

Emerging molecular technologies in renal cell carcinoma: liquid biopsy

Alessia Cimadamore¹, Silvia Gasparrini¹, Francesco Massari², Matteo Santoni³, Liang Cheng⁴, Antonio Lopez-Beltran⁵, Marina Scarpelli¹ and Rodolfo Montironi¹.

¹ Section of Pathological Anatomy, Polytechnic University of the Marche Region, School of Medicine, United Hospitals, Ancona, Italy; ²Division of Oncology, S.Orsola-Malpighi Hospital, Bologna, Italy; ³Oncology Unit, Macerata Hospital, Macerata, Italy; ⁴Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, USA, and ⁵Department of Surgery, Cordoba University Medical School, Cordoba, Spain,

Short title: Liquid biopsy in renal cell carcinoma.

Keywords: renal cell carcinoma, circulating DNA, CTC, diagnosis, follow-up, genetic alteration, target therapy.

Corresponding author: Rodolfo Montironi, MD, Pathological Anatomy, Polytechnic University of the Marche Region, School of Medicine, United Hospitals, Via Conca 71, I-60126, Ancona, Italy, Phone: +39 071 5964830, Fax +39 071 889985, E-mail: r.montironi@univpm.it

Abstract

Liquid biopsy, based on the circulating tumor cells (CTCs) and cell-free nucleic acids has potential applications at multiple points throughout the natural course of cancer, from diagnosis to follow-up. The advantages of doing ctDNA assessment vs. tissue-based genomic profile are the minimal procedural risk, the possibility to serial testing in order to monitor disease-relapse and response to therapy over time and to reduce hospitalization costs during the entire process. However some critical issues related to ctDNA assays should be taken in consideration. The sensitivity of ctDNA assays depends on the assessment technique and genetic platforms used, on tumor-organ, stage, tumor heterogeneity, tumor clonality. The specificity is usually very high, whereas the concordance with tumor-based biopsy is generally low. In patients with renal cell carcinoma (RCC) qualitative analyses of ctDNA have been performed with interesting results regarding selective pressure from therapy, therapeutic resistance, exceptional treatment response to everolimus and mutations associated with aggressive behavior. Quantitative analyses showed variations of cfDNA levels at different tumor stage. Compared to CTC assay, ctDNA is more stable than cells and easier to isolate. Splice variants, information at single-cell level and functional assays along with proteomics, transcriptomics and metabolomics studies can be performed only in CTCs.

Introduction

Liquid biopsy, based on the circulating tumor cells (CTCs) and cell-free nucleic acids (i.e., circulating cell-free DNA (ccfDNA), circulating tumor DNA (ctDNA), circulating cell-free RNA (ccfRNA) or extracellular vesicles and their cargo) from blood and urine, has received great attention because of its potential tool for monitoring the disease status in patients with urogenital cancers, including renal cell carcinoma (RCC)[1]. Examining all these circulating biomarkers are beyond the scope of this review, which will focus on ctDNA in RCC.

The advantages of doing ctDNA assessment versus tissue based genomic profile are the minimal invasive procedures and the consequent minimal procedural risk, the possibility to serial testing in order to monitor disease-relapse and response to therapy over time and to reduce hospitalization costs during the entire process, from diagnosis to follow-up. From a pathological point of view, ctDNA is more likely to represent whole tumor instead that only one tumor section, it also permit to evaluate the possible changes in tumor genes expression and enlighten mechanisms underlying resistance to therapy. [1].

