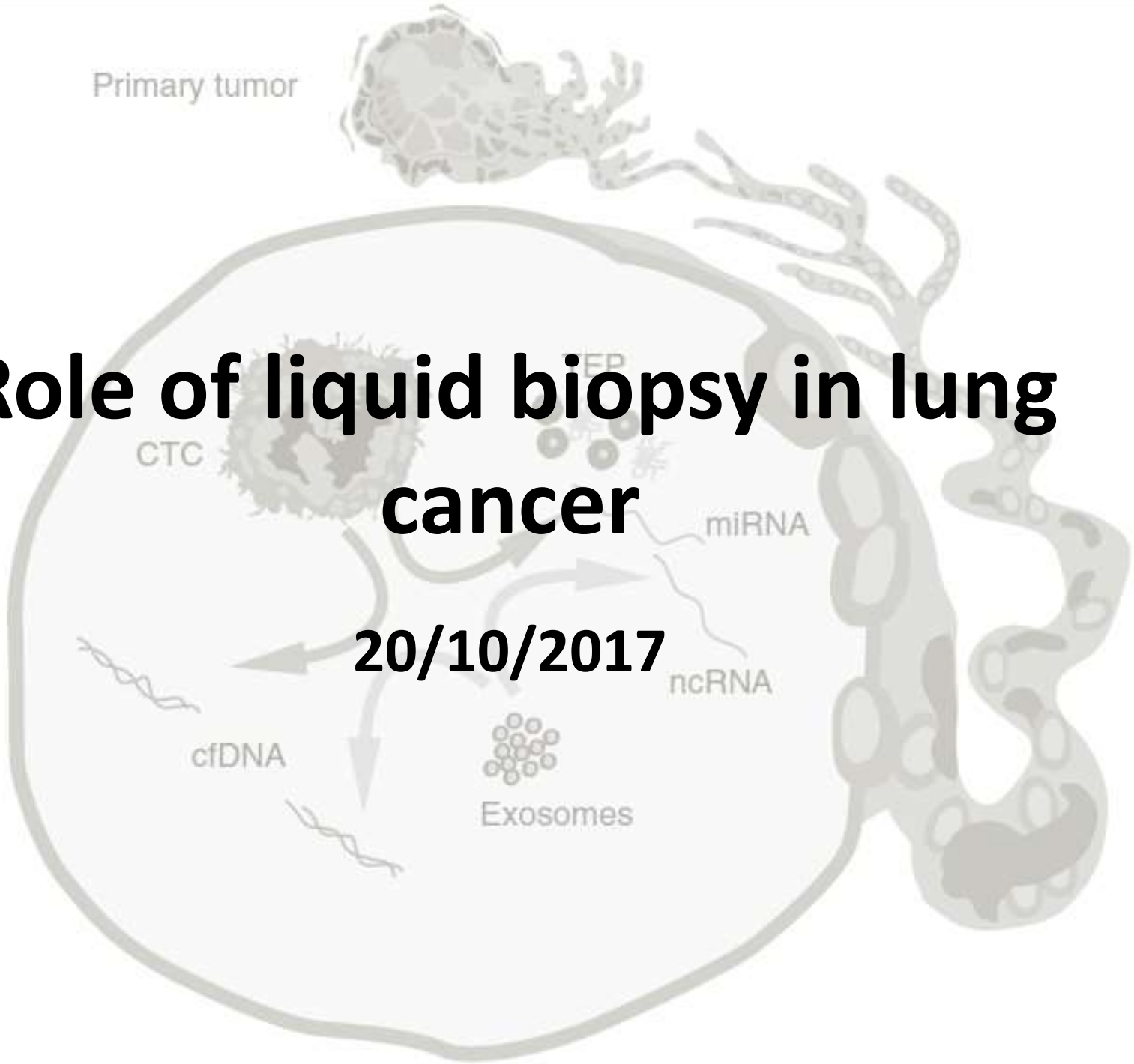


Role of liquid biopsy in lung cancer

20/10/2017

Overview of Seminar

- Background
- Concept of liquid biopsy
- Different Biological Components of Liquid Biopsy
- Various samples used in liquid biopsy
- EGFR mutation detection using liquid biopsy
- Analysis of ALK Status
- Uses
- Limitations
- Future perspectives

Background

- Diagnosis of lung cancer using biopsy tissue samples from primary tumour or metastatic lesion:
  - Histopathology
  - Immunohistochemistry (IHC)
  - Molecular profiles of solid tumours: EGFR mutation, ALK rearrangement, rearrangements in ROS1, RET, and NTKR, other genomic alterations such as BRAF, HER2, and MET

Background

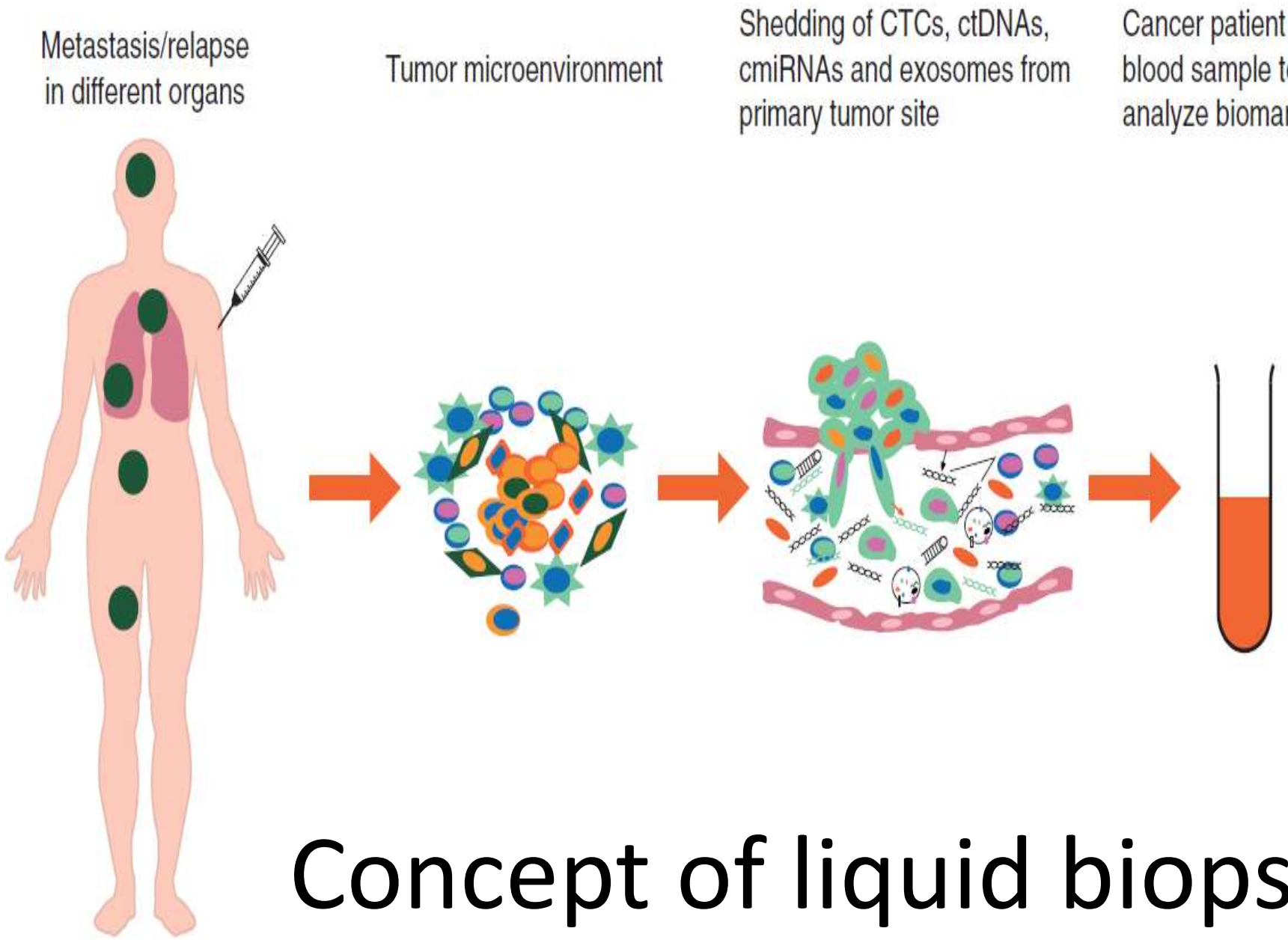
- Tissue-based tumour diagnosis limitations:
  - Invasive
  - Costly
  - Subject to sampling bias
  - Accessibility: Tumour lesions are in difficult anatomical locations, such as mid-lung or retroperitoneum
  - Safety questionable: Regarding sampling of tumours surrounding major vessels or in deep regions of brain, or in patients with major comorbidities

Background

- Provide only snapshot of tumour heterogeneity
- Inability to capture all the variability in gene expression
- Unfeasibility of performing multiple longitudinal tests to follow tumour evolution and thereby expose mechanisms of secondary resistance to treatment

Background

- Ability of tumours to **evolve in response** to wide variety of **endogenous and exogenous selective pressure**s has several implications:
  - Firstly, the **genetic make-up** of individual cancers is **highly heterogeneous**;
  - Secondly, within single patient, **distinct metastatic** lesions can be **molecularly divergent**;
  - Thirdly, **therapeutic stress** exerted on tumour cells, particularly by targeted drugs, can **dynamically modify genomic landscape** of tumour



Concept of liquid biopsy

Liquid biopsy

- Human blood samples contain- cell-free DNA (cfDNA) and RNA (cfRNA); proteins; cells; and vesicles (such as exosomes)— that can originate from different tissues, including cancers
- Genomic profiles of circulating cell-free tumour DNA (ctDNA) have been shown to closely match the corresponding tumours
- Analyses of circulating nucleic acids is referred to as ‘liquid biopsies’

Alix-Panabières, C. et. al. Annu. Rev. Med. 63, 199–215 (2012)

Liquid biopsy

- In addition to blood, several other body fluids, such as urine, saliva, pleural effusions, and cerebrospinal fluid, can contain tumour-derived genetic information
- Molecular profiles gathered from ctDNA can be complemented with those obtained through analysis of circulating tumour cells (CTCs), as well as RNA, proteins, and exosomes

