LOH) are another two types of genetic alterations in tumor that lead to misinterpretation. Many studies have shown that these abnormalities could be found in serum samples from patients with HCC (56,57), malignant mucosal melanoma (MMM) (58), glioma (59) and other tumor types. LOH at microsatellite markers in the plasma may also have potential correlation with diagnosis and prognosis of cancer.

Conclusions

"Liquid biopsy"—ctDNA detection has great potential in cancer diagnose, monitoring, predicting survival. Nevertheless, false-positive results exist. The major challenge with analysis of ctDNA is assay sensitivity and specificity. On this account, multigene panel analysis of ctDNA may lead to increased test sensitivity. However, evidence support this assumption is inadequate. Several hurdles still exist such as the lack of consensus in technical approaches of choice, preferable sample type, storage conditions, candidate molecules and suitable detection technique, *et al.* At the same time, the standardization of all experimental steps of techniques should be emphasized. A gap exists between reality and our hope of this "liquid biopsy". Consequently, the development of appropriate biomarkers and more accurate detection methods based on ctDNAs could benefit a lot to cancer patients and may as a result improve the clinical outcome in the near future.

Acknowledgements

This work was supported by Research and Innovation Project for College Graduates of Jiangsu Province (KYLX_0955), a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) (JX10231801), and grants from Key Academic Discipline of Jiangsu Province "Medical Aspects of Specific Environments".

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References

1. Alix-Panabieres C, Pantel K. The circulating tumor cells: liquid biopsy of cancer. Klin Lab Diagn 2014;(4):60-4.
2. Gold B, Cankovic M, Furtado LV, et al. Do circulating tumor cells, exosomes, and circulating tumor nucleic acids have clinical utility? A report of the association for molecular pathology. J Mol Diagn 2015;17:209-24.
3. Patel KM, Tsui DW. The translational potential of circulating tumour DNA in oncology. Clin Biochem 2015. [Epub ahead of print].
4. Scartozzi M, Giampieri R, Maccaroni E, et al. Phosphorylated AKT and MAPK expression in primary tumours and in corresponding metastases and clinical outcome in colorectal cancer patients receiving irinotecan-