Critical issues related to ctDNA assays

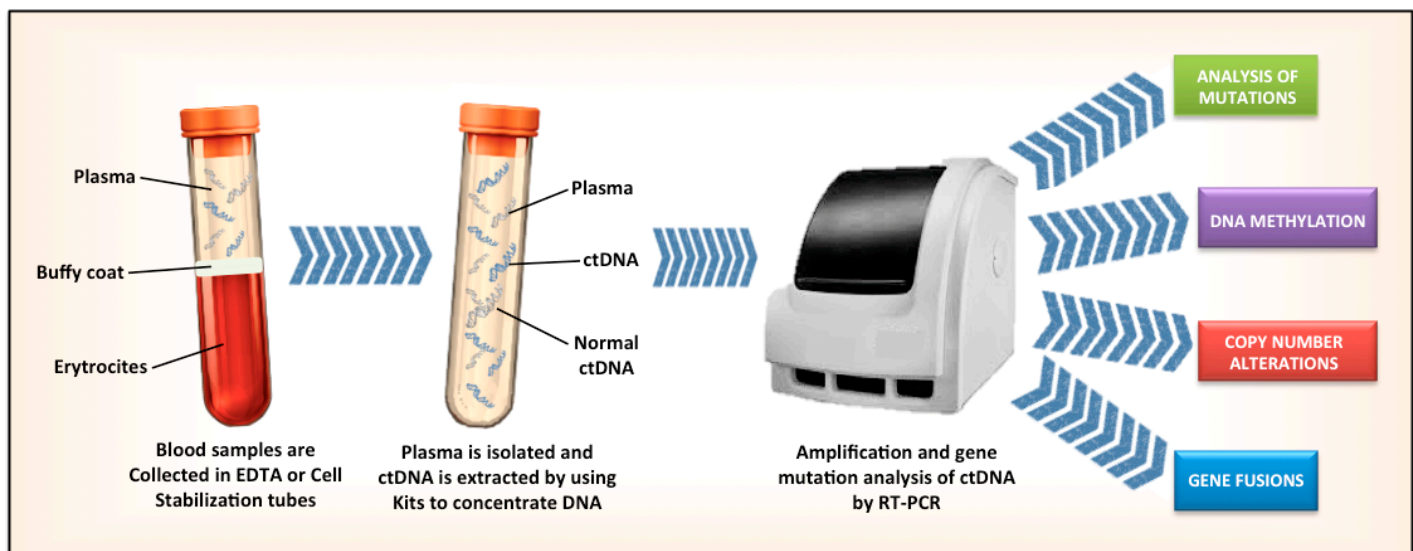
Genomic profiles of liquid biopsy have been shown to match very closely those of the corresponding tumors [2]; however, all ctDNA assays have a considerable rate of discordance with tissue testing. Except for cancer histotypes harboring a specific genetic alteration, ctDNA do not correlate directly with an histologic or cellular phenotype. Indeed, in lung cancer studies, using tissue genotyping as the reference standard, the sensitivities in finding a specific mutation is moderate (averaged 66%), while the specificity is usually very high (averaged 96%)[3–7]. Same results have been obtained by Hao and colleagues in a meta-analysis on 1,812 patients with colorectal cancer[8]. The sensitivity of ctDNA assays in detecting ctDNA in the plasma

depends on the assessment technique and genetic platforms used, on tumor-organ, stage, tumor heterogeneity, tumor clonality[9].

Commercial available platforms for assessing ctDNA detects distinct classes of genetic alterations (single nucleotide variants, insertions/deletions (indels), copy number amplifications, fusions) in different numbers of cancer-related genes. Some clinical research studies sequence specific genes of interest applying hotspots panels [10–12]. The assay can be targeted for a single or a small number of variants, or aiming for a broader coverage[13,14].

The process of genomic testing start with a blood draw in two streck tubes, DNA fragments undergo real-time or digital PCR or to Next Generation Sequence (NGS) analysis. The results are digital sequences analyzed to identify genetic alterations, quantitative mutant allele fractions and gene copy numbers. Different assays may have different lower limits of detection or interrogate different genomic regions (**Figure 1**).

Figure 1. Steps in the isolation of cell tumor DNA in the blood.



Independently of analytical factors, several biological elements may affect the sensitivity of the test. It is demonstrated that the concentration of ctDNA increased with stage; in fact the fractions of patients with cancer of any type, with detectable ctDNA was 47%, 55%, 69% and 82% for patients with stage I,II,III and IV respectively[15]. It is also demonstrated that ctDNA levels decrease when a tumor is responding to therapy[16,17] and can be used as biomarker of response to therapy[18].

Regarding tumor site, detectable levels of ctDNA was found in more than 75% of patients with metastatic cancer of pancreas, bladder, colon, breast, liver, stomach, and in less than 50% of patients with metastatic RCC, prostate and thyroid tumor.[15] The absence of a specific therapy-driving gene alteration could preclude the possibility of a patient to be treated with a target therapy. The undetected results should be confirmed with analysis of tumor tissue sample. A diagnostic approach relying only on ctDNA analysis could fail to identify relevant information in driving patient treatment decisions. Presence of detectable ctDNA has been proposed as a fourth parameter in a modified staging system: TNMB. Paralleling the 'M' category, initial categorization may be defined as the absence ('B0') or presence ('B1') of detectable ctDNA[19].