Different Biological Components of Liquid Biopsy

- Circulating tumor cells (CTCs)
- Microvesicles- Exosomes
- Cell free circulating tumor DNA (ctDNA)
- Cell free circulating tumor RNA (ctRNA): miRNAs and long non-coding RNA (lncRNA)

Circulating tumor cells (CTCs)

- CTCs are tumour cells: Intravasated or been passively shed from the primary tumour and/or metastatic lesions into the bloodstream
- Existance of CTCs was first reported in 1869 by Australian physician Thomas Ashworth
- But their clinical utility was not appreciated until the late 1990s

Ashworth, T. R. Australian Med. J.14, 146–147 (1869)
Krebs, M. G. et. al. Ther. Adv. Med. Oncol. 2, 351–365 (2010)

CTCs

- Abundance of CTCs in the blood is low (approximately 1 cell per 1×10^9 blood cells in patients with metastatic cancer), however, and varies between tumour types
- Number of CTCs detected has been associated with treatment outcomes and overall survival

Haber, D. A. et. Al. Cancer Discov. 4, 650–661 (2014)
Krebs, M. G. et al. Nat. Rev. Clin. Oncol. 11, 129–144 (2014)

CTCs

- CTCs can be isolated through:
  - **Negative-enrichment**: based on their size and other biophysical properties (loss of deformability, higher density and electrical charges), or by
  - **Positive enrichment**: using markers commonly expressed on surface of these cells, such as epithelial cell adhesion molecule (EpCAM)
- Size-based selection methods exploit fact that CTCs are usually larger than normal blood cells

Alix-Panabières, C. et. al. Nat. Rev. Cancer 14, 623–631 (2014)
Lin, H. K. et al. Clin. Cancer Res. 16, 5011–5018 (2010)

CTC detection

- Basic goal of CTC isolation is to **accurately and precisely discriminate** between CTCs and various types of cells in blood
- **Scarcity of the cells** presents an inherent technological **challenge**
- Refining CTC isolation been primarily driven by approach of **antibody-based or size-dependent principles**, relying on microfluidics to achieve the goal

CTC detection

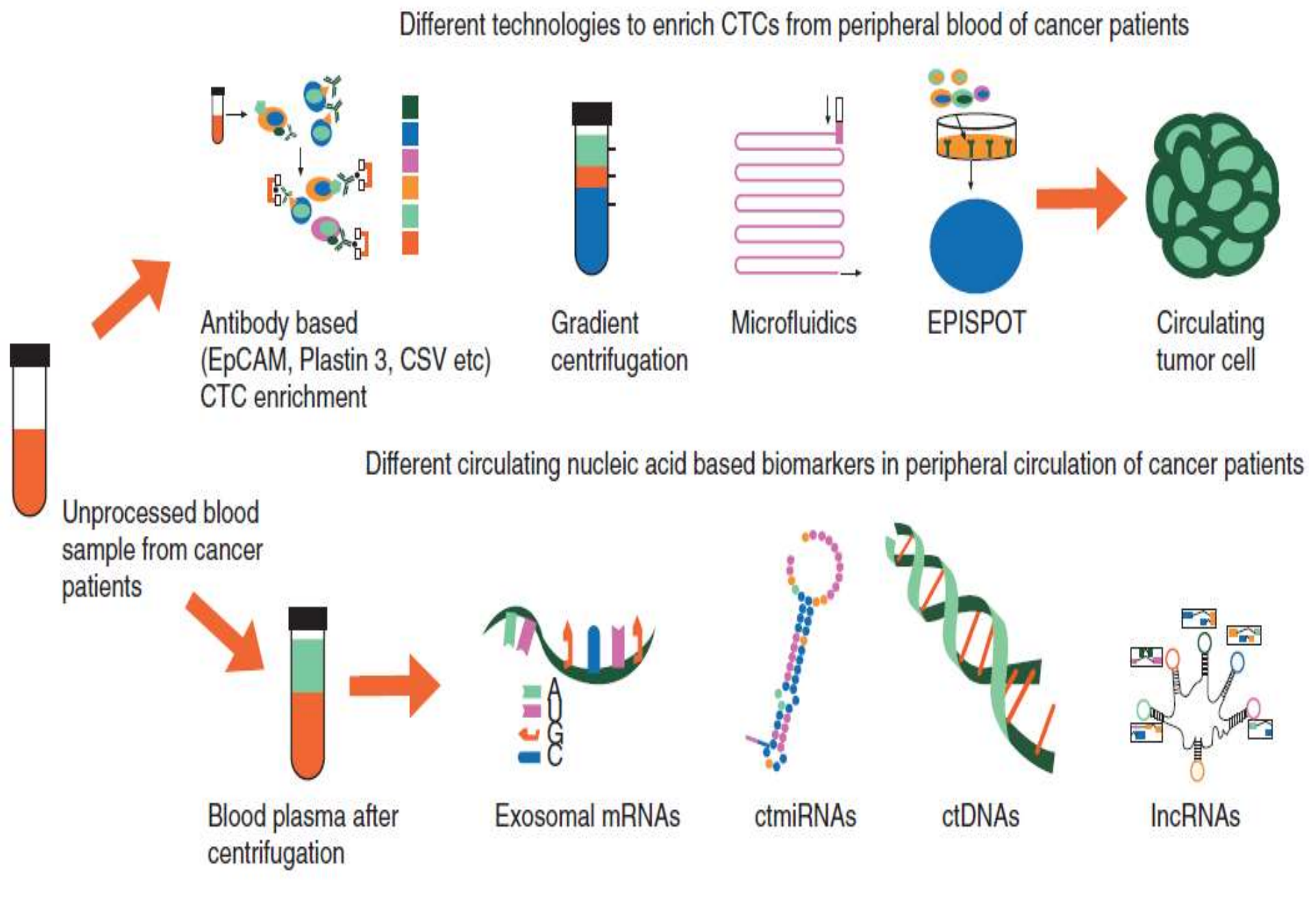
- Most prominent of antibody-based approach relies on epithelial cell adhesion molecule (EpCAM)
- **EpCAM-based CTC isolation**: Technology based on this protein and subsequent staining and validation with cytokeratin (CK; positive) and CD45 (negative) has been developed (CellSearch, Veridex, Raritan, NJ)
- **FDA** has **approved** "CellSearch system"
- Approval has led to its proliferative use and sets the benchmark for other CTC isolation technologies

Patriarca C. et al. Cancer Treat Rev 2012; 38(1): 68–75

CTC detection

- **CellSearch technology** have caused many to question the reliability of this test as both EpCAM and CKs are epithelial markers which are lost after EMT
- To address shortcomings, **Plastin3** identified as a metastasis-specific marker in colorectal cancer (CRC) and breast cancer patients
- Other technologies relies on universally-accepted mesenchymal marker **vimentin**

Grover PK. et al. Ann Oncol 2014; 25(8): 1506–1516


Different technologies to enrich CTCs from peripheral blood of cancer patients
Antibody based
(EpCAM, Plastin 3, CSV etc)
CTC enrichment
Gradient
centrifugation
Microfluidics
EPISPOT
Circulating
tumor cell
Different circulating nucleic acid based biomarkers in peripheral circulation of cancer patients
Unprocessed blood
sample from cancer
patients
Blood plasma after
centrifugation
Exosomal mRNAs
ctmiRNAs
ctDNAs
lncRNAs

CTC detection

1. **CellSearch platform** (Veridex, Raritan, NJ): method based on detection of epithelial cells that express epithelial cell adhesion molecule (EpCAM)
2. Enrichment technique based on blood filtration (**ISET** [isolation by size of epithelial tumor cells])
3. Fluorescent in situ hybridization (FISH) method on filters **(filter-adapted FISH [FA-FISH]):** optimized for high cell recovery

Uses

- CTCs represent noninvasive and easily accessible source of tumor material for assessing **predictive molecular biomarkers and screening** patients eligible for **targeted treatments**
- CTC levels were observed to be **prognostic** in various metastatic solid tumors, including NSCLC and SCLC
- CTC numbers after treatment can be **predictive of response to therapy and treatment outcomes**

CTCs

- CTCs as biomarkers and their **clinical utility**: At present, clinical value of CTC analysis remains **controversial**
- CTC numbers are highly variable between different tumour types, and are subject to biases relating to variety of CTC-detection methods used

Circulating tumor DNA (ctDNA)

- Circulating DNA can either be borne from tumor cells, exosomes or be found as cell free DNA (cfDNA) also referred to as ctDNA, (cfDNA can include non-tumor related DNA)
- DNA fragments maintain epigenetic marks from original tumor, contributing factor motivating ctDNA analysis

ctDNA

- Existence of cfDNA was **first described in 1948 by Mandel and Metais**, who detected non-cell-bound nucleic acids in bloodstream of individuals with cancer
- In 1977, **Leon** and co-workers reported **higher amount of cfDNA in patients with cancer** than in individuals without cancer

Mandel, P. & Metais, P. C. R. Seances Soc. Biol. Fil. 142, 241–243 (1948)
Leon, S. A. et al. Cancer Res. 37, 646–650 (1977)

ctDNA

- Triggered further research: **Stroun** and colleagues discovered that **tumour-related genetic alterations were present in cfDNA** of patients with cancer
- Other research confirmed that various types of **neoplastic genomic alterations** (mutations in oncogenes and/or tumour-suppressor genes, microsatellite instability, and epigenetic changes) **can be detected in 'ctDNA'**

Stroun, M. et al. Oncology 46, 318–322 (1989)
Fujiwara, K. et al. Clin. Cancer Res. 11, 1219–1225 (2005)

ctDNA

- Exact mechanisms by which ctDNA is released into blood remain to be clarified
- In addition to **apoptosis**, DNA fragments can be released from cancer cells via other mechanisms, including **necrosis**
- **Macrophages** seem to have role in release of tumour DNA fragments in circulation, via phagocytosis of necrotic neoplastic cells

Jahr, S. et al. Cancer Res. 61, 1659–1665 (2001)
Diehl, F. et al. Proc. Natl Acad. Sci. USA 102, 16368–16373 (2005)

ctDNA

- For ctDNA analyses, plasma samples are preferable to serum samples:
  - Overall quantity of cfDNA is 2–24-fold higher in the serum, owing to extensive contamination of DNA released from immune cells that are lysed during clotting process
- **Plasma has proved to be superior source** of ctDNA owing to lower background levels wild-type DNA

El Messaoudi, S. et. Al. *Clin. Chim. Acta 424, 222–230 (2013)*

ctDNA

- **Half-life** of non-exosomal ctDNA is estimated to be **several hours**, and therefore provides **opportunities for tumor monitoring, and therapeutic and surgical response**
- Rapid clearance has been shown to stratify risk of recurrence after resection with curative intent

How to detect ctDNA?