cetuximab. J Transl Med 2012;10:71.
5. Olivier M, Hollstein M, Hainaut P. TP53 mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harb Perspect Biol 2010;2:a001008.
6. Tomilo M, Williams PD, Bowden ET, et al. Discovery and characterization of driver MAPK and PI3K pathway mutations in tumors and association with drug response in cell lines. Cancer Res 2013;73:Abstract 2897.
7. Mäbert K, Cojoc M, Peitzsch C, et al. Cancer biomarker discovery: current status and future perspectives. Int J Radiat Biol 2014;90:659-77.
8. Tomioka N, Morita K, Kobayashi N, et al. Array comparative genomic hybridization analysis revealed four genomic prognostic biomarkers for primary gastric cancers. Cancer Genet Cytogenet 2010;201:6-14.
9. LaFramboise T. Single nucleotide polymorphism arrays: a decade of biological, computational and technological advances. Nucleic Acids Res 2009;37:4181-93.
10. Shu XO, Long J, Lu W, et al. Novel genetic markers of breast cancer survival identified by a genome-wide association study. Cancer Res 2012;72:1182-9.
11. Bush WS, Moore JH. Chapter 11: Genome-wide association studies. PLoS Comput Biol 2012;8:e1002822.
12. Forshew T, Murtaza M, Parkinson C, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Sci Transl Med 2012;4:136ra68.
13. Leary RJ, Sausen M, Kinde I, et al. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Sci Transl Med 2012;4:162ra154.
14. Chan KC, Jiang P, Zheng YW, et al. Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumoral heterogeneity by massively parallel sequencing. Clin Chem 2013;59:211-24.
15. Heitzer E, Ulz P, Belic J, et al. Tumor-associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing. Genome Med 2013;5:30.
16. Leary RJ, Kinde I, Diehl F, et al. Development of personalized tumor biomarkers using massively parallel sequencing. Sci Transl Med 2010;2:20ra14.
17. Murtaza M, Dawson SJ, Tsui DW, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 2013;497:108-12.
18. Sorenson GD, Pribish DM, Valone FH, et al. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol Biomarkers Prev 1994;3:67-71.
19. Jiao L, Zhu J, Hassan MM, et al. K-ras mutation and p16 and preproenkephalin promoter hypermethylation in plasma DNA of pancreatic cancer patients: in relation to cigarette smoking. Pancreas 2007;34:55-62.
20. Maire F, Micard S, Hammel P, et al. Differential diagnosis between chronic pancreatitis and pancreatic cancer: value of the detection of KRAS2 mutations in circulating DNA. Br J Cancer 2002;87:551-4.
21. Castells A, Puig P, Móra J, et al. K-ras mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: diagnostic utility and prognostic significance. J Clin Oncol 1999;17:578-84.
22. Liggett T, Melnikov A, Yi QL, et al. Differential methylation of cell-free circulating DNA among patients with pancreatic cancer versus chronic pancreatitis. Cancer 2010;116:1674-80.
23. Hsieh JS, Lin SR, Chang MY, et al. APC, K-ras, and p53 gene mutations in colorectal cancer patients: correlation to clinicopathologic features and postoperative surveillance. Am Surg 2005;71:336-43.
24. Lecomte T, Ceze N, Dorval E, et al. Circulating free tumor DNA and colorectal cancer. Gastroenterol Clin Biol 2010;34:662-81.
25. Kopreski MS, Benko FA, Borys DJ, et al. Somatic mutation screening: identification of individuals harboring K-ras mutations with the use of plasma DNA. J Natl Cancer Inst 2000;92:918-23.
26. Lecomte T, Berger A, Zinzindohoué F, et al. Detection of free-circulating tumor-associated DNA in plasma of colorectal cancer patients and its association with prognosis. Int J Cancer 2002;100:542-8.
27. Bazan V, Bruno L, Augello C, et al. Molecular detection of TP53, Ki-Ras and p16INK4A promoter methylation in plasma of patients with colorectal cancer and its association with prognosis. Results of a 3-year GOIM (Gruppo Oncologico dell'Italia Meridionale) prospective study. Ann Oncol 2006;17 Suppl 7:vii84-90.
28. Diaz LA Jr, Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature 2012;486:537-40.
29. Wang JY, Hsieh JS, Chang MY, et al. Molecular detection of APC, K- ras, and p53 mutations in the serum of colorectal cancer patients as circulating biomarkers. World J Surg 2004;28:721-6.

30. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042-54.
31. Siegel R, Ma J, Zou Z, et al. Cancer statistics, 2014. CA Cancer J Clin 2014;64:9-29.
32. Tóth K, Galamb O, Spisák S, et al. The influence of methylated septin 9 gene on RNA and protein level in colorectal cancer. Pathol Oncol Res 2011;17:503-9.
33. Grützmann R, Molnar B, Pilarsky C, et al. Sensitive detection of colorectal cancer in peripheral blood by septin 9 DNA methylation assay. PLoS One 2008;3:e3759.
34. deVos T, Tetzner R, Model F, et al. Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. Clin Chem 2009;55:1337-46.
35. Warren JD, Xiong W, Bunker AM, et al. Septin 9 methylated DNA is a sensitive and specific blood test for colorectal cancer. BMC Med 2011;9:133.
36. Church TR, Wandell M, Lofton-Day C, et al. Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. Gut 2014;63:317-25.
37. Cassinotti E, Melson J, Liggett T, et al. DNA methylation patterns in blood of patients with colorectal cancer and adenomatous colorectal polyps. Int J Cancer 2012;131:1153-7.
38. Pack SC, Kim HR, Lim SW, et al. Usefulness of plasma epigenetic changes of five major genes involved in the pathogenesis of colorectal cancer. Int J Colorectal Dis 2013;28:139-47.
39. Philipp AB, Stieber P, Nagel D, et al. Prognostic role of methylated free circulating DNA in colorectal cancer. Int J Cancer 2012;131:2308-19.
40. Gezer U, Mert U, Ozgür E, et al. Correlation of histone methyl marks with circulating nucleosomes in blood plasma of cancer patients. Oncol Lett 2012;3:1095-8.
41. Liggett TE, Melnikov AA, Marks JR, et al. Methylation patterns in cell-free plasma DNA reflect removal of the primary tumor and drug treatment of breast cancer patients. Int J Cancer 2011;128:492-9.
42. Martínez-Galán J, Torres-Torres B, Núñez MI, et al. ESR1 gene promoter region methylation in free circulating DNA and its correlation with estrogen receptor protein expression in tumor tissue in breast cancer patients. BMC Cancer 2014;14:59.
43. Dulaimi E, Hillinck J, Ibanez de Caceres I, et al. Tumor suppressor gene promoter hypermethylation in serum of breast cancer patients. Clin Cancer Res 2004;10:6189-93.
44. Sharma G, Mirza S, Parshad R, et al. CpG hypomethylation of MDR1 gene in tumor and serum of invasive ductal breast carcinoma patients. Clin Biochem 2010;43:373-9.
45. Mirza S, Sharma G, Parshad R, et al. Clinical significance of Stratifin, ERalpha and PR promoter methylation in tumor and serum DNA in Indian breast cancer patients. Clin Biochem 2010;43:380-6.
46. Sharma G, Mirza S, Parshad R, et al. Clinical significance of promoter hypermethylation of DNA repair genes in tumor and serum DNA in invasive ductal breast carcinoma patients. Life Sci 2010;87:83-91.
47. Knoepp UW, Ravenel JG. CT and PET imaging in non-small cell lung cancer. Crit Rev Oncol Hematol 2006;58:15-30.
48. Zhang Y, Wang R, Song H, et al. Methylation of multiple genes as a candidate biomarker in non-small cell lung cancer. Cancer Lett 2011;303:21-8.
49. Usadel H, Brabender J, Danenberg KD, et al. Quantitative adenomatous polyposis coli promoter methylation analysis in tumor tissue, serum, and plasma DNA of patients with lung cancer.Cancer Res 2002;62:371-5.
50. Wang YC, Hsu HS, Chen TP, et al. Molecular diagnostic markers for lung cancer in sputum and plasma. Ann N Y Acad Sci 2006;1075:179-84.
51. Rykova EY, Skvortsova TE, Laktionov PP, et al. Investigation of tumor-derived extracellular DNA in blood of cancer patients by methylation-specific PCR. Nucleosides Nucleotides Nucleic Acids 2004;23:855-9.
52. Ponomaryova AA, Rykova EY, Cherdyntseva NV, et al. Potentialities of aberrantly methylated circulating DNA for diagnostics and post-treatment follow-up of lung cancer patients. Lung Cancer 2013;81:397-403.
53. Kneip C, Schmidt B, Seegebarth A, et al. SHOX2 DNA methylation is a biomarker for the diagnosis of lung cancer in plasma. J Thorac Oncol 2011;6:1632-8.
54. Vinayanuwattikun C, Sriuranpong V, Tanasanvimon S, et al. Epithelial-specific methylation marker: a potential plasma biomarker in advanced non-small cell lung cancer. J Thorac Oncol 2011;6:1818-25.
55. Salazar F, Molina MA, Sanchez-Ronco M, et al. First-line therapy and methylation status of CHFR in serum influence outcome to chemotherapy versus EGFR tyrosine kinase inhibitors as second-line therapy in stage IV non-small-cell lung cancer patients. Lung Cancer 2011;72:84-91.
56. Pang JZ, Qin LX, Ren N, et al. Microsatellite alterations

of circulating DNA in the plasma of patients with hepatocellular carcinoma. Zhonghua Yi Xue Za Zhi 2006;86:1662-5.
57. Pang JZ, Qin LX, Wang QQ, et al. Loss of heterozygosity of plasma circulating DNA from hepatocellular carcinoma patients and its clinical significance. Zhonghua Gan Zang Bing Za Zhi 2007;15:906-9.
58. Nakamoto D, Yamamoto N, Takagi R, et al. Detection of microsatellite alterations in plasma DNA of malignant mucosal melanoma using whole genome amplification. Bull Tokyo Dent Coll 2008;49:77-87.
59. Lavon I, Refael M, Zelikovitch B, et al. Serum DNA can define tumor-specific genetic and epigenetic markers in gliomas of various grades. Neuro Oncol 2010;12:173-80.

Cite this article as: Ma M, Zhu H, Zhang C, Sun X, Gao X, Chen G. "Liquid biopsy"—ctDNA detection with great potential and challenges. Ann Transl Med 2015;3(16):235. doi: 10.3978/j.issn.2305-5839.2015.09.29