Concordance between tissue based- biopsy and ctDNA

Comparing genetic alterations (GA) detected by NGS in tumor tissue and ctDNA, Hahn et al. found that the median mutation rate for tissue NGS was 10.0 and for ctDNA was 2.2 but the concordance rate was only 8,6% [20]. Such results can be partially explained by presence of subclones, the time between tumor tissue and ctDNA NGS (mean time: 22 months, range 0–70 months) and by the fact that some patients (3 out of 19) received treatment in the period between the tests. Similar results were also obtained by Chae in 28 patients with advanced solid tumors, in which more than 50% of mutations detected in

either technique were not detected using the other biopsy technique, proposing “a potential complementary role of each assay.”[21]

In the study of Hahn, all of 19 metastatic RCC patients have GA detected on tissue based NGS while only 13 (68%) have GA at ctDNA NGS analysis. Six patients have no GA detected on ctDNA NGS assay. These so-called “negative” patients may comprehend patients with undetectable ctDNA in the metastatic phase, patients with GA not covered by the genetic platforms, or samples undergoing assay errors. Furthermore, among patients with GA detected by both biopsy methods, only five have the same mutations they had in the tissue-based NGS, all affecting *TP53* and *VHL* genes. *VHL* is a truncal mutation in mRCC and its aberrations are conserved events across all clones. However, the concordance rate for *VHL* is only 50%. According to these results, it is quite unlikely to monitor disease progression or relapse with only one gene-specific test in the follow-up.

Qualitative analyses of ctDNA

Pal and colleagues analyzed ctDNA in 220 patients with mRCC. 79% of patients had GA detected, the most frequent involving TP53 (35%), VHL (23%), EGFR (17%), NF1 (16%), and ARID1A (12%), with a preponderance of single nucleotide variants and small indels. They also compared GA detected in ctDNA analysis in first-line patients (sunitinib and pazopanib) and in later-lines patients (nivolumab, everolimus, axitinib, and cabozantinib). They note a higher frequency of TP53 (49% vs 24%, $p = 0.02$), NF1 (20% vs 3%, $p = 0.01$) alterations in later-line therapy group with subsequent VEGF-directed therapy. These findings raise the hypothesis that certain GAs may arise as a consequence of selective pressure from therapy and may have a role in therapeutic resistance[22].

Searching for specific mutations in molecular pathways known to be related to

sensitivity to therapy is functionally relevant to guide treatment decision. Sensitivity to everolimus seems to be related to presence of TSC1/TSC2/MTOR alterations. Regardless of histology, incidence of these mutations have been found in one third of patients with clinical benefit and in any of patient with non-clinical benefit[23]. In a recent report of five long-term responders mRCC patients treated with mTOR inhibitor, Voss et al. found genomic alterations with activating effect on mTOR signaling in two genes, TSC1 and MTOR, offering plausible explanation for exceptional treatment response [24]. TSC1 and TSC2 may be screened as predictive biomarkers of everolimus response in RCC patients who progressed on VEGF-targeted therapy.

Identification of specific mutations or a set of mutations associate with an aggressive tumor behavior such as *SETD2*, *PBRM1*, and *BAP1* could be relevant to clinicians considering using liquid biopsy to guide treatment of mRCC[25,26]. Al-Qassab and colleagues interrogate these prognostic genes along with other commonly mutated genes in RCC creating a panel of 14 genes. Twenty out of 30 preoperative RCC patients had detectable GA, comprehending nonsynonymous, frameshift, stopgain, or splice site mutations, in all genes assayed. Even patients with early stage disease and with 1.1 x 0.7 x 0.5 cm mass had detectable mutations in ctDNA[27].

Quantitative analyses of ctDNA

Diagnostic value of cfDNA was showed measuring cfDNA levels of healthy individual comparing to cfDNA in patients with RCC[28]. Measuring cfDNA fragments derived from cell apoptosis and those derived from cell necrosis, Hauser et al. developed a test with sensitivity of 68-57% and specificity of 70,4-81,5% respectively[29].