Table 1: Method or platform of CtDNA for detecting EGFR mutations and their associated sensitivity and application in lung cancer

Platform	Sensitivity (% mutant DNA)	Targeting mutation	Reference
Mass spectrometry	1%–10%	Known only	Arcila et al [110], Brevet et al [111].
NGS	1%–10%		Uchida et al [112]
Cobas, Therascreen, ARMS	1%–3%	Known only	Mok et al [80], Pasquale et al [113], Goto et al [17] Douillard et al [75] Li et al [114] Wang et al [19] Kuang et al [115]
PNA–LNA PCR clamp	2%	Known only	Kim [116] Kim [117].
TAM-Seq	2%	Known and new	Forshew [33]
EFIRM	1%–2%	Known only	Wei et al [40]
Digital PCR	<0.1%	Known only	Isobe et al [118]
BEAM	<0.1%		Taniguchi et al [119]
CAPP-Seq	~0.02%	Known and new	Newman et al [36]
iDES-enhanced CAPP-Seq	0.0025%	Known and new	Newman et al [37]

Table 2 | **Comparison of technologies for ctDNA analysis**[1]

Approach	Method	Technology	LoD	Advantages	Disadvantages
Candidate-gene analysis	qPCR*	PNA clamp-PCR‡ (REF. 104)	0.1%	• Rapid • High sensitivity • Suitable for the detection of specific point mutations, copy-number variations, short indels, and gene fusions • No bioinformatic analysis • Cost-effective	• Only enables monitoring of known mutations
		LNA/DNA-PCR‡ (REF. 208)	0.1%		
		ARMS[209]	0.05–0.1%		
		COLD-PCR‡ (REF. 210)	0.1–0.01%		
	Digital PCR	BEAMing[211]	0.01%		
		ddPCR[212–214]	0.001%		
	NA	InPlex§ (REFS 133,215,216)	<0.01%		
		Endpoint PCR¶ (REF. 156)	<0.0001%		
Deep-sequencing	Targeted	AmpliSeq[217]	>2%	• Does not require any prior knowledge of the molecular alteration	• Longer time needed to obtain, process and analyse results than that needed for candidate-gene analysis • Bioinformatic expertise required • Expensive
		TAm-Seq[218]	>2%		
		SAFE-SeqS[219]	0.1%		
		Guardant360 digital sequencing test[197]	<0.1%		
		CAPP-Seq[134]	0.01%		
		iDES[220]	<0.01%		
		PARE[221]	0.001%		
	WES (nontargeted)	NA	>1–3%		
	WGS (nontargeted)	Digital karyotyping[221–223]	0.001%		
		PARE[221,224,225]	0.001%		

Circulating tumor RNA (ctRNAs)

- **In 1996,** the detection of circulating tumour-associated mRNA: **first described** in blood of patients with melanoma
- Soon, other forms of RNAs-- miRNAs and long noncoding RNAs (lncRNAs)— identified in circulation of patients with solid cancers
- Somatic mutations in DNA only represent a subset of molecular alterations associated with cancers, and do not fully recapitulate changes in gene-expression profiles that might result from epigenetic alterations

Stevens, G. L. et al. Cancer Epidemiol. Biomarkers Prev. 5, 293–296 (1996)
Taylor, D. D. et al. Gynecol. Oncol. 110, 13–21 (2008)

ctRNAs

- miRNAs are most abundant and stable cfRNA molecules in blood, carried in exosomes, apoptotic bodies, protein–miRNA complexes, and tumour-educated platelets (TEP)
- Landscape of miRNAs in blood seems to correlate with that of solid tumours from which they originate
- Amount and composition of exosomal miRNAs differs between patients with cancer and individuals without cancer, implicating miRNAs as potential non-invasive diagnostic biomarkers

Joosse, S. A. et al. Cancer Cell 28, 552–554 (2015)
Mitchell, P. S. et al. Proc. Natl Acad. Sci. USA 105, 10513–10518 (2008)
Rabinowits, G. et al. Clin. Lung Cancer 10, 42–46 (2009)

ctRNAs

- Most of the **analyses of mRNA and miRNA** in blood remain **exploratory,** and validation in clinical studies with standardized protocols is required to substantiate the value of cfRNAs in the clinical setting
- The **clinical implications** of cfRNA are **currently unclear**

Exosomes

- Several types of microvesicles are released from non-neoplastic and tumour cells into extracellular space and body fluids
- Extracellular vesicles (EVs) can be classified into two groups:
  - the first comprises microvesicles shed directly from cell membrane via budding;
  - the second consists of exosomes, which are exuded via exocytosis when multivesicular bodies (MVBs) fuse with plasma membrane
- Exosomes were first described in 1983 by Pan and Johnstone, EVs of 40–100 nm in size

Heijnen, H. F. et. Al. Blood 94, 3791–3799 (1999)
Pan, B. T. et. al. Cell 33, 967–978 (1983)
Simons, M. et. al. Curr. Opin. Cell Biol. 21, 575–581 (2009)

Exosomes

- Content of exosomes includes: proteins, lipids, DNA, mRNA, and LncRNA
- Exosomes can be extracted from body fluids by normal density-gradient centrifugation
- Alternatively, exosomes can be isolated through ultracentrifugation, visualized by transmission microscopy, or selected based on the presence of specific protein markers, such as the tetraspanin proteins CD63, CD9, and CD81

Lobb, R. J. et al. J. Extracell. Vesicles 4, 27031 (2015)

Exosomes

- Exosomes and other EVs could potentially be exploited as cancer biomarkers
- Exosomal miRNAs seem to be involved in disease progression; can stimulate angiogenesis and promote metastasis
- Harvesting of exosomes from biological fluids enables isolation and subsequent analysis of mRNA→ detection of mutations, splice variants, and gene fusions, as well as gene-expression profiling

Zhang, J. et al. Genomics Proteomics Bioinformatics 13, 17–24 (2015)
Rani, S. et al. Methods Mol. Biol. 784, 181–195 (2011)

Exosomes

- In comparison with ctDNA fragments, of which only two copies are essentially present in the tumour cell of origin, mRNA originating from a highly expressed gene could occur in **thousands of copies per cell** and might be shed into the circulation (within EVs or as cfRNA) at higher concentrations
- Therefore, **analysis of exosomal mRNA** might be **advantageous**, especially in patients with limited amounts of detectable ctDNA

Table 1 | **Comparison between the applications of ctDNA, CTCs, and exosomes**

	ctDNA/RNA	CTCs	Exosomes
Potential to fully recapitulate spatial and temporal tumour heterogeneity	Yes[3,4,164]	No	No
Assesment of pre/post-analytical variability	Yes[12,60]	Yes[201]	Yes[35]
Detection of somatic mutations, InDels, copy-number alterations and gene-fusions	Yes[1–3,7,10,11,60,64,71,72,75,92,125,129–132,148,149,151–156,158,189,202]	Yes[13,19,21]	Yes[203,204]
Evaluation of methylation patterns	Yes[137–142,145,146]	Yes[205]	Yes[206]
Analysis of mRNA/miRNA/lncRNA/RNA splice variants	Yes[45,49]	Yes[20]	Yes[40,43,46,51]
Analysis of RNA expression	No	Yes[19,207]	Yes[50,86]
Cell morphology and functional studies *ex vivo*	No	Yes[26–34]	No
Demonstration of signal colocalization	No	Yes[121]	No
Proteomics analysis	No	Yes[116–118]	Yes[50]

'Yes' indicates that the approach is feasible, possible, and/or published studies are available; 'No' indicates that the application is not feasible and/or no studies are available. CTCs, circulating tumour cells; ctDNA, circulating tumour DNA; InDels, DNA insertions and/or deletions; lncRNA, long noncoding RNA; mRNA, messenger RNA; miRNA, microRNA.

Liquid biopsy of other body fluids

- Besides blood, several other body fluids such as urine, saliva, pleural effusions, and cerebrospinal fluid (CSF), as well as stool, have been shown to contain tumour-derived genetic material
- Ability to exploit liquid biopsies for diagnostic purposes will further expand in the future
- In patients with lung cancer, ctDNA has also been detected in bronchial washings and pleural fluids

ORIGINAL ARTICLE

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IASLC

A Highly Sensitive and Quantitative Test Platform for Detection of NSCLC *EGFR* Mutations in Urine and Plasma

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- Reckamp and co-workers reported ability to detect **mutations in exons 19, 20, and 21 of *EGFR* *in both plasma and matched urine samples*** *from patients enrolled on the* ***phase I/II TIGER-X trial*** *(NCT01526928) of* ***rociletinib*** *in previously treated patients with EGFR-mutated advanced-stage non-small-cell lung cancer (NSCLC)*

Urine as sample

- Using the mutation status of 60 evaluable tissue samples as a reference, the sensitivity of EGFR-mutation detection in urine was **72% (34 of 47 patients) for T790M, 75% (12 of 16 patients) for L858R, and 67% (28 of 42 patients) for exon 19 deletions**
- Remarkably, combined urine and plasma testing resulted in **identification of 12 additional T790M-positive** patients, in whom T790M mutations were **missed using tumour tissue analysis**
- Indicating that urine ctDNA analysis might provide **complementary information** about patient's mutational status that is not captured by plasma or tissue tests

Reckamp, K. L. et al. Thorac. Oncol. 11, 1690–1700 (2016)

Urine as sample

- Quantification of tumour-derived tr-DNA in urine is **technically challenging**, mainly to **low amounts present**
- Continuous development of DNA-amplification and sequencing technologies will probably facilitate this approach
- Moreover, liquid biopsy of urine could be preferable to use of other body fluids, as this approach is **truly non-invasive alternative** to biopsy sampling

CSF as sample

- Potential of CSF-ctDNA for characterizing and monitoring brain tumours investigated, in comparison with use of plasma ctDNA
- Matched samples of CSF, plasma, and tumour tissue DNA from patients with glioblastoma, medulloblastoma, or brain metastases from lung or breast cancer analysed
- **ctDNA derived from tumours** located in CNS was found to be **more abundant in CSF than in plasma**, and CSF-ctDNA could be used to detect somatic mutations as well as to longitudinally monitor tumour burden
- Additional studies are needed