CpG island hypermethylation of cfDNA in patients with RCC is a potential

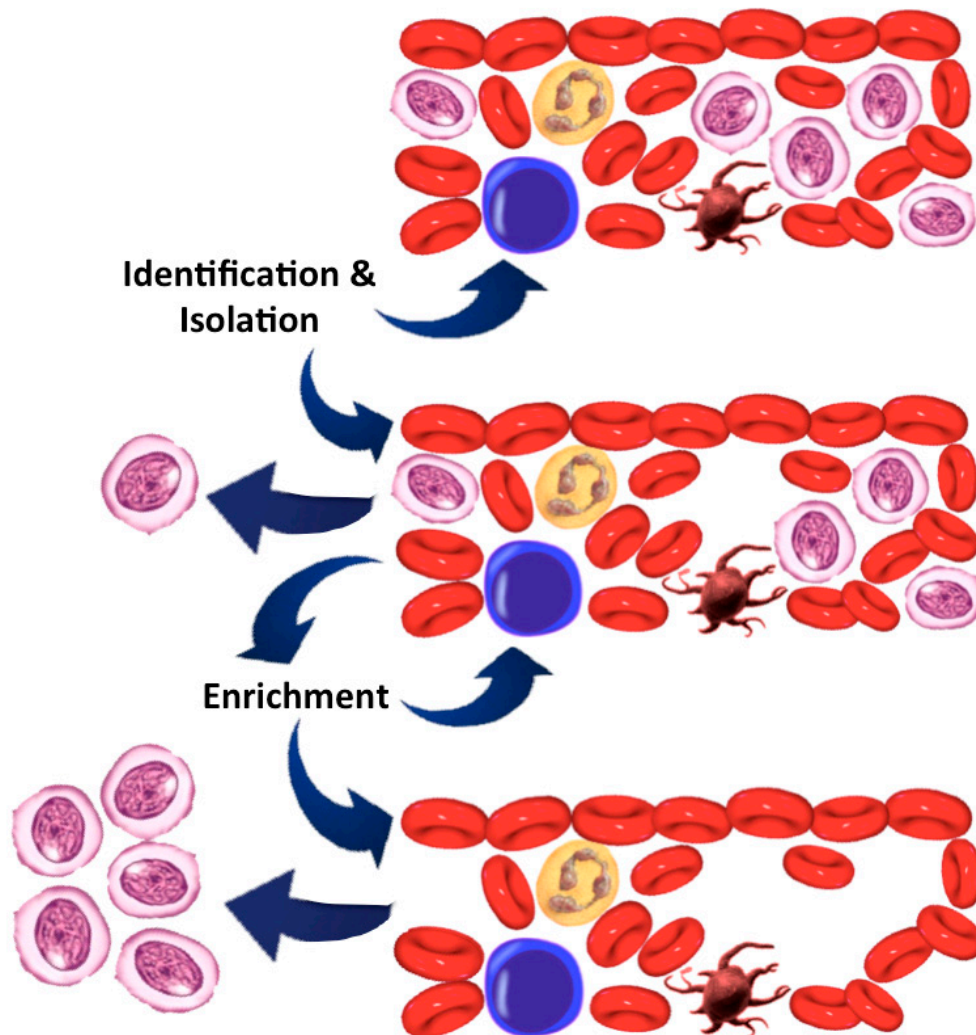
diagnostic biomarker. Methylation levels of CpG islands of RASSF1A, FHIT, and APC genes has been detected in cfDNA of renal cancer patients[30]. Combined analysis of methylation frequency of multiple genes reach a sensitivity of 62,9% and a specificity of 87%. DNA hypermethylation of APC gene also correlated with advanced tumor stage[31].

cfDNA is also a potential surveillance biomarker for disease recurrence in patient with localized disease. Postoperative recurrence could be monitored quantitatively with cfDNA instead with a specific genetic test. The pre-surgical level of plasma cfDNA in patients with metastatic clear cell RCC was significantly increased compared to patients with localized cRCC or controls.

Patients with a high cfDNA value had a significantly higher recurrence rate than those with a low plasma cfDNA level before and after nephrectomy ($p = 0.018$)[32]. Moreover, quantification of plasma cfDNA permit to predict therapeutic efficacy of sorafenib on mRCC. Quantitative real-time PCR at six different time-points was performed to analyze concentration of cfDNA in mRCC patients receiving sorafenib. Patients with remission or stable disease had a significant lower level of cfDNA compared to patients in progression[33].

Comparison with CTC Assay

Nucleic acids have a half-life in the circulation ranging from 15 minutes to several hours, are more stable than cells or RNA and technically easier to isolate than CTCs (**Figure 2**).

Figure 2. Identification and enumeration of circulating tumour cells

CtDNA represent a pool of cells, give an insight in the repertoire of genetic tumor alterations and on the number and properties of subclones. However, ctDNA in most cases requires *a priori* knowledge of the target of interest and not all DNA mutations are expressed. It have to be isolated from a vast amount of wild type cfDNA, also detected in healthy individuals in minor levels compared to cancer patients. As response-to-therapy biomarker, it is still unclear if cancer cells shed ctDNA because they are dying from

therapy or if because they are resistant to therapy. Cytotoxic chemotherapy induce leukocyte and erythrocyte apoptosis, which leads to a release of cfDNA into plasma, a potential confounder factor. On the other hand, CTCs are either apoptotic or viable, but only viable CTCs are important for developing metastases and are therefore of special attention.