De Mattos-Arruda, L. et al. Nat. Commun. 6, 8839 (2015)

Pleural effusion fluids and bronchial washings

- Pleural effusion fluid and bronchial washing samples collected with physiological saline solutions→ used in diagnosing cancers of respiratory system
- Detection of EGFR mutations through cytology is feasible, difficult owing to limited number of cancer cells that are available for analysis
- In alternative approach, Kimura et al. assessed feasibility of detection of **activating EGFR exon 18–21 mutations in cfDNA present in pleural effusion fluid** from patients with NSCLC
- Findings suggested, EGFR mutation status can be accurately ascertained using tumour-derived cfDNA from pleural effusion fluid, and correlated with responsiveness to EGFR tyrosine-kinase inhibitors (TKIs)

Kimura, H. et al. Br. J. Cancer 95, 1390–1395 (2006)
Soh, J. et al. Int. J. Cancer 119, 2353–2358 (2006)

Pleural effusion fluids and bronchial washings

- In another study, feasibility of identifying EGFR mutations in tumour-derived DNA collected through bronchial washings, termed **cytology cell-free DNA (ccfDNA)**, was examined
- Results demonstrated **high sensitivity and specificity (88% and 100%, respectively**) of this approach compared with analysis of DNA from tumour tissue, suggesting that activating EGFR mutations can be accurately detected in ccfDNA
- ccfDNA might be valuable alternative to cytological samples, although larger investigations are needed to validate

Kawahara, A. et al. Cancer Cytopathol. 123, 620–628 (2015)

Pleural effusion

- Limited number of studies have investigated the **diagnostic, prognostic, or predictive value of miRNAs in pleural effusion** fluid from patients with NSCLC
- In the study, authors found that a signature comprising five miRNAs in the effusion samples was predictive of overall survival of patients with NSCLC and malignant pleural effusion

Han, H. S. et al. Int. J. Cancer 133, 645–652 (2013)
Wang, T. et al. PLoS ONE 7, e43268 (2012)

EGFR detection using liquid biopsy

Clinical Cancer Research

Imaging, Diagnosis, Prognosis

Detection of Epidermal Growth Factor Receptor Mutations in Serum as a Predictor of the Response to Gefitinib in Patients with Non–Small-Cell Lung Cancer

Hideharu Kimura, Kazuo Kasahara, Makoto Kawaishi, Hideo Kunitoh, Tomohide Tamura, Brian Holloway, and Kazuto Nishio

DOI: 10.1158/1078-0432.CCR-05-2324 Published July 2006

- First attempt at tissue–plasma pairwise comparison of EGFR mutations reported decade ago
- Since then, reports from numerous studies, including IGNYTE and ASSESS (two large, multinational, diagnostic, noncomparative intervention trials)

Kimura, H. et al. Clin. Cancer Res. 12, 3915–3921 (2006)
Han, B. et al. Ann. Oncol. 26 (Suppl.1), 29–44 (2015)
Reck, M. et al. Ann. Oncol. 26 (Suppl.1), i57–i61 (2015)

EGFR detection using liquid biopsy

- *Two meta-analyses, have confirmed that EGFR-sensitizing mutations are detectable in ctDNA with high specificity (>93%), but improvable sensitivity (<70%), making plasma genotyping of NSCLCs viable alternative to tissue-based genotyping when the latter is not feasible*

Luo, J. et al. Sci. Rep. 4, 6269 (2014)
Qiu, M. et al. Cancer Epidemiol. Biomarkers Prev. 24, 206–212 (2015)

EGFR detection using liquid biopsy

- **EURTAC** was the first trial to investigate **ctDNA as surrogate** for EGFR testing of tissue biopsy samples and effect of liquid biopsy approach on outcomes
- Randomly assigned **173 patients with activating EGFR mutations to receive erlotinib or a platinum-based chemotherapy doublet**, with analysis of EGFR mutations in serum or plasma DNA included as a secondary end point
- Multiplex real-time PCR assay for **EGFR exon 19 deletions and L858R mutation** used, and able to show that blood and tissue biomarkers had **comparable predictive power**
- Found that **EGFR L858R mutation**, whether detected in tissue DNA or ctDNA, **negatively affected** median PFS and overall survival in erlotinib-treated patients compared with outcomes of patients with tumours harbouring exon 19 deletions

Karachaliou, N. et al. JAMA Oncol. 1, 149–157 (2015)

EGFR detection using liquid biopsy

- Mok and colleagues used **cobas EGFR Blood Test** to evaluate predictive biomarkers in **FASTACT-2 trial** of induction chemotherapy **plus either erlotinib or placebo**, followed by erlotinib or placebo maintenance treatment
- The test enabled detection of G719A/C/S mutations in exons 18; 29 deletions in exon 19; S768I and T790M mutations and five insertions in exon 20; and L858R and L861Q mutations in exon 21
- Testing of samples from approximately 50% of randomized patients revealed that **baseline EGFR-mutation-positive** patients who **became EGFR-negative in plasma ctDNA** at the end of the induction period had **better outcomes**, in terms of PFS and overall survival, than those who remained EGFR-mutation-positive

Mok, T. et al. Clin. Cancer Res. 21, 3196–3203 (2015)

EGFR detection using liquid biopsy

- Most-valuable asset of blood-based EGFR-mutation test is capacity to **monitor for resistance-associated mutations**, such as the T790M gatekeeper mutation, because of their impact on ongoing treatment decision-making and patient survival
- In a seminal study by **Oxnard et al**., investigators retrospectively established that patients with **T790M-positive tumours** treated with **osimertinib**, third-generation EGFR TKI that binds irreversibly to EGFRs harbouring common activating mutations and the T790M resistance mutation, had **equivalent outcomes whether the mutation was detected in DNA from the tumour tissue or the blood**

Oxnard, G. R. et al. J. Clin. Oncol. 34, 3375–3382 (2016)

EGFR detection using liquid biopsy

- Oxnard group **prospectively confirmed** and expanded these findings in patients with **newly diagnosed NSCLC** and those with **acquired resistance** to EGFR-TKI therapy
- Patients underwent **concomitant tissue and blood DNA genotyping** using the cobas Blood Test, followed by longitudinal blood-based monitoring using the same test for *EGFR exon 19 deletions, L858R and T790M mutations, and/or all KRAS codon 12 (G12X) alterations*
- ***PPV of plasma ctDNA** analysis was **100%** for EGFR 19 deletions, EGFR L858R, and KRAS G12X, and **79% for T790M**; **sensitivity** for the EGFR mutations was in the range of **74–88%**, and was **64%** for the KRAS mutations*

Sacher, A. G. et al. JAMA Oncol. 2, 1014–1022 (2016)

EGFR detection using liquid biopsy

- **Sensitivity** for both EGFR and KRAS **was higher** among patients with **multiple metastatic sites** and in those with hepatic or bone metastases than in patients with a single metastatic site outside of these organs
- The assay **also enabled detection** of EGFR T790M mutations **missed using tissue genotyping** owing to tumour heterogeneity in patients with resistant disease
- **High specificity** and a **short median turnaround time** of testing (12 days; range 1–54 days) make this assay a **promising tool** for guiding precision medicine

Sacher, A. G. et al. JAMA Oncol. 2, 1014–1022 (2016)

EGFR detection using liquid biopsy

- In the USA and Korea, **the umbrella LUNAR trial** is expected to enrol **thousands** of individuals to demonstrate, firstly, the **feasibility** and, secondly, the **efficacy** of early detection of breast, ovarian, lung, colorectal and pancreatic cancers
- The LUNAR trial will **integrate** the use of cfDNA analyses, imaging, and germ line risk assessment, and has already collected samples from multiple trial sites

EGFR detection using liquid biopsy

- To date, **liquid biopsy has entered clinical practice only for the management of NSCLC**
- In **January 2015**, **the EMA** granted In Vitro Diagnostic Medical Device (IVD) marketing approval in Europe to the **therascreen EGFR** RGQ PCR Kit, and
- The following year, the **FDA approved the cobas EGFR Mutation Test v2** (an updated version of cobas EGFR Mutation Test) for use in the USA
- Both of these IVDs can detect EGFR mutations in plasma ctDNA with **comparable accuracy** to that of **bidirectional Sanger sequencing of DNA** from tumour tissue specimens, as established within the framework of large clinical trials of small-molecule EGFR TKIs in patients with EGFR-mutated NSCLC

EGFR detection using liquid biopsy

- The **therascreen assay**: enables detection of 19 different exon 19 deletions and three distinct exon 20 insertions, as well as L858R, L861Q, G719X, S768I, and T790M mutations in EGFR
- In the **Lung-LUX3 trial**, the efficacy of frontline chemotherapy was compared with that of **afatinib** (an irreversible second-generation EGFR TKI), the **overall percentage agreement (OPA) between therascreen test and tissue-based test results was 92.2% (95% CI 89.0–94.8%)**
- For 27 of 28 discordant sample pairs (96%), presence of an **EGFR mutation was detected in plasma ctDNA**, but not in DNA from tumour tissue samples

Sequist, L. V. et al. J. Clin. Oncol. 31, 3327–3334 (2013)

EGFR detection using liquid biopsy

- Clinical utility of the therascreen test confirmed in a **phase IV trial of gefitinib**, in which **12 of 201 patients** with **no tumour tissue** available for genotyping were found to harbour **EGFR mutations using this liquid** biopsy assay
- But, the test had **34% false-negative rate** (36 out of 105 patients tested postive for EGFR-mutations in tissue DNA, but had no EGFR mutations detected in plasma cfDNA)
- Therefore, EMA has amended detection of EGFR mutations in ctDNA should **only be attempted for patients without an evaluable tumour sample**

Douillard, J. Y. et al. Br. J. Cancer 110, 55–62 (2014)

EGFR detection using liquid biopsy

- **cobas EGFR Mutation Test v2:** identification of 42 EGFR mutations, including exon 19 deletions, exon 20 insertions, and S768I, L861Q, L585R and T790M mutations, in both tissue and plasma
- The **plasma** test, is FDA-approved as companion diagnostic for determining **eligibility of patients with NSCLC for erlotinib treatment** based only on the **presence of EGFR exon 19 deletions and the L858R substitution** mutation
- Patients who test **negative for these alterations** must undergo **routine biopsy sampling** and testing for EGFR mutations in formalin-fixed paraffin-embedded tissue samples

EGFR detection using liquid biopsy

- Performance of **cobas EGFR Mutation Test v2 in detecting T790M resistance mutation in ctDNA**: evaluated in pooled retrospective analysis of two single-arm phase II registration studies of osimertinib (**AURA extension, NCT01802632; AURA2, NCT02094261)**
- **Positive percentage agreement and negative percentage** agreement between the findings of tissue and plasma testings was **61.4% and 78.6%,** respectively, similar to the results obtained for detection of exon 19 deletions and L585R mutations in ctDNA using same test

Jenkins, S. et al. J. Thorac. Oncol. 11 (Suppl. 4), S57–S166. (2016)

EGFR detection using liquid biopsy

- The **Guardant360 test** is quite sensitive (mutated ctDNA accounting for 0.4% of total cfDNA in blood could be detected)
- enabled the detection of **resistance mutations in EGFR, ALK, and KRAS** that were **not detectable** in the matched **tumour biopsy samples** in **almost one-third** of the patients
- Overall, the test identified molecular alteration in **64%** of patients, including 362 patients with NSCLC with no available tumour tissue, which could **potentially be targeted** using an FDA-approved drug or an experimental drug currently being tested in a clinical trial

Zill, O. A. et al.]. J. Clin. Oncol. 34 (Suppl.), LBA11501 (2016)

Analysis of ALK Status using liquid biopsy

- Recent studies have evaluated **possibility of also using liquid biopsy approach to detect an ALK rearrangement** and/or the emergence during inhibitor treatment of some **resistance mutations** in ALK
- Assessments can be performed by studying circulating tumor cells by fluorescent in situ hybridization and by immunocytochemistry and/or after the isolation of RNA from plasma samples, free or associated with platelets
- The liquid biopsy may **be complementary or sometimes alternative method** for the assessment of the ALK status in certain NSCLC patients, as well as a **non-invasive approach for early detection** of ALK mutations

Analysis of ALK Status using liquid biopsy

- Currently, analysis for the EML4-ALK status is mainly performed by immunohistochemistry (IHC) and confirmed by FISH analysis from tissue biopsies
- This status can also be detected on cytological samples by immunochemistry (ICC) and/or by FISH analysis
- Presence of an EML4-ALK rearrangement leads to targeted treatment

Analysis of ALK Status using liquid biopsy

- In 2012, a study conducted by Ilie et al. showed very **good agreement** between the presence of EML4-ALK rearrangements in **tissue biopsies and in CTCs**
- Second study using ISET method also showed a **correlation** between presence of an **EML4-ALK rearrangement in tissues and CTCs** and a correlation with **disappearance** of CTCs with a **good response to targeted therapy**

Ilie, M. et al. Ann. Oncol. 2012, 23, 2907–2913
Pailler, E. et al. J. Clin. Oncol. 2013, 31, 2273–2281

Analysis of ALK Status using liquid biopsy

- Using same ISET method for CTCs detection, recent study looked for the **prognostic value of patients with CTCs** having ALK-copy number gain (CNG) at baseline and treated with **crizotinib**
- In this study, **dynamic change in the numbers of CTCs** with ALK-CNG was a **predictive biomarker for crizotinib efficacy** in ALK-rearranged NSCLC patients
- Another study confirmed correlation between **positive EML4-ALK** status detected in **tissues** and **in CTCs detected by FISH** analysis
- This latter study showed **persistence of or an increase** in number of ALK positive CTCs on FISH analysis **correlated with tumor progression** in patients on treatment with crizotinib, in particular with the **appearance of new molecular profile**

Pailler, E, et al. Cancer Res. 2017, 77, 2222–2230
Tan, C. L. et al. Oncotarget 2016, 7, 23251–23262

Analysis of ALK Status with Plasma

- Free plasma DNA is routinely used to analyze mutations in EGFR in patients with advanced stage lung cancer
- In contrast, plasma RNA is **not used routinely**, or very rarely used, for detection of EML4-ALK rearrangements

Analysis of the Status of ALK in Platelets

- **Tumor-educated platelets** when isolated, constitute an **enriched source of tumor RNA for detection of EML4-ALK rearrangements**
- Recent study showed that this approach, **very specific, more sensitive** than those used for plasma RNA
- The **persistence of EML4-ALK rearrangements in platelets** of patients treated with crizotinib is associated with **tumor progression and poor prognosis**
- The **reappearance of this fusion transcript** in platelets is **sign of resistance to crizotinib** and it occurs several months before progression is detected on radiological examination

Nilsson, R.J. et al. Oncotarget 2016, 7, 1066–1075

Analysis of ALK Status using liquid biopsy

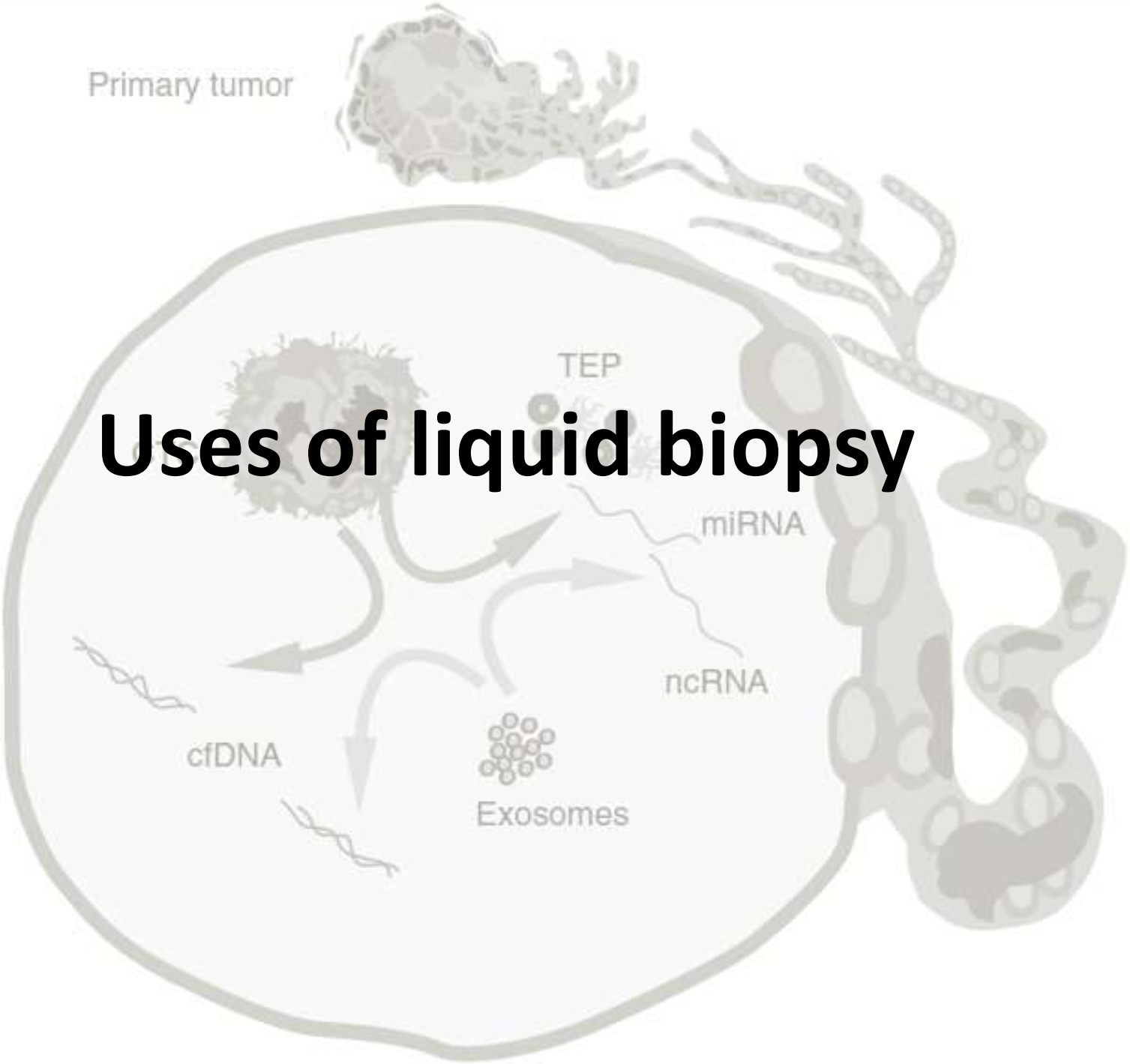
- Despite a frequent **effective therapeutic response** in the **first months of administration** of targeted therapeutics, patients with an ALK rearrangement **relapse or progress**, due to **emergence of mutations** in ALK
- Detection of these mutations leads to **changes in the targeted therapeutics**
- **Depending on treatment** used to target an ALK rearrangement, **resistance mutations** that emerge on the ALK gene can be **different**
- For example, the **L1196M mutation** often occurs after treatment with **crizotinib**, G1202R after ceritinib or alectinib, F1174C after ceretinib and I1171T/N/S after alectinib treatment

Gainor, J.F. et al. Cancer Discov. 2016, 6, 1118–1133

Table 1. The pros and the cons of anaplastic lymphoma kinase (*ALK*) gene analysis with a liquid biopsy in non small cell lung cancer patients.