In metastatic breast cancer, ctDNA is more sensitive than Cell Search CTC Assay, a better biomarker in monitoring tumor dynamics showing a greater correlation with changes in tumor burden, than did CA 15-3 or circulating tumor cells[34]. On the other hand, CTC can be used for functional assays (DNA, RNA, protein) and can be cultured to evaluate drug resistance *in vitro* or *in vivo*. Intact CTCs are rare events, isolation is technically challenging.

Current systems of isolation rely on methods based on physical differences between hematopoietic cells and tumor cells and on immune cytokeratin expression such as the epithelial cell adhesion molecule (EpCAM). Level of expression of cytokeratin is a sampling bias; thus, when the tumor cells go through epithelial mesenchymal transition (EMT), as occur in sarcomatoid changes in RCC[35,36], they lost cytokeratin expression leading to a partial or complete switch to a mesenchymal phenotype and so become undetectable at CTCs analysis. To overcome this bias, Ivonne et al. developed a CTC detection technique based on multi-parameter immunofluorescence microscopy (MPIM) that consist of epithelial markers such as CK or EpCAM and cells with mesenchymal and stem cell-like properties[37].

A new combination of cell surface markers comprising CA9 and CD147 as alternative CTC-detective antigens have been developed for RCC patients and demonstrated significantly higher efficiency compared to the conventional EpCAM-based

method [38]. However, certain assays cannot be applicable to ctDNA, such as splice variants, information at single-cell level and functional assays. Analytes i.e. proteins, metabolites and RNA can be found only in CTCs [39,40].

Conclusion

Application of liquid biopsy in the clinical scenario pave the way for a new research field. While identification of predictive marker, prognostication, classification of molecular subtypes can be accessible using both tissue (solid biopsy) and CTCs/ctDNA, tracking of clonal evolution over time, early identification of resistance mechanisms, monitoring treatment response, detection of recurrence and residual disease can be possible only with liquid biopsy in a non-invasive manner.

Many questions and challenges to implementation of liquid biopsies are currently under investigation along with emerging liquid biopsy analytes (i.e. EVs, cfRNAs, branched- chain amino acids (BCAAs), proteins, tumour- educated platelets. A better understanding of the mechanisms involved in release of liquid biopsy constituents and adoption of developing integrative multidimensional profiling approaches will be crucial to solving these challenges.

AUTHOR CONTRIBUTIONS

Conceptualization, R.M. and M.Sc.; Writing – Original Draft Preparation, A.C.; Review & Editing, S.G., F.M., M.Sa.; Supervision, L.C. and A.L.B.

FUNDING STATEMENT

This paper was not funded.

DECLARATION OF INTEREST

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

- [1] Bergerot, P.G.; Hahn, A.W.; Bergerot, C.D.; Jones, J.; Pal, S.K. The Role of Circulating Tumor DNA in Renal Cell Carcinoma. *Curr. Treat. Options Oncol.* **2018**, 19,10. doi: 10.1007/s11864-018-0530-4.
- [2] Siravegna, G.; Marsoni, S.; Siena, S.; Bardelli, A. Integrating liquid biopsies into the management of cancer. *Nat. Rev. Clin. Oncol.* **2017**,14,531-548.doi: 10.1038/nrclinonc.2017.14
- [3] Douillard, J.; Ostoros, G.; Cobo, M.; Ciuleanu, T.; Cole, R.; McWalter, G.; Walker, J.; Dearden, S.; Webster, A.; Milenkova, T.; McCormack, R. Gefitinib Treatment in EGFR Mutated Caucasian NSCLC. *J. Thorac. Oncol.* **2014**,9,1345-53. doi: 10.1097/JTO.0000000000000263.
- [4] Kukita, Y.; Uchida, J.; Oba, S.; Nishino, K.; Kumagai, T.; Taniguchi, K.; Okuyama, T.; Imamura, F.; Kato, K. Quantitative identification of mutant alleles derived from lung

cancer in plasma cell-free DNA via anomaly detection using deep sequencing data. *PLoS One*. **2013**,8,e81468. doi: 10.1371/journal.pone.0081468