Pros	
	Non-invasive method
	Easily repeatable
	Monitoring for early detection of mutation onset in the *ALK* gene
	Alternative approach to a tissue biopsy when:
	Presence of a non-biopsiable tumor site
	Patient is fragile
	Presence of a weak percentage of tumor cells
	Poor quality of extracted RNA
Cons	
	ALK status assessment in circulating tumor cells is not available routinely
	The quality of the results is strongly related to some pre analytical parameters:
	Volume of blood sample
	Tubes used for blood collection
	Delay for transfer to the laboratory
	Centrifugation procedure
	Temperature of plasma/platelets storage
	Large variability of amount of CTCs and free tumor RNA and platelets associated tumor RNA according to the patient

Uses of liquid biopsy



ctDNA in cancer diagnosis

- Quantitative analysis of cfDNA can be used to assess tumour **burden**
- Potentially be used to determine if patient is **disease free** after curative surgery
- When levels of cfDNA are coupled with identification **of somatic mutations** (that is, focusing on ctDNA), they provide valuable diagnostic information
- **Correlation** between mutations present in ctDNA and tumour tissue samples is increasingly important in diagnosing specific molecular tumour subtypes, with implications for precision medicine

Methylation profiles in ctDNA

- **Predicting response** to chemotherapy
- Promoter hypermethylation at specific CpG sites associated with tumour- suppressor genes occurs in many cancers; therefore, methylated ctDNA is a promising biomarker
- Several studies have compared aberrant methylation in tumour tissues and matched ctDNA from blood samples, in settings such as lung cancer

MRD monitoring and early diagnosis of relapse— ctDNA as prognostic biomarker

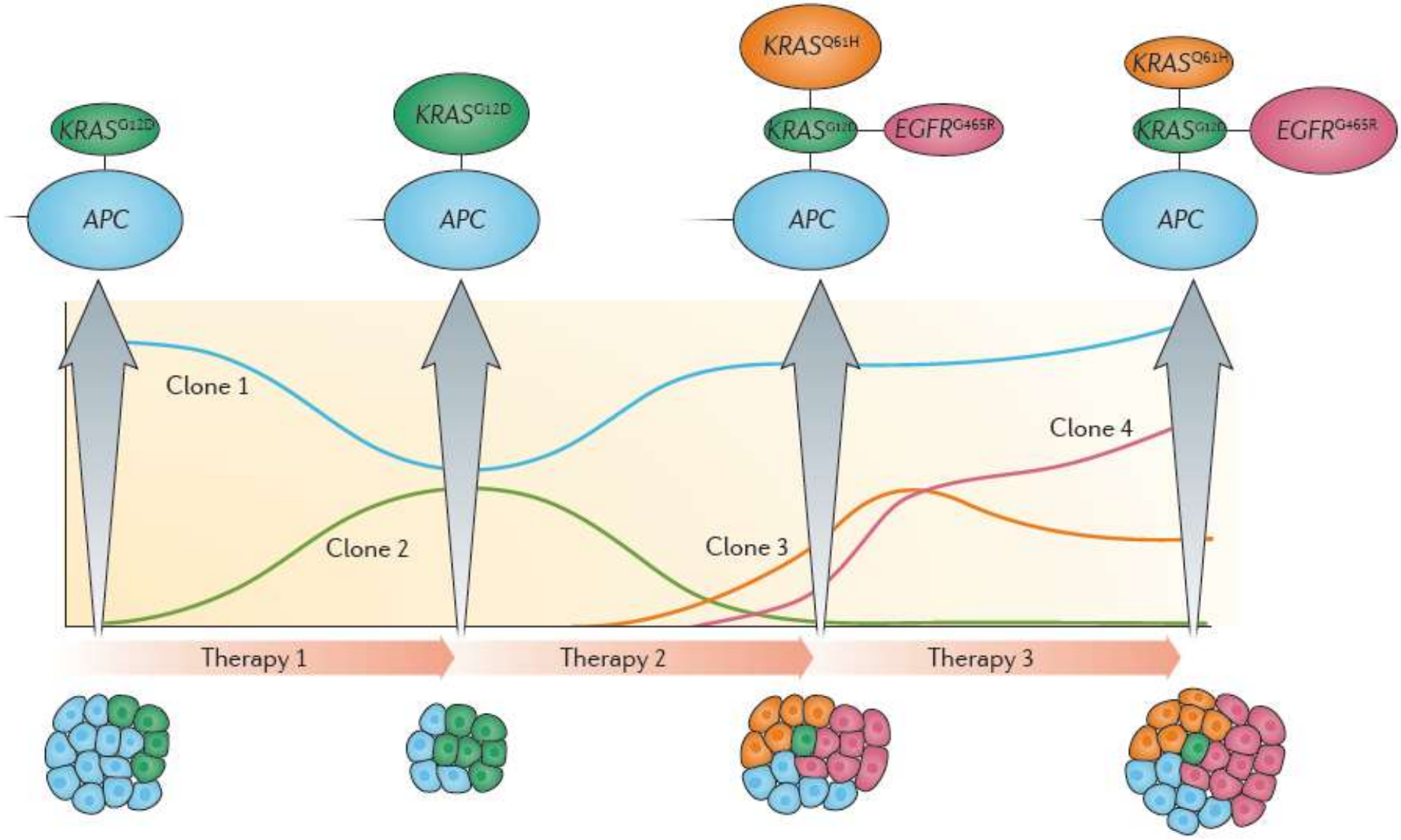
- Liquid biopsy approaches well-suited to measuring MRD, as **residual tumour components can be detected with high sensitivity**, and data from proof-of-concept studies have shown that ctDNA levels can be used to monitor MRD following surgery or other curative treatments
- **ctDNA surveillance**, aimed at **identifying recurrence** in patients with no evidence of disease after primary treatment with curative intent, is **key use of liquid biopsy** in clinical trials

MRD monitoring

- As for the early diagnosis of cancer, whether identification of MRD-positive patients at increased risk of relapse can improve patient outcomes through early diagnosis of relapse and proactive '**consolidative' or 'rescue' treatment** remains to be clarified
- Monitoring of MRD also raises the possibility of **de-escalating treatment in MRD-negative** patients
- Liquid biopsies can be applied to the **monitoring of response and/or resistance** to systemic therapy

Dynamic clonal evolution

- Detection of oncogenic gene rearrangements in ctDNA is another exciting area of research
- In addition to monitoring response and resistance, this liquid biopsy approach enabled characterization of previously unknown mutations that confer **resistance**
- Liquid biopsies can be used to **monitor dynamic clonal evolution in response to selective pressures** exerted by therapy
- ctDNA has been analysed in order to **genotype cancers and track clonal evolution at multiple points before, during, and after treatment**



Monitoring clonal evolution using liquid biopsies

Advantages of liquid biopsy

- Much less invasive than primary tumor biopsies
- Multiple clinical applications: including characterization of molecular profiles in line of tissue, monitoring response to therapy, detection of minimal residual disease, and tumor evolution with therapy
- Snapshot of circulating genome and transcriptome can be more representative of different regions of a heterogeneous tumor
- Helps identification and emergence of therapeutic resistance much earlier than solid tissue biopsies
- Longitudinal follow up possible

Limitations

- Limitation of liquid biopsy approaches is that transcriptome profiling and gene-expression analysis are not always possible, as cfRNA is present in insufficient amounts in plasma and other body fluids
- ctDNA is fragmented and highly under-represented compared with germ line cfDNA12
- Only a limited number of CTCs can be isolated from a blood sample
- Thus, analysis of tumour material obtained by liquid biopsies requires highly sensitive assays

Limitations

- Standardization of the blood collection procedure to improve the stability of samples at room temperature (reducing preanalytical variability)
- Defining ctDNA quantification methods
- Standardization of ctDNA isolation to improve yield; and
- Improving the sensitivity of ctDNA detection for rare molecular alterations in order to anticipate drug resistance

Future perspectives

- Technological progress and prolific use of such techniques holds great promise for the future of cancer treatment and individualized care
- Larger studies needed to validate
- Standard technique of molecular detection

ESMO

ORIGINAL ARTICLE

Annals of Oncology 28: 136–141, 2017
doi:10.1093/annonc/mdw531
Published online 6 October 2016

Monitoring of somatic mutations in circulating cell-free DNA by digital PCR and next-generation sequencing during afatinib treatment in patients with lung adenocarcinoma positive for *EGFR* activating mutations

E. Iwama[1,2], K. Sakai[3], K. Azuma[4], T. Harada[2], D. Harada[5], K. Nosaki[6], K. Hotta[7], F. Ohyanagi[8], T. Kurata[9], T. Fukuhara[10], H. Akamatsu[11], K. Goto[12], T. Shimose[13], J. Kishimoto[14], Y. Nakanishi[2], K. Nishio[3] & I. Okamoto[2]*

Background: Analysis of circulating cell-free DNA (cfDNA) is under intensive investigation for its potential to identify tumor somatic mutations. We have now explored the usefulness of such liquid biopsy testing with both the digital polymerase chain reaction (dPCR) and next-generation sequencing (NGS) during treatment of patients with the epidermal growth factor receptor (EGFR) inhibitor afatinib.

Patients and methods: Eligible patients had advanced lung adenocarcinoma with *EGFR* activating mutations and were treated with afatinib. Plasma samples were collected before and during (4 and 24 weeks) afatinib treatment as well as at disease progression. Tumor and plasma DNA were analyzed by dPCR and NGS.

Results: Thirty-five patients were enrolled. The objective response rate and median progression-free survival (PFS) were 77.1% and 13.8 months, respectively. Tumor and plasma DNA were available for 32 patients. dPCR and NGS detected *EGFR* activating mutations in 81.3% and 71.9% of baseline cfDNA samples, respectively. In 19 patients treated with afatinib for ≥24 weeks, the number of *EGFR* mutant alleles detected in cfDNA by dPCR declined rapidly and markedly after treatment onset, becoming undetectable or detectable at only a low copy number (<10 copies per milliliter) at 4 weeks. Median PFS was slightly longer for patients with undetectable *EGFR* mutant alleles in cfDNA at 4 weeks than for those in whom such alleles were detectable (14.3 versus 10.0 months). A total of 45 somatic mutations was identified in baseline tumor DNA, and 30 (66.7%) of these mutations were identified in cfDNA by NGS. Allele frequency for somatic mutations in cfDNA determined by NGS changed concordantly during afatinib treatment with the number of *EGFR* mutant alleles determined by dPCR.

Conclusions: Monitoring of cfDNA by dPCR is informative for prediction of afatinib efficacy, whereas that by NGS is reliable and has the potential to identify mechanisms of treatment resistance.

Key words: afatinib, epidermal growth factor receptor (EGFR), mutation, circulating cell-free DNA (cfDNA), digital polymerase chain reaction (dPCR), next-generation sequencing (NGS)

ESMO GOOD SCIENCE BETTER MEDICINE BEST PRACTICE

ORIGINAL ARTICLE

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doi:10.1093/annonc/mdw670
Published online 19 December 2016

Multiplex *KRAS*$^{G12/G13}$ mutation testing of unamplified cell-free DNA from the plasma of patients with advanced cancers using droplet digital polymerase chain reaction

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Background: Cell-free DNA (cfDNA) from plasma offers easily obtainable material for *KRAS* mutation analysis. Novel, multiplex, and accurate diagnostic systems using small amounts of DNA are needed to further the use of plasma cfDNA testing in personalized therapy.