- [5] Li, X.; Ren, R.; Ren, S.; Chen, X.; Cai, W.; Zhou, F.; Zhang, Y.; Su, C.; Zhao, C.; Li, J.; Cheng, N.; Zhao, M.; Zhou, C. Peripheral blood for epidermal growth factor receptor mutati detection in non-small cell lung cancer patients. *Transl. Oncol.* **2014**,7,341-8. doi: 10.1016/j.tranon.2014.04.006
- [6] Mok, T.; Wu, Y.L.; Lee, J.S.; Yu, C.J.; Sriuranpong, V.; Sandoval-Tan, J.; Ladrera, G.; Thongprasert, S.; Srimuninnimit, V.; Liao, M.; et al. Detection and dynamic changes of EGFR mutations from circulating tumor DNA as a predictor of survival outcomes in NSCLC Patients treated with first-line intercalated erlotinib and chemotherapy. *Clin. Cancer Res.* **2015**, 21,3196-203. doi: 10.1158/1078-0432.CCR-14-2594.
- [7] Oxnard, G.R.; Paweletz, C.P.; Kuang, Y.; Mach, S.L.; O'Connell, A.; Messineo, M.M.; Luke, J.J.; Butaney, M.; Kirschmeier, P.; Jackman, D.M.; Jänne, P.A. Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. *Clin. Cancer Res.* **2014**,20,1698-1705. doi: 10.1158/1078-0432.CCR-13-2482
- [8] Hao, Y.X.; Fu, Q.; Guo, Y.Y.; Ye, M.; Zhao, H.X.; Wang, Q.; Peng, X.M.; Li, Q.W.; Wang, R.L.; Xiao, W.H. Effectiveness of circulating tumor DNA for detection of KRAS gene mutations in colorectal cancer patients: a meta-analysis. *Onco. Targets Ther.* **2017**,10,945-953. doi: 10.2147/OTT.S123954
- [9] Merker, J.D.; Oxnard, G.R.; Compton, C.; Diehn, M.; Hurley, P.; Lazar, A.J.; Lindeman, N.; Lockwood, C.M.; Rai, A.J.; Schilsky, R.L.; et al. Circulating Tumor DNA Analysis in Patients With Cancer: American Society of Clinical Oncology and College of American

- Pathologists Joint Review. *Arch. Pathol. Lab. Med.* **2018**,142,1242-1253. doi: 10.5858/arpa.2018-0901-SA.
- [10] Mellert, H.; Foreman, T.; Jackson, L.; Maar, D.; Thurston, S.; Koch, K.; Weaver, A.; Cooper, S.; Dupuis, N.; Sathyanarayana, U.G.; et al. Development and Clinical Utility of a Blood-Based Test Service for the Rapid Identification of Actionable Mutations in Non-Small Cell Lung Carcinoma. *J. Mol. Diagn.* **2017**,19,404-416. doi: 10.1016/j.jmoldx.2016.11.004
- [11] Colwell J. Illumina Spin-off to Develop Early-Detection Test. *Cancer Discov.* **2016**,6,OF3. doi: 10.1158/2159-8290.CD-NB2016-015.
- [12] Lanman, R.B.; Mortimer, S.A.; Zill, O.A.; Sebisano, D.; Lopez, R.; Blau, S.; Collisson, E.A.; Divers, S.G.; Hoon, D.S.; Kopetz, E.S.; et al. Analytical and clinical validation of a digital sequencing panel for quantitative, highly accurate evaluation of cell-free circulating tumor DNA. *PLoS One.* **2015**,10,e0140712. doi: 10.1371/journal.pone.0140712.
- [13] Oxnard, G.R.; Paweletz, C.P.; Sholl, L.M. Genomic analysis of plasma cell-free DNA in patients with cancer. *JAMA Oncol.* **2017**,3,740-741. doi: 10.1001/jamaoncol.2016.2835.
- [14] Kuderer, N.M.; Burton, K.A.; Blau, S.; Rose, A.L.; Parker, S.; Lyman, G.H.; Blau, C.A. Comparison of 2 Commercially Available Next-Generation Sequencing Platforms in Oncology. *JAMA Oncol.* **2017**,3,996-998. doi: 10.1001/jamaoncol.2016.4983.
- [15] Bettegowda, C.; Sausen, M.; Leary, R.J.; Kinde, I.; Wang, Y.; Agrawal, N.; Bartlett, B.R.; Wang, H.; Lubner, B.; Alani, R.M.; et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci. Transl. Med.* **2014**,6,224ra24. doi: 10.1126/scitranslmed.3007094.

- [16] Gray, E.S.; Rizos, H.; Reid, A.L.; Boyd, S.C.; Pereira, M.R.; Lo, J.; Tembe, V.; Freeman, J.; Lee, J.H.; Scolyer, R.A.; et al. Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. *Oncotarget*. **2015**,6,42008-18. doi: 10.18632/oncotarget.5788.
- [17] Iijima, Y.; Hirotsu, Y.; Amemiya, K.; Ooka, Y.; Mochizuki, H.; Oyama, T.; Nakagomi, T.; Uchida, Y.; Kobayashi, Y.; Tsutsui, T.; et al. Very early response of circulating tumour-derived DNA in plasma predicts efficacy of nivolumab treatment in patients with non-small cell lung cancer. *Eur. J. Cancer*. **2017**,86,349-357. doi: 10.1016/j.ejca.2017.09.004
- [18] Ball, M.W.; Gorin, M.A.; Guner, G.; Pierorazio, P.M.; Netto, G.; Paller, C.J.; Hammers, H.J.; Diaz, L.A.; Allaf, M.E. Circulating Tumor DNA as a Marker of Therapeutic Response in Patients With Renal Cell Carcinoma: A Pilot Study. *Clin. Genitourin. Cancer*. **2016**,14,e515-e520. doi: 10.1016/j.clgc.2016.03.019.
- [19] Yang, M.; Forbes, M.E.; Bitting, R.L.; O'Neill, S.S.; Chou, P.C.; Topaloglu, U.; Miller, L.D.; Hawkins, G.A.; Grant, S.C.; DeYoung, B.R.; et al. Incorporating blood-based liquid biopsy information into cancer staging: Time for a TNMB system? *Ann. Oncol.* **2018**,29,311-323. doi: 10.1093/annonc/mdx766.
- [20] Hahn, A.W.; Gill, D.M.; Maughan, B.; Agarwal, A.; Arjyal, L.; Gupta, S.; Streeter, J.; Bailey, E.; Pal, S.K.; Agarwal, N.. Correlation of genomic alterations assessed by next-generation sequencing (NGS) of tumor tissue DNA and circulating tumor DNA (ctDNA) in metastatic renal cell carcinoma (mRCC): potential clinical implications. *Oncotarget*. **2017** May,8,33614-33620. doi: 10.18632/oncotarget.16833.
- [21] Chae, Y.K.; Davis, A.A.; Carneiro, B.A.; Chandra, S.; Mohindra, N.; Kalyan, A.; Kaplan, J.; Matsangou, M.; Pai, S.; Costa, R.; et al. Concordance between genomic alterations

assessed by next-generation sequencing in tumor tissue or circulating cell-free DNA. *Oncotarget*. **2016**,7,65364-65373. doi: 10.18632/oncotarget.11692.

- [22] Pal, S.K.; Sonpavde, G.; Agarwal, N.; Vogelzang, N.J.; Srinivas, S.; Haas, N.B.; Signoretti, S.; McGregor, B.A.; Jones, J.; Lanman, R.B.; et al. Evolution of Circulating Tumor DNA Profile from First-line to Subsequent Therapy in Metastatic Renal Cell Carcinoma. *Eur. Urol.* **2017**,72,557-564. doi: 10.1016/j.eururo.2017.03.046.
- [23] Lim, S.M.; Park, H.S.; Kim, S.; Kim, S.; Ali, S.M.; Greenbowe, J.R.; Yang, I.S.; Kwon, N.J.; Lee, J.L.; Ryu, M.H.; et al. Next-generation sequencing reveals somatic mutations that confer exceptional response to everolimus. *Oncotarget*. **2016**,7,10547-56. doi: 10.18632/oncotarget.7234.
- [24] Voss, M.H.; Hakimi, A.A.; Pham, C.G.; Brannon, A.R.; Chen, Y.B.; Cunha, L.F.; Akin, O.; Liu, H.; Takeda, S.; Scott, S.N.; et al. Tumor genetic analyses of patients with metastatic renal cell carcinoma and extended benefit from mTOR inhibitor therapy. *Clin. Cancer Res.* **2014**,20,1955-64. doi: 10.1158/1078-0432.CCR-13-2345.
- [25] Piva, F.; Santoni, M.; Matrana, M.R.; Satti, S.; Giulietti, M.; Occhipinti, G.; Massari, F.; Cheng, L.; Lopez-Beltran, A.; Scarpelli, M.; et al. BAP1, PBRM1 and SETD2 in clear-cell renal cell carcinoma: molecular diagnostics and possible targets for personalized therapies. *Expert Rev. Mol. Diagn.* **2015**,15,1201-10. doi: 10.1586/14737159.2015.1068122.
- [26] Cimadamore, A.; Gasparini, S.; Santoni, M.; Cheng, L.; Lopez-Beltran, A.; Battelli, N.; Massari, F.; Giunchi, F.; Fiorentino, M.; Scarpelli, M.; Montironi, R. Biomarkers of aggressiveness in genitourinary tumors with emphasis on kidney, bladder, and prostate cancer. *Expert. Rev. Mol. Diagn.* **2018**,18,645-655. doi: 10.1080/14737159.2018.1490179.