Patients and methods: Samples of 16 ng of unamplified plasma cfDNA from 121 patients with diverse progressing advanced cancers were tested with a *KRAS*$^{G12/G13}$ multiplex assay to detect the seven most common mutations in the hotspot of exon 2 using droplet digital polymerase chain reaction (ddPCR). The results were retrospectively compared to mutation analysis of archival primary or metastatic tumor tissue obtained at different points of clinical care.

Results: Eighty-eight patients (73%) had *KRAS*$^{G12/G13}$ mutations in archival tumor specimens collected on average 18.5 months before plasma analysis, and 78 patients (64%) had *KRAS*$^{G12/G13}$ mutations in plasma cfDNA samples. The two methods had initial overall agreement in 103 (85%) patients (kappa, 0.66; ddPCR sensitivity, 84%; ddPCR specificity, 88%). Of the 18 discordant cases, 12 (67%) were resolved by increasing the amount of cfDNA, using mutation-specific probes, or re-testing the tumor tissue, yielding overall agreement in 115 patients (95%; kappa 0.87; ddPCR sensitivity, 96%; ddPCR specificity, 94%). The presence of $\geq$ 6.2% of *KRAS*$^{G12/G13}$ cfDNA in the wild-type background was associated with shorter survival ($P = 0.001$).

Conclusion(s): Multiplex detection of *KRAS*$^{G12/G13}$ mutations in a small amount of unamplified plasma cfDNA using ddPCR has good sensitivity and specificity and good concordance with conventional clinical mutation testing of archival specimens. A higher percentage of mutant *KRAS*$^{G12/G13}$ in cfDNA corresponded with shorter survival.

Key words: cell-free DNA, droplet digital PCR, KRAS, multiplex

ESMO GOOD SCIENCE BETTER MEDICINE BEST PRACTICE

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doi:10.1093/annonc/mdx017
Published online 19 January 2017

ORIGINAL ARTICLE

Osimertinib benefit in *EGFR*-mutant NSCLC patients with *T790M*-mutation detected by circulating tumour DNA

J. Remon[1], C. Caramella[2], C. Jovelet[3], L. Lacroix[3], A. Lawson[4], S. Smalley[4], K. Howarth[4], D. Gale[4,5], E. Green[4], V. Plagnol[4], N. Rosenfeld[4,5,6], D. Planchard[1], M. V. Bluthgen[1], A. Gazzah[1], C. Pannet[1], C. Nicotra[1], E. Auclin[1], J. C. Soria[1,7] & B. Besse[1,7*]

- Assessed the feasibility of **identifying T790M mutations in ctDNA isolated from blood samples** in cohort of EGFR-mutant NSCLC patients with **progression under first- or second-generation** EGFR TKIs **without tissue biopsy at progression**, in order to detect acquired resistance
- The **efficacy** of **osimertinib** in the ctDNA T790M-positive NSCLC patients was **assessed**

ESMO GOOD SCIENCE BETTER MEDICINE BEST PRACTICE

Annals of Oncology 28: 784–790, 2017
doi:10.1093/annonc/mdx017
Published online 19 January 2017

ORIGINAL ARTICLE

Osimertinib benefit in *EGFR*-mutant NSCLC patients with *T790M*-mutation detected by circulating tumour DNA

J. Remon[1], C. Caramella[2], C. Jovelet[3], L. Lacroix[3], A. Lawson[4], S. Smalley[4], K. Howarth[4], D. Gale[4,5], E. Green[4], V. Plagnol[4], N. Rosenfeld[4,5,6], D. Planchard[1], M. V. Bluthgen[1], A. Gazzah[1], C. Pannet[1], C. Nicotra[1], E. Auclin[1], J. C. Soria[1,7] & B. Besse[1,7*]

Background: Approximately 50% of epidermal growth factor receptor (*EGFR*) mutant non-small cell lung cancer (NSCLC) patients treated with EGFR tyrosine kinase inhibitors (TKIs) will acquire resistance by the *T790M* mutation. Osimertinib is the standard of care in this situation. The present study assesses the efficacy of osimertinib when *T790M* status is determined in circulating cell-free tumour DNA (*ct*DNA) from blood samples in progressing advanced *EGFR*-mutant NSCLC patients.

Material and methods: *ct*DNA *T790M* mutational status was assessed by Inivata InVision™ (eTAm-Seq™) assay in 48 *EGFR*-mutant advanced NSCLC patients with acquired resistance to EGFR TKIs without a tissue biopsy between April 2015 and April 2016. Progressing *T790M*-positive NSCLC patients received osimertinib (80 mg daily). The objectives were to assess the response rate to osimertinib according to Response Evaluation Criteria in Solid Tumours (RECIST) 1.1, the progression-free survival (PFS) on osimertinib, and the percentage of *T790M* positive in *ct*DNA.

Results: The *ct*DNA *T790M* mutation was detected in 50% of NSCLC patients. Among assessable patients, osimertinib gave a partial response rate of 62.5% and a stable disease rate of 37.5%. All responses were confirmed responses. After median follow up of 8 months, median PFS by RECIST criteria was not achieved (95% CI: 4–NA), with 6- and 12-months PFS of 66.7% and 52%, respectively.

Conclusion(s): *ct*DNA from liquid biopsy can be used as a surrogate marker for *T790M* in tumour tissue.

Key words: EGFR mutation, *T790M*, osimertinib, lung cancer, ctDNA liquid biopsies

ORIGINAL ARTICLE

Plasma ctDNA Analysis for Detection of the *EGFR* T790M Mutation in Patients with Advanced Non-Small Cell Lung Cancer

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Available online - 17 April 2017

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Suresh S. Ramalingam, MD, PhD,[c] Karen Yu, BA,[d] Sabina Patel, PhD,[e]
Susie Weston, BSc,[a] Rachel Hodge, MSc,[e] Mireille Cantarini, MD,[a]
Pasi A. Jänne, MD, PhD,[f] Tetsuya Mitsudomi, MD, PhD,[g] Glenwood D. Goss, MD[h]

ABSTRACT

Introduction: Tu
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after previous EGFR tyrosine kinase inhibitor therapy was investigated to determine eligibility for osimertinib treatment.

Methods: Matched tumor tissue and plasma samples from patients screened for the AURA extension and AURA2 phase II studies were tested for *EGFR* mutations by using tissue- and plasma-based cobas EGFR mutation tests. Plasma test performance was assessed by using the cobas tissue test and a next-generation sequencing method (MiSeq [Illumina Inc, San Diego, CA]) as references. The objective response al review, was with a plasma ided matched nalysis of the

Conclusions: The cobas plasma test detected the T790M mutation in 61% of tumor tissue T790M mutation–positive patients. To mitigate the risk of false-negative plasma results, patients with a negative plasma result should undergo a tissue test where feasible.

positive and negative percent agreements between the cobas plasma and tissue tests for detection of T790M mutation were 61% and 79%, respectively. Comparing cobas plasma test with next-generation sequencing demonstrated positive and negative percent agreements of 90% or higher. The objective response rate was 64% (95% confidence interval: 57–70) in T790M mutation–positive patients by both cobas tissue and plasma tests (evaluable for response).

Table 2. Percent Agreement of the cobas Plasma Test with the cobas Tissue Test as a Reference Method for the Detection of *EGFR* T790M, L858R, and Exon 19 Deletion

	Percent Agreement (95% CI)								
	T790M			L858R			Exon 19 Deletion		
	AURA Extension (n = 210)	AURA2 (n = 341)	Pooled AURA Extension and AURA2 (n = 551)	AURA Extension (n = 210)	AURA2 (n = 341)	Pooled AURA Extension and AURA2 (n = 551)	AURA Extension (n = 210)	AURA2 (n = 341)	Pooled AURA Extension and AURA2 (n = 551)
PPA	64 (57-71)	59 (52-65)	61 (57-66)	75 (61-85)	76 (67-84)	76 (69-82)	88 (81-93)	83 (77-88)	85 (81-89)
NPA	–[a]	80 (72-87)	79 (70-85)	99 (95-100)	98 (95-99)	98 (96-99)	98 (92-100)	98 (94-100)	98 (95-100)
OPA	65 (58-71)	66 (61-71)	65 (61-69)	92 (88-96)	90 (86-93)	91 (88-93)	91 (86-94)	89 (86-93)	90 (87-92)

[a]Not calculated because of the low number of samples (total <20).

PPA, positive percent agreement (sensitivity); NPA, negative percent agreement (specificity); OPA, overall percent agreement (concordance).

Table 3. Concordance between the cobas *EGFR* Mutation Test and MiSeq NGS for Tumor Tissue and Plasma Tests from the AURA Extension and AURA2 Pooled Analysis Data Set

Percent Agreement (95% CI)	cobas Tissue Test vs. NGS Tissue Analysis (Reference) for the Detection of T790M (n = 673)	cobas Plasma Test vs. NGS Plasma Analysis (Reference [n = 562]) for the Detection of		
		T790M	Exon 19 Deletion	L858R
PPA	90 (87-93)	93 (89-96)	95 (92-98)	93 (87-97)
NPA	98 (94-99)	92 (88-95)	91 (87-94)	97 (95-98)
OPA	92 (90-94)	92 (90-94)	93 (91-95)	96 (94-97)

NGS, next-generation sequencing; CI, confidence interval; PPA, positive percent agreement (sensitivity); NPA, negative percent agreement (specificity); OPA, overall percent agreement (concordance).

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Published online 29 April 2017

ORIGINAL ARTICLE

Circulating tumor DNA changes for early monitoring of anti-PD1 immunotherapy: a proof-of-concept study

L. Cabel[1,2], F. Riva[2], V. Servois[3], A. Livartowski[1], C. Daniel[1], A. Rampanou[2], O. Lantz[4], E. Romano[1,4], M. Milder[4], B. Buecher[1], S. Piperno-Neumann[1], V. Bernard[5], S. Baulande[5], I. Bieche[6], J. Y. Pierga[1,2,7], C. Proudhon[2] & F.-C. Bidard[1,2,8*]

Departments of [1]Medical Oncology; [2]Circulating Tumor Biomarkers Laboratory; [3]Radiology; [4]INSERM U932 & CIC-BT 1428; [5]ICGex Next-Generation Sequencing Platform; [6]Genetics, Institut Curie, Paris, France; [7]Université Paris Descartes, Médecine, Paris; [8]Université Paris-Saclay, Médecine, Saint Cloud, France

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Background: Recent clinical results support the use of new immune checkpoint blockers (ICB), such as anti-PD-1 (e.g. nivolumab and pembrolizumab) and anti-PD-L1 antibodies. Radiological evaluation of ICB efficacy during therapy is challenging due to tumor immune infiltration. Changes of circulating tumor DNA (ctDNA) levels during therapy could be a promising tool for very accurate monitoring of treatment efficacy, but data are lacking with ICB.