- [27] Al-Qassab, U.; Lorentz, C.A.; Laganosky, D.; Ogan, K.; Master, V.; Pattaras, J.; Issa, M.; Keith, C.; Roberts, D.; Rossi, M.; Bergquist, S.; Goecks, J.; Arnold, R. (PNFBA-12) Liquid biopsy for renal cell carcinoma. *J. Urol.* **2017**,197,e913–e914. <https://doi.org/10.1016/j.juro.2017.02.3241>.
- [28] Lu, H.; Busch, J.; Jung, M.; Rabenhorst, S.; Ralla, B.; Kilic, E.; Mergemeier, S.; Budach, N.; Fendler, A.; Jung, K. Diagnostic and prognostic potential of circulating cell-free genomic and mitochondrial DNA fragments in clear cell renal cell carcinoma patients. *Clin. Chim. Acta.* **2016**,452,109-19. doi: 10.1016/j.cca.2015.11.009.
- [29] Hauser, S.; Zahalka, T.; Ellinger, J.; Fechner, G.; Heukamp, L.C.; Von Ruecker, A.; Müller, S.C.; Bastian, P.J. Cell-free circulating DNA: Diagnostic value in patients with renal cell cancer. *Anticancer Res.* **2010**,30,2785-9.
- [30] Skrypkina, I.; Tsyba, L.; Onyshchenko, K.; Morderer, D.; Kashparova, O.; Nikolaienko, O.; Panasenko, G.; Vozianov, S.; Romanenko, A.; Rynditch, A. Concentration and Methylation of Cell-Free DNA from Blood Plasma as Diagnostic Markers of Renal Cancer. *Dis. Markers.* **2016**,2016,3693096. DOI: 10.1155/2016/3693096
- [31] Hauser, S.; Zahalka, T.; Fechner, G.; Müller, S.C.; Ellinger, J. Serum DNA hypermethylation in patients with kidney cancer: Results of a prospective study. *Anticancer Res.* **2013**,33,4651-6.
- [32] Wan, J.; Zhu, L.; Jiang, Z.; Cheng, K. Monitoring of plasma cell-free DNA in predicting postoperative recurrence of clear cell renal cell carcinoma. *Urol. Int.* **2013**,91,273-8. doi: 10.1159/000351409.
- [33] Feng, G.; Ye, X.; Fang, F.; Pu, C.; Huang, H.; Li, G. Quantification of plasma cell-free DNA in predicting therapeutic efficacy of sorafenib on metastatic clear cell renal cell carcinoma. *Dis. Markers.* **2013**,34,105-11. doi: 10.3233/DMA-120950.

- [34] Dawson, S.J.; Tsui, D.W.; Murtaza, M.; Biggs, H.; Rueda, O.M.; Chin, S.F.; Dunning, M.J.; Gale, D.; Forshew, T.; Mahler-Araujo, B.; et al. Analysis of Circulating Tumor DNA to Monitor Metastatic Breast Cancer. *N. Engl. J. Med.* **2013**, 368, 1199-209. doi: 10.1056/NEJMoa1213261.
- [35] He, H.; Magi-Galluzzi, C. Epithelial-to-mesenchymal transition in renal neoplasms. *Adv. Anat. Pathol.* **2014**, 21, 174-80. doi: 10.1097/PAP.0000000000000018.
- [36] Conant, J.L.; Peng, Z.; Evans, M.F.; Naud, S.; Cooper, K. Sarcomatoid renal cell carcinoma is an example of epithelial-mesenchymal transition. *J. Clin. Pathol.* **2011**, 64, 1088-92. doi: 10.1136/jclinpath-2011-200216.
- [37] Nel, I.; Gauler, T.C.; Bublit, K.; Lazaridis, L.; Goergens, A.; Giebel, B.; Schuler, M.; Hoffmann, A.C. Circulating tumor cell composition in renal cell carcinoma. *PLoS One.* **2016**, 11, e0153018. doi: 10.1371/journal.pone.0153018.
- [38] Liu, S.; Tian, Z.; Zhang, L.; Hou, S.; Hu, S.; Wu, J.; Jing, Y.; Sun, H.; Yu, F.; Zhao, L.; et al. Combined cell surface carbonic anhydrase 9 and CD147 antigens enable high-efficiency capture of circulating tumor cells in clear cell renal cell carcinoma patients. *Oncotarget.* **2016**, 7, 59877-59891. doi: 10.18632/oncotarget.10979.
- [39] Corcoran, R.B.; Chabner, B.A. Application of Cell-free DNA Analysis to Cancer Treatment. *N. Engl. J. Med.* **2018**, 379, 1754-1765. doi: 10.1056/NEJMra1706174.
- [40] Heitzer, E.; Haque, I.S.; Roberts, C.E.; Speicher, M.R. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat. Rev. Genet.* **2018**. doi: 10.1038/s41576-018-0071-5. "in press"