Patients and methods: This prospective pilot study was conducted in patients with nonsmall cell lung cancer, uveal melanoma, or microsatellite-instable colorectal cancer treated by nivolumab or pembrolizumab monotherapy at Institut Curie. ctDNA levels were assessed at baseline and after 8 weeks (w8) by bidirectional pyrophosphorolysis-activated polymerization, droplet digital PCR or next-generation sequencing depending on the mutation type. Radiological evaluation of efficacy of treatment was carried out by using immune-related response criteria.

Results: ctDNA was detected at baseline in 10 out of 15 patients. At w8, a significant correlation ($r = 0.86$; $P = 0.002$) was observed between synchronous changes in ctDNA levels and tumor size. Patients in whom ctDNA levels became undetectable at w8 presented a marked and lasting response to therapy. ctDNA detection at w8 was also a significant prognostic factor in terms of progression-free survival (hazard ratio = 10.2; 95% confidence interval 2.5–41, $P < 0.001$) and overall survival (hazard ratio = 15; 95% confidence interval 2.5–94.9, $P = 0.004$).

Conclusion: This proof-of-principle study is the first to demonstrate that quantitative ctDNA monitoring is a valuable tool to assess tumor response in patients treated with anti-PD-1 drugs.

Key words: circulating tumor DNA, immune therapy, nivolumab, pembrolizumab, biomarker

ESMO

ORIGINAL ARTICLE

Annals of Oncology 28: 1523–1531, 2017
doi:10.1093/annonc/mdx156
Published online 19 June 2017

A prospective examination of circulating tumor cell profiles in non-small-cell lung cancer molecular subgroups

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Background: We report the first study examining the clinical, numerical and biological properties of circulating tumor cells according to molecular subtypes of non-small-cell lung cancer.

Patients and methods: 125 patients with treatment-naïve stage IIIb-IV NSCLC were prospectively recruited for CellSearch analysis. Anti-vimentin antibody was included for examination of CTCs to assess their mesenchymal character. Associations of total CTCs and vimentin-positive (vim +) CTCs with clinical characteristics, tumor genotype, and survival were assessed.

Results: 51/125 patients (40.8%) were total CTC+ and 26/125 (20.8%) were vim CTC+ at baseline. Multivariate analysis showed patients with ≥5 total CTCs had significantly reduced OS (HR 0.55, 95% CI 0.33–0.92, $P = 0.022$) but not PFS (HR 0.68, 95% CI 0.42–1.1, $P = 0.118$) compared to patients with <5 total CTCs. No OS difference was evident between vim+ CTC and vim- negative CTC patients overall (HR 1.24, 95% CI 0.67–2.28, $P = 0.494$), but after subdivision according to NSCLC driver mutation, we found an increase of vim+ CTCs in the *EGFR*-mutated subgroup ($N = 21/94$ patients; mean 1.24 vs 1.22 vim+ CTCs, $P = 0.013$), a reduction of total CTCs in the *ALK*-rearranged subgroup ($N = 13/90$ patients; mean 1.69 vs 5.82 total CTCs, $P = 0.029$), and a total absence of vim+ CTCs in *KRAS*-mutated adenocarcinomas ($N = 19/78$ patients; mean 0 vs 1.4 vim+ CTCs, $P = 0.006$).

Conclusions: We validate that the baseline presence of ≥5 total CTCs in advanced NSCLC confers a poor prognosis. CTCs from *EGFR*-mutant NSCLC express epithelial–mesenchymal transition characteristics, not seen in CTCs from patients with *KRAS*-mutant adenocarcinoma.

List of ongoing oncology clinical trials incorporating cfDNA analysis

ClinicalTrials.gov reference	Status	Disease setting	Sample size	cfDNA sequencing technique	Main objectives of the trial
NCT00290355	Completed	NSCLC (stage I–II)	182	NR	Descriptive: plasma cfDNA levels during adjuvant treatment with GSK 249553 (vaccine comprising recombinant MAGE-3 epitope fused to a *Haemophilus Influenzae* protein D antigen)
NCT00213798	Completed	NSCLC	200	NR	Descriptive: plasma cfDNA genotyping during treatment and follow-up evaluation
NCT01147562	Active, recruiting	NSCLC (stage I)	150	NR	Concordance: plasma and saliva cfDNA genotyping vs matched tumour DNA
NCT00997334	Active, not recruiting	NSCLC	60	NR	Descriptive: plasma cfDNA alterations and CTC DNA alterations before, during, and at progression on erlotinib treatment in patients with *EGFR*-mutated disease
NCT02127359	Active, not recruiting	Lung cancer CRC	400	WES	Feasibility of analyzing plasma cfDNA by WES Concordance: WES of plasma cfDNA vs matched tumour DNA

ClinicalTrials.gov reference	Status	Disease setting	Sample size	cfDNA sequencing technique	Main objectives of the trial
NCT01930474	Active, recruiting	NSCLC	200	ddPCR	Clinical validation: sensitivity of ddPCR of cfDNA for detection of *EGFR* mutations and *ALK* fusions in longitudinal plasma specimens, and analysis of temporal changes in these genes Clinical utility: detection of alteration(s) using NGS as predictive biomarker of drug resistance
NCT02038647	Active, not recruiting	SCLC	178	NR	Clinical utility: genotyping of plasma cfDNA and CTC DNA as predictive biomarkers of response to alisertib (AURORA kinase A inhibitor)
NCT02169349	Active, recruiting	NSCLC	100	NGS	Clinical validation (assay development): assessment of *EGFR* mutations in plasma cfDNA for EGFR-TKI treated patients
NCT02186236	Active, recruiting	NSCLC; CRC	200	ddPCR (Trovagene)	Concordance: *EGFR* mutations (NSCLC) and *RAS*/*RAF* mutations (CRC) in urine cfDNA vs matched tumour DNA; EGFR mutation in plasma cfDNA vs matched CTC DNA
NCT02284633	Active, recruiting	Lung cancer	200	NR	Clinical utility: plasma levels of *EGFR*-mutated cfDNA a predictive biomarker of response in patients with *EGFR*-mutated tumour DNA

ClinicalTrials.gov reference	Status	Disease setting	Sample size	cfDNA sequencing technique	Main objectives of the trial
NCT02179528	Not yet recruiting	SCLC	210	NR	Descriptive: plasma cfDNA genotyping for potential *post-hoc* patient stratification
NCT02281214	Active, recruiting	NSCLC	180	NGS	Concordance: between plasma cfDNA and matched tumour DNA according to a lung cancer gene panel (*EGFR, KRAS, BRAF, PI3K, HER2, AKT1*, and *ALK* mutations, and *ALK* and *ROS1* rearrangements) Clinical utility: plasma cfDNA lung panel findings as a predictive biomarkers for resistant genotype at progression in patients treated with EGFR-targeted therapy or chemotherapy
NCT02422628	Active, recruiting	Lung cancer	400	NR	Concordance: *EGFR* mutations in plasma cfDNA vs matched CTCs DNA and tumour DNA
NCT02418234	Completed	EGFR-TKI-resistant NSCLC	300	ARMS and ddPCR	Concordance: *EGFR* T790M mutations in plasma cfDNA by 2 different methods vs matched tumour DNA
NCT02645318	Completed	NSCLC (stage I–II)	95	NR	Concordance: plasma cfDNA vs matched tumour DNA (aberrations not specified) Clinical Utility: plasma cfDNA (aberrations not specified) as a predictive biomarker of recurrence (end point: DFS)

ClinicalTrials.gov reference	Status	Disease setting	Sample size	cfDNA sequencing technique	Main objectives of the trial
NCT02511288	Active, recruiting	Metastatic NSCLC	200	ddPCR	Concordance: *EGFR* mutations and other lung-cancer-associated alterations in plasma cfDNA vs matched tumour DNA
NCT02544633	Active, recruiting	NSCLC	200	NR	Diagnostic: *MET* mutations and amplifications in plasma cfDNA for selection of patients with *MET*-positive tumours. Concordance: *MET* mutations and amplifications in plasma cfDNA vs tumour DNA
NCT02623257	Not yet recruiting	NSCLC	1,000	ARMS	Clinical validation: *EGFR* mutations in plasma cfDNA by ARMS. Clinical utility: EGFR mutations in plasma cfDNA as predictive biomarker for response to chemotherapy
NCT02597738	Active, recruiting	Lung cancer	90	NR	Discovery: blood and tissue genotyping in patient with advanced-stage lung cancer (longitudinal sampling) vs nonsmokers with chronic inflammatory disease vs smokers without lung cancer
NCT02629523	Not yet recruiting	Lung cancer	21	NR	Diagnostic: *EGFR* mutation in plasma cfDNA in patient selection for afatinib treatment
NCT02696525	Not yet recruiting	NSCLC	145	NR	Concordance: plasma/urine cfDNA 3 days before and after surgery vs matched surgical tumour DNA (aberrations not specified) Clinical utility (surveillance): plasma cfDNA as predictive biomarker for locoregional recurrence and metastasis vs gold standard tumour markers and imaging

Take Home Message

- Patient selection is central to the success of targeted therapy; identification of tumour-specific molecular landscapes is pivotal to guiding treatment choices
- Genomic landscape of each individual tumour is heterogeneous and changes over time as a result of clonal evolution imposed on cancer cells by selective pressures, including targeted therapy
- Longitudinal surveillance of clonal evolution is essential for precision medicine, but cannot be effectively achieved using tissue biopsy specimens, owing to sampling issues

Take Home Message

- Blood of patients with cancer contains diverse tumour-derived materials, including circulating cell-free tumour DNA (ctDNA), circulating tumour cells, and exosomes
- The sampling and analysis of ctDNA or other circulating tumour components present in biological fluids, termed ‘liquid biopsy’, enables minimally invasive monitoring of tumour evolution over time in clinic
- Two different liquid biopsy companion diagnostic tests for EGFR mutations in plasma ctDNA have been approved by the regulatory agencies in Europe and the USA for the selection of patients with non-small-cell lung cancer for anti-EGFR treatment in clinical practice
- Liquid biopsy is emerging as a promising tool in management of lung cancer

Thank You....